TYPE 2 DIABETES EXACERBATES BONE LOSS IN NATIVE AMERICAN WOMEN

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CHAPTER I

INTRODUCTION

Background

Native Americans are often recognized for honoring traditions and preserving the culture of individual tribes (1-3). However, in recent generations there has been an extrinsic attribute invading Native cultures in the form of pervasive health disparities and chronic disease. Numerous obstacles including mistrust from previous scientific exploitation, linguistic and cultural dissimilarities, lack of culturally grounded theory and methods, and limited or selective access to community members, have impeded research in this population (4). One of these chronic diseases is type 2 diabetes which is associated with a state of low-grade, chronic systemic inflammation (5). Evidence demonstrates a relationship between type 2 diabetes and multiple microvascular (e.g. retinopathy and neuropathy) and macrovascular complications (e.g. coronary heart disease) and, more recently, an increased risk of bone fracture has been suggested (6-8). Because the prevalence of type 2 diabetes is high among Native Americans (i.e. ~ 2 times the national average) (9) and the availability of evidence regarding bone health is limited in this population, there is an emergent need to investigate the relationship between bone health and type 2 diabetes in Native Americans.

Osteoporosis is a skeletal condition often characterized by normally mineralized bone tissue but

decreased bone mass (10). The structural integrity of trabecular, or spongy bone may be compromised, causing it to lose elasticity and cortical bone, may become more porous and thin. While thinner bone alone is not necessarily more prone to fracture, bone that is both thinner and has compromised biomechanical properties is more prone to fracture (11). Osteoporosis is reportedly responsible for more than 1.5 million fractures in the U.S. annually and carries an estimated health care cost of $16.9 billion making this a major medical problem (12). Lifestyle factors including smoking, low levels of weight-bearing physical activity and compromised nutritional status can further contribute to fracture risk. Estimates from the 2004 Surgeon General's Report (13) indicate half of all women >50 years of age will experience an osteoporosis-related fracture during their lifetime.

Osteoporosis is diagnosed based on the occurrence of osteoporotic-related fracture or by assessment of bone mineral density (BMD), which accounts for approximately 70% bone strength, and fracture incidence (14). According to the World Health Organization (WHO) criteria, individuals with a BMD 2.5 standard deviations or more below the mean value for young, healthy, Caucasian women (i.e. T-score of < -2.5 SD) are considered osteoporotic (15;16). The most widely validated method for assessing BMD is an areal assessment dual energy X-ray absorptiometry (DXA) (17).

A misconception exists that osteoporosis is always a result of bone loss, when in fact bone loss is a common occurrence as both women and men age. Women however, typically have a lower peak bone mass than men (18) and experience rapid bone loss during the first 5-10 years of the postmenopausal period (19). The combination of bone loss relative to peak bone mass and the rate of postmenopausal bone loss results in an increased risk for fracture in women earlier than men. The hormonal changes associated with menopause (e.g., decrease in estrogen and increase in follicle stimulating hormone) can lead to a disruption of normal bone metabolism which is described as a dynamic, lifelong process involving the coordinated activities of bone-resorping osteoclasts and bone-forming osteoblasts (20). The bone remodeling cycle is regulated not only by hormones, but also cytokines (e.g., interleukin or

(IL)-6 and tumor necrosis factor (or TNF-α) and growth factors (21;22). These regulatory proteins can be influenced by a number of factors, including nutritional status (e.g. compromised vitamin D status), physical activity, age and illness, which subsequently alter bone turnover (23). When considering that type 2 diabetes also has a profound impact on many of these same nutritional, cytokine and hormonal factors the consequences on bone remodeling have the potential to be significant (24). This relationship is in part the impetus behind an increasing interest in the effects of type 2 diabetes on bone health.

Several studies have provided clinical evidence of an increased fracture risk in type 2 diabetics compared to the general public (7;8;25-31). Results of the Iowa Women's Health Study reported a 1.70-fold increased risk of hip fracture in post-menopausal women with type 2 diabetes compared to their non-diabetic counterparts (25). Duration of diabetes appeared causative to even higher risk for hip fracture, considering those women 13 to 40 years post-diagnosis had a 2.30-fold higher risk for hip fracture relative to women without diabetes. The increased risk for hip fracture was observed in both obese participants, known to benefit from greater skeletal loading, as well as non-obese women with type 2 diabetes (25). A higher fracture risk among type 2 diabetics was also found in the Health, Aging and Body Composition Study (Health ABC) (26). Results of this biracial cohort of elderly men and women demonstrated that type 2 diabetes was associated with a 64% increase in incident clinical fractures compared to non-diabetics. Participants in this study with type 2 diabetes had similar BMD and significantly higher body weight, BMI, lean and fat mass, visceral fat and fasting insulin compared to their non-diabetic counterparts. These studies provide data in support of an increased risk of fracture in the type 2 diabetic population.

Native Americans are a high-risk population for type 2 diabetes. Data from the 2005 Indian Health Services (IHS) user population database indicate that 16.5% of the total adult population served by IHS had diagnosed diabetes compared to 7.8% of the total U.S. adult population (9). In addition to diabetes, factors such as smoking (32) and low serum vitamin D (33) contribute as risk factors for

bone fracture in this population. Unfortunately, there is a paucity of data regarding Native American women and bone health substantiating increased risk. In the National Osteoporosis Risk Assessment (NORA), Native Americans represented 0.9% of the total population. Results from this study indicated that body weight could explain differences in BMD found among White, Native American, Hispanic and Asian women (34). Among limitations identified in this study were selection bias due to eligibility criteria, reliance on self-reported information and insufficient numbers (34). Other data from the Women's Health Initiative (WHI) suggest that overall, American Indian/Alaska Natives (AI/AN) and non-Hispanic white women had similar BMDs, after adjusting for age, education, and years of hormone therapy (35). Both the NORA and WHI reports represent large scale research studies that acknowledge as limitations the low or insufficient number of Native American participants and eligibility criteria excluding diabetics from participation.

Problem Statement:

Despite these efforts to examine the risk of osteoporosis in Native American's, the issue of fracture risk and the potential influence of type 2 diabetes on bone health in this population remains to be addressed.

Purpose:

The purpose of this study is to examine the effects of type 2 diabetes and diabetes duration on bone health compared to non-diabetics in Native American women over 50 years of age. To accomplish this purpose the following hypotheses have been developed.

Hypotheses and Specific Aims:

Hypothesis 1: Change in BMD from baseline to final visit, one year later, will be greater in those women with type 2 diabetes and especially those women who have been diabetic for ten or more

years compared to their non-diabetic counterparts. To test this hypothesis the following specific aims have been developed:

Specific Aim 1a: To examine differences in change in BMD between diabetics and non diabetics by performing DXA scans of the three principal sites of osteoporotic fracture, spine, total hip and forearm, at baseline and at one year follow-up.

Specific Aim 1b: To examine differences in change in BMD between diabetics stratified by duration of diabetes diagnosis (<10 or ≥10 years) and non diabetics over a one year period.

Hypothesis 2: Type 2 diabetics will demonstrate alterations in bone metabolism consistent with increased bone resorption and decreased bone formation rates from baseline to final visit, compared to non-diabetics. These alterations in bone metabolism will be more pronounced in longer duration type 2 diabetics (i.e., ≥ 10 yrs). The following aims have been developed to test this hypothesis:

Specific Aim 2a: To evaluate differences over time in serum markers of bone resorption, as indicated by C-telopeptide or CTX in type 2 diabetics compared to non diabetics.

Specific Aim 2b: To evaluate differences over time in serum markers of bone formation as indicated by bone-specific alkaline phosphatase (BAP) in type 2 diabetics compared to non diabetics.

Specific Aim 2c: To evaluate differences over time in serum CTX and BAP in diabetics stratified by duration of diabetes diagnosis (<10 or ≥10 years) compared to non diabetics.

Hypothesis 3: The mechanisms by which type 2 diabetics will experience accelerated bone loss will be mediated by an increased inflammatory state and compromised vitamin D status. The increased inflammatory state and compromise in vitamin D status will be exacerbated in longer duration diabetics. The following aims have been developed to test this hypothesis:

Specific Aim 3a: To examine differences in change in serum IL-6 and TNF-α in type 2 diabetics vs. non-diabetics between baseline and final visits.

Specific Aim 3b: To examine differences in change in monocyte and lymphocyte percentage and absolute counts based on complete blood counts (CBC) in type 2 diabetics vs. non-diabetics between baseline and final visits.

Specific Aim 3c: To evaluate differences in change in vitamin D status via 25-hydroxyvitamin D3, in type 2 diabetics vs. non-diabetics over time.

Specific Aim 3d: To examine differences in change in inflammatory indices and vitamin D status when type 2 diabetics are stratified by duration of diabetes diagnosis (<10 or ≥10 years).

Limitations:

Some of the limitations of this study include external validity due to the small (N=123), unique sample (stratified by diabetes diagnosis) available for the study. Therefore, results may not be generalizable beyond the specific population from which the sample was drawn. Additionally, the accuracy of results such as medical history, medications and supplements, and calcium intake was dependent on the self-report of the participants. Data provided that were crucial to the accuracy of this study included diabetes diagnosis and blood quantum information.

Delimitations:

This study is delimited to Native American women, who were 50 years of age and older and eligible to receive services at an Indian Health Clinic. The study was delimited to examination of changes in BMD, measures of bone formation and resorption, vitamin D status and markers of inflammation over the duration of one year to determine the impact of type 2 diabetes on bone health. Due to the large number of potential participants in the study population, recruitment efforts were concentrated within the state of Oklahoma.

CHAPTER II

REVIEW OF LITERATURE

Bone Tissue

Bone is a living, dynamic tissue that is constantly renewed, resulting in complete turnover of the adult skeleton every 10 years (36). Bone tissue is generally classified into two types, cortical and trabecular bone, which are identical in their chemical composition. Cortical bone is a dense, compact structure, highly resistant to bending and torsion, and except for the periosteum, has a slow turnover rate. It constitutes the diaphysis of long bones and the outer part of all skeletal structures. It functions primarily to provide mechanical strength and protection to vital organs. Trabecular bone is less dense, more elastic and has a higher turnover rate than cortical bone. Anatomical sites rich in trabecular bone include the epiphyses and metaphyses of the long bones and it is also the major component of the ribs, the shoulder blades and the flat bones of the skull. In addition to providing strength and support for the body, bone serves as a site for development and storage of blood cells. Bone accumulates micro-damage from loading, but is unique in its ability to self-repair (37-39). The complexities and processes involved in the anabolic and catabolic aspects of bone metabolism (i.e., formation by osteoblasts and resorption by osteoclasts), and the influence of nutritional and environmental factors all contribute to the susceptibility for diseases and disorders to potentially affect bone health.

Osteoporosis Defined

Osteoporosis is a skeletal disorder characterized by compromised bone strength with a consequent increased risk of fracture (40;41). Prediction of fracture risk is a key element in fracture prevention. As a result, the definition of osteoporosis has evolved over the years in an effort to improve predictive ability.

In 1941 Fuller Albright, a research endocrinologist, observed thinning bones in women following menopause (42). He was the first to propose that estrogen deficiency played a primary role in postmenopausal osteoporosis. Albright's definition of osteoporosis was described as "too little calcified bone" due to the osteoblast's inability to lay sufficient osseous matrix (42).

It was not until 1994 that osteoporosis was formally defined by the World Health Organization (WHO) in terms of BMD and fracture history (15). This was a result of the efforts of an international panel of scientific experts to assess fracture risk and its application to screening for postmenopausal osteoporosis. The panel considered several approaches to defining osteoporosis based on bone mineral measurement, but each failed to overcome the problem of overlap in BMD between those who have and those who have not suffered fragility fractures. The determination was made that bone mineral assessment may provide an index of risk, much as hypercholesterolemia is a risk for coronary heart disease, but that index was not reflective of all elements of risk. Subsequently the panel established four general diagnostic categories for women based on bone mineral content and proximity to the young adult reference mean. These categories include normal, osteopenia, osteoporosis and severe osteoporosis (43). The panel further emphasized the importance of distinguishing between the diagnostic use of BMD measurements by providing information concerning the presence or absence of osteoporosis with the cut-off values chosen, and the prognostic use whereby bone density values are considered a risk factor (43).

Risk Factors for Osteoporosis

The 1994 report of the international WHO conference identified the role of osteoporosis in predicting fracture risk (43). Because no obvious warning signs precede the clinical manifestation of osteoporosis, identification of risk factors for reducing fracture risk was considered of great importance.

Thus osteoporosis, once viewed primarily as a natural occurrence of aging, was now recognized as a condition that can stem from suboptimal bone mineralization during childhood and adolescence (44-47), or from bone loss later in life. Bone health is influenced by factors both modifiable and non-modifiable.

Modifiable Risk Factors

Among the factors affecting bone health that are modified or controlled is diet. A diet low in calcium and vitamin D has been shown to increase risk of osteoporosis and fractures (48-51). A low calcium intake can contribute to osteoporosis and fracture risk by enhancing parathyroid hormone (PTH) release from the parathyroid glands, resulting in excessive bone turnover in favor of bone resorption and eventual bone loss. Studies have demonstrated that calcium supplementation for elderly women are associated with a reduction in bone resorption and a partial suppression of serum PTH (52;53).

Vitamin D, well known for enhancing calcium absorption in the gut, is also critical for maintenance of bone mass through its actions on other cellular processes such as bone mineralization. Vitamin D facilitates bone mineralization at the osteoblast level by enhancing differentiation and stimulates proximal tubular phosphate reabsorption in the kidney. The latter function enhances bone mineralization by contributing phosphate, one of the two principal crystalline salts deposited in the mineralized matrix of bone. The combination of calcium and phosphate with hydroxide form hydroxyapatite crystals, which constitutes the mineral phase of

bone tissue (54). A meta-analysis performed by Papadimitropoulos et al. (55), examined all randomized placebo-controlled trials of vitamin D and its analogs over the last two decades. They reported a significant reduction in spine fractures (i.e., 37% risk reduction) in women taking vitamin D compared to placebo. Other research supporting beneficial effects of dietary calcium and vitamin D includes Chapuy et al. (48) who dispensed 800 international units (IU) of cholecalciferol or vitamin D3 and 1200 mg of calcium to nursing home residents for 18 months. A 35% reduction in the occurrence of hip fractures was reported with this supplement regimen (48). Similarly, Dawson-Hughes et al. (49) reported that elderly men and women treated with 700 IU of cholecalciferol plus 500 mg of calcium citrate a nearly 50% reduction in nonvertebral fractures (49).Thus supporting the effectiveness of adequate consumption of calcium and vitamin D.

Physical activity is also a modifiable risk factor that affects bone remodeling (56-60). The microgravity environment of space flight is an extreme example of deficient weight-bearing activity, which results in a significant decrease in bone formation (61;62). Skeletal unloading has been shown to decrease osteoblast activity and number (63-70), which according to Garetto et al. (71) is likely due to a decreased proliferation of osteoprogenitor cells. The weightless environment of space has also been shown by Smith et al. (72) to affect bone resorption. This was demonstrated based on astronauts' post flight elevation (50-125%) in urinary collagen cross-links (N-telopeptide [NTX], deoxypyridinoline [DPD], and pyridinoline [PYD]). A somewhat less extreme example of the effect of inactivity is found in bed rest where the rate of loss has been observed to be one to two percent per month (73). In contrast, Howe et al. (74) demonstrated in an extensive review that weight-bearing exercise had a significantly greater effect on BMD in post-menopausal women compared with controls who did not exercise.

Non-Modifiable Risk Factors

Apart from modifiable risk factors are those that cannot be controlled. These risk factors include among others, gender, age and race. There is evidence to support the relationship between menopausal status and BMD thus contributing to the greater risk of development of osteoporosis in women than men. Results of numerous studies have demonstrated a negative association between either menopausal status or years post-menopause and BMD (75-80). Two separate studies of the same population reported that post-menopausal status was associated with a decrease in both femoral neck and spinal BMD (75;76). Mizuno et al. (80) also reported that after menopause, BMD of the lumbar spine (L2-4) decreased more rapidly than other anatomical sites. The correlation coefficient between lumbar spine BMD and lumbar spine/total body BMD ratio was 0.746, indicating that a decreasing ratio of lumbar spine (L2-4) BMD was more prominent than that of total body BMD. Perimenopause, defined as the two to eight year period preceding menopause and one year following the last period, is marked by an increase in bone loss which is a result of increased bone resorption (81). Greater bone loss is observed in trabecular-rich regions compared to sites higher in cortical bone due to the larger surface area over which osteoclasts can attach and degrade bone. The result of perimenopausal bone loss is demonstrated in the fractures occurring in early menopause in the trabecular-rich skeletal regions of the distal forearm and vertebrae (82).

Although differences in BMD between men and women may explain in part the gender-related differences in fracture rates, it is also possible that differences in both BMD and fracture rates may be attributed to differences in bone size and geometry (83-86). The geometry and structure of bone have been increasingly recognized as important risk factors for fracture including the role of puberty on bone growth. It has been shown that the length and width of bone increase progressively throughout the pre-pubertal period, in both sexes (87). Because boys enter puberty approximately two years later than girls, boys can acquire greater long bone length before puberty

(88). Gender-related differences in bone width are more apparent after puberty as demonstrated by periosteal growth. This enlargement in bone diameter is the result of bone formation beneath the periosteal envelope causing a widening of the diaphysis of the bone and stimulating longitudinal bone growth (88). This process is accelerated by puberty in males, but inhibited during puberty in females due to the increases in circulating estrogen (87). These differences in bone development contribute to the greater attainment of peak bone mass in males which is considered a major determinant of bone mass and fracture risk in later life (89).

Age represents another non-modifiable risk factor for osteoporosis. A progressive reduction in BMD with aging has been documented at nearly every skeletal site (90), however fracture risk has shown an increase with age independent of BMD (91). Other factors influenced by age include degree of mineralization, microfracture, number and frequency, skeletal geometry and periosteal response to trabecular bone loss. Additionally, greater periosteal apposition has been demonstrated more in aging men than women. This contributes to a greater net bone loss in women compared with men (92) which may in turn propagate the higher fracture rate seen in women.

Race is another risk factor for osteoporosis. Although each group is comprised of unique characteristics that set them apart from the others, one of the challenges of examining racial differences is the absence of an obvious or uniform method of classification of individuals into such groups. Racial differences in bone health have been associated with several key features. Differences in body size (93;94), bone size (95), rate of skeletal loss (96-99), and hip geometry (100-103) have all been reported to partially explain observed differences in fracture risk among races. These differences have multifactorial explanations including differences in bone metabolism (104) and pubertal onset (105). Longitudinal data with baseline BMD and fracture outcomes for nonwhites are limited and it is also unknown whether T-scores obtained by BMD measurement in nonwhite women have the same value in terms of fracture prediction (106). It is

important therefore, for future osteoporosis research to continue to examine variables that contribute to observed racial differences.

Gastrointestinal conditions are also included among the non-modifiable risk factors for osteoporosis. Underlying in many conditions is malabsorption of vitamins and minerals, particularly vitamin D (107) and calcium (108). There is consistent evidence of a reduction in BMD in patients with both Crohn's disease and ulcerative colitis resulting from a decrease in bone formation and an increase in bone resorption (109-111). Calcium malabsorption in gastrointestinal conditions has been associated with steatorrhea, alteration in the calcium-transport mechanisms, and lack of vitamin D (112-116). Contributing to vitamin D malabsorption are chronic pancreatic insufficiency, intrinsic small bowel disease, disorders of the biliary tract, and surgical bypass procedures of the jejunum and ileum. Leichtmann et al. (117) reported the small intestine involvement in Crohn's disease leads to an increase in vitamin D malabsorption and subsequently increased bone loss. In fact vitamin D deficiency in Crohn's has been shown to be a predictive factor for osteoporosis and osteopenia (117;118).

In addition to malabsorption, gastrointestinal conditions including inflammatory bowel disease (IBD) and celiac disease increase fracture risk through the chronic release of pro-inflammatory cytokines by immunologically competent cells. Osteotropic cytokines such as TNF-α and IL-6 are involved in both normal and abnormal bone remodeling, and increased cytokine production in chronic inflammatory diseases is associated with increased bone loss (119;120).

Endocrine disorders contribute to osteoporosis risk via the role of hormonal regulation of the bone remodeling cycle. For example, the chronic secretion of PTH (i.e., hyperparathyroidism) or the continuous infusion of PTH can lead to decalcification of bone and loss of bone mass. The regulation of calcium homeostasis is a process maintained in large part by PTH via secretion in response to very small decrements in blood ionized calcium. The action of PTH on calcium

homeostasis is accomplished by: 1) promotion of bone resorption (i.e., release of calcium from the skeletal reservoir); 2) induction of renal calcium conservation and phosphate excretion; and 3) indirect enhancement of intestinal calcium absorption by increasing the renal production of the active vitamin D metabolite, $1,25(OH)_2$ vitamin D. PTH has been shown *in vivo* to increase the numbers of osteoclasts as well as the resorptive activity of preformed osteoclasts (121).

One hormone that contributes as a risk factor for osteoporosis post-menopause is estrogen. A major physiological effect of this hormone is inhibition of bone resorption (122), as such it plays a regulatory role in osteoclast apoptosis (123). In estrogen deficiency, an increased number of osteoclasts and their extended longevity lead to increased bone resorption. In response to the increased bone resorption, there is increased bone formation and a high-turnover state develops favoring resorption and leading to rapid bone loss and perforation of the trabecular plates (124). In addition, estrogen elicits a protective effect on bone through its ability to decrease pro-osteoclastogenic cytokines. For example decreases in estrogen levels at menopause have been associated with an increase in serum interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 7 (IL-7) and TNF-α which are known to promote osteoclast differentiation and activity (125-127).

Another hormone influencing bone remodeling is insulin. Current evidence suggests insulin impacts bone development and physiology by regulating osteoblast function in the following manner. Osteoblasts express a functional insulin receptor (IR) and when primary osteoblasts or osteoblast-like cell lines are exposed to physiological levels of insulin, increases are observed in bone anabolic activity such as collagen synthesis (128;129) and alkaline phosphatase production (130). In addition, it has been demonstrated that patients with type 1 diabetes develop early onset osteopenia or osteoporosis (131;132), experience increased risk of fragility fracture (25;133) and exhibit poor bone healing and regeneration after injury (134). A recent discovery by Fulzele et al. (135) demonstrated that insulin suppresses the runt-related transcription factor 2 (Runx2) inhibitor Twist-related protein 2 (Twist2). Runx2 is a key transcription factor regulating gene

expression for several key bone proteins, including osteocalcin, osteopontin and osteoprotegeron (136-139). Twist2 inhibition in turn promotes osteoblast differentiation necessary for normal bone formation. In addition, previous clinical studies clearly demonstrate that an oral glucose load which increases insulin secretion suppresses markers of bone resorption by 50% (140). These findings provide a basis for the discrepancy in BMD between the type 1and type 2 diabetic populations which is related to their opposing insulin-secretory states (i.e. hypoinsulinemia vs. hyperinsulinemia).

Obesity and Type 2 Diabetes

Obesity is a major public health problem in our society. According to data from the 1999-2000 National Health and Nutrition Examination Survey (NHANES) (141) almost 65% of the adult population in the United States is overweight or obese (body mass index [BMI] 25-29.9; $\geq$ 30kg/m^2). Health concerns related to increased prevalence of obesity stem from the accompanying increased risk of chronic disease, particularly type 2 diabetes (142;143).

In general, obesity is defined as excess adipose tissue. This tissue is found in the human body in two types, brown adipose tissue and white adipose tissue. Adipocytes found in brown adipose tissue are specialized primarily for non-shivering thermogenesis (144). Brown fat depots are present in human infants and recent evidence suggests that dispersed brown adipocytes might persist in adults (145). In contrast, white adipose tissue is the predominant type found in adults and is located just beneath the skin as subcutaneous fat, around the organs as visceral fat and within the bone marrow. Adipocytes found in white adipose tissue store dietary energy in the form of triglycerides, predominantly in single large, lipid droplets. The droplets' unique structure allow triglycerides to be rapidly hydrolyzed by lipases in a process known as lipolysis, and the resulting fatty acids are transported to other tissues to be oxidized in mitochondria for energy.

Adipose tissue is now considered the largest endocrine organ in the body (146).The endocrine action of adipose tissue is via adipocyte secretion of a number of protein factors, collectively referred to as adipokines which include growth factors (e.g., transforming growth factor (TGF)-ß) and hormones (e.g., leptin and adiponectin). Leptin is well known for its regulatory role in energy metabolism by stimulating energy expenditure, inhibiting food intake and restoring euglycemia. In obesity however, leptin resistance often limits the effectiveness of this process (147-149). In contrast to leptin, adiponectin has been associated with an anti-inflammatory action (150) and when increased in plasma, has been independently associated with reduced risk of type 2 diabetes in healthy individuals (151).

Two other cytokines secreted by adipose tissue are interleukin (IL)-6 and TNF-α. Serum levels of these pro-inflammatory cytokines have been reported to be elevated in the obese (152;153), contributing to the concept that obesity and diabetes are characterized by a state of chronic low-grade inflammation (153-155).

A number of studies have demonstrated that TNF-α can impair insulin signaling in hepatocytes and adipose tissue (156-158). The underlying mechanism involves the inhibition of insulin receptor substrate (IRS) signaling capability. IRS proteins act as mediators of insulin signaling playing a central role in maintaining basic cellular functions such as growth and metabolism by acting as docking proteins between the insulin receptor and a complex network of intracellular signaling molecules. The inhibition of this signaling is thought to be achieved through the TNF-α activation of serine kinases such as the c-Jun-N-terminal kinase (JNK) (159;160). Although understanding of the signaling network of JNKs continues to evolve (161), JNK1 knockout mice have been shown to exhibit decreased adiposity and significantly increased insulin sensitivity (162). TNF-α also affects insulin action by reducing fatty acid oxidation in hepatocytes (163) and skeletal muscle (164). These affects are mediated by suppression of adenosine monophosphate-activated protein kinase (AMPK) via the up-regulation of protein phosphatase 2C (PP2C). In

skeletal muscles, AMPK stimulates glucose transport and fatty acid oxidation. In the liver, it decreases cholesterol and triglyceride synthesis, glucose output and augments fatty acid oxidation. The reduced rates of fatty acid oxidation subsequently increase accumulation of bioactive lipids, such as diacyglycerols (164), which in turn activates protein kinase C and inhibits IRS function (165).

The association between elevated serum IL-6 and insulin resistance is supported by epidemiological and genetic studies. IL-6 has been demonstrated to inhibit the insulin signaling pathway in adipocytes by up-regulating suppressor of cytokine signaling (SOCS) 3 expression, which in turn impairs insulin-induced insulin receptor and IRS-1 phosphorylation (166-168), resulting in increased insulin resistance. Clinically, plasma IL-6 has been shown to positively correlate with both obesity and insulin resistance (169;170). In fact, Pradhan et al. (171) found elevated plasma IL-6 to be a predictive marker in the development of type 2 diabetes. The association between IL-6 and type 2 diabetes remained positive after adjusting for BMI, family history of diabetes, smoking, physical activity, alcohol use, and hormone replacement therapy. This finding supports the possibility of a causal relationship between IL-6 and the development of diabetes.

Although it may appear logical that adipose tissue expression of adipocytokines plays a role in the development of type 2 diabetes based on total adiposity, other factors such as the size of adipocytes and fat distribution may also contribute to insulin resistance. Recently, the influence of adipocyte size has been given much attention from the scientific community due to the discovery that larger adipocytes are more likely to become insulin resistant (172). For instance, in Pima Indians, who have a very high prevalence rate of type 2 diabetes, larger adipocytes were shown to have a greater propensity for insulin resistance, which led to reduced triglyceride and glucose clearance (173). These data indicated that large adipocytes and increased circulating fatty acids were independent predictors of diabetes risk in this population.

It has also been suggested that insulin resistance can be attributed to a defect in subcutaneous adipose tissue's ability to expand appropriately as indicated by telomere length (174-176), independent of body weight (177). In fact, hyperplastic obesity is typically more metabolically benign in terms of insulin sensitivity than fat hypertrophy (178). A study by McLaughlin et al. (179) aimed at identifying insulin-resistant individuals, found that 17% of the overweight and obese subjects were relatively insulin sensitive. Moreover, Karelis et al. (180) reported that approximately 20% of the general population were obese but metabolically healthy, which reinforces the influence of body composition in the development of diabetes.

Distribution of adipose tissue is also an important influence on insulin sensitivity. One area that has been studied for its influence on insulin resistance is adipose tissue of the visceral depots (181). Increased adiposity of the intra-abdominal fat area (IAFA) was found by Boyko et al. (182) to be predictive of diabetes incidence. This observation was independent of other measures of total and regional adiposity, family history of diabetes, gender, correlates of insulin resistance (fasting C-peptide) and glycemia (fasting glucose). This is consistent with other studies that have demonstrated visceral adipose tissue was significantly correlated with both insulin resistance and type 2 diabetes (183-186). The primary regional difference between the two compartments appears to be in the rate of lipolysis (187). The rate of release of free fatty acids (FFAs) from stored triglycerides is higher in visceral adipocytes than subcutaneous where anti-lipolytic hormones, such as insulin, have a more pronounced effect (188). Because visceral fat drains into the portal vein, rapid visceral lipid metabolism results in the delivery of excessive amounts of FFA concentrations to the liver. This in turn leads to stimulation of gluconeogenesis, increased triglyceride synthesis and inhibition of insulin clearance. The result of these metabolic alterations may ultimately lead to the development of hyperglycemia and hyperinsulinemia (188).

Obesity and Bone Health

Obesity and its association with chronic diseases such as type 2 diabetes, hypertension, coronary heart disease and some cancers pose a serious health threat to our society (142;143). The association between obesity and skeletal health may not be as obvious. This is due in part to higher BMI values having been traditionally considered a protective feature against osteoporotic related fracture risk (189-192). In fact body weight and BMI are positively correlated to BMD (193-196) in adults (193;197-199). The increasing prevalence of obesity in our society however, does not support the beneficial effects of increased BMI on fracture prevention.

Epidemiologic studies have demonstrated some common genetic determinants between obesity and osteoporosis. Both adipocytes and osteoblasts originate from common progenitor, pluripotent mesenchymal stromal cells (MSC) (200-202). The fate of these cells' differentiation is largely determined by the expression of transcription factors, Runx2 and peroxisome proliferator-activated receptor gamma (PPARγ). These transcription factors act as molecular switches to promote the direction of differentiation of precursor cells into osteoblast or adipocyte lineages, respectively (203-205). Animal models have demonstrated that decreased PPARγ activity leads to increased number of osteoblasts and bone mass (206;207). Inversely, increased PPARγ activity, related to antidiabetic treatment with the thiazolidinedione (TZD) drug rosiglitazone, has resulted in significant decreases in BMD, bone volume and changes in bone microarchitecture (208-211). This bone loss has been associated with a decreased number of osteoblasts and an increased number of adipocytes within the bone marrow (198;201). PPARγ expression has been found at its highest levels in white adipose tissue (212-215).

Another feature that highlights the relationship between obesity and the bone remodeling cycle is hormonal. The hormone leptin is secreted by adipocytes and well known for its role in the regulation of appetite and energy expenditure (216). More recently leptin has been examined for

its role in bone remodeling. The effects of leptin via a central hypothalamic pathway were first reported by Ducy et al. (216), wherein he described the action of leptin on neurons in the hypothalamus which has since been proposed to regulate bone mass as well (217). The mechanism by which leptin influences bone metabolism is at least in part through activation of hypothalamic nerves. This in turn stimulates sympathetic nerves extending into the bone and promoting the release of the neurotransmitter noradrenaline (217). Noradrenaline stimulates ß2-adrenergic receptors (ADRB2). ADRB2 upregulation decreases osteoblast activity and bone formation, and increases bone resorption via receptor activator for nuclear factor κB (NF-κB) ligand (RANKL) production, which ultimately leads to trabecular bone loss (216-222). These complex interactions between fat, brain and bone are further explained by the effects of pro-inflammatory mediators (e.g., TNF-α and IL-6) on bone metabolism (223).

Obesity, IL-6 and Bone

Adipose tissue is responsible for secretion of one third of all circulating IL-6 which explains why overweight and obese adults and children generally have elevated serum IL-6 (153;224). Cytokines that promote bone resorption such as TNF-α and IL-1 also reportedly stimulate the synthesis of IL-6 in osteoblasts (225-227). In the context of chronic inflammation, the role of IL-6 is central to the pathogenesis of bone loss, exerting its effects as a potent stimulator of osteoclast-induced bone resorption (228). These effects are demonstrated in the osteopenia observed in transgenic mice overexpressing IL-6, characterized by severe alterations in cortical and trabecular bone microarchitecture. Also reported is the uncoupling of bone formation and resorption, evidenced by decreased osteoblast and increased osteoclast number and activity (228). The role of IL-6 in osteoporosis has been further highlighted in a study of the expression of key regulatory molecules of bone remodeling in fragility fracture patients who underwent total hip arthroplasty as a result of a femoral neck fracture (229). The fracture groups' expression of RANK and IL-6 were significantly elevated compared with an age-matched control group. IL-6

mRNA levels associated strongly with bone mRNA levels in the fracture group, but not in the control group (229). These findings suggest an association between IL-6 and the RANKL/RANK pathway and are consistent with studies in murine osteoblastic cell lines, where IL-6 has been shown to induce RANKL mRNA expression (230). It is thought that IL-6 utilizes the RANK/RANKL/OPG interaction to exert an indirect effect on osteoclasts by promoting activation and subsequent bone resorption. This effect has been proposed to occur via an interaction between IL-6 and osteoblasts, which may lead to increased osteoblastic RANKL production (231).

The effects of estrogen on IL-6 are seen in postmenopausal women as an increased secretion rate of IL-6 compared with baseline levels in cells from premenopausal women. Notably, the increase is found in the early post-menopause phase, but not in late years (232-234). Jilka et al.(125) have proposed that estrogen's affect on IL-6 is through inhibition of TNF-α, and IL-1 stimulated IL-6 gene transcription by binding the estrogen receptor ligand complex to NF-kB, therefore preventing binding to the IL-6 promoter (235-240). Conversely, in an estrogen deficient state, the inhibitory effects of estrogens are removed, resulting in enhanced osteoclast development in the marrow. It is this increase in osteoclastogenesis that is responsible for the increased bone resorption and hence the loss of bone in the post menopausal state. This effect was demonstrated in ovariectomized mice where the increase in osteoclast number was prevented by treatment with a neutralizing anti-IL-6 antibody *in vivo* and in *ex vivo* cultures (126).

Obesity, TNF-α and Bone

TNF-α is another proinflammatory cytokine expressed and secreted by adipose tissue (241;242). Though numerous factors contribute to bone loss, TNF-α plays a central role in the pathophysiology by increasing bone resorption while simultaneously inhibiting bone-forming osteoblasts (243-250). The influence of TNF-α is exerted through increased osteoclastogenesis,

decreased osteoblastogenesis and inducing vitamin D resistance (251). The effects of TNF-α serve as potent stimuli for bone loss that ultimately lead to bone microarchitectural deterioration and increased fracture risk that has been demonstrated in conditions such as rheumatoid arthritis (RA), periodontitis, orthopedic implant loosening, and other forms of chronic inflammatory osteolysis (252-257).

The role of TNF-α as a stimulator of osteoclastogenesis has been confirmed by numerous investigators (125;258;259). Hematopoietic stem cells differentiate along the myelo-monocytic lineage toward an osteoclast phenotype under the influence of macrophage colony stimulating factor (M-CSF) and RANKL. RANKL is essential for the induction of osteoclast differentiation and supports survival of the mature functional osteoclast (260). TNF-α closely regulates RANK/RANKL-induced osteoclastogenesis, markedly increasing RANKL expression via its TNF type 1 receptor (TNFr1) previously shown to promote osteoclastogenesis (261;262). Following TNF-α and RANKL binding with their respective receptors, the transcription factor NF-κB enters the nucleus and activates genes coding for the mature osteoclasts including tartrate-resistant acid phosphatase (TRAP), and the receptors for calcitonin and vitronectin (263-266). NF-κB functions are required for osteoclast/macrophage development as evidenced in mice lacking the p50 and p52 subunits of NF-κB who fail to generate mature osteoclasts, leading to severe osteopetrosis or elevated BMD (266).

TNF-α has also been shown to impair the differentiation and function of osteoblasts (252). One mechanism by which TNF-α impairs bone formation is through the inhibition of osteoblast differentiation by suppression of Runx2 (267). Runx2 is a critical transcription factor in the regulation of MSC toward an osteoblast lineage (136;268) and required for the expression of alkaline phosphatase (138;139;170;269;270), an important enzyme involved in mineral deposition (271;272). Mice lacking tissue non-specific alkaline phosphatase have impaired mineralization

(271). One of the hallmarks of bone formation is the increased expression of bone alkaline phosphatase (BAP) and bone specific isoforms can be measured in the serum (273).

A second mechanism by which TNF-α affects osteoblasts is by inducing resistance to 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) the biologically active form of vitamin D. 1,25-(OH)2D3 is responsible for maintaining serum calcium and phosphorus in adequate concentrations to allow mineralization of the bone matrix (274). 1,25-(OH)2D3 also promotes differentiation of osteoblasts (275) and stimulates osteoblast expression of bone-specific alkaline phosphatase (121;276-278). TNF-α, however, has been shown to decrease the number of vitamin D receptors (VDR) post-transcriptionally and also 1,25(OH)2D3-stimulated receptor transactivation in osteoblasts (279;280). VDR's are required for normal 1,25(OH)2D3 function and vitamin D deficiencies and mutations in VDR lead to osteomalacia characterized by insufficient mineralization (281). Clearly, a greater understanding of the relationship between bone and fat at a molecular and cellular level would generate a better understanding of such processes as adipogenesis, lipid metabolism, and glucose homeostasis.

Type 2 Diabetes and Bone Integrity

Although type 2 diabetes is often accompanied by normal or even high BMD, diabetes is associated with long-term increased risk of fracture (7;8). Until recently, diabetes was not generally considered a risk factor for fracture (282), and the studies designed to evaluate this association often produced conflicting results (8;29;283). In the Rotterdam Study (29), evidence for an association between type 2 diabetes and elevated bone density was found at the proximal femur and lumbar spine in both men and women. A lower frequency of fractures in women with type 2 diabetes was also reported, however, no consideration was given to duration of diabetes. Heath et al.(283) reported an elevated risk of ankle fractures among women with type 2 diabetes

but did not find a higher risk for other fracture sites. These results were not adjusted for body size or BMD.

To further examine the relationship between type 2 diabetes and risk of fractures among older women, Schwartz et al. (8) analyzed prospective data from the Study of Osteoporotic Fractures (SOF). In this cohort of 9,704 non-black women, aged 65 years and older, it was found that despite the elevated BMD in diabetic women, their risk of all non-spine fractures was increased above non-diabetic women. The increase in fracture risk has been reported to occur at approximately 12–14 years post diagnosis of diabetes (284). The apparent effects of prolonged diabetes were also seen in the Iowa Women's Health Study where diabetics 13-40 years post diagnosis displayed a much higher risk for hip fracture (RR 2.30, 95% CI 1.39-3.81) than non-diabetics (25). Similarly, increased fracture risk was also reported in the Rochester Epidemiology Project (285). In this study, relative risk estimates for overall fracture risk in type 2 diabetics were greater in the follow-up period beyond 10 years than in the first decade. For hip fractures specifically, the relative risks were 0.8 (95% CI, 0.6–1.1) for early follow-up, and 1.5 (95% CI, 1.1–2.0) for late follow-up. Among women, the estimated relative risk for hip fracture in late follow-up was 1.5 (95% CI, 1.04–2.1), whereas it was not increased in the first decade after the diagnosis of diabetes (SIR, 0.7; 95% CI, 0.5–1.03). A limitation of the previous studies is the omission of biochemical markers of bone turnover providing evidence of the metabolic changes in bone that contribute to the increased fracture risk.

The factors that contribute to greater risk of fractures reported in patients with advanced diabetes is uncertain, but may be due to prolonged exposure to hyperglycemia, the pro-inflammatory state, as well as detrimental effects of advanced glycation end products (AGE's) on bone. Whether it is a single factor or a combination of several factor, a disconnect seems to occur between fracture risk and BMD in this patient population.

Factors Affecting Bone Strength

Structural and Material Properties of Bone

Contributing factors that determine bone strength are not only the quantity of mineralized tissue, but also the quality of the bone (40). Subsequently, bone quality is determined by biomechanical properties and describes a relationship between forces (loads) applied to bone and the resulting deformation. It is important to further delineate biomechanical behaviors as material or structural. The former is described independent of its geometry and shape and reflects the intrinsic property of the matrix (i.e., mineral and protein matrix), whereas the whole-bone structural behavior is determined by different types of loads (e.g., bending or torsion). The outcomes of these two measures are influenced by both material properties and geometric distribution of tissue.

Advanced Glycation End Products and Bone Strength

Several studies have suggested that some of the variation in bone quality may occur within the material properties of the collagenous protein matrix (40;286-290). More specifically, accumulation of AGE's in bone collagen matrix has been linked to skeletal fragility (288;291;292). Type I collagen fibers, the basic building block of the bone protein matrix network, are packed together to form collagen fibrils, arranged in a three-dimensional concentric weave in bone (293;294). Their mechanical strength depends on a highly regulated mechanism of intermolecular cross-linking that improves bone's toughness or capacity to absorb energy (288). These collagen cross-links can be formed enzymatically (295-299) and by glycosylation or oxidation induce the formation of AGE's (291;300).

The mechanical effects of a collagen defect are most evident in diseases such as osteogenesis imperfecta (OI), where mutations in the type I procollagen gene leads to a marked increase in risk of fracture.

In contrast, the accumulation of excessive AGE cross-linking within collagen fibers is thought to deteriorate the mechanical function of bone (301). *In vitro* the cross-links are mediated by nonenzymatic glycation of bone collagen which is highly correlated with the stiffness of the organic matrix of bone (291). In turn, the increased stiffness of the organic matrix has been shown to reduce measures of collagen deformation and microcracking (291). Bone derives its resistance to fracture from collagen deformation (302) and from its ability to form microcracks during crack propagation (303). Collagen deformation and microcracking are the primary mechanisms of toughening in bone (304). Therefore, it is likely that *in vivo* accumulation of nonenzymatic glycation cross-links in collagen (305;306) may explain the loss of bone toughness based on a stiffer collagen network and loss of the collagen and micro-crack-based toughening mechanisms.

The formation and accumulation of AGE's occurs with aging at a constant but slow rate (307-309). This process is remarkably accelerated in type 2 diabetes due to the increased availability of glucose (310). The AGE, pentosidine, has been shown to accumulate within collagen fibers in senescence as a result of glycation and oxidation. The result of pentosidine accumulation is decreased mechanical properties of bone, in response to the reduction of collagen fiber elasticity (287;288;296). This effect was demonstrated by Saito et al. (311) in animals when comparing the bone content of pentosidine in non-diabetic Wistar rats to the spontaneously diabetic WBN/Kob rats. The Wistar rats served as age-matched controls and exhibited gradually increasing pentosidine accumulation in the bone with age, while the WBN/Kob rats increased pentosidine only after the onset of diabetes. Three-point bending test demonstrated the WBN/Kob rats experienced further reduction of cortical bone mechanical properties when compared to the subclinical diabetic stage. The compromise in bone biomechanical properties in the WBN/Kob rats occurred with no significant decrement in BMD (311). A similar decrease in bone biomechanical properties was reported in a study by Verhaeghe et al. (312) where diabetic

animals experienced a decrease in bone strength and toughness, and increased stiffness compared to the control cohort. Importantly, these changes in biomechanical properties were observed without a significant decrease in BMD or bone mineral content (BMC) (312).

Assessment of Bone Strength in Humans

Results of mechanical tests in humans are limited due to the invasive nature of some procedures, but promising techniques including microindentation and nanoindentation are under development. Although outcomes remain to be validated, the former represents a first step toward *in vivo* characterization of tissue material properties. Other advantages include the relative ease of testing and the ability to make measurements in multiple locations within the tissue. A disadvantage of this technique is that its sole outcome is the tissue hardness (310). Nanoindentation advantages include the capability to measure the material properties of microstructural features such as lamellae (314;315) and to detect localized changes in bone material properties induced by disease or drug treatment (316).

Continued advances in mechanical assessment will only bolster a growing scientific interest in bone health. Furthermore, accumulating evidence highlights the need for a closer examination of the deleterious effects of diminishing bone quality to better understand the compromise in bone strength associated with type 2 diabetes.

Type 2 Diabetes and Bone Metabolism

Although patients with type 2 diabetes do not necessarily show a reduction in BMD, fracture risks are known to increase compared to their non-diabetic counterparts (27;30;317). The increase however occurs with duration and has shown to present approximately 10 years post-diagnosis (7;8;284;285). Though the exact mechanisms responsible for this alteration in metabolism remain in question, there are many factors that, over time, contribute to the uncoupling of bone formation and resorption and consequently to the pathogenesis of fractures. Among these factors are the

effects of chronic inflammation (5) and AGE accumulation on osteoclasts and osteoblasts, the result of which lead to poor bone quality and impaired micro and macroarchitecture (318). In addition, insulin has been reported to exert an effect on bone metabolism (319).

As previously mentioned, the diabetes-induced proinflammatory mediators TNF-α and IL-6 effect bone metabolism. One effect of IL-6 is through stimulation of osteoclast-induced bone resorption (228). An indirect effect utilizes the RANK/RANKL/OPG interaction on osteoclasts which promotes activation and subsequent bone resorption. The interaction is thought to be between IL-6 and osteoblasts, and results in an increased osteoblastic RANKL production (231). The influence of TNF-α is demonstrated through increased osteoclastogenesis, decreased osteoblastogenesis and induction of vitamin D resistance (251). These effects are potent stimuli for bone loss that ultimately lead to deterioration of bone microarchitecture and increased fracture risk (253-257).

The accumulation of AGE's in diabetic conditions is a key mechanism involved in the induction of oxidative stress via reactive oxygen species (ROS) (310). AGE's bind to AGE receptor (RAGE) which results in the generation of intracellular ROS through a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The result of the increased ROS production on bone is seen in the inhibition of osteoblastic differentiation, increased osteoblast apoptosis and enhanced osteoclast activity (320). Mody et al. (321) demonstrated the influence of oxidative stress on osteoblast differentiation and activity using xanthine/xanthine oxidase (XXO) or hydrogen peroxide (H_2O_2) in a pre-osteoblast cell line (i.e. MC3T3-E1) and a bone marrow stromal cell line (i.e. M2-10B4). The pro-oxidants inhibited differentiation of osteoblasts, as assessed by their effect on alkaline phosphatase, a marker of osteoblast activity, reduced mineralization (321) and induced osteoblast DNA damage and apoptosis via activation of caspase-3 (322;323).

Results presented by Cortizo et al. (324) indicated that exposure of osteoblast-like cells to AGE modified proteins that regulate the expression of RAGE. Although RAGE was found to be expressed in all stages of osteoblastic development, it was only observed to regulate activation of extracellular signal-regulated kinases (ERK) at the later stages of development, when osteoblasts had matured. When mature MC3T3E1 osteoblasts were cultured 1–3 weeks in continuous contact with AGE-collagen to assimilate a chronic AGE's/RAGE interaction, the AGE's decreased osteoblastic ERK activation which was associated with a decrease in both cell survival and calcified nodule formation (325).

In addition to the effects on the osteoblast, oxidative stress has also been associated with enhanced bone resorption (326). Jagger et al. (327) recently reported that ROS may not increase *in vivo* bone resorption directly but rather indirectly by stimulating TNF-α expression. They suggest that ROS might augment osteoclast formation by directly acting on the intracellular signaling systems responsible for increased osteoclast formation.

Results of *in vitro* insulin analysis demonstrate an effect on bone metabolism as well. Reports of both decreased resorptive activity of osteoclasts and inhibition of osteoblast apoptosis in the presence of insulin have been documented (328-330). Insulin action on bone may involve direct signaling through the insulin receptor, activation of bone anabolic IGF-1 signaling by binding to IGF-1 receptor, or synergistic effects with other anabolic agents such as parathyroid hormone (PTH) (330;331). The role of insulin on bone metabolism is important due to the state of hyperinsulinemia experienced in the early onset of type 2 diabetes (332-334).

Native Americans and Osteoporosis Risk

To date, the majority of the osteoporosis-related research has focused on Caucasian populations with limited information available on other high risk ethnic groups such as Native Americans (335) and Canadian Aboriginals (336). The First Nations Bone Health Study (336) was performed

in Canada to determine whether racial differences in body composition affect differences in BMD between Canadian White and Aboriginal women. Differences in BMD were not found between the two groups which left the question regarding the high fracture risk reported in Aboriginal women unanswered. The authors of the study indicated that there were several limitations, including a relatively small cohort and the study was underpowered for conducting age-specific analyses. Two large scale studies with relatively small Native American representation are the National Osteoporosis Risk Assessment (NORA) Study (34) and the Women's Health Initiative (WHI) Study (35). The purpose of NORA was to describe the relationship of BMD and 1-year fracture risk in a cohort of postmenopausal women of varied racial background. Findings from this study indicated that Native American's risk for osteoporosis is at least as great as their Caucasian counterparts. The data also revealed a self-reported fracture history of 15.3% among Native Americans, the highest group percentage in the cohort (34). The WHI Study was designed to compare BMD and determine differences between postmenopausal Native American and white women. Wampler and colleagues (35) found that mean total hip, spine, and whole body BMD's of Native American and white women were similar after controlling for age and BMI.

Limited information is available relative to the prevalence of osteoporosis, rate of bone loss and the influence of lifestyle factors in this population. Several risk factors, characteristic of Native Americans, contribute to a possible explanation as to why they may be considered at high risk for osteoporosis or increased fracture. Obesity is a significant health issue among Native Americans who have a 1.6 times greater likelihood of obesity than Non-Hispanic whites. According to the Centers for Disease Control (CDC) in 2007, the age-adjusted percentage of Native American persons 18 years of age and over who were obese (i.e. BMI of $\geq$ 30) was 33.2% compared to 24.8% of whites (337), and the prevalence of obesity continues to increase (338). This observation was supported by data from the WHI Study where obesity (BMI $\geq$ 30 kg/m^2) was twice as prevalent in Native American women (50%) than in non-Hispanic white women (25%)

(35). Another contributing factor in the Native American community affecting bone health is low serum vitamin D (33). An individual's vitamin D status depends mostly on exposure to sunlight and to a lesser extent on dietary intake of vitamin D. The synthesis of vitamin D by skin exposed to sunlight varies considerably due to factors such as skin pigmentation (339). Darker skin pigmentation results in lower cutaneous synthesis of cholecalciferol (339). This may contribute to findings by Perry et al. (33) of a decreasing serum 1,25-(OH)2D with increasing age in Native American women (33). A limited intake of dairy products, secondary to lactose malabsorption, may also exacerbate decreased serum vitamin D. Although the vitamin does not naturally occur in dairy products, it is a primary dietary food source due to its fortification. A study by Johnson et al. (340) demonstrated a 93% prevalence rate of lactose malabsorption among Native Americans (340). Vitamin D insufficiency has also been associated with diabetes (341). This becomes an important factor when considering Native Americans are a high-risk population for type 2 diabetes (9).

Statistics regarding Native American's risk for type 2 diabetes are alarming. Data from the 2005 IHS user database report 16.5% of the total adult population served by IHS had diagnosed diabetes (9). This is compared to 6.6% prevalence in non-Hispanic whites 20 years and older according to 2004–2006 national survey data (141).

Given the prevalence of type 2 diabetes (9), obesity (337) and decreased vitamin D status (33) in the Native American population, there is sufficient evidence in support of a thorough examination of these factor's and their metabolic impact on bone loss. The limited information available in this population imposes a daunting task to obtain basic knowledge of BMD, bone metabolism and inflammation for a better understanding of their association to bone health.

CHAPTER III

METHODOLOGY

Research Design

This study was a longitudinal, causal-comparison design examining the extent to which type 2 diabetes alters bone density and metabolism in Native American women. Outcome measures were obtained at two time points (baseline and at one year follow-up or final visit) to examine changes occurring in bone formation, bone resorption and BMD between type 2 diabetics and non-diabetics that may have resulted from inflammatory processes. This subset of a larger, two year study to examine the incidence of osteoporosis in Native Americans, included those participants completing the first two visits (n=123). Only Native American women, defined by their eligibility to receive services at an Indian Health Clinic, who were 50 years of age or older and committed to all study visits**,** were included. Excluded from the sample due to the weight limitations of the DXA instrumentation were those whose body weight was 300 pounds or greater. Pregnant women, as determined by urine pregnancy test, were also excluded due to risk of radiation exposure to the embryo/fetus.

Participant recruitment involved a collaborative effort between the General Clinical Research Center's (GCRC) Special Populations Core and area Indian Health Clinic's medical treatment teams including physicians, nursing and auxiliary staff. The IHS clinics were provided information regarding the research study and were requested to inform eligible patients about

the opportunity. In addition, the clinics agreed to post research flyers and assist interested participants in contacting the GCRC. Potential participants contacted the GCRC to schedule an appointment and receive initial instruction regarding participation. Follow-up visits were scheduled via telephone calls and email if requested by the participant. Follow-up visits were analogous to baseline and procedures performed at baseline (i.e. DXA scan, relevant medical history and anthropometric measures) were repeated. Serum samples collected at each visit were processed and stored for batch measures of indicators of bone metabolism (e.g. bone specific alkaline phosphatase and C-telopeptide), 25-hydroxy vitamin D3, and inflammatory mediators (e.g. TNF-α and IL-6).

All participants were encouraged to return to their respective clinics to discuss the results of their screening with their primary care physician.

The study was approved by the Institutional Review Board (IRB) of the University of Oklahoma Health Sciences Center, IRB#13281 in reciprocal agreement with the Oklahoma State University IRB #HE0840 and Indian Health Services IRB #P-07-03-OK.

Data collection

Upon arrival, informed consent was obtained and participants were asked about their tribal heritage and categorized by blood quantum (bq): bq1 represents <50%, bq2 represents 50-100% (where bq=100% is a full blood Native American). Relevant medical history and anthropometric measures were collected by the nursing staff. In consideration of cultural diversity, the blood draw was designated optional for participation and required specific selection on the subject consent.

Medical History and Supplement Use: Information obtained included medical history and medication and supplement use. The same form was used at the final visit to identify changes that may have occurred over time.

Anthropometric Measurements: Height and weight were measured at both visits. The protocol for assessing anthropometric measurements was adopted from the NHANES III survey. Waist and hip circumference were also measured to evaluate waist-to-hip ratio.

DXA Evaluation of Bone Density and Body Composition: The DXA measurements of each subject included the lumbar spine (L1-L4), hip, forearm, and whole body scans using a HOLOGIC 4500QDR/Delphi instrument (HOLOGIC, Inc., Waltham, MA). Available software calculates BMD (gm/cm^2) by dividing BMC (in grams of calcium hydroxyapatite) by the area (cm^2) of interest. Specific criteria for positioning subjects and for scan analysis were followed according to guidelines set forth by HOLOGIC. Instrument calibration, maintenance, and quality control were strictly maintained. All DXA scans were performed by the same certified bone densitometrist.

Serum Biomarkers and Complete Blood Count (CBC): Participants were presented the option of providing a blood specimen for serum analyses of inflammatory mediators, indices of bone metabolism, 25 (OH) vitamin D_3, as well as an aliquot of whole blood for a CBC. Venous blood was collected at baseline and follow-up visits. Serum was separated within two hours of collection and aliquots stored at -80°C until the time of analysis.

Serum bone-specific alkaline phosphatase (BAP) was measured as an indicator of bone formation using commercially available enzyme immunoassay (EIA) kits (MicroVue, Quidel Corporation, San Diego, CA). Intra-assay coefficients of variation (CV) were 5.8%, 3.9%, and 5.2% at low, medium, and high concentrations, respectively. Inter-assay CV's were 5.2%, 7.6%, and 5.0% at low, medium, and high concentrations, respectively.

Serum CTX was measured as an indicator of bone resorption using a commercially available ELISA assay (Immunodiagnostic Systems Inc. UK). Intra-assay CV's were 3.0%, 1.7%, and

1.8% at low, medium, and high concentrations, respectively. Inter-assay CV's were 10.9%, 9.7%, and 2.5% at low, medium, and high concentrations, respectively.

Alterations in vitamin D status were assessed by measuring serum $25(OH)D_3$ using commercially available EIA kits (Immunodiagnostic Systems Inc. UK). The intra-assay CV's were 5.3%, 5.6%, and 6.7% at low, medium, and high concentrations, respectively. Inter-assay CV's were 4.6%, 6.4%, and 8.7% at low, medium, and high concentrations, respectively.

To examine the effects of inflammatory mediators, serum TNF-α and IL-6 were assessed. Serum IL-6 was measured using an ultra-sensitive sandwich-type ELISA assay (R&D Systems, Minneapolis, MN). The intra-assay CV's were 6.9%, 7.8%, and 7.4% at low, medium, and high concentrations, respectively. Inter-assay CV's were 9.6%, 7.2%, and 6.5% at low, medium, and high concentrations, respectively.

Serum TNF- α was measured by an ultra-sensitive ELISA sandwich assay (R&D Systems, Minneapolis, MN). Intra-assay CV's were 8.5%, 4.3%, and 3.1% at low, medium, and high concentrations, respectively. Inter-assay CV's were 10.6%, 7.3%, and 7.4% at low, medium, and high concentrations, respectively.

A CBC, including total white cell counts and differentials were performed on fresh, whole blood samples to investigate the relationships between total and differential white cell populations, osteoporosis risk and inflammation.

Data analysis

The data were analyzed using SAS version 9.2 (SAS Institute, Inc., Cary, NC). Descriptive statistics were calculated for all variables in individuals with and without type 2 diabetes, stratified by duration of diabetes diagnosis (<10 or ≥ 10 years) and included means and standard errors. A gamma statistic was used to determine frequency of osteopenia and osteoporosis at the

hip, spine and forearm sites in the population. Analysis of variance (ANOVA) was performed to detect differences between groups at baseline and final time points and change in measures of BMD, bone resorption and formation biomarkers, inflammatory markers and vitamin D status, followed by pair-wise comparisons. Primary outcome variables of interest were change in BMD observed from baseline, in the lumbar spine, hip and forearm. The secondary or explanatory outcome variables were biochemical markers of bone formation and bone resorption (i.e. BAP and CTX), inflammatory mediators (i.e. TNF-α and IL-6) and vitamin D status. Covariates including age and blood quantum were considered. Pearson correlation analyses were performed among the dependent and independent variables (e.g. vitamin D status vs. change in BMD). For all analyses, alpha was set at 0.05 for statistical significance.

CHAPTER IV

FINDINGS

Description of the Study Population

In this longitudinal study, 123 Native American women completed a baseline and final visit. Baseline characteristics of participants without a previous diagnosis of type 2 diabetes (non DM) and those with a previous diagnosis of type 2 diabetes mellitus (all DM) can be found in **Table 1.** Additionally, the all DM group is shown when stratified by years post diagnosis as either < 10 years (DM< 10) or ≥ 10 years (DM≥ 10) due to the potential for increased risk of fracture with longer diabetes duration. Mean participant age for the non DM and the all DM groups was 61.02 ± 1.02 and 63.01 ± 1.06 years, respectively and did not differ from one another. A comparison of years post menopause between groups also found no differences. When the diabetics were stratified by duration of diabetes, there remained no differences between groups in mean age, an unexpected outcome, as longevity of diagnosis may assume an advanced age. Not surprising however, were the anthropometric measures that were significantly higher in the all DM group, including weight, BMI and waist/hip ratio (WHR). Although these measures were not unexpected, they are relevant to this study due to the effects of weight-bearing on bone and chronic inflammation associated with obesity, especially increased central adiposity. The BMI results demonstrate that though the non DM group was categorically overweight (i.e., 25-29.9), the BMI of the all DM group was still higher and considered clinically obese (i.e., ≥ 30). Not only

Table 1. Baseline Descriptive Characteristics of Groups According to Diabetes Mellitus (DM) Status.

	Non DM[1] (n=79)	All DM[2a] (n=44)	DM<10[3] (n=19)	DM≥10[4] (n=21)	*P* (1vs2)	*P* (1vs3vs4)
Age (years)	61.02±1.02	63.01±1.06	63.2±1.63	61.8±1.47	0.210	0.601
Years Post Menopause	14.2±1.30	15.5±2.0	16.2±2.3	14.1±3.6	0.560	0.788
Smoking[b] (%)	23±5	26±7	39±12	15±8	0.679	0.212
Packs/day	0.60±0.12	0.59±0.12	0.54±0.13	0.57±0.43	0.976	0.962
Anthropometrics						
Height (cm)	163±0.67	160.7±0.92	160±1.52	161.8±1.33	0.051	0.160
Weight (kg)	74.9±1.70	88.8±2.22	86.6±3.88	91.7±2.68	<0.001	<0.001*†
Body Mass Index	28.2±0.62	34.5±0.90	33.9±1.6	35.1±1.1	<0.001	<0.001*†
Waist/hip ratio	0.87±0.01	0.91±0.01	0.91±0.01	0.92±0.02	0.002	0.003*†
Trunk fat (kg)	15.8±0.68	20.9±0.95	20.6±1.71	21.3±1.11	<0.001	<0.001*†
Body fat (kg)	33.2±1.09	35.1±1.82	35.0±3.26	35.0±2.34	0.333	0.675
Body lean (kg)	44.1±0.71	44.8±0.99	44.1±1.57	45.2±1.45	0.567	0.786
Daily Calcium intake						
From food (mg)	974±63	811±56	902±84	755±80	0.082	0.195
From supplements (mg)	450±83	410±101	630±206	227±81	0.762	0.152
Total (mg)	1266±101	1126±109	1392±198	939±121	0.372	0.176
Blood Quantum by DM Category[c]	35±0.05	64±0.07	53±0.12	76±0.10	0.002	0.003†
1= <50%	n=52	n=16	n=9	n=5		
2= ≥50%	n=27	n=28	n=10	n=16		

Data presented as mean ± SE unless otherwise noted.

Non diabetic = non DM, all diabetic group = all DM, DM<10 = diagnosed diabetes <10 years, DM ≥10 = diagnosed diabetes ≥10 years

[a] Diabetes duration unknown in 4 participants.

[b] Data presented as percent use ± SE.

[c] Data presented as percent of participants categorized as BQ 2 (≥50%), ± SE, within each DM category.

* Denotes statistically significant differences (ANOVA, $p<0.05$) between non DM and DM<10

† Denotes statistically significant differences (ANOVA, $p<0.05$) between non DM and DM≥10

were there differences in weight and BMI between the all diabetic and non-diabetic groups, but the distribution of weight differed as well. This was demonstrated by the WHR which showed the increase in the deposition of adipose tissue in the abdominal region compared to the hips. This was further supported by a significantly greater amount of trunk fat in the all DM group when compared to the non DM. Also expected, these anthropometric differences remained following the stratification of the all DM group by diabetes duration.

Apart from anthropometric measurements, few baseline differences existed between groups despite comparisons of mean calcium intake and smoking predilection. One notable difference however, was blood quantum (BQ). That is to say, a greater number of individuals with a higher percent BQ were in the all DM group compared to the non DM group with 28 of the 44 participants (64%) at BQ $\geq$50% (p=0.002). When the all DM group was stratified by duration, the difference remained and 16 of the 21 participants (76%) in the DM$\geq$10 were BQ $\geq$50%.

Results of a self-reported medical history questionnaire demonstrated few significant differences between non DM and all DM groups **(Table 2)**. This included medications such as bisphosphonates and hormone replacement therapies which can have beneficial effects on bone as well as fracture history and previous osteoporosis diagnosis. Although no significant differences existed between the non DM and all DM groups in terms of fracture history report, when the all DM group was stratified by diabetes duration, the self-report of vertebral fracture incidence was significantly higher in the DM<10 than either the non DM or DM$\geq$10 groups (p=0.021). As anticipated, thiazolidinedione (TZD) use was significantly higher in the all DM group than the non DM group (2% vs11% p=0.039) and this medication has been shown to have a deleterious effect on bone. Comparisons of the frequency of osteoporosis between groups based on WHO T-score classifications the hip, spine and forearm, demonstrate that there was no significant difference in the prevalence of osteoporosis due to diabetes or duration of diabetes at any of the sites **(Table 3).** These data do suggest a discrepancy between self-reported osteoporosis and

Table 2. Self-Reported Osteoporosis Diagnosis, Fracture History and Medication Use of Groups by DM Status at Baseline.

	Non DM[1] (n=79)	All DM[2a] (n=44)	DM<10[3] (n=19)	DM≥10[4] (n=21)	*P* (1vs2)	*P* (1vs3vs4)
Osteoporosis Diagnosis[b]	11±0.04	16±0.06	11±0.07	14±0.08	0.479	0.922
Fracture History[b]						
Vertebral fracture	3±2	7±4	16±9	0	0.259	0.021*‡
Hip fracture	3±2	0	0	0	0.291	0.604
Medication Use[c]						
Bisphosphonate	8±0.03	5±0.04	12±0.08	0	0.654	0.393
Hormone Replacement Therapy	15±0.04	13±0.06	12±0.08	18±0.10	0.783	0.893
Thyroid hormone	26±0.05	32±0.08	24±0.11	35±0.12	0.528	0.695
Selective Estrogen Receptor (SERMs)	3±0.02	0	0	0	0.283	0.599
Thiazolidinedione's (TZDs)	2±0.02	11±0.05	6±0.06	18±0.10	0.039	0.024†
Selective Serotonin Reuptake Inhibitors (SSRIs)	26±0.05	21±0.07	18±0.10	24±0.11	0.593	0.788

Non diabetic = non DM[1], all diabetic group = all DM[2], diagnosed diabetes <10 years = DM<10[3], diagnosed diabetes ≥10 years = DM ≥10[4]

[a] Diabetes duration unknown in 4 participants.

[b] Data presented as percent occurrence, ± SE.

[c] Data presented as percent use, ± SE.

* Denotes statistically significant differences ($p<0.05$) between non DM and DM<10

† Denotes statistically significant differences ($p<0.05$) between non DM and DM≥10

‡ Denotes statistically significant differences ($p<0.05$) between DM<10 and DM≥10

Table 3. Frequency of Osteopenia and Osteoporosis of the Hip, Spine and Forearm by Diabetes Status.

	Non DM[1] (n=74)			All DM[2] (n=38)			DM<10[3] (n=17)			DM≥10[4] (n=21)			*P* (1vs2)	*P* (1vs3vs4)
Classification by T-score[a]	**N**	**O**	**Op**	**N**	**O**	**Op**	**N**	**O**	**Op**	**N**	**O**	**Op**		
Total Hip	47	24	3	28	9	1	13	3	1	15	6	0	0.554	0.643
Total Spine	48	22	4	25	12	1	11	6	0	14	6	1	0.793	0.896
Total Forearm	24	38	12	14	15	9	7	4	4	7	9	5	0.441	0.750

Non diabetic = non DM[1], all diabetic group = all DM[2], diagnosed diabetes <10 years = DM<10[3], diagnosed diabetes ≥10 years = DM ≥10[4]

[a] Classification by T-score defined by the World Health Organization:
N = normal (> -1 standard deviation below the mean);
O = osteopenia (between -1 and -2.5 standard deviations below the mean);
Op = osteoporosis (< -2.5 standard deviations below the mean)

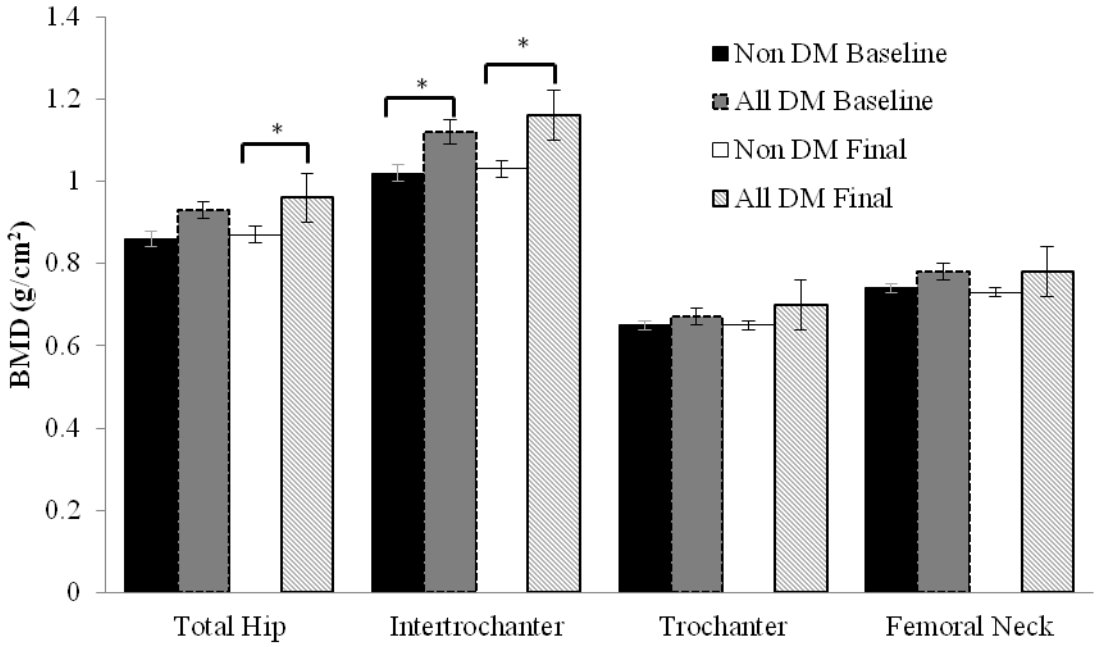

Figure 1. BMD of the total hip, intertrochanter, trochanter and femoral neck regions of the hip. Comparisons were made between non diabetic (Non DM) and all diabetic (All DM) groups at baseline and final visit. Bars indicate mean ± SE and * indicates statistically significant difference ($p<0.05$) between groups.

osteoporosis observed in this study. The frequency of T-scores in the osteoporotic range is greater than identification by the participants, most notably in the forearm.

Bone Densitometry

Results of DXA measurements revealed significant differences in the hip region between the non DM and all DM groups **(Figure 1)**. BMD was significantly higher in the all DM group compared to the non DM group at both time points in the intertrochanter and at the final visit in the total hip. In contrast, no significant differences in BMD were observed in either the lumbar spine or distal forearm **(Figure 2)**. Differences in BMD between groups can often be explained by alterations in BMA and/or BMC, but no significant differences were observed in either of these measures at the intertrochanter or total hip at either time point in this study **(Appendix A)**. This

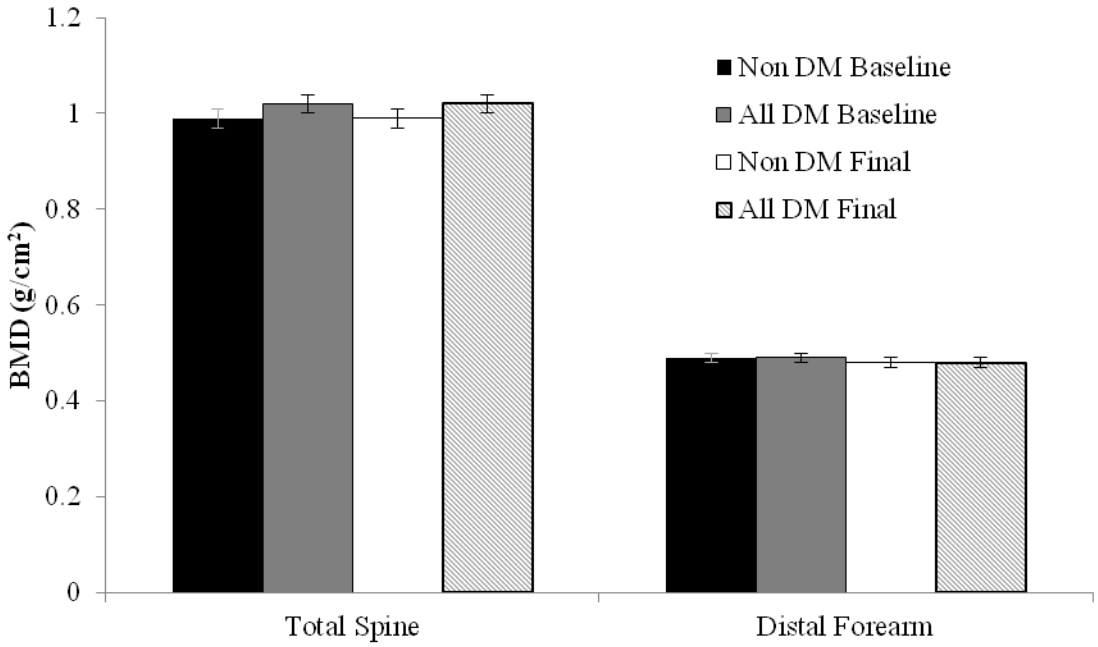

Figure 2. BMD of the total lumbar spine and distal forearm. Comparisons were made between non diabetic (Non DM) and all diabetic (All DM) groups at baseline and final visit. No statistically significant differences ($p<0.05$) were observed between non DM and all DM groups at either time point. Bars indicate mean ± SE.

observation suggests that modest changes in BMA and BMC were likely responsible for the significant change in BMD. To examine the effects of diabetes duration on BMD in the hip, the all DM group was stratified **(Figure 3)**. A significantly higher BMD value was observed in the DM<10 group compared to the non DM and DM ≥10 groups in the final visit at both the intertrochanter and total hip sites. Stratification also revealed a significantly higher BMD in the trochanter region in the DM<10 group compared to the non DM and DM ≥10 groups. No statistically significant differences were noted in either the lumbar spine or forearm with stratification by duration of diabetes diagnosis. When adjustments were made for age and BQ, the BMD differences between groups observed at the hip (i.e., total hip, intertrochanter and trochanter) were not altered, but the increase in femoral neck BMD in the DM < 10 group compared to the non DM and DM ≥ 10 groups did reach a level of statistical significance ($p=0.035$) (*data not shown*).

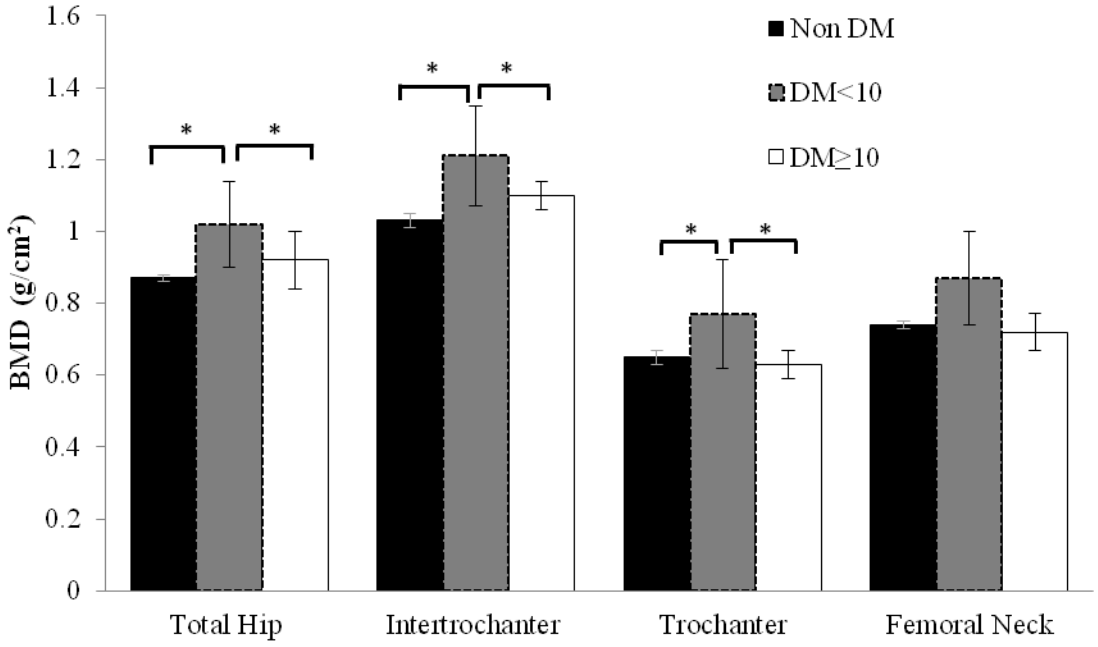

Figure 3. BMD values of total hip, intertrochanter and trochanter at final visit. Comparisons were made between non diabetic (Non DM), diabetic less than 10 years duration from diagnosis (DM<10) and diabetic 10 years or greater post-diagnosis (DM≥10) groups. Bars indicate mean ± SE and statistically significant differences (p<0.05) denoted *.

Based on the usefulness of the WHO classifications of T-scores as an indicator of fracture risk (17), it is important to note that both total hip and intertrochanter T-scores were significantly greater in the all DM group at both time points as was the femoral neck T-score greater in the same group at the final visit. Results of the forearm measures actually showed the mean T-score for all groups (i.e., non DM, all DM, DM<10, and DM≥10 groups) to be in the osteopenic range (i.e., T-score <-1.0) at both baseline and final visits. Results were similar when adjusted for age and BQ as well. Findings in the lumbar spine more closely resembled those of the forearm than the greater weight-bearing sites of the hip in that no significant differences in T-scores existed between any of the groups.

DXA results at each site were also analyzed to assess percent change over time (**Table 4)**. The only statistically significant differences noted were the increased rate of change in the all DM

Table 4. Percent Change in Bone Mineral Area, Content, Density and T-scores from Baseline to Final Visit According to Diabetes Status.

	Non DM[1] (n=79)	All DM[2a] (n=44)	DM<10[3] (n=19)	DM≥10[4] (n=21)	*P* (1vs2)	*P* (1vs3vs4)
Total Hip						
BMA	1.005±<.004	1.011±0.007	1.019±0.012	1.006±0.007	0.239	0.318
BMC	1.012±0.006	1.009±0.010	1.030±0.019	0.994±0.010	0.952	0.259
BMD	1.007±<.004	1.033±0.036	1.088±0.078	0.988±0.007	0.688	0.024*‡
T-score	1.012±0.088	0.952±0.132	0.757±0.151	1.163±0.239	0.640	0.338
Intertrochanter						
BMA	1.015±0.009	1.021±0.014	1.034±0.025	1.017±0.017	0.717	0.686
BMC	1.023±0.010	1.019±0.015	1.047±0.028	1.003±0.016	0.859	0.328
BMD	1.008±0.004	1.018±0.030	1.074±0.062	0.970±0.019	0.669	0.022*‡
T-score	1.070±0.065	0.940±0.159	0.980±0.224	0.906±0.268	0.373	0.676
Total Spine						
BMA	0.999±0.003	1.001±0.006	0.992±0.010	1.008±0.010	0.534	0.150
BMC	0.999±0.005	1.003±0.009	0.995±0.016	1.014±0.012	0.454	0.241
BMD	0.999±0.004	1.003±0.005	1.005±0.009	1.006±0.007	0.488	0.600
T-score	1.023±0.120	0.699±0.136	0.526±0.272	0.815±0.122	0.090	0.146
Forearm						
BMA	1.004±0.002	0.997±0.003	0.994±0.005	1.001±0.004	0.026	0.085
BMC	0.994±0.003	0.989±0.004	0.994±0.007	0.988±0.006	0.162	0.217
BMD	0.990±0.002	0.991±0.003	0.998±0.005	0.987±0.004	0.820	0.157
T-score	0.999±0.124	1.019±0.114	1.020±0.034	0.877±0.219	0.921	0.881

Data are presented as percent change ± SE.

Non diabetic = non DM, all diabetic group = all DM, DM<10 = diagnosed diabetes <10 years, DM ≥10 = diagnosed diabetes ≥10 years

[a] Diabetes duration unknown in 4 participants.

* Denotes statistically significant differences ($p<0.05$) between non DM and DM<10

‡ Denotes statistically significant differences ($p<0.05$) between DM<10 and DM≥10

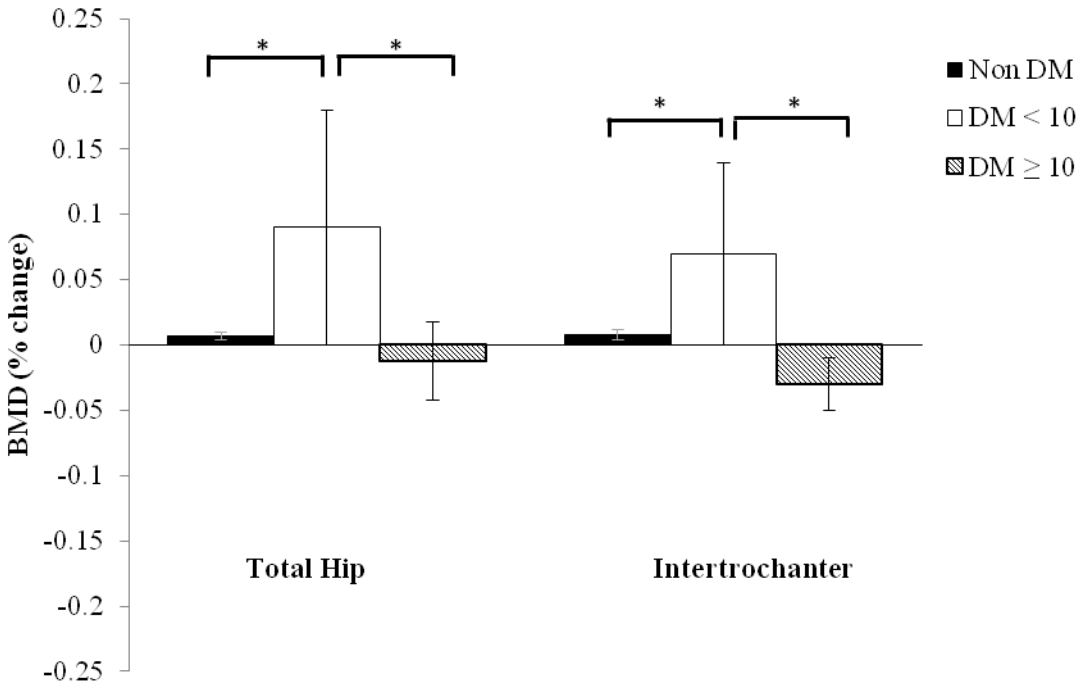

Figure 4. Percent change in BMD of total hip and intertrochanter region of the hip over one year. Comparisons were made between non diabetic (Non DM), diabetic less than 10 years duration from diagnosis (DM<10) and diabetic 10 years or greater post-diagnosis (DM≥10) groups. Bars indicate percent change ± SE and statistically significant differences (p<0.05) denoted between*.

group compared to the non DM group in the BMA of the forearm (p=0.026) and the femoral neck T-score (p=0.033) (*data not shown*). Both of these affects were lost when the all DM group was stratified by diabetes duration. An interesting observation resulting from the stratification was a change in BMD in the total hip and intertrochanter regions **(Figure 4)**. Here the percent change in the DM<10 group was significantly greater than the change observed in the BMD of the non DM and DM ≥10 groups. This result was not seen in the lumbar spine or forearm.

Biochemical Markers of Bone Metabolism

Baseline and final visit comparisons of serum CTX were made to examine differences between diabetics and non-diabetics and the effects of diabetes duration on bone resorption and bone

formation. A significantly lower serum CTX was observed in the all DM group compared to the non DM group at the baseline (p=0.006), but not at the final visit (**Figure 5)**. Following stratification of the all DM group, a significantly lower concentration of CTX in the DM<10 group compared to the non DM group (p=0.031) was observed. Statistical significance was not reached when assessing the percent change in CTX between the non DM and all DM groups (p=0.072) or when the all DM group was stratified by diabetes duration (p=0.295) (*data not shown*).

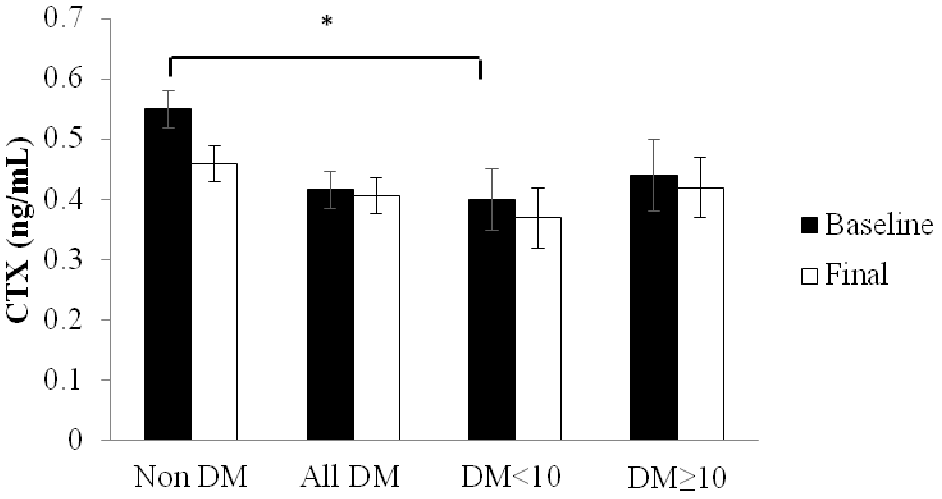

Figure 5. Serum C-telopeptide of type I collagen (CTX) concentrations at baseline and final visits. Comparisons were made between non diabetic (Non DM), all diabetic (All DM), diabetic less than 10 years duration from diagnosis (DM<10) and diabetic 10 years or greater post-diagnosis (DM≥10) groups. Significance ($p < 0.05$) denoted between Non DM and DM<10 by *. Bars indicate mean ± SE.

Serum BAP reflects osteoblast activity, and was therefore used as an indicator of bone formation. Comparisons of serum BAP between diabetics and non-diabetics at baseline and final visits were not different, nor did they differ following stratification of the all DM group by duration of diabetes diagnosis (**Figure 6)**. Additionally, no differences were observed when serum BAP was evaluated based on the percent change over time.

Negative correlations were observed in the all DM group between CTX and forearm BMD at both time points but no significant relationship in percent change between CTX and BAP and BMD at any site was noted. When the all DM group was stratified by diabetes duration, a single

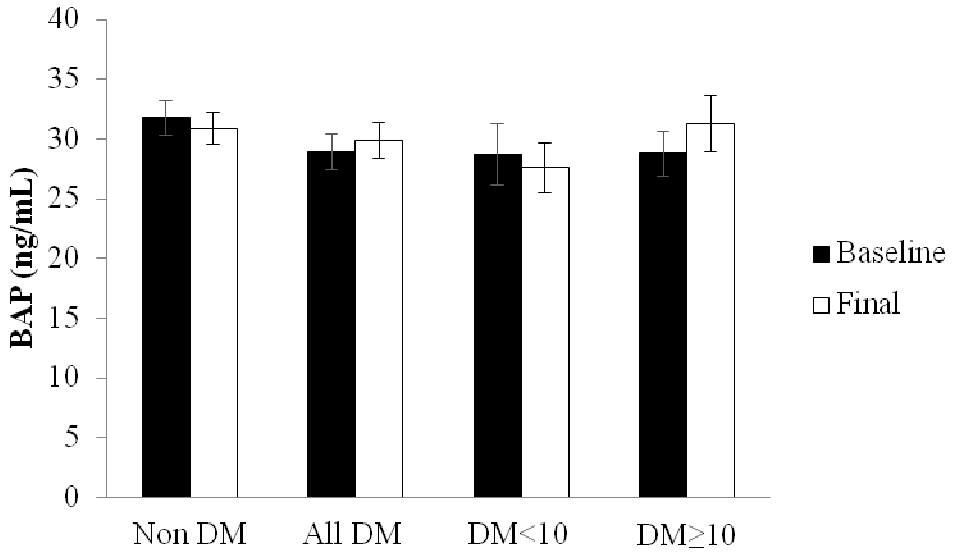

Figure 6. Serum bone alkaline phosphatase (BAP) concentrations at baseline and final visit. Comparisons were made between non diabetic (Non DM), all diabetic (All DM), diabetic less than 10 years duration from diagnosis (DM<10) and diabetic 10 years or greater post-diagnosis (DM≥10) groups. No statistically significant differences ($p<0.05$) were observed between groups at either time point. Bars indicate mean ± SE.

correlation was noted as a negative relationship between CTX and forearm BMD at baseline in the DM <10 group. These data indicate that the only site in which the anticipated negative relationship between bone resorption and BMD was observed was at the least weight-bearing skeletal site.

Cytokines and Complete Blood Count

Results of the serum cytokine TNF-α (**Figure 7**) assessment revealed no significant differences between the all DM and non DM groups, or when the all DM group was stratified by diabetes

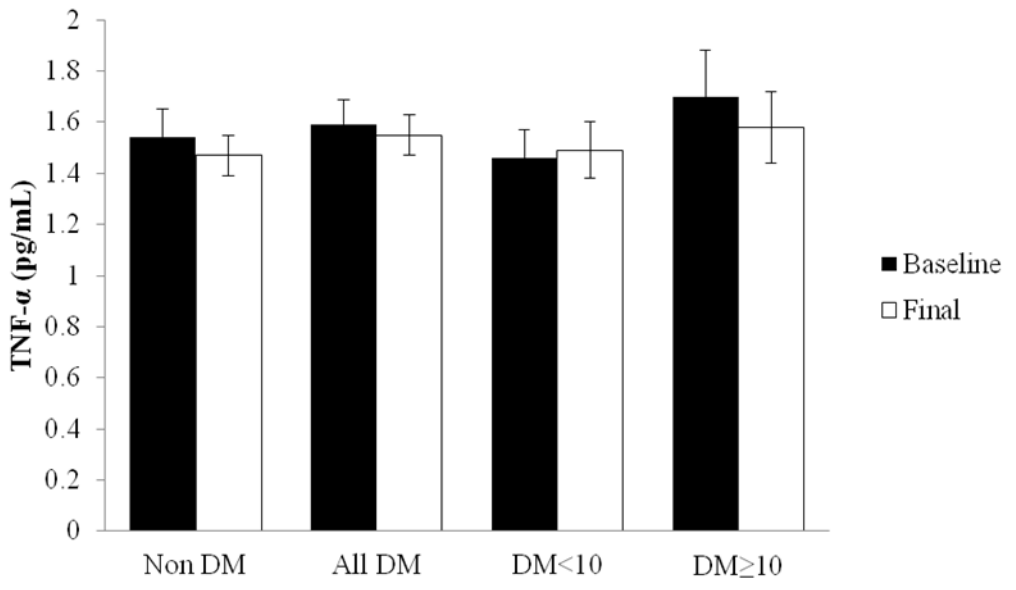

Figure 7. Serum TNF-α concentrations at baseline and final visit for non diabetic (Non DM), all diabetic (All DM), diabetic less than 10 years duration from diagnosis (DM<10) and diabetic 10 years or greater post-diagnosis (DM≥10) groups. No statistically significant differences ($p<0.05$) were observed between groups at either time point. Bars indicate mean ± SE.

duration at either the baseline or final visit. In addition no observable differences in TNF-α and IL-6 were noted between groups when results were assessed for percent change (*data not shown*).When the cytokine IL-6 (**Figure 8**) was assessed, results again revealed no significant differences between the all DM and non DM groups, or when the all DM group was stratified by diabetes duration at either the baseline or final visits.

Lymphocyte percent was significantly lower in the all DM group compared to the non DM group at the final visit, but this effect was lost when the diabetic group was stratified **(Table 5)**. No other differences in lymphocyte absolute counts or percent were observed between groups. Monocyte absolute counts and percent were not different between any groups. It is important to note that all mean lymphocyte and monocyte counts were within normal limits.

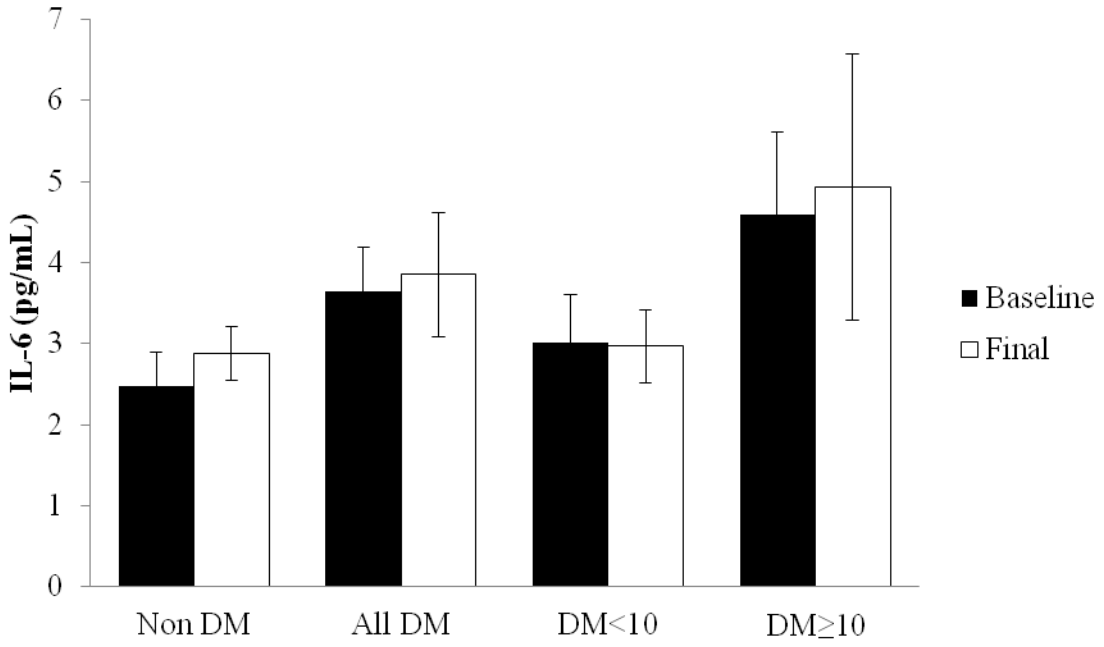

Figure 8. Serum IL-6 concentrations at baseline and final visit for non-diabetic (Non DM), all diabetic (All DM), diabetic less than 10 years duration from diagnosis (DM<10) and diabetic 10 years or greater post-diagnosis (DM≥10) groups. No statistically significant differences (p<0.05) were observed between groups at either time point Bars indicate mean ± SE.

The pro-inflammatory cytokines present in chronic disease such as diabetes have also been associated with a decrease in bone formation and increase in bone resorption that may ultimately lead to bone loss. A positive correlation between TNF-α and CTX (p=0.005) was demonstrated at baseline in the non DM group but not in the all DM group **(Figure 9)**. No correlation between BAP and TNF-α was observed in either the non diabetic or all diabetic groups *(data not shown)*. Positive correlations between BAP and CTX were noted in the non DM, all DM and DM<10 groups at baseline, however, this was not the case for the DM≥10 group *(data not shown)*. Somewhat unexpectedly, no correlations between metabolic markers were identified in the DM≥10 group at either visit.

Table 5. Serum Markers of Inflammation, Lymphocyte and Monocyte Counts at Baseline and Final Visit According to Diabetes Status.

	Non DM[1] (n=79)	All DM[2a] (n= 44)	DM<10[3] (n= 19)	DM≥10[4] (n= 21)	*P* (1vs2)	*P* (1vs3vs4)
Pro-inflammatory Cytokines						
Tumor Necrosis Factor (TNF)-α						
Baseline (pg/mL)	1.54±0.11	1.59±0.10	1.46±0.11	1.7±0.18	0.733	0.619
Final (pg/mL)	1.47±0.08	1.55±0.08	1.49±0.11	1.58±0.14	0.609	0.873
Interleukin (IL)-6						
Baseline (pg/mL)	2.48±0.42	3.64±0.55	3.0±0.59	4.6±1.02	0.102	0.088
Final (pg/mL)	2.88±0.33	3.85±0.77	3.0±0.45	4.9±1.6	0.171	0.096
Blood Count Percent						
LYMPH						
Baseline (%)	30.3±0.92	28.8±1.1	28.2±1.3	30±1.7	0.367	0.537
Final (%)	31.5±1.05	28.6±0.86	27.6±1.09	29.5±1.5	0.041	0.097
MONO %						
Baseline (%)	7.3±0.25	7.0±0.28	7.1±0.49	6.9±0.4	0.523	0.762
Final (%)	7.4±0.26	7.5±0.39	8±0.66	7.1±0.51	0.998	0.486
Blood Count Absolute Counts						
LYMPH #						
Baseline (µL)	1.95±0.07	2.04±0.12	1.9±0.13	2.2±0.22	0.537	0.358
Final (µL)	1.94±0.07	2.0±0.14	1.8±0.11	2.1±0.28	0.696	0.380
MONO #						
Baseline (µL)	0.47±0.02	0.48±0.03	0.44±0.04	0.51±0.05	0.773	0.473
Final (µL)	0.46±0.02	0.49±0.02	0.49±0.03	0.49±0.04	0.236	0.533

Data presented as mean ± SE. Non diabetic = non DM, all diabetic group = all DM, DM<10 = diagnosed diabetes <10 years, DM ≥10 = diagnosed diabetes ≥10 years. [a] Diabetes duration unknown in 4 participants. No statistical differences were found ($p<0.05$).

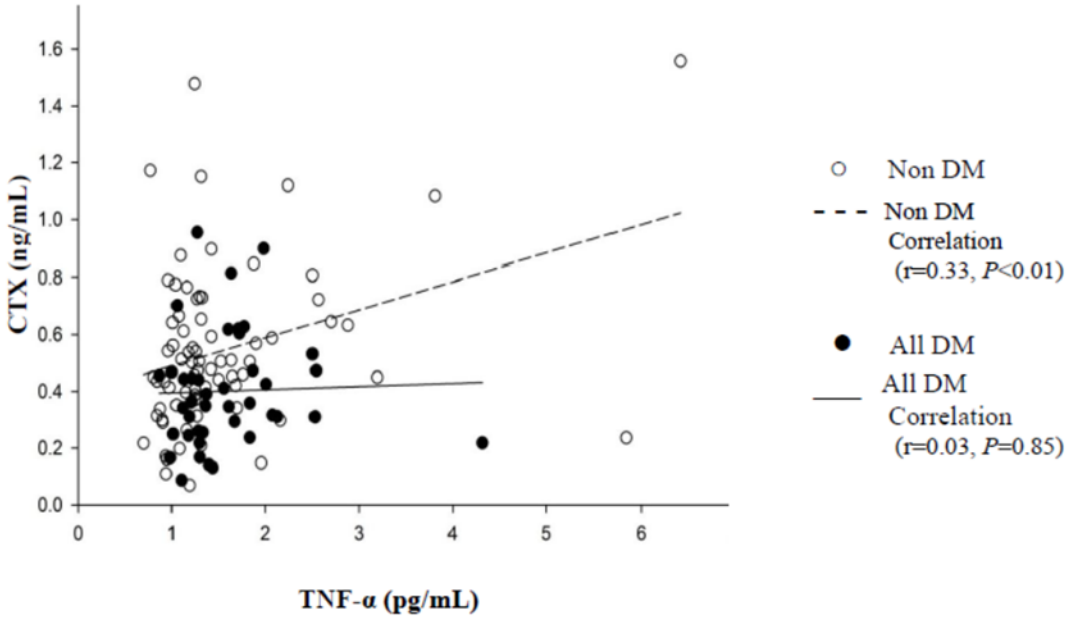

Figure 9. Correlation of CTX to TNF-α in the non diabetic (Non DM) group and the all diabetic (All DM) group at baseline visit. A significant positive correlation was observed in the Non DM group that was not present in the All DM group. Linear correlations were evaluated by Pearson's correlation coefficient.

Vitamin D

Given vitamin D's relationship with calcium homeostasis and bone metabolism, differences in serum 25-hydroxy vitamin D were examined between groups. No differences in serum vitamin D between any of the groups were noted at either time point **(Figure 10)**. Based on the mean value of the non DM group and the DM<10, both groups' vitamin D status exceeded 50 nmol/L, the serum value recommended by the Institute of Medicine (IOM) to sustain bone density, calcium absorption, and to minimize risk of osteomalacia and rickets (342). However, the DM≥10 group in this study did not reach recommended serum 25-hydroxy vitamin D levels at either time point which suggests that the longer duration diabetic may be at greater risk for compromised vitamin

status. Data from the Third National Health and Nutrition Examination Survey (NHANES III) demonstrate the national average serum 25- hydroxyvitamin D in similar age (i.e., 70.6 nmol/L) and diabetes (i.e., 63.4 nmol/L) (343) categories exceed that of the non DM and all DM groups.

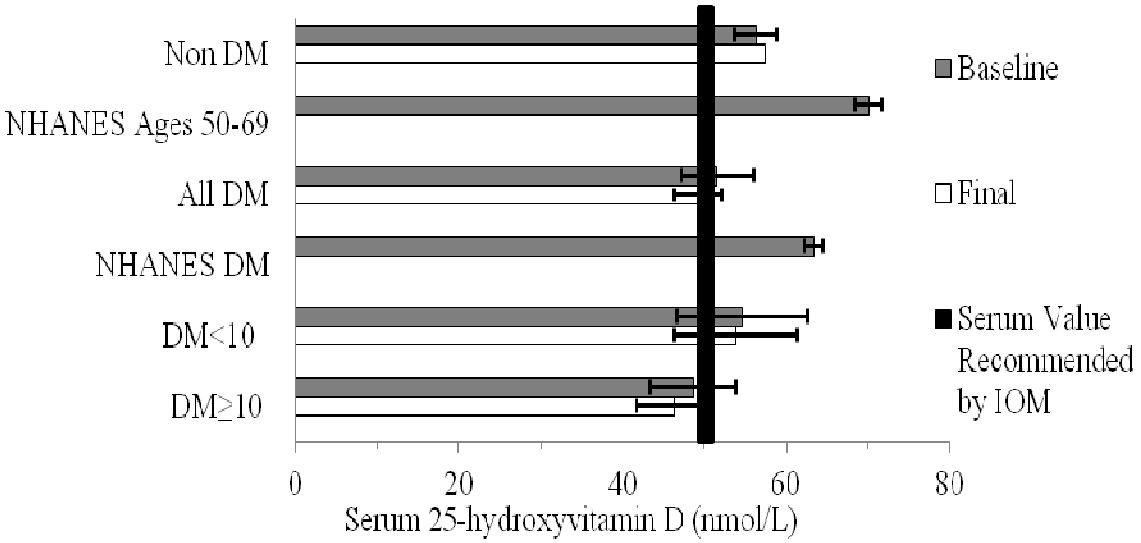

Figure 10. Serum 25-hydroxyvitamin D concentrations of non diabetic (Non DM), all diabetic (All DM), diabetic less than 10 years from diagnosis (DM<10) and diabetic 10 years or greater post-diagnosis (DM≥10) groups at baseline and final visits. For visual comparison, results from the Third National Health and Nutrition Examination Survey (NHANES III) of adults 50-69 years of age from NHANES III (NHANES Ages 50-69) and NHANES III adults diagnosed with diabetes (NHANES DM) are shown. The black vertical bar at 50 nmol/L 25-hydroxy vitamin D indicates Institute of Medicine (IOM) recommended serum levels. Bars indicate mean ± SE.

CHAPTER V

DISCUSSION

This study was designed to examine the effects of type 2 diabetes and diabetes duration on bone health in Native American women. It is important to note that this study was part of a larger study to examine the incidence of osteoporosis in Native American women over the age of 50 in the state of Oklahoma. BMD was the primary outcome variable in this study and represents a means of assessing fracture risk.

The findings of this study demonstrate that BMD differences did occur between diabetics and non-diabetics and more specifically in the hip regions. This effect appears to be biphasic as BMD was increased in the hip region the first ten years following diagnosis and decreased in the same region, after the initial decade. Although greater rate of change in BMD in the DM group was anticipated, it was not expected to continue to increase. This demonstration of both greater BMD and increased rate of change in the DM<10 group suggests that detrimental metabolic changes in bone, specific to the hip regions, occurred at some point after the first decade following diabetes diagnosis. A possible explanation for this biphasic effect could be related to the compensatory hyperinsulinemia accompanying the onset of type 2 diabetes in an effort to lower blood glucose levels (332-334). The ability of insulin to stimulate collagen production by osteoblasts has been

well documented and the effect has been observed at physiological insulin concentrations (133;344-346).

The increased BMD in the DM<10 group however, was characterized by no change in serum BAP and decreased serum CTX compared to the other groups which suggests that bone formation was constant while resorption was attenuated. This effect has been documented in transgenic L-SACC1 mice with liver-specific overexpression of mutant of the Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1). These mice have decreased CTX that was attributed to impaired insulin clearance. The resulting high levels of insulin in this animal model were shown to affect recruitment and differentiation of osteoclasts by impairing RANKL signaling (319). Although insulin was not measured in the current study, a similar impaired insulin clearance has been previously reported in type 2 diabetics (347;348)

Over time a decline in β cell number and function results in decreased circulating insulin. This may explain the latter phase of this effect when BMD was reduced in the DM ≥10 longer duration diabetics and the rate of change returns to one more closely related to the non-diabetic group. With diabetes duration, the bone made dense by resorption impairment in the first decade is more likely to have structural irregularities affecting strength. This could be due in part to the accumulation of AGEs that have been observed in obese mouse models of type 2 diabetes and resulted in reduced bone strength (349). The alterations in BMD observed in the current study are in agreement with other studies conceding that the onset of increased fracture risk (8;285) and even fracture incidence (25;284) in type 2 diabetes occurs 10 years or more post-diagnosis. This has also been demonstrated specific to race in populations including Asian (350), Hispanic (351) and Norwegian (352;353) where results show that the risk of hip fracture was higher among people with diabetes than without and this risk increased with duration of diabetes diagnosis. Differences in self-reported fracture of the hip were not detected between groups in the current study, although participants were asked to respond to questions regarding fracture history.

Importantly, this study was not powered to evaluate fracture incidence and thus the absence of a difference between groups could be expected. Emerging evidence in studies powered to detect differences in fracture incidence however, has shown that patients with type 2 diabetes have higher fracture rates (1.4-fold) in spite of the absence of a reduction in BMD (30). This suggests that BMD values in diabetics, 10 years or longer, that are similar to non diabetics of a similar age group may not adequately reflect bone fragility.

In this study, differences in BMD between groups were observed in the hip regions alone and could be attributed to this regions primary function of weight bearing. Weight was not considered as a covariate in the current study due to its influence on type 2 diabetes. The lumbar spine also has a role in weight bearing, but has been shown to respond to different forces than those applied to the hips. If differences in the hip were due to weight bearing influences, this would explain why forearm BMD's were similar between groups.

Although BMD remains a useful measurement for fracture prediction in the non-diabetic population, these findings suggest a need to clarify the use of standard methods for assessing fracture risk using BMD in type 2 diabetes due to biphasic effects of diabetes with duration.

At this time it remains unclear exactly why BMD is not an accurate predictor of long-term fracture risk in the diabetic population. Animal studies using experimental models of diabetes have suggested that with diabetes duration bone structure is altered in a way that increases stiffness and compromise overall bone strength (291). This idea of compromised bone quality provides a possible explanation of the paradox of an increased risk of fractures in type-2 diabetics in the presence of normal or elevated BMD (354;355).

Examination of the current findings revealed a significantly lower serum CTX in the DM<10 group when compared to the non-diabetic group at some time points. It could be postulated that the lower concentration of CTX seen in the DM<10 group was responsible for the increased

BMD in the hip regions. Consensus has yet to be reached as to the explanation of the effects of diabetes on bone metabolism. Oz et al. (356) examined bone biomarkers in men and women, diabetic and controls and observed that the serum BAP and CTX in the diabetics were significantly lower than the non diabetic controls. When the same markers were evaluated according to gender, it was discovered that only CTX was reduced in the male diabetic population whereas the diabetic women experienced a reduction in BAP only. Other studies have reported significantly elevated BAP in type 2 diabetics over non diabetic counterparts (357-359). Bone biochemical markers have been shown to differ among ethnic groups (360), but no data is available to determine if differences exists among Native American populations. The bone biochemical marker data in this study suggest a decrease in bone resorption with no change in bone formation in the DM<10 group, but further examination of bone biochemical markers in Native American women is warranted.

No differences between groups were observed in serum IL-6 or TNF-α. In addition, no correlations existed between markers of metabolism and cytokines in the diabetic groups. Although numerous factors can influence serum levels of these cytokines it is possible that the observed outcomes were an indication that mechanisms affecting bone metabolism in non-diabetic models may not function the same in a diabetic environment. It is noteworthy that much of the research related to the effects of TNF-α and IL-6 have been demonstrated in non-diabetic models. An example of this influence of TNF-α on increased osteoclastogenesis, has been demonstrated in *in vitro* models (361;362), and with TNF-α infusion in normal (363;364) and nude mice (365). In addition to its effects on osteoclastogenesis, TNF-α also suppresses osteoblastogenesis as demonstrated *in vitro* (256-259) and in transgenic mouse models (366). A similar outcome was reported with IL-6 overexpression in transgenic mice resulting in increased osteoclast and decreased osteoblast number and activity (228). However, in all of these cases, the effects of TNF-α and IL-6 were reported in the context of a non-diabetic model. These examples

illustrate that less is known about the relationship between inflammatory mediators and bone metabolic processes in the diabetic. Based on observations such as the biphasic response of BMD in the hip in this study, it appears that type 2 diabetes potentially represents a unique scenario relative to bone metabolism. Therefore more research is required so that therapeutic approaches can be developed to prevent or reduce the number of fractures in this population.

Identifying potentially modifiable risk factors for fracture in Native Americans with type 2 diabetes is of major importance for future diabetes health care initiatives. Important to that task is exploring the metabolic differences and the factors that contribute to these differences. It is also important to consider outcomes from the current study in context of other published reports. For example, TNF-α has been shown to influence osteoclastogenesis, a finding supported by the positive correlation of CTX to TNF-α in the non-diabetics of the current study. However, this does not hold true in this diabetic population. It is also plausible that an inflammatory biomarker such as TNF-α may not be different between these groups due to innate differences among Native Americans. Reference standards, (e.g. T-scores) are often based on Caucasian populations (15;16) making race-related deviations conceivable, a concept evidenced in fact by the higher average C reactive protein (CRP) concentrations seen in this population (367). This non-diagnostic test is used for the detection of inflammation and must be compared to appropriate ethnic normative reference data to ensure relevance. In addition to the inflammatory markers, low serum vitamin D in Native Americans has been reported (33), but the mean serum concentrations of the diabetic, non-diabetic and the group as a whole in this study were within the current IOM recommendation. Results of the self-reported calcium intake did not differ between groups and should be examined as well as physical activity, specifically weight-bearing for greater understanding of the role they play specific to bone health in Native Americans. Perhaps further exploration into race-related norms is in order to determine the usefulness of standard measures and their role in chronic disease.

Currently patients with type 2 diabetes represent a diagnostic and prognostic dilemma because the value of BMD measurement in predicting osteoporotic fractures may be limited by decreased bone quality. Given the prevalence of type 2 diabetes in Native Americans, this population is at great risk. This necessitates exploration into underlying alterations in bone metabolism responsible for the biphasic response observed in this study and a greater understanding of the contributing risk factors. Improving understanding of the mechanisms involved will provide guidance toward better methods of assessment and treatment more appropriate than BMD may be discovered. It is necessary to recognize the need for not only different methods of risk assessment but different options of reducing risk as well. Therefore, evidence-based guidelines of fracture risk management, especially in Native Americans with diabetes are warranted.

CHAPTER VI

CONCLUSION

Summary

Recent evidence suggests a relationship between type 2 diabetes and an increased risk of fracture. Native Americans are known to have a high prevalence of type 2 diabetes (i.e. ~2 times the national average), but the availability of evidence regarding fracture risk is limited in this population. This study was designed to examine the extent to which type 2 diabetes affects change in BMD and bone metabolism in Native American women. Participants included Native American women (n=123) 50 years of age and older, defined by their eligibility to receive services at an Indian Health Clinic. Of the total, 36% (n=44) reported a diagnosis of type 2 diabetes to which the all DM group was stratified by years post-diagnosis due to the potential for increased risk of fracture with longer diabetes duration. Baseline and final visits included DXA scans to determine BMD, relevant medical history and anthropometric measures. In addition, an optional serum sample was collected for measures of bone metabolism indicators, 25-hydroxyvitamin D3, and inflammatory mediators (e.g. TNF-α and IL-6).

DXA results revealed increased BMD in the hip regions of the DM group and more specifically the DM<10 years duration group. In fact the stratification of the diabetes group by duration

supports the possibility of a biphasic effect evidenced by increasing BMD in the hip region the first 10 years post-diagnosis, followed by significant decreases in the same region after the first decade following diabetes diagnosis. No differences in BMD were observed in the spine or forearm regions. The only difference observed in bone metabolism was decreased CTX in the all DM group. No differences were observed between groups in inflammatory mediators or vitamin D concentrations. These findings suggest a need to clarify the use of standard methods for assessing fracture risk using BMD in type 2 diabetes due to biphasic effects of diabetes with duration. Additionally, mechanisms affecting bone metabolism in non DM models may not function similarly in a diabetic environment. The differences occurring over time between the stratified diabetes group, is a possible indication that mechanisms are modified with disease duration. Finally, it remains to be determined if differences in this study, or lack of differences, is due to innate differences within the Native American population.

Purpose

The purpose of this study is to examine the effects of type 2 diabetes and diabetes duration on bone health compared to non-diabetics in Native American women over 50 years of age.

Hypothesis 1: Change in BMD from baseline to final visit, one year later, will be greater in those women with type 2 diabetes and especially those women who have been diabetic for ten or more years compared to their non-diabetic counterparts.

No statistically significant difference in percent change in BMD was observed between the non DM and all DM groups. When the all DM group was stratified by duration of DM diagnosis, however, the DM<10 group demonstrated a significantly greater increase in BMD in the total hip and intertrochanter regions compared to both the non DM and the DM≥10 groups. The DM≥10

group demonstrated a decrease in BMD compared to the DM<10 at the same sites. Therefore we rejected the null hypothesis based on the stratification of the DM group by diabetes duration.

Hypothesis 2: Type 2 diabetics will demonstrate alterations in bone metabolism consistent with increased bone resorption and decreased bone formation rates from baseline to final visit, compared to non-diabetics. These alterations in bone metabolism will be more pronounced in longer duration type 2 diabetics (i.e., $\geq$ 10 yrs).

No statistically significant difference in bone resorption or formation was observed between the non DM and all DM groups. When the all DM group was stratified by duration of DM diagnosis no differences were observed when compared to the non DM group. We failed to reject the null hypothesis.

Hypothesis 3: The mechanisms by which type 2 diabetics will experience accelerated bone loss will be mediated by an increased inflammatory state and compromised vitamin D status. The increased inflammatory state and compromise in vitamin D status will be exacerbated in longer duration diabetics.

No statistically significant difference in the inflammatory mediators TNF-α or IL-6 was observed between the non DM and all DM groups. No statistically significant difference in serum 25-hydroxy vitamin D was observed between the non DM and all DM groups. When the all DM group was stratified by duration of DM diagnosis no differences were observed when compared to the non DM group in either inflammatory mediator or serum 25-hydroxy vitamin D. We failed to reject the null hypothesis.

Recommendations for Future Research

As the prevalence of type 2 diabetes continues to rise, acknowledging the numerous complications and developing effective prevention and treatment strategies becomes increasingly

important. The recently discovered relationship between diabetes and bone introduces the increased risk of fracture as another complication that needs to be considered clinically. Important next steps will include exploration into factors affecting bone strength. This will require the use of animal models as well as techniques to determine alterations in bone biomechanical properties in humans. A better understanding of the influences of insulin and glucose control on biochemical markers of bone turnover is also needed. Impaired insulin clearance has been observed in type 2 diabetes and recently been associated with decreased bone resorption. Monitoring insulin levels with disease progression, in conjunction with bone biochemical markers, may provide new insight into metabolic changes in bone over time.

Another important point to consider is that the current literature focused on fracture risk and type2 diabetes only delineates type 2 diabetics by the number of years post-diagnosis. Other factors such as glucose control, the role of renal function and physical activity may also contribute to the ultimate effects of type 2 diabetes on bone health and fracture risk. Future studies should take these factors into consideration.

Lastly, it will also be essential in this pursuit to identify differences inherent to specific populations. Relatively little is known about Native Americans and osteoporosis risk. Therefore it is important to establish population based norms for different ethnic groups not only for BMD but also biochemical markers of bone metabolism.

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APPPENDICES

Appendix A. DXA Measurements of the Hip Regions at Baseline and Final Visit According to DM Status.

	Non DM[1]	All DM[2]	DM<10[3]	DM≥10[4]	*P* (1vs2)	*P* (1vs3vs4)
Total Hip						
Baseline						
BMA (cm^2)	33.8±0.37	33.2±0.40	33.0±0.73	33.0±0.52	0.283	0.423
BMC (g)	29.0±0.56	30.8±0.91	30.2±1.46	30.8±1.27	0.087	0.338
T-score	-0.67±0.12	-0.14±0.19	-0.25±0.28	-0.10±0.28	0.014	0.075
Final						
BMA (cm^2)	33.9±0.37	33.5±0.45	33.7±0.79	33.2±0.52	0.444	0.629
BMC (g)	29.3±0.57	31.1±0.97	31.1±1.55	30.8±1.39	0.078	0.286
T-score	-0.63±0.13	-0.13±0.19	-0.17±0.29	-0.13±0.28	0.021	0.103
Intertrochanter						
Baseline						
BMA (cm^2)	18.1±0.29	17.6±0.37	17.5±0.74	17.6±0.36	0.302	0.529
BMC (g)	18.4±0.40	19.7±0.67	19.2±1.17	19.8±0.86	0.070	0.285
T-score	-0.51±0.12	0.13±0.17	-0.01±0.26	0.16±0.26	0.003	0.023†
Final						
BMA (cm^2)	18.3±0.28	17.8±0.39	17.9±0.75	17.7±0.37	0.326	0.606
BMC (g)	18.7±0.41	20.0±0.71	20.0±1.19	19.9±0.98	0.080	0.283
T-score	-0.4 ±0.12	0.14±0.17	0.10±0.27	0.12±0.25	0.004	0.034*
Trochanter						
Baseline						
BMA (cm^2)	10.7±0.14	10.6±0.23	10.6±0.45	10.4±0.25	0.674	0.694
BMC (g)	6.91±0.15	7.13±0.26	7.19±0.42	6.96±0.36	0.449	0.779
T-score	-0.55±0.12	-0.29±0.18	-0.30±0.24	-0.32±0.30	0.225	0.542
Final						
BMA (cm^2)	10.7±0.15	10.7±0.24	10.8±0.41	10.5±0.33	0.995	0.889
BMC (g)	6.92±0.15	7.18±0.27	7.27±0.44	7.01±0.39	0.363	0.659
T-score	-0.53±0.12	-0.33±0.18	-0.31±0.25	-0.39±0.31	0.359	0.698

Data presented as mean ± SE. Non diabetic = non DM, all diabetic group = all DM, DM<10 = diagnosed diabetes <10 years, DM ≥10 = diagnosed diabetes ≥10 years. [a] Diabetes duration unknown in 4 participants. ANOVA showed a statistical difference across all three groups (ANOVA, $p<0.05$) with significant pairwise post-hoc tests denoted between: * non DM and DM<10, † non DM and DM≥10

Appendix B. DXA Measurements of the Lumbar Spine and Forearm at Baseline and Final Visit According to DM Status.

	Non DM[1]	All DM[2]	DM<10[3]	DM≥10[4]	*P*	*P*
Total Spine						
Baseline						
BMA (cm^2)	58.1±0.51	57.1±0.97	57.2±1.46	57.3±1.52	0.287	0.687
BMC (g)	57.8±1.15	58.2±1.85	57.1±2.00	59.1±3.38	0.841	0.847
T-score	-0.50±0.15	-0.29±0.20	-0.44±0.23	-0.22±0.34	0.377	0.686
Final						
BMA (cm^2)	58.1±0.50	57.2±0.97	56.8±1.49	57.9±1.51	0.404	0.626
BMC (g)	57.5±1.15	58.8±1.82	56.9±2.14	60.7±3.28	0.545	0.457
T-score	-0.55±0.14	-0.23±0.19	-0.43±0.25	-0.07±0.32	0.191	0.335
Forearm						
Baseline						
BMA (cm^2)	23.5±0.22	24.1±0.34	23.9±0.51	24.4±0.52	0.138	0.257
BMC (g)	11.5±0.21	11.8±0.27	11.8±0.37	11.8±0.45	0.413	0.761
T-score	-1.47±0.14	-1.44±0.18	-1.35±0.29	-1.58±0.25	0.918	0.849
Final						
BMA (cm^2)	23.6±0.22	23.9±0.34	23.8±0.50	24.1±0.54	0.438	0.639
BMC (g)	11.4±0.21	11.7±0.28	11.7±0.37	11.7±0.47	0.475	0.778
T-score	-1.57±0.14	-1.49±0.18	-1.36±0.29	-1.61±0.25	0.724	0.783

Data presented as mean ± SE.

Non diabetic = non DM, all diabetic group = all DM, DM<10 = diagnosed diabetes <10 years, DM ≥10 = diagnosed diabetes ≥10 years

[a]Diabetes duration unknown in 4 participants.

No statistical differences were found (ANOVA, $p<0.05$).

Appendix C. Consent Form.

CONSENT FORM

University of Oklahoma Health Sciences Center (OUHSC)
Oklahoma Native American Women's Osteoporosis Screening Study
Sponsor: Proctor and Gamble
Principal Investigator: Mary Baker, MD

This is a research study. Research studies include only individuals who choose to take part in them. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?
You are being asked to take part in this study because you are a Native American woman 50 years of age or older who is qualified to receive services at Oklahoma Indian Health Clinics.

Why Is This Study Being Done?
Osteoporosis is a disease in which bones become weaker and are more likely to break. Currently, there is not much information available about osteoporosis risk in Native Americans and factors that may hinder access to treatment. The purpose of this study is to determine how common osteoporosis is in Native American women who receive treatment at Oklahoma Indian Health Clinics. Additionally, we want to better understand the factors such as diet, exercise and other health problems that may affect a persons risk for osteoporosis

How Many People Will Take Part In The Study?
About 300 people will take part in this study that is being conducted in the Oklahoma City area.

What Is Involved In The Study?
This project will require that you come to the General Clinical Research Center (GCRC) on the University of Oklahoma Health Sciences Center (OUHSC) campus in Oklahoma City for an osteoporosis screen and then return 2 more times for follow-up visits, 1 and 2 years later. The initial visit will take 1-2 hours and during this time you will do the following:

- complete a questionnaire related to your medical history. You will be asked about medications and supplements that you take, medical conditions that your doctor has told you that you have, lifestyle habits (for example - do you smoke?) and questions related to pregnancies and breastfeeding.
- measure your height, weight, waist and hip circumference
- complete a questionnaire related to how much you exercise and what types of exercise you have done over the past year.
- measure your skin tone - for this you will have a photo taken of the inner portion of your (dominant) forearm using a Smartpointe Color Reader, this is the same instrument used in the cosmetic industry to assess clients skin tone color
- a bone density (a type of x-ray) scan of your whole body and some specific sites such as your back, hip and arm will be done to measure the bones in your body - for this

you will lie on an x-ray table and once you are positioned it will take a few seconds for the machine to pass over your body and take measurements of your bone.

- receive instructions and materials for completing a 3-day food record that you will return in the mail.
- be given some educational material about osteoporosis and how you can reduce your chances of having osteoporosis.
- You have the option to do a blood test – we will take about 2 tablespoons (30 cc) of your blood at each visit for a total of 6 tablespoons (90cc) over the course of the 2 year study. We will measure chemicals in your body that provide information about the quality of your bones, the way you body handles calcium, and how much inflammation you have. Blood samples will be labeled with your study number and the date the sample was collected. All samples will be handled by trained study personnel only and will be stored in very cold temperature freezers in the principal investigators laboratory for up to 5 years. At the end of the 5 year period any remaining samples that have not used in the analysis will be destroyed by the principal investigator and the chairman of the Oklahoma City Area Indian Health Service Institutional Review Board will be notified. You can still participate in the study even if you don't want to do this blood test.

Blood Test: ____Yes ____ No _____Initial

If you indicate that you have been diagnosed with Type II diabetes, we will ask your physician at the clinic to provide us with the results of your most recent hemoglobin A1c test (if they are available). This will provide us with important information about your glucose levels over several months.

At your follow-up visits to the GCRC (1 year later and 2 years later) you will be asked similar questions regarding your medical history and physical activity habits, have repeated height, weight, and bone density scans performed, and complete another 3-day food record. You will also be asked specific questions about use of medication for osteoporosis.

How Long Will I Be In The Study?
We think that you will be in the study for about 2 years and during that time you will be asked to come to the GCRC on the OUHSC campus three times. You can stop participating in this study at any time. If you do decide to stop participating in the study, we encourage you to talk to the researchers and your regular doctor first. There may be anticipated circumstances under which your participation may be terminated by the investigator without regard to your consent.

What Are The Risks of The Study?
The procedures may involve risks that are currently unforeseeable. You will undergo a bone density scan of your entire body, and specific sites including your hip, spine and forearm. If you agree to participate in this research, you will receive radiation exposure from 12 DEXA scans (a type of x-ray) that you will not receive if you choose not to participate. The radiation exposure that you will receive from these DEXA scans is approximately 2% of the amount of radiation

exposure that an x-ray technologist is permitted to receive in one year. If you agree to participate in the blood tests, the risks of drawing blood include pain at the needle site, bruising, feeling faint, and a slight risk of infection. As with any research study, there may be additional risks that we don't know about yet. You will be informed of any such risks if we learn about any.

You must not be pregnant while participating in this study. Due to the radiation exposure from the bone density scans, there may be some risks to an embryo or fetus, including birth defects. If you are considered of childbearing age (less than two years since your last period) you will be asked to do a pregnancy test (urine test) prior to entrance into the study and each of the follow-up visits. If you become pregnant during this study, you will be withdrawn from the study. The study physician will assist you in getting obstetrical care. Payment for all aspects of obstetrical, child, or related care will be your responsibility.

Are There Benefits to Taking Part in The Study?
If you agree to take part in this study, there may or may not be direct medical benefit to you. If you are in the study, you will have the opportunity to have several bone density scans at no charge, a test that is not currently available at your clinic. You will also get all of the study's dietary and clinical tests at no cost. We hope that the information learned from this study will benefit other patients with this disease in the future.

What Other Options Are There?
You may choose not to participate in the study. If so, you can get a bone density scan at some local hospitals and clinics for a fee. Please talk to your regular doctor about this option.

What About Confidentiality?
Efforts will be made to keep your personal information confidential. All of your private health information will be kept in locked file cabinets. Data will be entered into a password protected database and files managed by the principal investigator. All records will be kept confidential and participants assigned a study number. The master list of study participants and their study number will be kept in a locked file cabinet in the principal investigator's office with limited access. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

There are organizations that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include the US Food & Drug Administration, the National Institutes of Health and the OUHSC Institutional Review Board.

To help us protect your privacy, we have obtained a Certificate of Confidentiality from the National Institutes of Health. With this Certificate, the researchers cannot be forced to disclose information that may identify you, even by a court subpoena, in any federal, state, or local civil, criminal, administrative, legislative, or other proceedings. The researchers will use the

Certificate to resist any demands for information that would identify you, except as explained below.

You should understand that a Certificate of Confidentiality does not prevent you or a member of your family from voluntarily releasing information about yourself or your involvement in this research. If an insurer, employer, or other person obtains your written consent to receive research information, then the researchers may not use the Certificate to withhold that information.

The Certificate of Confidentiality does not prevent the researchers from disclosing voluntarily, without your consent, information that would identify you as a participant in the research project under the following circumstances: child abuse, intent to hurt self or others.

What Are the Costs?
You can participate in this study with no cost to you. All study procedures including the bone density scans, dietary evaluation, physical activity assessment, educational materials and optional blood tests will be provided at no cost to you.

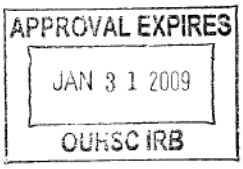
Will I Be Paid For Participating in This Study?
We will give you $25 after you complete the first visit and at each of the 1 year and 2 year follow-up visits to help make up for your time and travel expenses. Also, if you agree to have your blood drawn for the optional blood tests you will receive an additional $25 dollars after each visit. There is a total of up to $150.

What if I am Injured or Become Ill While Participating in this Study?
In the case of injury or illness resulting from this study, emergency medical treatment is available. OUHSC, GCRC, Proctor and Gamble, and the Indian Health Service have no policy or plan to pay for any injuries you might receive as a result of participating in this research protocol.

What Are My Rights As a Participant?
Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. If you agree to take part and then decide against it, you can withdraw for any reason; however, please be sure to discuss leaving the study with the principal investigator or your regular physician. Leaving the study will not result in any penalty or loss of benefits that you would otherwise receive.

We will tell you about any new information that may affect your health, welfare or willingness to stay in this study.

You have the right to access the medical information that has been collected about you as a part of this research study. However, you agree that you may not have access to this medical information until the entire research study has completely finished and you consent to this temporary restriction.



Whom Do I Call If I have Questions or Problems?
If you have questions, concerns or complaints about the study or have a research-related injury, contact Dr. Brenda Smith at 405-744-3866 or 405-878-6364 after hours.

For questions about your rights as a research subject, contact the OUHSC Director, Human Research Participant Protection Program at 405-271-2045 or Dr. Travis Watts, Chairman of the Oklahoma City Area Indian Health Service Institutional Review Board at 405-951-3829.

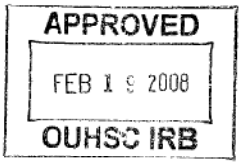
Signature:
By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

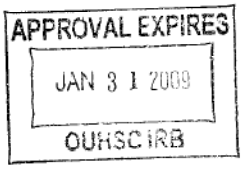
I agree to participate in this study:

Research Subject: _____
Date:_____

Subject's Printed Name _____

Person Obtaining Informed Consent:_____
Date:_____





Appendix D. Medical History Form.

IRB No: 13281

Oklahoma Native American Women's Osteoporosis Screening Study

Study ID: _____ **Date:** _____ **Primary Care Physician:** _____
Date of Birth: _____ **Bloodline:** (Indicate tribe & percentage) _____

Social History: (Circle Y for yes and N for no)
Do you currently smoke? Y N If yes, ____packs/day Do you use other forms of tobacco? Y N
Do you drink alcohol? Y N If yes, how often/how much: _____/_____
Do you have any special needs related to your culture? _____

Education: (Circle the highest level) Grade: 1 2 3 4 5 6 7 8 9 10 11 12 College: 1 2 3 4 5+

Allergies: (List any drug, food, contact, or environmental substance you have had an allergic reaction)

_____
_____

Medications & Supplements: (List any prescription, over-the-counter, herbal, vitamin, or nutritional supplement you are currently taking and the approximate amounts.)

1)	4)	7)
2)	5)	8)
3)	6)	9)

Past Hospitalizations or Surgeries: (List all illnesses/surgeries for which you have been hospitalized and the approximate date.)

1)	4)
2)	5)
3)	6)

Reproductive History: (Circle Y for yes and N for no)
Have you ever been pregnant: Y N If so, how many times? _____

How many children? _____ What are your children's' ages? _____

What was your age at the time of delivery of your first child? _____

Did you breastfeed your children? Y N

Did you use oral contraceptives? Y N If so, for approximately how many years? _____

Do you currently have regular menstrual period? Y N

What was the date of your most recent menstrual period? _____

Have you ever taken (female) hormone replacement? Y N How long did you take hormones?_____

Medical History: (For each illness or condition that you have had diagnosed by a physician, indicate how long ago you were diagnosed in the box to the right.)

Osteoporosis	
Vertebral fracture, x-ray confirmed	
Hip replacement	
Wrist Fracture	
Hip fracture	
Bone loss of the jaw	
Diabetes Mellitus (Type I	
Diabetes Mellitus (Type II)	
Elevated cholesterol	
High blood pressure	
Myocardial Infarction (heart attack)	
Angina pectoris	
Coronary bypass or angioplasty	
Congestive heart failure	
Stroke (CVA)	
Peripheral artery disease or claudication of legs (not varicose veins)	
Carotid surgery	
Pulmonary embolus	
Cholecystectomy	
Glaucoma	
Macular degeneration	
Cataract	
Liver disease (such as cirrhosis)	

Asthma	
Emphysma	
Chronic bronchitis	
Tuberculosis	
Depression, clinician diagnosed	
Osteoarthritis	
Breast cancer	
Cancer of the uterus (endometrium)	
Cancer of the ovary	
Cancer of the colon	
Cancer of the lung	
Melanoma	
Basal cell skin cancer	
Squamous cell skin cancer	
Other cancer (specify)	
Rheumatoid Arthritis	
SLE (systemic lupus)	
Lactose intolerance	
Irritable Bowel Syndrome	
Ulcerative colitis/Crohn's	
Kidney stones	
Renal disease	

Participant's Signature: _____ RN Signature: _____

Relevant Medical History Form GCRC -	Participant ID

Appendix E. Tribal Representation Form.

Tribal representation: Please circle all that apply and write in the percent blood you have for each tribe.
example: (Apache) 1/4, (Ponca) 1/8, (Seminole) 1/2

Oklahoma Based Tribes:

Absentee Shawnee Tribe: _____ Alabama Quassarte Tribal Town: _____

Apache Tribe: _____ Caddo Tribe: _____

Cherokee Nation: _____ Cheyenne-Arapaho Tribes: _____

Chickasaw Nation: _____ Choctaw Nation: _____

Citizen Potawatomi Nation: _____ Comanche Nation: _____

Delaware Nation: _____ Eastern Shawnee Tribe: _____

Fort Sill Apache Tribe: _____ Iowa Tribe of Oklahoma: _____

Kaw Nation of Oklahoma: _____ Kialegee Tribal Town: _____

Kickapoo Tribe of Oklahoma: _____ Kiowa Tribe: _____

Miami Nation: _____ Modoc Tribe: _____

Muscogee Creek Nation: _____ Osage Nation: _____

Otoe-Missouria Tribe: _____ Ottawa Tribe: _____

Pawnee Nation of Oklahoma: _____ Peoria Tribe of Indians of Oklahoma: _____

Ponca Nation: _____ Quapaw Tribe of Oklahoma: _____

Sac & Fox Nation: _____ Seminole Nation: _____

Shawnee Tribe: _____ Seneca-Cayuga Tribe of Oklahoma: _____

Thlopthlocco Tribal Town: _____ Tonkawa Tribe: _____

Wichita & Affiliated Tribes: _____ United Keetoowah Band of Cherokees: _____

Wyandotte Nation: _____ Euchee Tribe of Indians: _____

Please list any other tribes you represent and blood quantum (example: Navajo ¼):

_____

Please circle what best describes your <u>total</u> Indian blood quantum:

Less than ¼

¼ to less than ½

½ to less than ¾

¾ to Full Blood

Please list the one tribe which you are enrolled in: _____

VITA

Misti J. Leyva

Candidate for the Degree of

Doctor of Philosophy

Thesis: TYPE 2 DIABETES EXACERBATES BONE LOSS IN NATIVE AMERICN WOMEN

Major Field: Nutritional Sciences

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in May, 2012.

Completed the requirements for the Master of Science in Nutritional Sciences at the University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma in 2001.

Completed the requirements for the Bachelor of Arts in Psychology at Texas Tech University, Lubbock, Texas in 1990.

Experience:

2011 Bionutritionist, Harold Hamm Diabetes Center at the University of Oklahoma Health Sciences Center, Oklahoma City, OK

2002-2011 Director of Nutrition Research, University of Oklahoma Health Sciences Center- General Clinical Research Center, Oklahoma City, OK

2007-Current Assistant Professor, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK

Professional Memberships:

2009-present American Society for Bone and Mineral Research

1999-present American Dietetic Association

1999-present Oklahoma Dietetic Association

Name: Misti J. Leyva Date of Degree: May, 2012

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: TYPE 2 DIABETES EXACERBATES BONE LOSS IN NATIVE AMERICAN WOMEN

Pages in Study: 104 Candidate for the Degree of Doctor of Philosophy

Major Field: Nutritional Sciences

Scope and Method of Study: Recent evidence suggests a relationship between type 2 diabetes and an increased risk of fracture. Native Americans are known to have a high prevalence of type 2 diabetes (i.e. ~2 times the national average), but the availability of evidence regarding fracture risk is limited in this population. This study was designed to examine the extent to which type 2 diabetes affects change in BMD and bone metabolism in Native American women. Participants included Native American women (n=123) 50 years of age and older, defined by their eligibility to receive services at an Indian Health Clinic. Of the total, 36% (n=44) reported a diagnosis of type 2 diabetes to which the DM group was stratified by years post-diagnosis due to the potential for increased risk of fracture with longer diabetes duration. Baseline and final visits included DXA scans to determine BMD, relevant medical history and anthropometric measures. In addition, an optional serum sample was collected for measures of bone metabolism indicators, 25-hydroxyvitamin D3, and inflammatory mediators (e.g. TNF-α and IL-6).

Findings and Conclusions: DXA results revealed increased BMD in the hip regions of the DM group and more specifically the DM<10 years duration group. In fact the stratification of the diabetes group by duration supports the possibility of a biphasic effect evidenced by increasing BMD in the hip region the first 10 years post-diagnosis, followed by significant decreases in the same region after the first decade following diabetes diagnosis. No differences in BMD were observed in the spine or forearm regions. The only difference observed in bone metabolism was decreased CTX in the all DM group. No differences were observed between groups in inflammatory mediators or vitamin D concentrations. These findings suggest a need to clarify the use of standard methods for assessing fracture risk using BMD in type 2 diabetes due to biphasic effects of diabetes with duration. Additionally, mechanisms affecting bone metabolism in non DM models may not function similarly in a diabetic environment. Finally, it remains to be determined if differences in this study, or lack of differences, is due to innate differences within the Native American population.

ADVISER'S APPROVAL: Brenda J. Smith