TYPE 1 DIABETES AND BONE MASS: INTERRELATIONSHIPS WITH NUTRIENT INTAKE AND PHYSICAL ACTIVITY IN CHILDREN AND WITH DIETARY FISH OIL IN WEANLING RATS

BY

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

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Abstract

An investigation was conducted in 8 to 18 year old children to determine interrelationships among Type 1 diabetes (DM 1), bone mass, nutrient intake and physical activity. Bone mineral content (BMC, g), bone area (BA, cm^2), and bone mineral density (BMD, g/cm^2) were measured by dual-energy x-ray absorptiometry in children with (n=27 females, 24 males) and without (n=34 females, 34 males) DM 1. Nutrient intake was assessed using 3-day food records and physical activity by a validated questionnaire. Data was analyzed by two-way ANOVA and correlation analysis. As a group, children with DM 1 did not have significantly lower BMC, BA, or BMD compared to age- and sex-matched control children, although there was a trend towards lower bone mass in boys with DM 1. Children with a disease duration of $\leq$ 1 year had significantly lower standardized scores for BMC and BA than children with a disease duration of > five years. Physical activity was significantly correlated with bone mass only in girls with DM 1. Intakes of calcium, phosphorus, vitamin D, and polyunsaturated fatty acids (PUFA) were not correlated or were negatively correlated with BMC, BA, and BMD, except in the boys with DM 1 where PUFA intake was positively correlated with standardized scores for BMC and BA. Thus it appears that bone mass is adversely affected in children with DM 1 prior to initiation of and adaptation to treatment. While boys appear to be more adversely affected by the disease than girls, bone mass was higher in boys with higher PUFA intakes.

Based on these results, a second investigation was undertaken to study further the role of insulin in bone development, and the effect of n-3 PUFA on bone development in insulin deficiency. Weanling male Sprague-Dawley rats (n=80) were randomly assigned to one of six groups: saline injection + pair-weigh (n=20) or ad lib feeding (n=20), or streptozotocin injection to induce insulin deficiency + ad lib feeding (n=40). Animals were fed diet containing either soy oil or fish oil (menhaden + corn oil). After 35 days, measurements included plasma $1,25(OH)_2D_3$ (nmol/L), plasma insulin-like growth factor I (IGF-I; pmol/L), plasma osteocalcin (OC; pmol/L), urinary N-telopeptide (NTx; nm BCE/mM creatinine), ex vivo release of prostaglandin E_2 (PGE_2) from femur (mmol/g), femur diaphysis calcium and phosphorus (mg/g), femur weight (g), and femur length (cm). Data was analyzed by two-way ANOVA. Insulin deficient rats had similar diaphysis calcium and phosphorus compared to insulin sufficient rats, but had lower vitamin D, IGF-I, femur weight and length, and higher NTx and PGE_2. The fish oil diet reduced PGE_2 and NTx in insulin deficient animals, but also reduced OC. Thus in weanling rats, insulin deficiency appears to promote a state of high bone turnover which may be moderated by dietary fish oil.

The results of these studies suggest that prior to establishment of and adaptation to treatment of Type 1 diabetes, metabolism is altered. This altered metabolism results in lower bone mass. Higher consumption of n-3 PUFA attenuates this effect on bone mass.

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LIST OF ABBREVIATIONS

AA:	arachidonic acid
AIN:	American Institute of Nutrition
ANOVA:	analysis of variance
BA:	bone area
BCE:	bone collagen equivalents
BMC:	bone mineral content
BMD:	bone mineral density
BMI:	body mass index
DCCT:	diabetes control and complications trial
DXA:	dual-energy x-ray absorptiometry
EPA:	eicosapentaenoic acid
HbA1c:	hemoglobin A1c
IGFBP:	insulin-like growth factor binding proteins
IGF-I:	insulin-like growth factor I
IP:	intra-peritoneal
IV:	intravenous
NTx:	N-telopeptide
OC:	osteocalcin
PGE:	prostaglandin E
pQCT:	peripheral quantitative computed tomography
PTH:	parathyroid hormone
PUFA:	polyunsaturated fatty acids
SD:	standard deviation

Preface

The role of insulin in bone development has been investigated in children and adults, as well as in animals (1-12). Insulin is known to be both directly and indirectly involved in bone development (1-2,8,13-4), and insulin deficiency has been shown to result in various metabolic derangements which themselves affect bone (7,15-24). Nonetheless, results from these studies have been conflicting, with some showing diminished bone mass in insulin deficiency (1-2,5-6) and others showing no effect (4,25). In addition, numerous, and sometimes contradictory, suggestions have been made as to the cause of the observed effects of insulin deficiency on bone (2-3,6-7,10,15,18-9,21-2,24,26), with new potential contributors still being discovered. It would appear there is still much to learn.

This thesis, designed to examine the role of insulin in bone development, contains two projects. The first project was a clinical investigation of children with Type 1 diabetes, and the second a basic investigation using a diabetes-induced rodent model. Since the projects were related but distinct, the methods, results, and interpretations for each have been included separately, with the rationale for the methods included in the methods section for each investigation. Throughout the thesis, diabetes refers specifically to Type 1 diabetes, and diabetic rats to streptozotocin-induced diabetes, unless otherwise indicated.

1. Introduction

Type 1 diabetes is a chronic autoimmune disorder which tends to develop during childhood (27). The disease occurs with destruction of pancreatic B-cells by the body's own antibodies. Since the pancreatic B-cells are the site of insulin production, an absolute or relative insulin deficiency results when these cells are destroyed (27-8). Because insulin is the primary hormone responsible for glucose uptake by the cells, lack of insulin secretion leads to hyperglycemia - an excess of glucose in the bloodstream. Elevated levels of blood glucose are linked to long-term complications. Many of these complications are well established, including retinopathy, neuropathy, and nephropathy, as well as increased risk of atherosclerosis. Results from the Diabetes Control and Complications Trial (DCCT, 29) showed that the risk of developing these complications can be reduced by more intensive insulin management. However, one potential complication that was not examined in the study, and one which remains controversial is that of diabetic osteopenia. While some researchers have not shown bone growth and development to be significantly different in individuals with Type 1 diabetes compared to those without Type 1 diabetes (4,25), most researchers have confirmed the presence of reduced bone mass in individuals with the disease (2,5-7). Numerous factors have been suggested as directly or indirectly causative: insulin deficiency itself, poor metabolic control, reduced levels of insulin-like growth factor I (IGF-I), and altered calcium, phosphorus, vitamin D, and/or parathyroid

hormone (PTH) metabolism. Type of dietary fat may also affect development and progression of diabetic osteopenia. Recent research has shown that n-3 polyunsaturated fatty acids (PUFA) can have a beneficial effect on bone formation and growth (30). While numerous studies have been conducted to examine the role of dietary fat in bone development, few of these studies have investigated the combined effects of insulin deficiency and dietary fat on bone.

2. Present State of Knowledge

2.1 Monitoring of Type 1 Diabetes

Generally, children with Type 1 diabetes are most successful in achieving glycemic control when they have access to a diabetes clinic or service designed specifically for children (31). According to Chiarelli et al. (32), a management plan for diabetes care and treatment must be established in order to achieve patient compliance. They suggest that treatment and care of Type 1 diabetes involves several components including self-monitoring, regular testing to monitor the development of complications, and an educational component conducted by a multidisciplinary team. Continued education of patients and their families is important to long-term management, with patient visits occurring every 1 to 3 months, and more frequently if necessary.

In Manitoba, children 0 to 18 years of age diagnosed with Type 1 diabetes are referred to The Manitoba Diabetes Education Resource for Children and Adolescents (DER-CA). Patients visit the clinic every 6 months to monitor

anthropometry, blood pressure and hemoglobin A_{1c} (HbA_{1c}). Visits are scheduled every 3 months for children whose HbA_{1c} exceeds 140% of the upper limit of normal. The more frequent visits are an attempt to teach the children strategies for good metabolic control.

Self-monitoring of various indicators of metabolic control is encouraged. Strip tests, requiring a drop of capillary blood from the finger, are available for blood glucose monitoring. Strip tests are also available to monitor glucosuria. In addition, self-monitoring for ketonuria, an indicator of insulin deficiency, can be done. Long-term biochemical indicators tested at the diabetes clinic are also a routine part of monitoring. HbA_{1c}, which indicates glycemic control over the past 1 to 3 months, remains the standard indicator for metabolic control. Regular physical examinations are also part of ongoing care (31-2).

2.2 Current Treatment Approaches

There are at present 2 basic approaches for using insulin as a treatment for Type 1 diabetes: conventional therapy and intensive therapy. Conventional therapy involves 1 or 2 daily insulin injections, with urinary or blood glucose monitoring occurring once daily. Intensive therapy, on the other hand, involves daily insulin injections 3 or more times per day; with this regimen, monitoring of urinary or blood glucose values occurs a minimum of 4 times daily. Results from the DCCT (29), showed that intensive therapy is significantly more effective than conventional therapy in maintaining glycemic control, as well as slowing the progression of diabetic complications in the form of retinopathy,

nephropathy, and neuropathy. Linear growth was not different between the 2 treatment groups; however, body mass index (BMI) increased significantly more in individuals on intensive therapy compared to those on conventional therapy. In fact, patients receiving intensive therapy were almost 2 times as likely to become overweight as patients receiving conventional therapy. No indication was given regarding the effect of each type of therapy on bone growth and development. Nonetheless, it is known that improved glycemic control has a positive effect on bone formation (4,6,21,33).

2.3 Diabetic Osteopenia

In 1979, Levin et al. (2) studied skeletal mass in 35 individuals with Type 1 diabetes. These researchers measured the radius of the non-dominant arm using photon absorptiometry. Two locations were measured – a metaphyseal site and a diaphyseal site. Results showed that more than half of the subjects had a forearm bone mass less than 90% that of age and sex matched controls. A longitudinal study conducted by McNair et al. (34) supports these results; bone mass was found to decrease by 10% in the first 5 years of the disease, with the rate of loss declining as duration of diabetes increased. Shore et al. (5) also used photon absorptiometry to measure bone mineral content (BMC), bone width (BW), and BMC/BW in the non-dominant radius, ulna, and humerus of 51 children with Type 1 diabetes. BMC was as much as 11% lower than that of age and sex matched controls. Another group of researchers measured ulnar length, bone width, and mineral content in

individuals with Type 1 diabetes aged 6 to 26 (35). On average, they found bone density to be 2 to 8% lower in individuals with diabetes compared to sex and race matched controls. Bone density was affected most in Caucasian females; almost half had bone density values less than 90% of control values. In terms of duration of disease, those with onset in the previous 5 years had the lowest bone density relative to controls. Hui et al. (36) reported reduced bone mass, but normal bone width in individuals with Type 1 diabetes measured 3 times per year for 1 to 4 years. Krakauer et al. (37) conducted a longitudinal study in which radial bone density was determined for patients with Type 1 diabetes and compared to that of nondiabetic controls. Measurements were repeated on a subset of the original group at 2.5 years and 12.5 years following baseline measurements. Results at baseline, short-term and long-term follow-up indicated that bone density in the diabetic subjects was significantly lower than in the control group. In addition, mineralizing surface and mineral apposition rate in trabecular bone were significantly reduced in the diabetic subjects at short-term follow-up.

Overall, previous research indicates that bone mass, BMC, bone mineral density (BMD), mineralizing surface, and mineral appositional rate are lower in individuals with Type 1 diabetes compared to those who do not have the disease. These complications appear to be most severe in the period shortly after onset, after which they may stabilize.

2.4 Fracture Risk

It is not clear whether these changes in bone in individuals with Type 1 diabetes result in increased risk of fracture. Some researchers report a significant increase in risk (13,22) while others report no increase (37-8). For those that showed elevated risk, other factors besides osteopenia may be involved; complications such as hypoglycemia, neuropathy, or vascular disease may increase risk of falling, thereby indirectly increasing risk of fracture (37-8). While the risk of fracture may not be higher in individuals with diabetes, among individuals with fractures there is increased prevalence of diabetes (37). In addition, fracture healing time is extended in individuals with diabetes by more than 50%; this may be due to accelerated collagen aging (38). It would appear therefore, that diabetic osteopenia has at least an indirect effect on overall risk of fracture.

2.5 Bone Development

2.5.1 Bone Growth and Mineralization

Bone formation occurs through one of three modes. Primary ossification, which promotes increased bone mass, consists of primary intramembranous ossification and primary endochondral ossification. Both involve formation of a honeycomb network of nonlamellar bone through replacement of tissue; in the former, the periosteum is replaced, while in the latter, growing cartilage is the target tissue. Old cartilage cells hypertrophy, mineralize, and eventually disintegrate and die, to be replaced by new cells. Blood vessels and

osteoprogenitor cells – precursors to osteoblastic cells - invade the spaces left by the disintegrated cells. Resorption also occurs at this time, resulting in tunnels called primitive haversian systems (39). The honeycomb network formed in primary ossification is strengthened through the addition of lamellar bone layers, one inside the other, to form primary osteons. This is secondary ossification. The third stage of ossification, remodelling, involves bone resorption followed by formation, thus creating secondary osteons (40).

Long bones tend to appear about the fourth week of embryonic development. At this time, they are simply a mass of tightly packed connective tissue, or mesenchyme, more or less in the shape of the bone. These cells develop into cartilage cells, undergoing primary endochondral ossification. A proliferative zone at the end of the bone, on either side of the central zone of hypertrophic cartilage, is the active site of cell multiplication (40). Growth occurs as primary endochondral ossification spreads toward the ends of the bone. A layer of cartilage on the end of the bone, epiphyseal cartilage, and the epiphyseal growth plate form the end of this expanding bone. Both forms of primary ossification occur as the bone continues to grow. Sometime before puberty, endochondral ossification begins at secondary sites in the epiphyseal cartilages; these sites enlarge to form 2 bony epiphyses. The epiphyseal growth plate and the layers of cartilage lining the joints separate the newly formed epiphyses from the metaphyses. During puberty, the growth plate disappears when the epiphyses successively fuse with the metaphysis and

diaphysis (40). Following puberty and continuing through adulthood, bone continues to be laid down at the endosteal surface. Later in adulthood, a resorptive phase occurs in which resorption of bone is greater than formation. In adolescents with Type 1 diabetes, however, bone apposition may be low (41).

Prior to puberty, gain in bone size appears to be the primary contributor to bone mass accumulation, while bone density gains are lesser contributors. Increases in true volumetric bone density, that is density adjusted for bone size, occur during pubertal growth and development. While a small gain in bone size adjusted BMD occurs in females following menarche, rates of BMC accumulation, bone expansion, and somatic growth declines. It has been suggested that this slight increase in BMD may be a sort of maturation and tightening of bone (42-3). This continues until the end of the third decade. Type 1 diabetes appears to adversely affect true bone density. Using peripheral quantitative computed tomography (pQCT), Lettgen et al. measured volumetric bone density in children with Type 1 diabetes, and found that compared to age- and sex-matched controls, the children with diabetes had significantly lower trabecular bone density (44).

2.5.2 Trabecular and Cortical Bone

Bone is composed of both trabecular and cortical components. Trabecular bone, which has a spongy consistency, forms thin plates that traverse the marrow spaces and form the interior meshwork of the bone. Trabecular bone

contributes only 20% of skeletal mass; however, it has an extremely large surface area. Cortical bone is compact, and lines the shafts of long bones to provide structural support (45-6). Lack of deposition may occur in either component or both. Lettgen et al. (44) recently compared BMD in 21 children and adolescents (aged 6.2 – 19.9 years) with Type 1 diabetes to BMD in age and sex matched controls. Sixteen of the subjects with Type 1 diabetes were on conventional therapy, while the remaining 5 were on intensive therapy. Using pQCT, the researchers measured trabecular and total bone. From these analyses, cortical bone density was calculated. Results indicated reduced bone density in individuals with Type 1 diabetes compared to controls. However, only trabecular bone density and not total or cortical bone density showed a significant reduction. This is in contrast to the previously cited study by Levin et al. (2), who found cortical bone mass to be significantly lower in individuals under 21 years of age, in addition to the significantly lower trabecular bone mass seen in all subjects. Santiago et al. (47) studied 107 children with Type 1 diabetes, and found that 75% of girls and 87% of boys with Type 1 diabetes had cortical bone thickness less than the mean for age adjusted normal values.

2.5.3 Bone Remodelling

The structure of bone is determined by the bone remodelling process, which is the result of the continuous and opposite actions of osteoblasts and osteoclasts. In children and adolescents, bone formation normally occurs at a

faster rate than bone resorption; thus there is a net gain of bone. However, it has been suggested that in Type 1 diabetes, this balance may shift, with the result that less bone is being laid down in relation to that being resorbed. Reduced bone density can therefore be due either to diminished bone formation or increased bone resorption (4). In diabetic osteopenia, the problem appears to be primarily one of reduced bone formation (13), although increased resorption has also been suggested (33,48). Reduced osteoblast number and activity have been found in humans with Type 1 diabetes, as indicated by low serum osteocalcin (13). A reduction in osteocalcin is especially prevalent in adolescents (15). This reduction in osteoblasts and their activity appears to be the primary cause of reduced bone formation (13). Bone formation rate may be reduced by as much as 25% following a significant reduction of mineralizing surface and mineral appositional rate in cancellous bone (37). According to Verhaeghe (10), the onset of diabetes represses skeletal growth, resulting in less bone mass. Lower BMD may then result from this lower build-up of bone mass (49), although normal amounts of mineral and matrix have been reported (10).

In insulin deficient BB rats, the number of osteoblasts, along with osteoid formation and bone mineral apposition rate are commonly reduced, while bone resorption is relatively less reduced (13). One study found osteoclast surfaces to be at the low end of the normal range, while the eroded surface of the bone was at the high end of normal, suggesting that resorption occurred, but was

not followed by formation (37). In another study, osteoblast and osteoid surfaces, along with daily bone mineral apposition rate were found to be 10 to 20% of that found in non-diabetic controls, while osteoclast surface showed a small reduction only (10,50). In addition, periosteal seam width and periosteal mineralization front width may be lower than controls by as much as 50%. Such changes in bone turnover are apparent after as little as 10 days duration of diabetes in rats (12), although in the short-term no differences are apparent between BB diabetic and control rats in terms of relative bone volume (50). During skeletal growth, this reduced bone formation and matrix apposition at the cellular level leads to diminished bone formation and growth at the tissue level compared to normal. If osteoid indexes are only modestly diminished, matrix apposition, and not mineralization appears to be the problem (37).

2.6 Dual-energy X-ray Absorptiometry

Dual-energy X-ray absorptiometry (DXA) has become a frequently used method of measuring bone mass. The DXA machine measures the attenuation of the x-ray beams that cross the tissue to determine the amount of bone mineral, lean mineral free mass, and fat mass in the whole body (51-2). In terms of bone, BMC and bone area (BA) are measured by DXA, and from them, bone density is calculated. Since the device is limited to a 2-dimensional display, however, determination of density provides area' density only (53). This measure is strongly influenced by height, and therefore must be adjusted for size. In comparisons with 3- and 4-component models, and

with bioelectrical impedance analysis, DXA has been found to be reliable and accurate (51-2,54). This is true for both genders, various races, and for a range of body sizes and musculoskeletal development (54). Ellis et al. measured body composition of young pigs with a weight range representative of the weight of 1 to 12 year old children (55). Precision was measured through repeat scans, and accuracy through wet chemical analysis of the pig carcass. While they did recommend that in comparing children of the same age measurements be adjusted for body weight, these researchers suggest that DXA is acceptable for measurement of body composition in children due to its precision and ease of use. Precision of DXA has been reported to be as low as 0.5% (56) with an accuracy of about 4% (57). Differences in measurement have been found between machines manufactured by different companies, probably due to varying data collection modes (pencil beam or array beam) (51,54). The radiation dose received during a whole body scan is well below the maximum limit (57). On the whole, DXA is a safe, simple, and relatively fast method of performing bone analysis, and is appropriate for use in children.

2.7 Growth

Linear growth, which is a reflection of bone growth, is affected by Type 1 diabetes. One group of researchers reported greater height in diabetic children compared to controls for a minimum of 3 years before onset of diabetes. This height difference peaked 1 to 2 years before onset of the

disease and then levelled off (58). Another study demonstrated that at diagnosis, and at the start of puberty, girls with Type 1 diabetes were taller than control subjects (59). Also, their bone age was more advanced than their chronological age (11.48 ± 1.01 years versus 10.93 ± 0.86 years). At final height, however, the girls with Type 1 diabetes were shorter than girls without Type 1 diabetes. Boys with Type 1 diabetes were also taller than controls at time of diagnosis, but height at onset of puberty and final height was similar to controls. Bone age in boys was somewhat less than chronological age (11.75 ± 1.07 years versus 12.1 ± 0.71 years). One reason for these gender differences may be that boys with Type 1 diabetes have a growth spurt similar to that of control subjects, while in girls with Type 1 diabetes the growth spurt is significantly lower than that of controls. However, age at onset appears to affect linear growth, and may do so differently in females than in males. Adult patients with prepubertal onset were significantly shorter than those with pubertal onset (60). In addition, standardized height was significantly lower in males with pubertal onset compared to females who developed diabetes during puberty, while height z-scores were similar in males and females with prepubertal onset. These differences may be explained by the timing of the growth spurt which occurs earlier in females than in males.

Weight is also affected in children with Type 1 diabetes. Results of the DCCT showed that adolescents receiving intensive therapy were twice as likely as those on conventional therapy to become overweight (29). Other

researchers have also reported a tendency for overweight in children with Type 1 diabetes (61-2). Standardized scores for weight increased over the 10 years following onset, with the greatest increases occurring during and after puberty. This increased weight gain may be related to metabolic control, or may be due to hormonal changes which affect growth (61).

2.8 Development of Peak Bone Mass

Since peak bone mass (PBM) is established mainly during the second decade when genetic factors affecting bone growth and development are strongest (63), individuals who develop diabetes in childhood or early adolescence may be at increased risk of diabetic osteopenia. In normal growth, the critical period for attainment of PBM occurs between ages 9 to 17. Approximately 45% of adult skeletal volume is formed during puberty, corresponding to a gain in bone density of 7 to 8% per year (43). Kordonouri et al. (64) compared children with prepubertal onset of diabetes to those with pubertal or post-pubertal onset of the disease. The course of the disease tended to be milder in the latter groups. For example, deterioration of metabolic control which occurred in the prepubertal group did not occur in the pubertal and post-pubertal groups; long-term control was also better in the pubertal and post-pubertal groups. In addition, exogenous insulin requirement was higher in the prepubertal group. In spite of these findings, values for bone age difference, calculated as the difference between bone age and chronological age, were similar for the prepubertal group compared to the

pubertal and post-pubertal groups. McNair (26) reported that patients who were diagnosed with Type 1 diabetes before the age of 20, and therefore had not completed bone mineral storage, had lower BMC than those with onset after age 20. Another group of researchers (41) found abnormalities in the endosteal surface suggesting inadequate bone growth. However, diminished bone growth alone does not account for the lower bone mass seen in diabetic osteopenia since differences in total bone mass are much greater than differences in bone length and diameter (13). Bone ossification and mineralization are also reduced and delayed (8), affected in part by metabolic control.

2.9 Factors Affecting Bone Development in Children with Type 1 Diabetes

The development and progression of diabetic osteopenia is multifactorial. While insulin deficiency itself is known to be a primary cause (1,7-8,13-4,24), other important factors include levels of IGF-1 (15,24,64-5), vitamin D (18-9), calcium (7,10), and parathyroid hormone (PTH) (20-3,66). In addition, degree of metabolic control (6,24) and duration of diabetes (2-3,26) play a role in the extent to which bones are affected by diabetes. More recently, dietary fat, in the form of n-3 and n-6 polyunsaturated fatty acids has been shown to have an effect on bone (8,30).

2.9.1 Metabolic Control

Several studies have shown that good metabolic control is linked to normal bone formation in Type 1 diabetes (4,6,21,33). Patients with poor metabolic

control were found to have reduced formation of osteoblast cells (24). Non-enzymatic glycosylation and collagen cross-linking have also been correlated with glycemic control, as has bone mineral composition (6,21). McNair et al. (6) performed a cross-sectional study of 215 patients with Type 1 diabetes. Using photon absorptiometry, they measured BMC in both forearms. Subjects were stratified into groups based on several indicators of metabolic control: serum C-peptide (an indicator of endogenous insulin secretion), insulin dose, and fasting blood glucose. The researchers found BMC to be lower by 21% in diabetics with poor metabolic control compared to sex- and age-matched controls, while those with good metabolic control had only 1% less BMC compared to the controls. However, maintenance of glycemic control may become difficult with the onset of puberty. Even in adolescents without Type 1 diabetes, insulin sensitivity and fasting insulin concentrations change substantially during pubertal development (16). Insulin resistance increases during puberty, reaching a peak near the end of pubertal development; this insulin resistance is greater in adolescents with Type 1 diabetes. Both insulin dose and HbA_{1C} have been shown to increase during pubertal growth (59,67). As previously mentioned, the DCCT (29) showed that intensive insulin treatment significantly improved glycemic control, measured by HbA_{1C}, compared to conventional treatment. However, even adolescents on intensive therapy had poorer metabolic control than a corresponding group of adults. As a result of poor control, net bone resorption may be increased in

individuals with Type 1 diabetes. The effect of this increased resorption on bone depends on when problems with control occur. Before closure of the growth plate, poor glycemic control impairs bone growth, while after closure, bone consolidation is diminished, leading to bone loss (37).

2.9.2 Duration of Disease

Another factor that can be taken into consideration when looking at diabetic osteopenia is duration of the disease. In the study by Levin et al. (2), those who had been diagnosed with Type 1 diabetes within the 5 years previous to the start of the study had lower bone mass compared to controls than those who had been diagnosed 5 to 10 or more than 10 years prior to the study. From these results, the researchers concluded that bone mass was already lower at time of clinical onset of Type 1 diabetes. In the work by Shore et al. (5), BMC and BMC/BW were significantly lower than controls regardless of whether the children had been diagnosed with Type 1 diabetes less than 1 year or more than 1 year before the start of the study. According to Melchior, Sorensen and Trop-Pedersen (3), appendicular bone mass is lower by 5 to 20% within the first few years of onset of Type 1 diabetes. McNair (26) found that the initiation of osteopenia corresponded with the onset of Type 1 diabetes, with a significant difference in BMC becoming apparent after 2 years. Bone mass remained low, but stabilized 3 to 5 years after onset of the disease. A follow-up study by McNair et al. (34) measured change in bone mass in C0 adults with Type 1 diabetes over a period of 1½ years. In order to relate

change in bone mass to duration of diabetes, subjects were grouped according to their median duration of the disease at the time of the study. Bone mass was significantly higher in patients who had had diabetes for less than 7 years compared to those who had had diabetes for longer than 7 years; however, bone loss over the 1½ years was 3 times higher in the former group than in the latter.

2.9.3 Insulin

Insulin plays an important role in bone formation. Researchers have found that insulin promotes amino acid uptake in bone, stimulates collagen synthesis, and enhances mesenchymal cell production and endochondral bone growth (1-2). Insulin deficiency results in a reduction of both growth plate thickness and longitudinal growth (13). Rats with streptozotocin-induced diabetes showed reduced and delayed cartilage formation due to diminished generation of mesenchymal cells (8). Collagen metabolism is also affected, since incorporation of proline into protein is stimulated by insulin (14). Reduced collagen synthesis may appear within 1 week of onset of Type 1 diabetes (12). Primarily type I collagen, the main structural protein of bone tissue, is affected (24). In addition, insulin receptors are present on osteoblastic cells, and insulin is reported to aid in the growth of an osteoblast-like cell line (7). According to Hough, insulin therapy normalizes histometric and biochemical markers of bone formation, resorption, and growth (1). In other words, insulin deficiency has a negative effect on the normal bone formation process, while

insulin repletion tends to have a positive effect (4). However, bone mineral loss was found to be 3 times greater in individuals with no residual beta-cell function compared to patients with unchanged or elevated insulin secretion, in spite of higher insulin dosage in the former patients (34).

Insulin has also been linked with increased intestinal absorption of calcium (2). This is significant since calcium absorption has been shown to be reduced in stz-injected and BB diabetic rats (8-9). Humans with Type 1 diabetes, on the other hand, have elevated rates of calcium absorption compared to non-diabetics (8). In addition, urinary excretion of calcium is elevated with elevated glucosuria and elevated hyperglycemia (21,66). Low bone turnover may contribute to this diabetes-induced hypercalciuria: although intestinal absorption of calcium is enhanced, the skeleton of the diabetic rat may not retain absorbed calcium, leading to urinary calcium excretion; insulin therapy corrects the defect (18). Insulin may also act directly on bone formation by influencing calcium transport at the site of bone formation (8). Since calcium is known to play a role in development and maintenance of bone mass, insulin can be seen to have both direct and indirect effects on bone.

2.9.4 Insulin-like Growth Factor-I

IGF-I is a growth hormone dependent polypeptide that functions in 3 ways; it mediates the growth-promoting action of growth hormone, it stimulates mitosis, and it regulates metabolism in a manner similar to that of insulin (17). Low insulin levels are linked to reduced insulin-like growth factor I (IGF-I)

activity. Insulin is an important regulator of IGF binding proteins (IGFBP), to which IGF-I is bound as it is transported in circulation. IGFBP regulate the bioavailability of IGF-I and may either inhibit or stimulate its action (17,59). IGFBP-3, the principal regulator of IGF-I actions, and IGFBP-5 may act in a stimulatory fashion, while IGFBP-I and IGFBP-4 appear to inhibit activity (20). Levels of IGFBP-3 have been reported to be both elevated and reduced in diabetes (17,20,68). Strasser-Vogel et al. observed elevated serum IGFBP-3 concentrations in both prepubertal and pubertal subjects with diabetes compared to reference values (17). This is in contrast to the findings of most researchers who have reported reduced IGFBP-3 in children with Type 1 diabetes (20,68).

Normally, serum concentration of IGF-I increases during childhood and puberty as a result of increased secretion of growth hormone, but in children and adolescents with diabetes IGF-I levels are reduced (15-7). Insulin therapy helps prevent this reduction. Compared to treated patients with diabetes, newly diagnosed individuals who had not yet received insulin treatment had lower IGF-I levels. Stage of pubertal growth also seems to affect IGF-I concentrations. While concentrations were reduced in prepubertal diabetic patients, they were normal in pubertal diabetics. This discrepancy may be the result of sex steroids which are known to stimulate IGF-I secretion (17).

Like insulin, IGF-I has both direct and indirect effects on bone. In individuals both with and without Type 1 diabetes, IGF-I is positively correlated

with bone formation markers, in particular plasma osteocalcin (15,64). Consequently, reduced IGF-I concentrations such as are seen in Type 1 diabetes are linked with reduced bone formation. Although serum concentrations of IGF-I are primarily indicative of hepatic synthesis and secretion, there is a relationship between IGF-I and osteoblasts. Osteoblasts also secrete IGF-I, which in part regulates osteoblast proliferation and maturation (15,24). In fact, IGF-I is seen as one of the primary osteoblast activators (69). It has been suggested that increased bone formation resulting from IGF-I treatment may be caused by increased osteoblast activity rather than increased number (64). In addition, IGF-I has been shown to play a role in stimulation of epiphyseal cartilage width; it also stimulates chondrocytes in the growth plate, resulting in longitudinal bone growth (70). In terms of BMD, a correlation with serum IGF-I has been shown in some studies (20), but not in others (65). One possible explanation for a lack of correlation is that serum IGF-I is either not a good indicator of IGF-I levels in bone, or it is not as important functionally as IGF-I formed in the bone (65,70).

2.9.5 Vitamin D

Alterations in vitamin D metabolism have been suggested as one possible cause of diabetic osteopenia (18-9). Vitamin D plays an important role in bone metabolism through regulation of calcium homeostasis. The active form of vitamin D, 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) has been shown to stimulate bone remodelling, possibly mediated by IGF-I (69). Treatment with

IGF-I (8μg kg^{-1} m^{-1}) was shown to increase levels of total serum $1,25(OH)_2D_3$ (64). IGF-I is also involved in regulation of 25-OHD-1α-hydroxylase, directly stimulating renal production via a calcium-dependent pathway, independently of PTH (64,71). Conversely, patients with osteoporosis treated with 1 or 2 μg/day of $1,25(OH)_2D_3$ had increased plasma levels of IGF-I, along with increased serum osteocalcin levels (69). Diabetic children treated with 1-alpha-hydroxy vitamin D_3 (1-a-OHD_3) had significantly increased bone density after 6 and 12 months (72). In spontaneously diabetic rats infused with $1,25(OH)_2D_3$ at levels of 15 or 30 ng/100g body weight per day, plasma osteocalcin levels were increased slightly compared to spontaneously diabetic rats infused with saline (73). However, mineral apposition rate was not affected by the infusion. Osteoblast, osteoid, and osteoclast surfaces, which were considerably lower than those of control rats, were also not affected by the treatment. It appears that sensitivity of osteoblasts to vitamin D depends on age of the individual, dose and route of administration of vitamin D, and length of vitamin D deficiency (69).

During the pubertal growth spurt, serum concentrations of $1,25(OH)_2D_3$ are increased, while 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) are decreased in comparison. Children with Type 1 diabetes, however, have reduced levels of plasma $1,25(OH)_2D_3$, and elevated levels of $24,25(OH)_2D_3$. Unbound plasma $1,25(OH)_2D_3$ is also reduced in diabetic children compared to age and sex matched controls (19). An increase in total, but not unbound plasma

$1,25(OH)_2D_3$ concentrations may indicate vitamin D resistance at the duodenal level (9). Resistance at the osteoblast level has also been suggested (10). In diabetic rats, both renal production and plasma levels of $1,25(OH)_2D_3$ levels are low (23). In addition, plasma osteocalcin was not increased in diabetic rats injected with $1,25(OH)_2D_3$, but was increased in injected nondiabetic rats.

2.9.6 Calcium

Altered calcium metabolism has also been implicated as a cause of diabetic osteopenia (7). The skeleton plays an active role in calcium metabolism, with 99% of absorbed calcium being taken up by the skeleton, most of it going to the trabeculae (74). Calcium metabolism, like skeletal modelling and growth, occurs at varying intensities depending on age, with adolescents having increased calcium requirements (75). Using calcium balance techniques, a significant positive relationship was shown between intake and retention of calcium (76); that is, high calcium intake was associated with high calcium retention and high skeletal retention of calcium in the young. With high calcium consumption, pubertal females retained 200 to 500 mg of calcium per day. However, calcium intakes in adolescence are frequently insufficient. Since there appears to be a direct effect of dietary calcium on skeletal retention, and since calcium is known to be a primary factor in attainment of peak bone mass, inadequate calcium intake may restrict longitudinal bone growth and adult height, and may result in lower bone density. Children and adolescents should therefore consume adequate

calcium to increase skeletal mass (76). In one study, bone mass in individuals consuming 450 mg of calcium per day was significantly lower than bone mass of individuals consuming 1200 mg of calcium per day; this difference in bone mass was established by age 30 (43). In another study, BMD in a calcium supplemented group was 1.4% higher on average than in the placebo group (77). However, these differences were significant for prepubertal pairs only. Lower osteocalcin concentrations were also found in the supplemented group, indicating a lower rate of bone turnover. Another longitudinal study showed higher bone mass and bone density in adolescents with high calcium intake (greater than 1600 mg per day) compared to adolescents with low calcium intake (less than 850 mg per day) (76).

While PTH and other hormones control calcium excretion, urinary calcium loss is elevated by high intakes of dietary sodium (78). In fact, dietary sodium has a larger influence on urinary calcium excretion than dietary calcium intake. In adolescents without diabetes, renal calcium excretion remains steady at approximately 127 mg per day, regardless of level of calcium intake (43). However, net absorption of calcium, which tends to be increased during periods of growth, increases further with increased intake, indicating a strong correlation between calcium retention and absorption. Since most of the calcium that is absorbed is retained, the skeleton may become saturated when intakes are adequate, thus supporting development of PBM. In adolescents with diabetes, on the other hand, while duodenal calcium absorption may

increase, elevated renal calcium excretion is common (66). This increased excretion of calcium may prevent skeletal saturation. In rats, diabetes results in delayed mineralization and lower amounts of calcium being deposited in bone. While the calcium content of the tibia increased in nondiabetic rats, diabetic rats showed no gain in bone calcium content over 3 weeks (10). It has been suggested that increased renal excretion may be due to a primary defect in bone formation which results in increased plasma calcium and subsequent reduced parathyroid function. Duration of diabetes and degree of metabolic control appear to play a role in this dysfunction.

2.9.7 Parathyroid Hormone

Along with vitamin D, PTH is a primary hormone regulating intestinal absorption, renal resorption, and bone turnover of calcium (79). Generally, PTH is secreted when extracellular calcium concentration decreases. The hormone acts to increase calcium levels through direct stimulation of bone and renal resorption of calcium, along with increased $1,25(OH)_2D_3$ production which in turn increases intestinal absorption (79). Osteoblast and kidney cell membranes contain receptors for PTH which activates cyclic adenosine monophosphate (cAMP) production. This in turn results in the actions of PTH in the intestine, kidney, and bone (79). Although osteoclasts are the cells involved in bone resorption, they have not been found to contain PTH receptors. PTH may stimulate resorption by increasing space available for osteoclasts through alteration of osteoblast shape. Another proposed

mechanism is that PTH stimulates release of a messenger from osteoblasts that enhances osteoclast activity (79).

Some researchers have found higher PTH levels in those with diabetes, suggesting mild secondary hyperparathyroidism (20). Other studies, however, have shown that individuals with Type 1 diabetes have reduced serum concentrations of N-terminal and intact PTH (14,22). This relative hypoparathyroidism may be correlated with duration of the disease and degree of metabolic control (22). PTH levels were reported to be lower in individuals with poor metabolic control and to improve, although not significantly, with improved blood glucose control (66). Besides metabolic control, a link also exists between PTH and IGF-I, with IGF-I mediating the action of PTH in bone cells (22).

In diabetic rats, the ability of PTH to stimulate formation of $1,25(OH)_2D_3$ is reduced (23). This may explain why renal $1,25(OH)_2D_3$ production is low even when serum PTH levels are normal. As a result of low renal $1,25(OH)_2D_3$, serum levels may also be reduced. Ultimately, this may be the cause of reduced calcium-binding protein and reduced intestinal calcium absorption seen in diabetic rats (23). Insulin has been shown to stimulate cAMP production, resulting in a reversal of the low cellular response to PTH (22).

2.9.8 Dietary Fat

Prostaglandins, which are synthesized from polyunsaturated fatty acids (PUFAs), are known to stimulate both bone formation and bone resorption

(30,80-2). Prostaglandin E_2 (PGE_2), derived from arachidonic acid (AA), appears to have the strongest effect. PGE_2 has been shown to activate periosteal and endocortical surface formation of long bones by recruiting and stimulating osteoprogenitor cells in the periosteum and mesenchyme (83). The biphasic effects of PGE_2 are mediated by length of exposure, the type of cell involved, and especially by PGE_2 concentration (80,82). Collagen synthesis was reduced in fetal rat calvariae exposed to high concentrations of PGE_2, while those exposed to moderate concentrations showed a delayed elevation in collagen synthesis (80). PGE_2 has also been shown to increase osteoblastic synthesis of IGF-I, likely as a result of increased intracellular cyclic adenosine mono-phosphate (cAMP) levels (80).

While PGE_2 has a strong influence on bone remodelling, other eicosanoids may be produced in bone which affect formation and resorption (82). Although much is known about the beneficial effects of n-3 fatty acids in heart disease and their role in immunosuppression (84), limited research has been conducted regarding the effect on bone of diets high in n-3 fatty acids. PGE_3 is derived from eicosapentaenoic acid (EPA), a metabolite of the n-3 fatty acid α-linolenic acid. While PGE_3 stimulated bone resorption in bone organ culture (81), growing rats supplemented with EPA had reduced bone resorption compared to those fed higher amounts of n-6 PUFA (85). In addition, n-3 PUFA may also activate bone formation (86). One group of researchers found that compared to chicks fed diets containing soybean oil (rich in n-6 PUFA),

chicks fed diets containing menhaden oil (rich in n-3 PUFA) had higher bone volume, trabecular number, and bone formation rates, and lower trabecular separation and mineralization lag time (30). In another study, EPA prevented loss of bone weight and bone strength in ovariectomized rats on low calcium diets (86). Although formation of PGE_3 from EPA does not occur as readily as PGE_2 formation from AA, there is a preference for n-3 fatty acids over n-6 fatty acids by the converting enzyme system. As a result, n-3 supplementation may affect the n-6 pathway, leading to less PGE_2 being synthesized (85,87). This may reduce the concentration of PGE_2 from levels at which it promotes bone resorption to levels at which bone formation predominates. Other possible mechanisms by which EPA affects bone have been suggested, including activation of bone formation by EPA itself, and augmentation of intestinal calcium absorption or reduction in calcium excretion (86). In fact, EPA has been shown to be negatively correlated with urinary calcium excretion (87-8). IGF-I concentrations are also affected by n-3 PUFA. At 14 and 21 days of age, chicks on a diet high in n-3 fatty acids (menhaden oil), had higher levels of plasma IGF-I than chicks on a diet high in n-6 fatty acids or a diet high in saturated fatty acids. At day 28, cortical bone and epiphyseal cartilage IGF-I concentrations were also highest in the menhaden oil group; however at 28 days of age, plasma concentrations of IGF-I remained unchanged in chicks on the menhaden oil diet, while levels in chicks on the other diets had increased (82).

2.9.9 Physical Activity

It is well accepted that weight-bearing physical activity contributes to bone mass (89-95). This effect may be due either to compressive gravitational forces or to the muscular pull on bone where muscle is attached to the periosteum (95). Both of these stresses stimulate osteoblast activity, resulting in increased BMC, bone width, and BMD (92,94-5). Ruiz et al. (89) measured BMD of the lumbar spine and upper femur in healthy children using DXA. The children were divided into two groups based on level of activity (1 to 3 hours per week versus 3 to 12 hours per week). Physical activity contributed significantly to BMD, especially in girls and during puberty, although clinical significance of the contribution was low. Gunnes and Lehmann also investigated the effect of physical activity on BMD in children (90). These researchers measured BMD in the non-dominant forearm of the children at baseline and 1 year follow-up. They found significantly higher gain in BMD in physical active children compared to sedentary children. However, they found no correlation between gain in BMD and physical activity in adolescent girls, suggesting that the effect of physical activity on gain in BMD does not extend past puberty, at least in females. While BMD may not continue to increase with increased physical activity post-puberty, the benefit of physical activity on BMD appears to remain. Madsen et al. found that female athletes 18 to 26 years of age had significantly higher BMC and BMD than height- and age-matched female controls (94). While many investigations into physical activity

and bone development have been carried out on healthy children, there appears to have been none conducted on children with diabetes.

3. Rationale, Hypotheses and Objectives

Previous research has established a solid base of information with respect to diabetic osteopenia. Children with Type 1 diabetes have lower bone mass and bone density than children of the same age and sex without the disease (5,35); mineralizing surface and apposition rate are also lower in children with diabetes (37,41). These changes in bone are most severe within 5 years of onset of the disease (26,34). Although differences have also been seen in cortical bone, it is primarily trabecular bone that is affected (44,47). Studies in both children and animals have shown that lower BMC and BMD may stem from reduced bone formation in relation to bone resorption which in turn may be a result of reduced osteoblast number or activity (13,15). In rats, diabetes has also been shown to impede bone mineralization (8,11) and reduce bone matrix formation and apposition rate (12). The pathogenesis of diabetic osteopenia is multifactorial. It includes reduced levels of plasma IGF-I and $1,25(OH)_2D_3$, elevated renal calcium excretion, and a reduced effectiveness of PTH in stimulating $1,25(OH)_2D_3$ formation (15-7,19,23,66). Level of residual insulin production, degree of metabolic control, and duration of disease also play a role (4,6,21,26,33-4).

Although much is known about diabetic osteopenia, numerous questions remain. Intensive management is known to reduce the risk of many of the complications associated with Type 1 diabetes; however, its effect on bone has not been investigated. Also, while many researchers have measured

bone development in children, few have done so in children with diabetes using dual-energy x-ray absorptiometry (DXA). This project will therefore investigate and compare bone development in children with and without Type 1 diabetes using DXA, and examine the effect of intensive management compared to conventional management. While the effects of physical activity on bone are well established (89-95), few researchers have examined the effect of physical activity on bone in children with diabetes. Thus the relationship of physical activity with bone development will be examined in children both with and without Type 1 diabetes. In addition, relationships between dietary factors such as phosphorus, vitamin D, and in particular PUFA, with BMC, BA, and BMD are not well established; these will also be investigated. While dietary fish oil appears to inhibit bone resorption (85), its effect on bone formation (both absolute and relative to resorption) is not as clear. Whether or not fish oil has similar effects on bone in insulin deficiency compared to insulin sufficiency also not been established. Bone resorption, formation, and mineral content, as well as other indicators of bone metabolism will therefore be investigated and compared in insulin sufficient and insulin deficient animals following consumption of fish oil.

Hypotheses:

1. Type 1 diabetes results in lower whole body BMC, BA, and BMD during the growth period.

2. In insulin deficiency, consumption of dietary fish oil elevates bone formation in relation to bone resorption through a PGE_2-dependent pathway.

Objectives: In growing children age 8 to 18 years:

1. To determine the relationship between Type 1 diabetes, its management and control and indices of bone mass.

2. To determine the relationship of dietary calcium, vitamin D, phosphorus and PUFA with BMC, BA, and BMD.

3. To determine the relationship of physical activity with BMC, BA, and BMD.

In diabetes-induced growing rodents:

1. To determine the effect of dietary fish oil consumption on mineral content and markers of bone formation and resorption.

4. Clinical Study: Methods

4.1 Rationale for Nutrient Intake Assessment: Common methods for assessing nutrient intake include diet histories, food frequency questionnaires, food records, and 24-hour recalls. As with adults, problems with dietary assessment in children include response bias, recall bias, and difficulty assessing portion size (96). In addition, limited knowledge of food and food preparation, and unfamiliarity with foods or ingredients in mixed dishes restrict accuracy of information collected from children (97). Twenty-four-hour recalls are relatively fast and easy to conduct, and despite the aforementioned problems, have been shown to be accurate when used with children or adolescents (96). Food records, on the other hand, are the most suitable means of assessment for individuals, in spite of the high respondent burden associated with them. Since the recording period follows shortly after eating, food records limit recall bias, and have been shown to be associated with fewer missing and phantom foods (foods reported but not actually eaten) (97). Thus food records are also appropriate for use with children and adolescents. In order to maximize the accuracy of the dietary information obtained from each subject, both a 24-hour recall and a 3-day food record were used in this research; however, for the purposes of analysis, only the 3-day food records were used.

4.2 Rationale for Physical Activity Assessment: Several methods are available to assess physical activity. These include physical activity recalls,

activity surveys, activity rating scales, direct observation, and mechanical or electronic monitors. For this study, self-reports were a feasible method of determining activity level in our subjects. Little information is available on the use or accuracy of a 24-hour activity recall, although a 7-day physical activity recall has been found to be a reliable and valid means of assessing physical activity in children and adolescents (98). Although children are able to recall physical activity for up to 7 days, the optimum time frame is 24 hours (98). Our use of a 24-hour activity recall was therefore appropriate; on its own, however, its reliability and validity was questionable. For that reason, a modifiable activity questionnaire for adolescents (99) was used in addition to the recall. This questionnaire has been shown to be reliable and valid for use with adolescents (99), in spite of a relatively heavy response burden.

4.3 Rationale for Bone Mass Assessment: Dual-energy X-ray absorptiometry (DXA) has become a frequently used method of measuring bone mass. Compared to other methods, DXA emits a low radiation dose (57), has excellent precision (56), and is a simple and fast means of analyzing bone. To determine bone mass, DXA measures BMC, which is the amount of mineral in the bone. Both BMC and unmineralized collagen fibres contribute to bone mass, but since BMC is the largest contributor, and is the measurement provided by the DXA scan, BMC was used in this study as a measure of bone mass. Total BMC depends on both bone area and bone density (how tightly packed the mineralized bone crystals are); therefore, both of these

measurements were also used as indicators of bone development in this study. While either whole body or regional scan can be performed, whole body measurements were chosen for this study due to the difficulty of interpreting regional scan. This difficulty in interpretation results from changing bone geometry during growth .

4.4 Ethical Approval : This study was approved by the University of Manitoba's Faculty of Medicine Committee on the use of Human Subjects in Research.

4.5 Recruitment: Recruitment of subjects took place over a period of 14 months. Children with Type 1 diabetes were recruited for the study from the DER-CA with the help of the dietitian, while children for the control group were recruited from the patient base of the Manitoba Clinic, with the aid of doctors at the clinic. In addition, the study was advertised both through the newsletter published every 3 months by the DER-CA and posters displayed at the clinic. Children who had had Type 1 diabetes for at least 4.5 months were included in the study, provided they did not have another chronic disease such as celiac disease, hypothyroidism, Addison's disease, Down's Syndrome, or cystic fibrosis. Children with normal growth and who were free of any major disease were included in the control group. For both the control and the diabetes groups, subjects were between the ages of 8 and 18 years. A lower age limit of 8 years of age was imposed based on the manufacturer's specifications that the DXA scan is most accurate in individuals weighing more than 30 kg. It was

assumed that most children 8 years of age and older would weigh at least 30 kg. Informed written consent from one parent of the child preceded participation in either group.

4.6 General Procedures: Children made one visit to the Manitoba Clinic. During this visit, height and weight were measured, a full body DXA scan was performed, a random a.m. urine sample was taken to measure urinary glucose, creatinine, calcium and phosphorus, and a 24-hour food and activity recall was conducted. In addition, children with diabetes were asked to provide their blood glucose levels and the type and amount of insulin injected, as well as the number of injections the day prior to their clinic visit. Following the visit, a 3-day food record (**Appendix A**) and a physical activity questionnaire (**Appendix B**) was mailed to each subject for further assessment of diet and physical activity. The most recent HbA1c values for each subject were provided by the DER-CA.

4.7 Hemoglobin A1c: HbA1c was measured at the Winnipeg Health Sciences Centre Laboratory using a boronate affinity binding assay. The assay measures percent glycated hemoglobin (Ghb), as well as percent HbA1c (100).

4.8 Height: Height was measured using a Harpenden stadiometer. The child stood facing the researcher, with feet flat on the floor, back flat against the wall, shoulders square, and head level. The bar of the stadiometer was then lowered to rest firmly on top of the child's head, and the height measurement

was read to the nearest 0.1 cm. The child stepped away from the wall and then back into position for a second measurement. Both measurements were taken with the child in sock-feet.

4.9 Weight: A standard upright balance was used to weigh each child. The child was weighed fully clothed, with only shoes and outerwear removed. With the child standing on the balance, the arm of the scale was balanced by sliding the weight to the appropriate notch. The weight measurement to the nearest 0.1 kg was then read. Only one weight measurement was taken.

4.10 Body Mass Index: To assess appropriateness of weight, BMI was calculated as weight/height^2 (kg/m^2).

4.11 Diet Information: Twenty-four hour recall - The child was asked to verbally list all food and beverages consumed during the previous day. Children who had difficulty with recall were aided by their parents. Food models and measuring cups and spoons were used by the researcher to determine quantities. For certain items not measurable by measuring cups or spoons such as baked goods or pizza slices, the child was asked to draw a picture of the actual size of the item consumed. Children were also asked about their use of vitamins or regular medications. To increase consistency of data collection, all diet information was collected by one individual.

Three-day food record - Following their visit to the clinic, children were provided with a 3-day food record in which they were asked to record all food and beverages, along with quantities consumed over 3 (non-consecutive) days

(**Appendix A**). As with the 24-hour recall, children were asked to draw a picture indicating the size of items such as baked goods. Self-addressed, stamped envelopes were provided to return the food records to the researchers.

Diet information was then entered into a computerized nutrient analysis program (E. Warwick, PEI, Canada), and analyzed for calcium, phosphorus, vitamin D, and PUFA content.

4.12 Physical Activity: Children were asked to verbally list all physical activity of the previous day (including activities such as walking, bike riding, active play at recess, phys ed class, and other sports and activities), and to state whether or not this was a typical day of activity. If the day was reported to be atypical, children were asked to describe a typical day of activity. As with the food recall, children were aided by their parents as necessary.

Following their clinic visit, children were provided with a physical activity questionnaire measuring type and amount of physical activity over the past year (**Appendix B**). Total hours of activity per week were calculated for the purpose of analysis.

4.13 Dual-energy X-ray Absorptiometry Scan: BA and bone composition were assessed by a whole body DXA scan (Hologic QDR4500W, Hologic Inc, Waltham, MA). Whole body measurements were used to determine total BMC, BA, and BMD of all subjects. Because DXA measures areal rather than volumetric density, BMC measurements were adjusted by calculating

BMC/height (g/cm) and BMC/weight (g/kg). Age is positively correlated with BMC, BA, and BMD; therefore, standardized z-scores were calculated for all three indices. In addition, BA for height and BMC for BA were calculated as standardized z-scores, using two separate data sets (101-2).

Children wore either shorts and a t-shirt or a hospital gown for the scan. All metal objects (rings, bracelets, earrings, etc.) were removed whenever possible. The child was positioned on his back, with arms not touching his body, fingers spread apart, knees unbent, and feet/toes pointed inward. In some cases, the child's feet were taped together to facilitate holding this position during the scan. Children were instructed to lie as still as possible, and to close their eyes to avoid retinal damage which could result from staring into the x-ray beam.

4.14 Calcium and Phosphorus: A random a.m. urine sample was assayed for calcium and phosphorus content as follows. Urine (0.25 mL) was placed in a glass test tube to which 0.5 mL concentrated HNO_3 (Fisher Scientific, Nepean, ON) was added. The tube was left in the fume hood overnight to allow the sample to dissolve completely. When the contents of the tube were clear in appearance, 9.5 mL of deionized water was added. The mixture was then transferred to a 20 mL scintillation vial for storage until calcium and phosphorus content were analyzed via emission spectrometry (Varian Liberty 200 ICP, Varian Canada, Mississauga, ON).

4.15 Glucose: Urinary glucose was measured using an adapted colourimetric microassay (Procedure number 510-A, Sigma Diagnostics, Inc., St. Louis, MO). The procedure is based on 2 coupled enzymatic reactions:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

$$H_2O_2 + \text{O-Dianisidine} \xrightarrow{\text{Peroxidase}} \text{Oxidized o-dianisidine}$$

(Colourless) (Brown)

The intensity of the colour that develops is directly proportional to the concentration of glucose in the sample.

Procedure: Random a.m. urine samples were diluted 2- to 1000-fold with deionized water, and the mixture was vortexed for 5 to 10 seconds to ensure uniformity. A standard curve was developed using 3 concentrations of standard: 5mg/dL (5 μL of 100 mg/dL standard + 95 μL deionized water), 10 mg/dL (10 μL of 100 mg/dL standard + 90 mL deionized water), and 15 mg/dL (15 μL of 100 mg/dL standard + 85 μL deionized water). Twenty μL of deionized water (blank), standard, or sample were pipetted in triplicate into the designated well of the microplate. Enzyme-colour reagent was made by combining 1 capsule peroxidase glucose oxidase (PGO) enzymes dissolved in 100 μL deionized water with 1 vial o-Dianisidine dihydrochloride reconstituted in 20 μL deionized water. Using a multi-channel pipet, 200 μL of the enzyme-colour reagent solution was added to each well. The microplate was covered

with foil and incubated at room temperature (18-26°C) for 45 minutes. Using the blank as a reference, the absorbance of the standard and samples were read at 450 nm on a SPECTRAmax 340 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). The standard curve was formed by plotting concentration of the standards against their absorbance. The concentration of the samples was calculated from this standard curve.

4.16 Creatinine: Urinary creatinine was measured using an adapted microassay (Procedure number 555, Sigma Diagnostics, Inc., St. Louis, MO). Creatinine, produced in kidney, liver, and pancreas, travels in the blood to organs such as muscle, where it is phosphorylated and stored. Muscle is also a storage site for free creatine which is converted to creatinine daily and excreted. The amount of creatine converted to creatinine is proportional to muscle mass, and in individuals with normal renal function, the amount excreted is relatively constant. As a result, creatinine clearance can be used to monitor renal function.

This assay is based on the Jaffe reaction in which a yellow/orange colour develops when alkaline picrate is added to the sample; however, the colour that develops due to the presence of creatinine is then destroyed by the addition of acid. The difference in colour before and after the addition of acid is measured, and is proportional to creatinine concentration.

Procedure: Random a.m. urine samples were diluted 5- to 80-fold with deionized water, and the mixture was vortexed for 5 to 10 seconds to ensure

uniformity. A standard curve was developed using 4 concentrations of standard: 2.5 mg/dL (25 μL of 10 mg/dL standard + 75 μL deionized water), 5.0 mg/dL (50 μL of 10 mg/dL standard + 50 μL deionized water), 7.5 mg/dL (75 μL of 10 mg/dL standard + 25 μL deionized water), and 10.0 mg/dL (100 μL of 10 mg/dL) standard. Twenty μL of deionized water (blank), standard or sample were pipetted into the designated well of the microplate. A 5:1 mixture of creatinine colour reagent and sodium hydroxide formed an alkaline picrate solution; 200 μL of this solution was added to each well using a multi-channel pipet. The microplate was incubated at room temperature (18-26°C) for 8 to 12 minutes, after which time the absorbance was read at 500 nm on a SPECTRAmax 340 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Acid reagent (7 μL) was added to each well, and mixed by carefully pipetting the contents of each well up and down. The plate was incubated at room temperature (18-26°C) for 5 minutes, and the absorbance was again read at 500 nm.

From the standard curve, which was plotted as concentration versus absorbance, the creatinine concentration of the samples was calculated as follows:

$$\text{Creatinine (mg/dL)} = \frac{\text{Initial - Final Sample Absorbance}}{\text{Initial - Final Standard Absorbance}} \times \text{Standard Concentration}$$

This value was multiplied by the dilution factor, and then multiplied by 88.4 to converted to mmol/L.

4.17 Statistical Analysis: Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego CA), and Sigma Stat (Jandel Scientific, San Rafael, CA). Two-tailed Pearson correlation analysis for parametric nominal data was conducted to determine relationships between indices of bone development and diet (calcium, vitamin D, phosphorus, and PUFA) and indices of bone development and physical activity. One-way analysis of variance (ANOVA) was performed to determine differences in bone development based on level of physical activity or diet, and, in the children with diabetes, duration of disease, and age at onset. Tukey's post-test was used to determine differences between means. Two-way ANOVA was conducted to study the effect of gender and presence or absence of diabetes on growth and development. Student-Newman-Keuls' test was used to determine differences between means. Best subsets regression analysis was performed to examine which factors most contribute to BMC and BMD. For all analyses, differences were considered significant at a p value < 0.05.

5. Clinical Study: Results

One hundred nineteen children were recruited for the study. The control group consisted of 34 females and 34 males, while there were 27 females and 24 males in the diabetes group. Subjects ranged in age from 8.3 to 17.4 years in the control group and from 8.9 to 17.0 years in the diabetes group. The mean ages of the control and diabetes groups were similar, as were the mean heights, weights, BMI, BMC, BA, and BMD for each group (**Table 5.1**). Z-scores for BMC, BA, and BMD, along with BMC corrected to height, weight, and BA, and BA corrected to height were also not different. Data from both groups was also comparable to reference data for BMC and BMD compiled by Faulkner et al. (102). These researchers used DXA to study 8 to 17 year old children and adolescents. In both the control and the diabetes groups, most children fell within the expected limits for BMC (**Figures 5.1 and 5.2**) and BMD (**Figures 5.3 and 5.4**) when compared to the reference children of the same age and gender.]

Although the children in this study showed expected bone development when compared to the reference data, there were some interesting trends. While all of the boys except for 2 with diabetes and 1 without the disease fell within the expected range, there was a trend towards lower BMC in the boys with diabetes. Sixty-seven percent of the boys with diabetes compared to 32% of the boys without diabetes had BMC less than the mean BMC for boys of their age (**Figure 5.1**). The opposite appeared to be true in the girls. Thirty-

seven percent of the girls with diabetes compared to 56% of girls without diabetes had a BMC less than the mean BMC for girls of the same age (**Figure 5.2**). The trend was similar for BMD. Thirty-three percent and 18% respectively of boys with and without diabetes had BMD less than the mean BMD for boys of the same age (**Figure 5.3**), while 19% and 38% respectively of girls with and without diabetes had BMD less than the mean BMD for girls of the same age (**Figure 5.4**).

For children with diabetes, mean insulin dosage (U/kg), HbA1c (%), morning blood glucose (mmol/L), creatinine (mmol/L), and urinary glucose, calcium and phosphorus corrected to creatinine (mmol/mmol) were calculated as indicators of metabolic control (**Table 5.2**). None of these factors were significantly correlated with bone development (**Table 5.3**).

Duration of diabetes has been shown to affect bone mass (2-3,26,34); however it is not clear at what point changes occur. Research suggests that changes are most likely either at onset (26) or within the first 5 years after onset (2-3). To determine the effect of duration of disease on growth and bone development of children with diabetes, and whether changes were more prevalent at onset or within 5 years after onset, data was divided into 4 groups of disease duration: 1.0 year or less, 1.1 to 5.0 years, 5.1 to 10.0 years, and more than 10.0 years. Because the mean age of the latter group was significantly higher than that of the other 3 groups, this group was not included in the analysis. Z-scores for BMC, BA, BMD, height, and weight were

analyzed by one-way ANOVA. There were no significant differences among the groups for BMD or height (**Table 5.4**). However, children who had had diabetes for 1.1- 5.0 years and 5.1 - 10.0 years had significantly higher z-scores for BMC and BA than those who had more recent onset of the disease. Children who had had diabetes for 5.1 to 10.0 years also had a significantly higher weight z-score than children in the other 2 groups. Since by grouping the data in this way the number of children in each group was not equal, data was also divided into quartiles for further analysis. There were no significant differences among any of the groups for BMC, BMD, height, or weight when the data was divided in this manner. (Data not shown).

To determine the effect of age at onset of diabetes on BMC, BA, BMD, and z-scores for each parameter, data was divided into quartiles based on the age of the child at onset and analyzed using 1-way ANOVA (**Table 5.5**). There were no significant differences among any of the groups for any of the parameters.

Fifty-two children (33 from the control group and 19 from the diabetes group) completed the physical activity questionnaire. While there were no significant differences in age between those who responded to the questionnaire (R) and those who did not (NR), there were significant differences in standardized score for BMC, BMD, and BA, with responders having lower scores than non-responders (Age - N: 12.8 $\pm$ 2.3 vs NR: 13.2 $\pm$ 2.3, p=0.83; BMC z-score - N: -0.4 $\pm$ 1.4 vs NR: 0.2 $\pm$ 1.0, p=0.002 ; BMD z-

score - N: 0.5 ± 1.0 vs NR: 1.0 ± 1.3, p=0.01; BA z-score - N: -0.4 ± 0.8 vs NR: -0.1 ± 1.1, p=0.03). Among those who responded, reported hours of activity ranged from 0.1 to 35.1 per week (**Table 5.6**). Boys in the control group reported the highest mean amount of physical activity, while girls in the control group reported the lowest. To study bone development based on level of activity, subject data was divided into quartiles based on reported total hours of physical activity per week and analyzed by one-way ANOVA. To increase power, and since physical activity is not known to affect bone development of children with and without diabetes differently, all subjects were grouped together for this analysis. BMC, BMC z-score, BA, BA z-score, BMD, or BMD z-score did not vary significantly with level of activity divided by quartiles (**Table 5.7**).

Correlation analysis was also conducted to examine the relationship between physical activity and various indicators of bone development (**Table 5.8**). For the females in the control group and the males in both the control and diabetes groups, no significant relationship was found between physical activity and any of the indicators of bone development. For the females with diabetes, there was a significant positive correlation of physical activity with BMC, BA, and BA z-score.

Seventy-one children (45 from the control group and 26 from the diabetes group) completed the 3-day food records. There were no significant differences between responders and non-responders in terms of age, BMC z-

score, BMD z-score, or BA z-score (Age - N: 13.0 ± 2.3 vs NR: 13.0 ±2.3, p=1.0; BMC z-score - N: -0.2 ± 1.5 vs NR: 0.3 ± 0.9, p=0.1; BMD z-score - N: 0.7 ± 1.1 vs NR: 1.1 ± 1.2, p=0.1; BA z-score - N: -0.3 ± 1.0 vs NR: 0.0 ± 1.0, p=0.3). For those who responded, analysis revealed mean intakes of calcium, vitamin D, and phosphorus at or above the RNI for all groups (**Table 5.9**). For calcium, intakes were close to the RNI for both males and females, while intakes for phosphorus were 1.5 to 2 times the RNI, and for vitamin D more than twice the RNI. For PUFA, mean intakes were above the RNI for all groups except the males in the control group, where they fell just below the recommended intakes. When tested by t-test, there were no significant differences between the control and diabetes groups in intake as a percentage of RNI for any of the diet components. Although mean intakes tended to meet or exceed the RNI, 40% of subjects did not meet the RNI for calcium, and 47% did not meet the RNI for PUFA. Only 10% and 13% of subjects did not meet the RNI for phosphorus and vitamin D respectively.

To determine bone development based on level of nutrient intake, data was divided into quartiles based on percentage of RNI consumed for calcium, vitamin D, phosphorus, and PUFA. Groups were then compared for BMC, BA, and BMD, as well as z-score for each indicator (**Tables 5.10-5.13**). Based on percentage of RNI consumed, there were no significant differences among the groups for any of the indicators of bone development measured. For calcium and phosphorus, the quartile with the lowest dietary intake was significantly

older than the quartile with the highest intakes of these nutrients. For vitamin D, the quartile with the lowest intake was significantly older than the quartile with the second highest intake as well as the group with the highest intake.

Dietary intakes of calcium, vitamin D, phosphorus, and PUFA as a percentage of RNI were tested for correlation with indices of bone development (**Tables 5.14-5.17**). For calcium, significant relationships were found only in the boys (**Table 5.14**). There were significant negative correlations between calcium intake and age, calcium intake and BMC, calcium intake and BMD, and calcium intake and BA in the control boys. In the boys with diabetes, there were significant negative correlations between calcium intake and age and calcium intake and BMC. For vitamin D, a significant correlation was found only in the boys with diabetes where intake was negatively correlated with age (**Table 5.15**). Negative correlations between phosphorus intake and age, phosphorus intake and BMC, phosphorus intake and BMD, and phosphorus intake and BA were found in the control girls (**Table 5.16**). In the girls with diabetes, phosphorus intake was positively correlated with BA z-score. In the boys with diabetes, phosphorus intake was negatively correlated with age, but positively correlated with BMD z-score. PUFA intake was positively correlated with BMC z-score and BA z-score in the boys with diabetes (**Table 5.17**).

Regression analysis was conducted to determine which factors most contribute to BMC in children with Type 1 diabetes (**Table 5.18**). Independent

variables included in the analysis were age (years), sex, BA (cm^2), height (cm), weight (kg), duration of diabetes (years), amount of insulin injected daily (U/kg), and HbA1c (%). Analysis yielded the following equation (R^2 = 0.959, R^2 adjusted = 0.956):

BMC = 1137 + 2.09(BA) - 1.69(height) - 1.19(insulin) - 1.96(HbA1c).

To determine which factors contribute most to BMD in children with Type 1 diabetes, age (years), height (cm), weight (kg), HbA1c (%), and daily insulin dosage (U/kg) were analyzed as independent variables (**Table 5.19**). The resulting equation was (R^2 = 0.607, R^2 adjusted = 0.562):

BMD = 1.08 + 0.02(age) + 0.01(weight) - 0.003(height)
- 0.05(insulin) - 0.02(HbA1c).

Table 5.1: Mean age and indicators of growth and bone development in females and males with and without Type 1 diabetes

	Females		Males	
	Control (n=34)	**Diabetes** (n=27)	**Control** (n=34)	**Diabetes** (n=24)
Age (years) (Age range)	13.1 (2.2) (8.3 - 16.7)	13.1 (2.0) (8.9 - 17.0)	12.6 (2.4) (8.6 - 17.4)	12.8 (3.3) (8.1 - 17.9)
Height (cm)	155.5 (12.8)	156.9 (10.3)	157.6 (14.9)	154.2 (34.6)
Height z-score	-0.2 (1.0)	-0.3 (0.9)	-0.1 (0.8)	-0.4 (1.0)
Weight (kg)	50.0 (14.4)	54.4 (14.2)	53.1 (18.2)	53.5 (21.0)
Weight z-score	0.0 (1.0)	0.3 (1.0)	0.5 (1.5)	0.2 (1.4)
BMI (kg/m^2)	20.2 (3.6)	21.8 (3.9)	20.8 (4.7)	19.9 (5.1)
BMC (g)	1519.7 (413.9)	1639.5 (375.8)	1645.0 (593.8)	1662.5 (623.3)
BMC z-score	-0.2 (0.9)	0.1 (0.8)	0.3 (1.0)	-0.2 (1.2)
BA (cm^2)	1584.7 (315.4)	1653.9 (258.1)	1654.9 (393.6)	1638.7 (487.1)
BA z-score	-0.5 (1.0)	-0.3 (0.9)	0.2 (0.9)	-0.3 (1.1)
BMD (g/cm^2)	0.946 (0.090)	0.981 (0.087)	0.971 (0.124)	0.991 (0.118)
BMD z-score	0.6 (1.1)	1.0 (1.0)	1.0 (1.2)	0.5 (1.5)
BMC/height (g/cm)	9.6 (2.0)	10.3 (1.8)	10.2 (2.9)	10.5 (2.7)
BMC/weight (g/kg)	30.8(4.4)	30.4 (3.8)	31.3 (4.8)	31.4 (3.0)
BA for height z-score	-0.7 (0.9)	-0.4 (0.7)	-0.1 (1.1)	-0.4 (1.0)
BMC for BA z-score	1.0 (1.4)	1.4 (1.4)	1.5 (1.6)	2.1 (2.4)

Data shown as mean (SD), except for age data which is shown as mean (SD), (range in years).
No significant differences as analyzed by 2-way ANOVA.

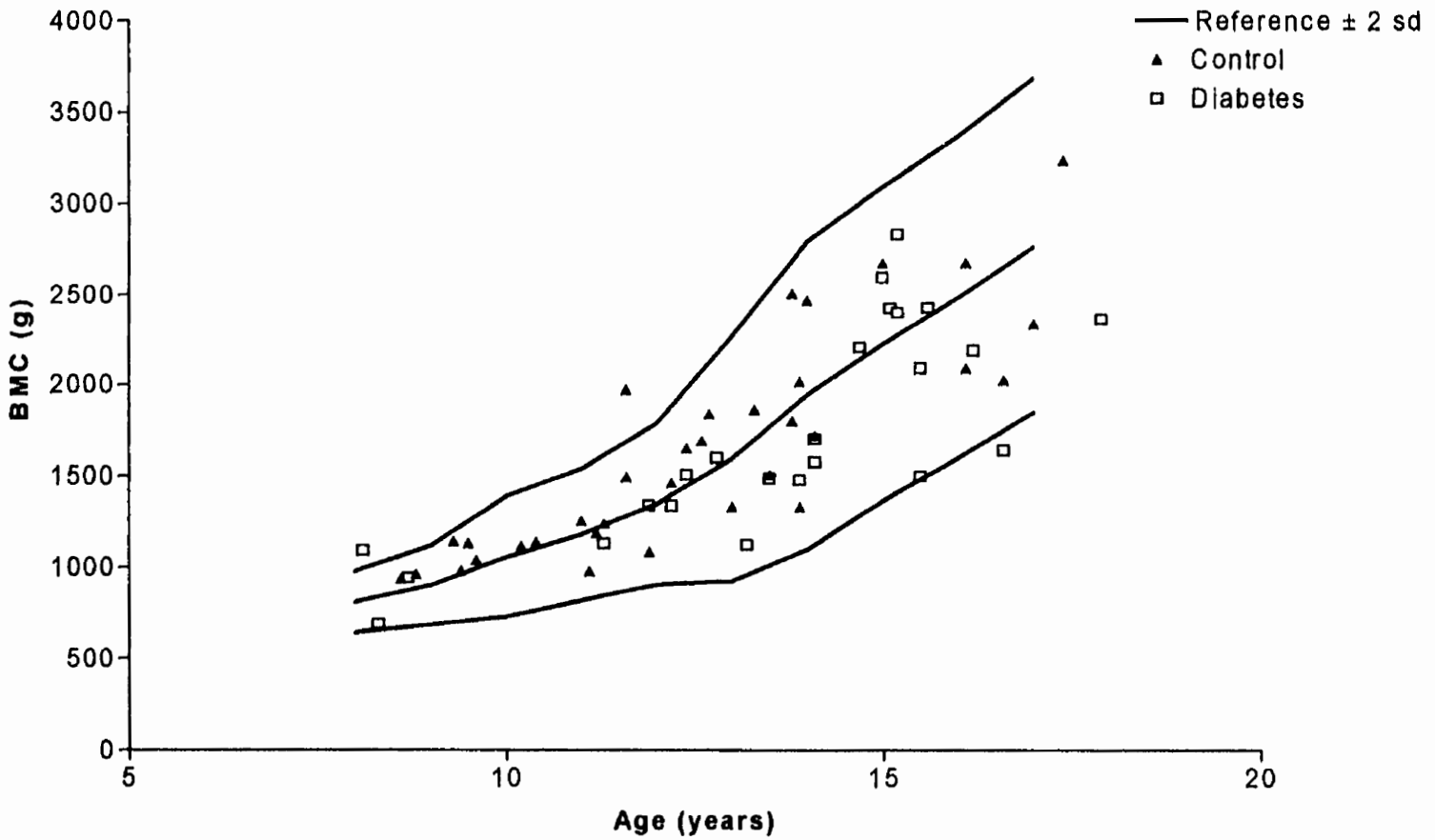

Figure 5.1: BMC in males with and without Type 1 diabetes compared to reference data (102) n = 34 (control), 24(diabetes)

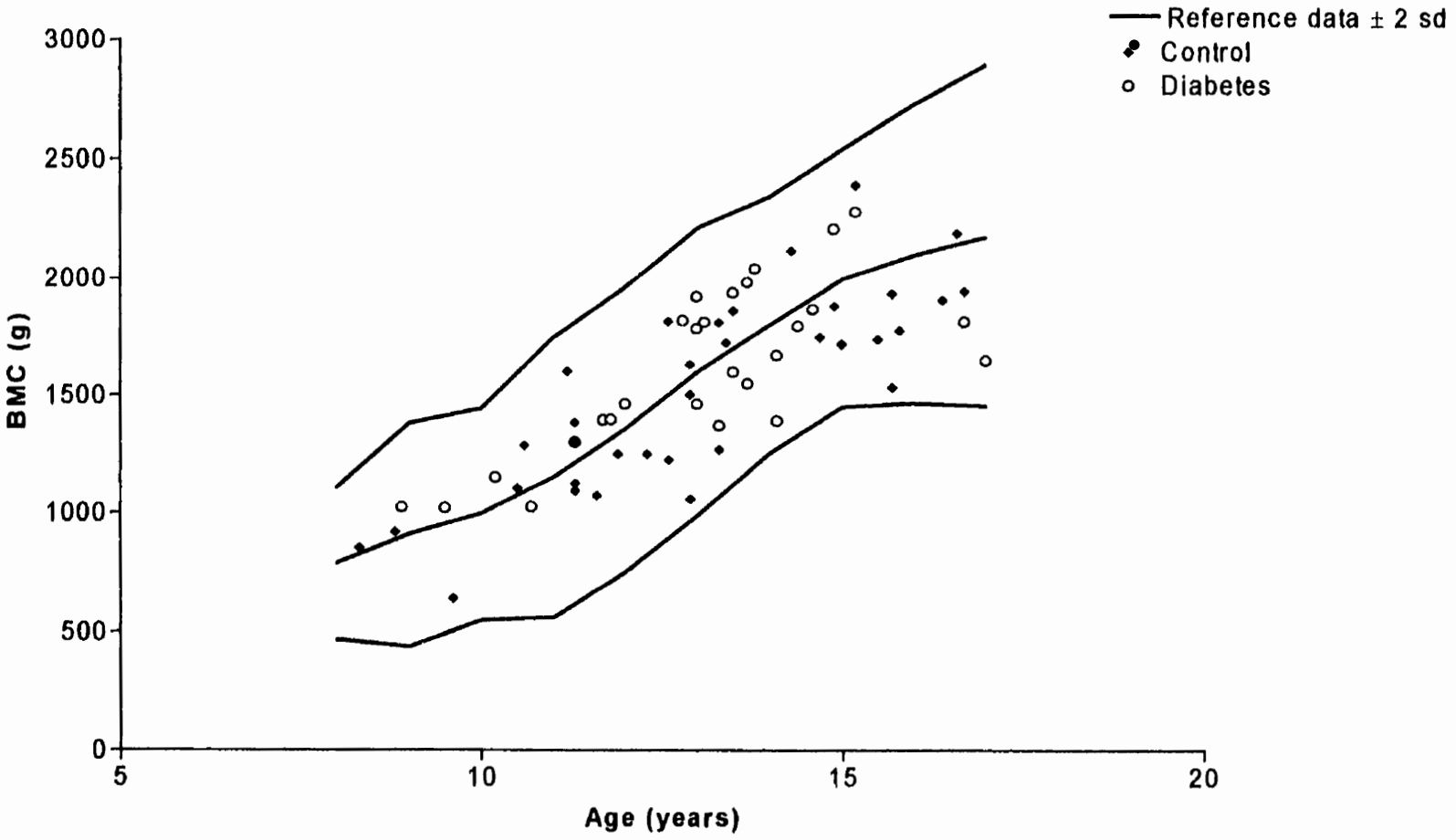

Figure 5.2: BMC in females with and without Type 1 diabetes compared to reference data (102) n = 34 (control), 27 (diabetes)

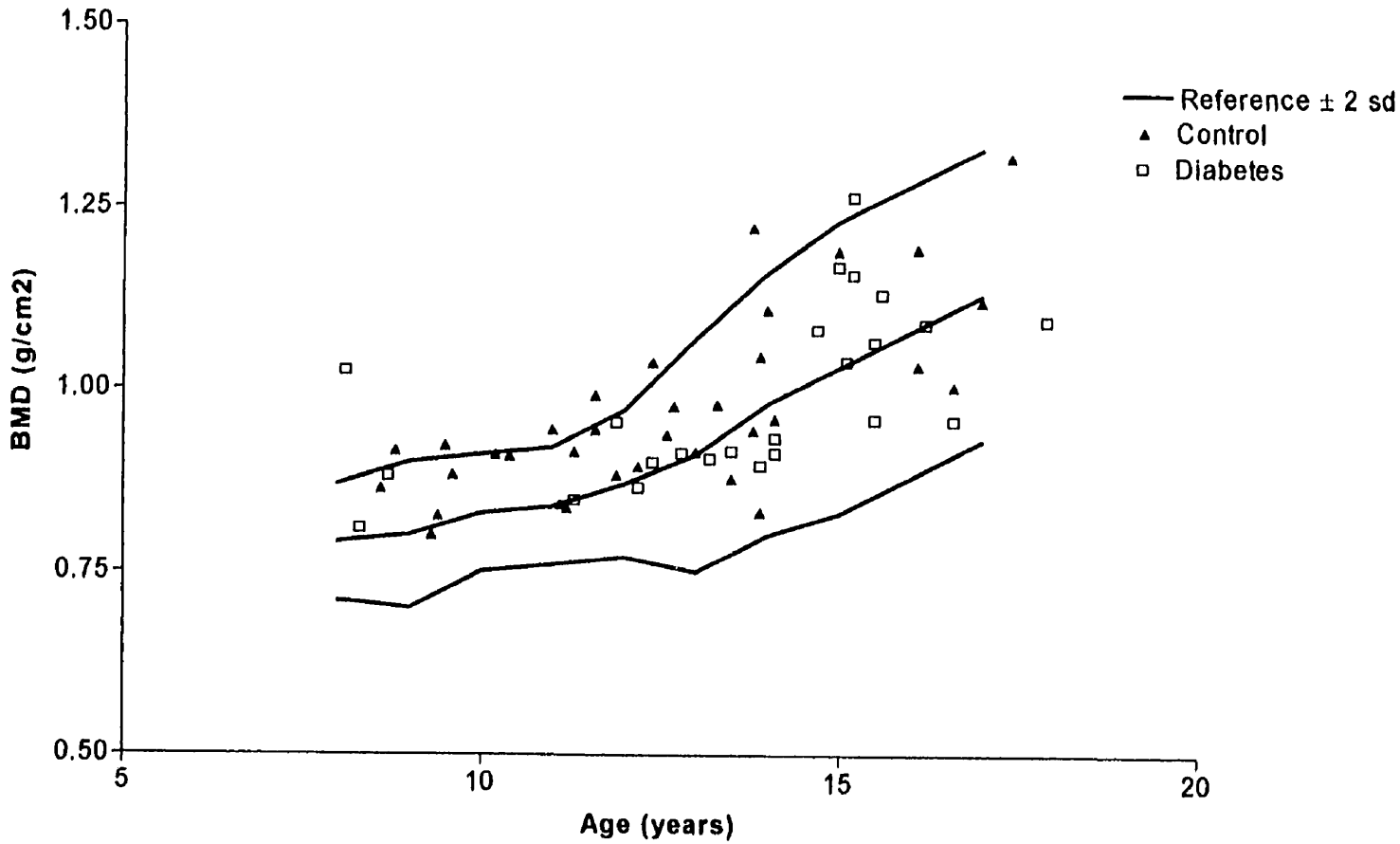

Figure 5.3: BMD in males with and without Type 1 diabetes compared to reference data (102) n = 34 (control), 24(diabetes)

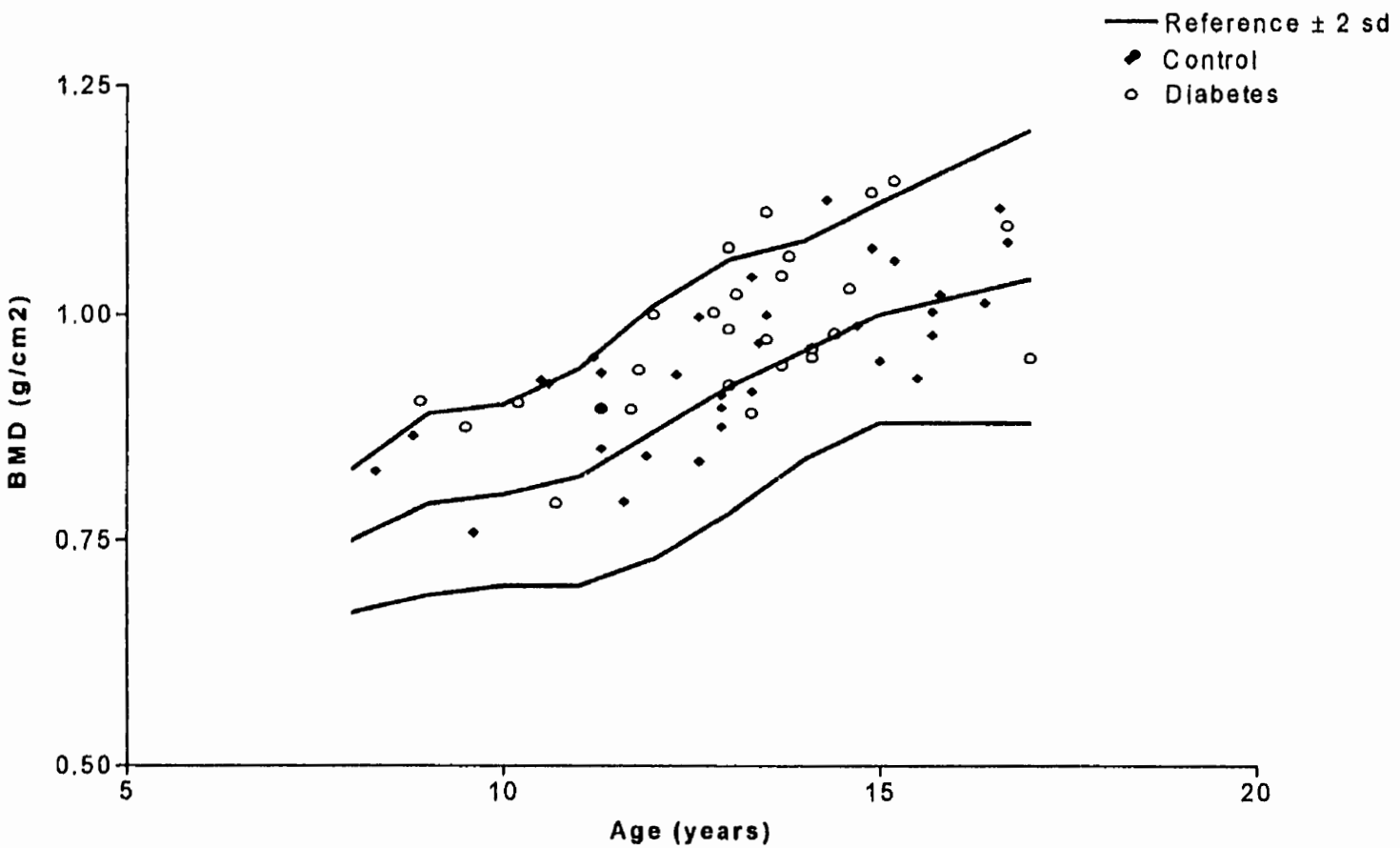

Figure 5.4: BMD in females with and without Type 1 diabetes compared to reference data (102) n = 34 (control), 27 (diabetes)

Table 5.2: Indicators of metabolic control in females and males with Type 1 diabetes

	Females (n=27)	**Males** (n=24)
Insulin (U/kg)	0.9 (0.3)	0.9 (0.4)
HbA1c (%)	7.8 (1.3)	7.9 (1.4)
a.m. Blood sugar (mmol/L)	9.5 (3.6)	11.2 (4.5)
Injection frequency (times/day)	2.7 (0.6)	2.6 (0.5)
2 injections/day (% of subjects)	37.0	43.5
3^{+} injections/day (% of subjects)	63.0	56.5
Urinary creatinine (mmol/L)	7.5 (4.6)	8.2 (7.8)
Urinary glucose/creatinine (mmol/mmol)	25.8 (31.5)	32.0 (46.4)
Urinary calcium/creatinine (mmol/mmol)	0.26 (0.24)	0.30 (0.32)
Urinary phosphorus/creatinine (mmol/mmol)	2.8 (2.5)	2.7 (2.2)

Data shown as mean (SD)

Table 5.3: Relationship of indicators of metabolic control with measures of bone development in females and males with Type 1 diabetes

	BMC n=27(F),24(M)	**BA** n=27(F),24(M)	**BMD** n=27(F),24(M)
Insulin dosage (U/kg)	r= 0.19 p= 0.19	r= 0.26 p= 0.07	r= 0.07 p= 0.61
HbA1c (%)	r= 0.11 p= 0.45	r= 0.17 p= 0.23	r= 0.001 p= 0.995
a.m. Blood glucose (mmol/L)	r= 0.16 p= 0.28	r= 0.16 p= 0.28	r= 0.13 p= 0.36
Urinary creatinine (mmol)	r= 0.18 p= 0.22	r= 0.19 p= 0.21	r= 0.16 p= 0.27
Urinary glucose/creatinine (mmol/mmol)	r= -0.14 p= 0.34	r= -0.11 p= 0.44	r= -0.17 p= 0.23
Urinary calcium/creatinine (mmol/mmol)	r= 0.01 p= 0.96	r= 0.01 p= 0.96	r= -0.03 p= 0.85
Urinary phosphorus/creatinine (mmol/mmol)	r= -0.19 p= 0.20	r= -0.13 p= 0.37	r= -0.23 p= 0.12

r= Pearson correlation coefficient
F- females, M- males

Table 5.4: Z-scores for BMC, BA, BMD, height, and weight of females and males with Type 1 diabetes based on duration of disease

Duration of Diabetes (years)	**0-1.0** n=3(F), 3(M)	**1.1-5.0** n=13(F), 12(M)	**5.1-10.0** n=9(F), 5(M)
Mean age (years)	13.1 (3.1)	13.0 (2.1)	13.1 (2.0)
BMC (g) p= 0.04	-0.8[a] (1.1)	0.2[ab] (1.0)	0.3[b] (0.6)
BA (cm^2) p= 0.002	-1.3 (1.3)[a]	-0.2 (0.7)[b]	0.2 (0.8)[b]
BMD (g/cm^2) p= 0.32	0.2 (1.0)	1.1 (1.5)	0.8 (0.8)
Height (cm) p=0.096	-0.8 (1.2)	-0.2 (0.8)	0.1 (0.7)
Weight (kg) p=0.001	-0.7[a] (0.7)	0.3[a] (0.9)	1.1[b] (1.2)

Duration of diabetes shown as range, age as mean (SD), all other data shown as mean z-score (SD).
Within rows, data with different superscripts are significantly different at $p < 0.05$ as analyzed by 1-way ANOVA.
F- females, M- males

Table 5.5: BMC, BA, BMD, and z-scores for BMC, BA, and BMD based on age at onset of diabetes

Age at onset (years)	**1.2 - 6.0** n=8(F), 5(M)	**6.1 - 8.9** n=5(F), 7(M)	**9.0 - 11.2** n=8(F), 4(M)	**11.3 - 16.5** n=6(F), 8(M)
Age (years)	12.6 (3.0)	13.1 (2.5)	12.9 (1.4)	14.7 (1.1)
BMC (g)	1518 (430.1)	1643 (548.3)	1687 (422.9)	1834 (474.4)
BMC z-score	0.3 (1.2)	-0.2 (1.0)	0.3 (0.5)	-0.4 (1.1)
BA (cm^2)	1554 (342.8)	1654 (393.6)	1703 (299.4)	1775 (264.5)
BA z-score	-0.3 (0.9)	-0.3 (1.0)	0.2 (0.7)	-0.8 (1.2)
BMD (g/cm^2)	1.0 (0.1)	1.0 (0.1)	1.0 (0.1)	1.0 (0.1)
BMD z-score	1.2 (1.6)	0.6 (1.1)	1.0 (1.0)	0.4 (1.3)

Age at onset shown as range, all other data shown as mean (SD).
Within rows, data with different superscripts are significantly different at $p < 0.05$ as analyzed by 1-way ANOVA.
F- females, M-males

Table 5.6: Hours of activity per week performed by females and males with and without Type 1 diabetes

	Females n=9(D), 14(C)		**Males** n=10(D),19(C)	
	Mean (hr/wk)	**Range** (hr/wk)	**Mean** (hr/wk)	**Range** (hr/wk)
Control	4.3 (3.2)	1.3 - 12.4	9.6 (9.0)	2.0 - 35.1
Diabetes	4.9 (3.5)	0.1 - 10.5	6.0 (5.2)	0.4 - 16.8

Data shown as mean (SD) and as range.
D- diabetes group, C- control group

Table 5.7: BMC, BA, BMD, and z-scores for BMC, BA, and BMD based on level of activity

Activity (hours/week)	**0 - 2.7** n=8(F), 5(M)	**2.8 - 4.2** n=5(F), 7(M)	**4.3 - 9.0** n=6(F), 7(M)	**9.1 - 35.1** n=4(F), 10(M)
Age (years)	13.2 (2.8)	11.8 (1.9)	12.2 (1.7)	14.0 (2.2)
BMC (g)	1491.0 (476.3)	1361.9 (432.7)	1415.2 (318.8)	1813.7 (491.6)
BMC z-score	-0.7 (0.6)	0.2 (1.1)	-0.2 (0.7)	-0.7 (2.3)
BA (cm^2)	1572.5 (391.3)	1456.6 (344.9)	1523.8 (262.8)	1786.6 (315.4)
BA z-score	-0.7 (0.8)	-0.5 (0.9)	-0.4 (1.2)	-0.1 (1.1)
BMD (g/cm^2)	0.931 (0.085)	0.918 (0.082)	0.923 (0.075)	1.001 (0.114)
BMD z-score	0.2 (0.8)	0.9 (1.1)	0.5 (1.3)	0.6 (1.1)

Data shown as mean (SD)
No significant differences as analyzed by 1-way ANOVA
F- females, M- males

Table 5.8: Relationship of hours of physical activity with indicators of bone development

	Females		Males	
	Control (n=14)	**Diabetes** (n=9)	**Control** (n=19)	**Diabetes** (n=10)
Age (years)	r= 0.14 p= 0.63	r= 0.19 p= 0.63	r= 0.02 p= 0.93	r= 0.18 p= 0.62
BMC (g)	r= 0.19 p= 0.51	**r= 0.70** **p= 0.04**	r= 0.21 p= 0.41	r= -0.04 p= 0.91
BMC z-score	r= 0.10 p= 0.74	r= 0.60 p= 0.09	r= -0.21 p= 0.41	r= -0.32 p= 0.37
BA (cm^2)	r= 0.10 p= 0.74	**r= 0.73** **p= 0.03**	r=0.16 p= 0.52	r= 0.02 p= 0.96
BA z-score	r= 0.01 p= 0.97	**r= 0.76** **p= 0.02**	r= 0.42 p= 0.08	r= -0.27 p= 0.45
BMD (g/cm^2)	r= 0.37 p= 0.19	r= 0.41 p= 0.27	r= 0.23 p= 0.35	r= -0.10 p= 0.78
BMD z-score	r= 0.17 p= 0.55	r= 0.22 p= 0.56	r= 0.13 p= 0.61	r= -0.55 p= 0.10

r= Pearson correlation coefficient
Significant correlations are shown in bold

Table 5.9: Mean % of RNI consumed for calcium, vitamin D, phosphorus, and PUFA according to 3-day food records

	Females		Males	
	Control (n=22)	Diabetes (n=13)	Control (n=23)	Diabetes (n=13)
Calcium	103.8 (32.6)	100.5 (48.9)	139.1 (49.4)	132.5 (47.2)
Vitamin D	278.2 (214.8)	285.2 (194.4)	226.6 (126.1)	221.3 (98.2)
Phosphorus	153.4 (43.1)	146.5 (54.8)	185.8 (68.3)	178.7 (67.1)
PUFA	124.9 (45.7)	123.9 (43.6)	94.1 (20.1)	119.8 (73.8)

Data shown as mean % RNI (SD)
No significant differences as analyzed by t-test

Table 5.10: Age, BMC, BA, and BMD and z-scores for BMC, BA, and BMD based on dietary calcium intake

Calcium Intake (% RNI)	**25.8 - 86.3** n=13(F), 5(M)	**86.4 - 109.6** n=12(F), 5(M)	**109.7 - 146.5** n=4(F), 14(M)	**146.6 - 248.7** n=6(F), 12(M)
Age (years)	13.8^{a} (2.3)	13.5^{ab} (1.8)	13.0^{ab} (2.0)	11.8^{b} (2.6)
BMC (g)	1765.0 (523.0)	1509.0 (288.3)	1604.0 (556.3)	1405.0 (495.4)
BMC z-score	-0.1 (1.1)	-0.6 (0.8)	-0.1 (0.7)	0.2 (1.1)
BA (cm^2)	1743.0 (372.4)	1599.0 (231.9)	1644 (351.6)	1490 (373.2)
BA z-score	-0.5 (1.3)	-0.8 (1.0)	-0.1 (0.9)	-0.0 (0.9)
BMD (g/cm^2)	0.994 (0.107)	0.938 (0.057)	0.995 (0.120)	0.924 (0.103)
BMD z-score	0.7 (1.2)	0.1 (1.0)	0.5 (0.9)	0.8 (1.2)

Data shown as mean (SD)
Within rows, data with different superscripts are significantly different at $p < 0.05$ as analyzed by 1-way ANOVA.
F- females, M-males

Table 5.11: Age, BMC, BA, and BMD and z-scores for BMC, BA, and BMD based on dietary vitamin D intake

Vitamin D Intake (% RNI)	**21.4 - 135.9** n=8(F), 9(M)	**136.0 - 199.9** n=9(F), 7(M)	**200.0 - 327.9** n=6(F), 11(M)	**328.0 - 688.0** n=8(F), 11(M)
Age (years)	14.4[a] (2.3)	13.2[ab] (2.2)	12.3[b] (2.0)	12.5[b] (2.1)
BMC (g)	1811.0 (515.8)	1414.0 (347.4)	1681.0 (533.4)	1446.0 (439.8)
BMC z-score	-0.4 (1.1)	0.3 (0.9)	0.1 (0.7)	-0.1 (1.2)
BA (cm^2)	1773.0 (303.4)	1697.0 (378.5)	1526.0 (289.7)	1518.0 (330.6)
BA z-score	-0.5 (1.3)	-0.1 (0.9)	-0.1 (0.6)	-0.3 (0.9)
BMD (g/cm^2)	1.006 (0.110)	0.970 (0.115)	0.919 (0.076)	0.940 (0.093)
BMD z-score	0.3 (1.2)	0.9 (0.9)	0.7 (1.2)	0.8 (1.7)

Data shown as mean (SD)
Within rows, data with different superscripts are significantly different at $p < 0.05$, as analyzed by 1-way ANOVA.
F- females, M-males

Table 5.12: Age, BMC, BA, and BMD and z-scores for BMC, BA, and BMD based on dietary phosphorus intake

Phosphorus Intake (% RNI)	**0.8 - 124.7** n=11(F), 7(M)	**124.8 - 163.7** n=12(F), 5(M)	**163.8 - 194.2** n=8(F), 10(M)	**194.3 - 308.8** n=4(F), 14(M)
Age (years)	14.3^{a} (2.1)	13.0^{ab} (2.3)	13.1^{ab} (1.8)	11.8^{b} (2.3)
BMC (g)	1702.0 (378.5)	1558.0 (489.8)	1626.0 (541.8)	1398.0 (514.5)
BMC z-score	-0.5 (1.0)	-0.2 (0.9)	-0.0 (1.0)	0.0 (1.1)
BA (cm^2)	1734.0 (292.0)	1600.0 (359.8)	1646.0 (313.6)	1496.0 (388.5)
BA z-score	-0.7 (1.2)	-0.5 (0.9)	-0.2 (1.1)	0.1 (1.0)
BMD (g/cm^2)	0.973 (0.062)	0.956 (0.097)	0.969 (0.131)	0.914 (0.101)
BMD z-score	0.2 (0.9)	0.6 (1.1)	0.7 (1.3)	0.5 (1.1)

Data shown as mean (SD)

Within rows, data with different superscripts are significantly different at $p < 0.05$, as analyzed by 1-way ANOVA.

F- females, M-males

Table 5.13: Age, BMC, BA, and BMD and z-scores for BMC, BA, and BMD based on dietary PUFA intake

PUFA Intake (% RNI)	**45.7 - 87.1** n=6(F), 12(M)	**87.2 - 102.8** n=7(F), 10(M)	**102.9 - 130.4** n=8(F), 9(M)	**130.5 - 275.5** n=14(F), 4(M)
Age (years)	13.4 (2.9)	13.2 (2.5)	12.3 (1.5)	13.5 (1.8)
BMC (g)	1568.0 (602.1)	1617.0 (521.8)	1452.0 (316.6)	1680.0 (464.2)
BMC z-score	-0.5 (1.1)	-0.2 (0.9)	0.1 (0.9)	-0.1 (0.9)
BA (cm^2)	1594.0 (411.8)	1633.0 (343.6)	1568.0 (259.2)	1711.0 (338.1)
BA z-score	-0.7 (1.1)	-0.4 (1.0)	-0.1 (0.9)	-0.1 (1.1)
BMD (g/cm^2)	0.959 (0.119)	0.972 (0.118)	0.920 (0.060)	0967 (0.097)
BMD z-score	0.3 (1.1)	0.6 (1.3)	0.6 (1.0)	0.5 (1.0)

Data shown as mean (SD)
No significant differences as analyzed by 1-way ANOVA
F- females, M-males

Table 5.14: Relationship of calcium intake as % RNI with indicators of bone development

	Females		Males	
	Control (n=22)	**Diabetes** (n=13)	**Control** (n=23)	**Diabetes** (n=13)
Age (years)	r= -0.14 p= 0.54	r= -0.14 p= 0.64	**r= -0.63** **p= 0.001**	**r= -0.70** **p= 0.007**
BMC (g)	r= -0.23 p= 0.29	r= 0.29 p= 0.34	**r= -0.49** **p= 0.02**	**r= -0.56** **p= 0.05**
BMC z-score	r= -0.30 p= 0.18	r= 0.43 p= 0.14	r= -0.11 p= 0.63	r= 0.19 p= 0.54
BA (cm^2)	r= -0.23 p= 0.29	r= 0.24 p= 0.43	**r= -0.51** **p= 0.01**	r= -0.55 p= 0.05
BA z-score	r= 0.06 p= 0.80	r= 0.48 p= 0.10	r= 0.17 p= 0.43	r= 0.24 p= 0.43
BMD (g/cm^2)	r= -0.17 p= 0.44	r= 0.30 p= 0.32	**r= -0.48** **p= 0.02**	r= -0.53 p= 0.06
BMD z-score	r= -0.21 p= 0.35	r= 0.32 p= 0.29	r= 0.06 p= 0.77	r= 0.44 p= 0.13

r= Pearson correlation coefficient
Significant correlations are shown in bold

Table 5.15: Relationship of vitamin D intake as % RNI with indicators of bone development

	Females		Males	
	Control (n=22)	Diabetes (n=12)	Control (n=22)	Diabetes (n=13)
Age (years)	r= -0.09 p= 0.68	r= -0.30 p= 0.34	r= -0.30 p= 0.18	**r= -0.64** **p= 0.02**
BMC (g)	r= -0.15 p= 0.50	r= -0.09 p= 0.78	r= -0.29 p= 0.18	r= -0.44 p= 0.13
BMC z-score	r= -0.12 p= 0.58	r= 0.27 p= 0.39	r= 0.16 p= 0.49	r= 0.15 p= 0.62
BA (cm^2)	r= -0.19 p= 0.41	r= -0.24 p= 0.46	r= -0.29 p= 0.19	r= -0.46 p= 0.12
BA z-score	r= -0.12 p= 0.59	r= 0.04 p= 0.89	r= 0.03 p= 0.88	r= 0.25 p= 0.41
BMD (g/cm^2)	r= -0.03 p= 0.89	r= 0.18 p= 0.58	r= -0.30 p= 0.17	r= -0.41 p= 0.17
BMD z-score	r= 0.01 p= 0.98	r= 0.44 p= 0.15	r= 0.04 p= 0.88	r= 0.41 p= 0.16

r= Pearson correlation coefficient
Significant correlations are shown in bold

Table 5.16: Relationship of phosphorus intake as % RNI with indicators of bone development

	Females		Males	
	Control (n=22)	**Diabetes** (n=13)	**Control** (n=23)	**Diabetes** (n=13)
Age (years)	**r= -0.52** **p= 0.013**	r= -0.13 p= 0.68	r= -0.17 p= 0.45	**r= -0.74** **p= 0.004**
BMC (g)	**r= -0.53** **p= 0.01**	r= 0.38 p= 0.20	r= -0.09 p= 0.69	r= -0.41 p= 0.16
BMC z-score	r= -0.09 p= 0.70	r= 0.48 p= 0.10	r= -0.39 p= 0.06	r= 0.49 p= 0.09
BA (cm^2)	**r= -0.51** **p= 0.01**	r= 0.36 p= 0.23	r= -0.09 p= 0.67	r= -0.43 p= 0.14
BA z-score	r= 0.05 p= 0.84	**r= 0.60** **p= 0.03**	r= 0.16 p= 0.48	r= 0.51 p= 0.08
BMD (g/cm^2)	**r= -0.49** **p= 0.03**	r= 0.33 p= 0.27	r= -0.09 p= 0.68	r= -0.38 p= 0.19
BMD z-score	r= 0.07 p= 0.75	r= 0.31 p= 0.31	r= 0.01 p= 0.97	**r= 0.73** **p= 0.005**

r= Pearson correlation coefficient
Significant correlations are shown in bold

Table 5.17: Relationship of PUFA intake as % RNI with indicators of bone development

	Females		Males	
	Control (n=22)	**Diabetes** (n=13)	**Control** (n=22)	**Diabetes** (n=13)
Age (years)	r= 0.10 p= 0.67	r= -0.11 p= 0.73	r= -0.35 p= 0.11	r= 0.03 p= 0.93
BMC (g)	r= -0.003 p=0.99	r= 0.22 p= 0.47	r= -0.10 p= 0.66	r= 0.34 p= 0.26
BMC z-score	r= -0.16 p= 0.47	r= 0.45 p= 0.13	r= -0.11 p= 0.63	**r= 0.61** **p= 0.03**
BA (cm^2)	r= 0.07 p= 0.76	r= 0.26 p= 0.39	r= -0.18 p= 0.42	r= 0.40 p= 0.18
BA z-score	r= 0.10 p= 0.64	r= 0.40 p= 0.18	r= 0.31 p= 0.17	**r= 0.62** **p= 0.02**
BMD (g/cm^2)	r= -0.22 p= 0.32	r= 0.09 p= 0.76	r= -0.005 p= 0.98	r= 0.21 p= 0.48
BMD z-score	r= -0.40 p= 0.06	r= 0.21 p= 0.48	r= 0.40 p= 0.07	r= 0.28 p= 0.35

r= Pearson correlation coefficient
Significant correlations are shown in bold

Table 5.18: Best subsets regression of factors related to BMC in females and males with Type 1 diabetes

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent BMC (g) R^2 = 0.959, R^2 adj. = 0.956				
Constant	1137	333.000	3.415	0.001
BA (cm^2)	2.085	0.137	15.226	<0.001
Height (cm)	-16.940	3.311	-5.116	<0.001
Insulin (U/kg)	-119.490	48.087	-2.485	0.017
HbA1c (%)	-19.619	10.005	-1.961	0.056

BMC = constant + BA (mean) - height (mean) - insulin(mean) - HbA1c (mean)
= 1137 + 2.085(1646.3) - 16.940(155.6) - 119.490(0.9) - 19.619(7.9)

Table 5.19: Best subsets regression of factors related to BMD in females and males with Type 1 diabetes

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent BMD (g/cm^2) R^2 = 0.607, R^2 adj. = 0.562				
Constant	1.080	0.196	5.495	< 0.001
Age (years)	0.016	0.008	2.068	0.045
Height (cm)	-0.003	0.002	-1.596	0.118
Weight (kg)	0.005	0.001	4.314	<0.001
Insulin (U/kg)	-0.047	0.033	-1.424	0.161
HbA1c (%)	-0.016	0.007	-2.272	0.028

BMD = constant + age(mean) - height(mean) + weight(mean) - insulin(mean) - HbA1c(mean)
= 1.080 + 0.016(13.0) - 0.003(156.6) + 0.005(54.0) - 0.047(0.9) - 0.016(7.8)

6. Clinical Study: Discussion

Results of the DCCT showed that risk of long-term complications in children and adolescents with Type 1 diabetes could be reduced through intensive insulin management (29). However, the study did not examine the effects of intensive management on bone development. While most investigations have shown bone mass to be lower in children with the disease compared to those without diabetes (2,5-7), some researchers found no differences between the two groups (4,25). In addition, while the role of calcium in bone development is well investigated (43,76-7), the relationship of other nutrients such as phosphorus, vitamin D, and PUFA with bone development is not established. Therefore, the hypothesis for the clinical portion of this thesis was: **Type 1 diabetes results in lower whole body BMC, BA, and BMD during the growth period.** The objectives of this investigation in growing children age 8 to 18 years were:

1. To determine the relationship between Type 1 diabetes, its management and control, and indices of bone mass.
2. To determine the relationship of dietary calcium, phosphorus, vitamin D, and PUFA with indices of bone mass.
3. To determine the relationship of physical activity with indices of bone mass.

Few, if any studies have assessed bone development in children with diabetes by performing a whole body scan using DXA. Lower bone mass has

been found in children and adults following measurement of the lumbar spine and femoral neck using DXA (103), the radius, ulna, and humerus using photon absorptiometry (2,6,35,37), the ultradistal forearm using pQCT (44), and the radius using a bone mineral analyzer (36). While whole body measurements are the best measurements for children, they may mask differences between groups as well as differences in the growth pattern at specific sites. Thus any differences that might exist between the control and diabetes groups in indicators of bone development such as BMC, BA, or BMD, may not be apparent due to the type of scan performed.

In this study, the children with diabetes as a group had whole body bone mass similar to that of the children without diabetes, as indicated by BMC, BA, BMD, and standardized scores for each parameter (**Table 5.1**). Both groups of children appeared to be undergoing expected bone growth and development, with values for bone indicators comparable to those of the reference data (101-2). However, bone development in boys with diabetes appears to be impeded in a higher proportion of subjects than for girls. Although the differences were not significant, boys with diabetes tended to have lower BMC and BMD compared to the boys in the control group (**Figures 5.1, 5.3**). This was not true for girls (**Figures 5.2, 5.4**). Santiago et al. (47) and Hough (104), measuring cortical thickness, also found that among children with diabetes, boys in particular were prone to have reduced bone mass, although in both studies the girls with diabetes were also affected. These

gender differences may be the result of the differential timing of the pubertal growth spurt. Santiago et al. found that skeletal maturation was delayed in a third of the boys and a fifth of the girls studied, although cortical thickness was lower than that of controls in almost half of the subjects with diabetes, even when corrected to calculated bone age (47). Differences in standardized bone mass between genders may also be due to differences in metabolic control. In the current study, while the difference was not significant, boys had a higher mean morning blood glucose value than girls, although insulin dosage and HbA1c values were the same (**Table 5.2**). While he did not distinguish between boys and girls in terms of metabolic control, Hough's findings were similar in that individuals with lower bone mass had significantly higher fasting blood glucose levels, but not significantly different insulin dosage or HbA1c levels (104).

Poor metabolic control has been cited as a contributing factor of diabetic osteopenia (4,6,21,33). As a group, the children with diabetes in this study appeared to be maintaining acceptable metabolic control (**Table 5.2**). Both the mean insulin dosage and mean HbA1c values fell within expected ranges for this age group. Insulin dosage for prepubertal children may range from 0.4 - 1.2 U/kg/d, and from 0.6 - 1.7 U/kg/d for pubertal children (67). Others have reported that prepubertal children generally use 0.5 units of insulin/kg or less, while pubertal children may use as much as 1.5-2.0 units/kg (16,37). Thus, given the age range of the children in this study, a mean insulin dosage of 0.9

U/kg/d is reasonable. This is also true for the mean HbA1c values of 7.8% and 7.9% found respectively in females and males with diabetes. HbA1c values for children who do not have diabetes tend to fall between 4.5-6.5%; however, for children with the disease, this will be elevated. Mean HbA1c values of 8.3% and 8.6% have been reported for children with diabetes in this age group (29,67). Other parameters were also at acceptable levels. For both calcium and phosphorus, levels of urinary excretion were within normal ranges, and lower than those reported for individuals with diabetes in other studies (72,103). It should be pointed out that while calcium excretion was corrected to creatinine to control for urinary dilution, correction to sodium might also have been useful, given the strong positive relationship between dietary sodium and calcium excretion (78).

None of the indicators of metabolic control (insulin dosage, morning blood glucose values, HbA1c, urinary glucose, urinary creatinine, urinary calcium, or urinary phosphorus) were significantly correlated with bone development (**Table 5.3**). However, this may be due to the relatively high level of metabolic control being maintained by the children with diabetes as a group. This being the case, the lack of observed difference in indicators of bone growth and development between the diabetes and control groups might be expected.

Although the children with diabetes as a group were maintaining acceptable metabolic control, control in individual children ranged from poor to good, as indicated by HbA1c values ranging from 4.8% to 11.7%. The extent

to which control was maintained appeared to have a small but significant effect on BMC (**Table 5.18**). While BA was the largest contributing factor to BMC, daily insulin dose and HbA1c also contributed a small amount, although the contribution of HbA1c was not statistically significant. As expected, higher HbA1c levels and insulin dose resulted in lower BMC. Like HbA1c, insulin dose is an indication of metabolic control, but it may also be a reflection of endogenous secretion. McNair et al. found that patients with increased insulin dosage but lower residual beta-cell function had increased bone mineral loss compared to patients with higher levels of insulin secretion (34). A reduction in growth plate thickness and in longitudinal growth has also been reported to occur with insulin deficiency (13). This may be due at least in part to altered bone formation, since insulin is involved in the progression of osteoblast-like cells (7). For BMD, HbA1c was a statistically significant contributing factor; however, its clinical significance is questionable (**Table 5.19**). This finding is supported by the results of several researchers who found no significant relationship between BMD and HbA1c (25,55,103). Another group of researchers found an inverse relationship between BMD and HbA1c (44).

A number of studies have shown that age at onset affects growth in children with diabetes (26,60,64). In particular, a distinction can be made between prepubertal and pubertal onset, with children in the former group more prone to display complications than those with pubertal onset. In this study, children were not classified according to stage of pubertal development

at onset, but were compared according to age at onset by quartiles. No significant differences were found between groups for BMC, BA, BMD or standardized scores for any of these indicators (**Table 5.5**). Although measured BMC and BA were within the expected 95% range (**Figures 5.1 - 5.4**), standardized scores for these parameters indicate there is a transition period during which bone development may be impeded.

Duration of diabetes has been shown in a number of studies to be a factor in diabetic osteopenia (2-3,6,26,34), although in this study few differences were found based on duration of disease. Compared to children who were within 1 year or less of onset, children with longer disease duration had higher z-scores for BMC, BA, and weight, although there were no differences between the groups for BMD or height z-scores (**Table 5.4**). These results suggest that BMC and BA are affected most at, or even prior to, onset, before a diagnosis has been made. The time from onset until approximately one year following diagnosis might be considered a transition period during which metabolic changes occur. These changes restrict growth and development, including growth and development of bones. As children adapt to insulin treatment and dietary changes, bone development appears to stabilize or even accelerate. Similar to the findings of Levin et al. and McNair et al. (2,26), children with the longest duration of the disease had higher mean z-scores for BMC and BA. Using photon absorptiometry, both groups of researchers found that subjects with recent onset of diabetes had lower bone mass in the forearm

compared to controls than those subjects who had a longer duration of the disease. However, when data from the current study was grouped by quartiles, no differences were found between any of the groups for BMC or BMD, signifying that duration of disease may not significantly affect bone development.

The trend towards higher weight gain in children with diabetes seen here (**Table 5.4**) has been observed in several studies (29,61-2). The DCCT group showed that adolescents who had been on intensive treatment for 5 years had a significantly greater increase in BMI than those who were on conventional treatment, with the intensively treated group twice as likely to become overweight (29). Other researchers have also observed this trend. Compared to siblings without diabetes, children and adolescents with the disease had significantly higher weight and BMI (61). While this gain in weight developed primarily during and post-puberty, even at onset children had a higher z-score for weight compared to controls. Girls in particular are prone to becoming overweight. The high prevalence of overweight in girls with diabetes compared to boys with diabetes may be explained by hormonal differences (62). Insulin sensitivity may differ between pubertal males and females, mediated by adrenal androgen metabolism. Eating disorders, more common in adolescent females than males, might also be a contributing factor.

This excess weight might be another explanation for the differences in bone mass seen between boys and girls with diabetes in this study. It is likely

that the trend towards elevated weight seen in the group with the longest duration of disease (**Table 5.4**) is due mainly to the larger proportion of females (nine) compared to males (five) in this group. In other words, the elevation in standardized weight with duration of disease may have been primarily due to high weight for age in the girls. It has been suggested that elevated body weight increases bone mass through the increased mechanical forces of bearing the excess weight (94). A study of obese children showed that compared to non-obese children, the former have higher total body BMC, possibly as a result of higher BA (105). An increase in both BMC and BA would result in little or no change in BMD which could explain the lack of significant difference in BMD between groups in the present study (**Table 5.4**).

Physical activity is known to have a positive weight-bearing effect on bone growth and development, although the amount and type of exercise, as well as how often it is performed are important (92-3). Weight-bearing activity is known to increase BMC, BA, and bone strength by promoting osteoblastic activity, and may be especially important in childhood and adolescence (92). In this study, reported activity levels in children with and without diabetes ranged from extremely low to very high (**Table 5.6**). In spite of the large differences in reported activity level, no significant differences were seen in indicators of bone development when subjects were grouped according to level of physical activity (**Table 5.7**). This is contrary to the findings of Welten et al. who demonstrated that weight-bearing activity is a significant contributor

to BMD (91). Another group of researchers found that over the period of 1 year, mean gain in BMD was 14-128% higher in physically active children and adolescents compared to inactive children and adolescents (90). In contrast, no relationship was found in the current study between physical activity and any of the indicators of bone development for control subjects or for boys with diabetes (**Table 5.8**). There was however, a significant relationship seen between physical activity and BMC, BA, and BA z-score in the females with diabetes. Interpretation of these results is made more difficult by the observation of lower standardized scores for BMC, BMD, and BA in subjects who responded to the survey compared to those who did not respond. It is possible that had the entire group responded to the survey and been included in the subsequent analysis that more expected results might have been found.

Dietary factors, including intakes of calcium and vitamin D are also known to be positively correlated with bone development (43,69,72-3,76-7). More recently, intakes of PUFA have been associated with elevated bone volume, weight, and strength, as well as elevated formation rates (30,86). However, no consistent pattern was found in this study between dietary factors and indicators of bone development. Significant positive relationships were seen in the males with diabetes between dietary PUFA and BMC z-score (**Table 5.17**), dietary PUFA and BA z-score (**Table 5.17**), and dietary phosphorus and BMD z-score (**Table 5.16**); in the females with diabetes there was a significant positive relationship between dietary phosphorus intake and BA z-score (**Table**

5.16). However, with these exceptions, no relationship, or a negative relationship was found (**Tables 5.14 - 5.17**). Welten et al. found no significant relationship between calcium intake and BMD of the lumbar spine in healthy adolescents and young adults, although subjects with the highest consumption of calcium tended to have higher BMD than the rest of the subjects (91). In contrast, Ruiz et al. found that calcium intake contributed significantly to BMD in the femur and spine in healthy prepubertal boys and girls, and in pubertal boys (92). In terms of level of intake, the results of the 3-day food records are not unexpected: while the mean intakes for all subjects are at or above the RNI for calcium, phosphorus, vitamin D, and PUFA, many individual subjects were not consuming adequate amounts of these nutrients (**Tables 5.10 - 5.13**). In this study, older children were less likely to meet the RNI for calcium (**Table 5.10**), vitamin D (**Table 5.11**), and phosphorus (**Table 5.12**) compared to younger children. However, when looking at the group as a whole, the effect of these nutrients on bone development may be minimal, since they are at the recommended amounts for optimal bone health.

In this study, diet was analyzed on the basis of intake as a percent of RNI. Since both nutrient intake and bone mass are positively correlated with age, it would be difficult to draw any meaningful conclusions from analysis of absolute intakes. However, there are also problems with analysis of RNI's since they increase in a stepwise fashion, while bone mass increases in a more linear

manner. In other words, the RNIs are not consistently proportionate to body size.

Results from the DCCT showed that intensive management of diabetes significantly slowed the progression of disease related complications such as retinopathy, neuropathy, and nephropathy. This intensive management involves 3 or more injections of insulin per day, compared to the 2 injections per day in conventional management. According to these guidelines, 63% of girls and 57% of boys in this study were on intensive management, with 37% and 43%, respectively receiving conventional treatment . Although no relationship was found between insulin dose and BMC, BA, or BMD (**Table 5.3**), it might be speculated that better management of the disease through improved insulin regimes does have a positive effect on bone development. Insulin therapy has been shown to normalize indicators of bone formation, resorption, and growth (1). Thus intensive management also appears to attenuate progression of less critical complications such as impaired growth and bone mass.

In conclusion, as a group, children with Type 1 diabetes did not have impaired bone development as indicated by BMC, BA, and BMD similar to children without the disease, although bone mass tended to be lower in boys with diabetes compared to boys without the disease. In addition, there appears to be a transition period extending from onset of the disease (prior to diagnosis) through the first year after diagnosis. Following this transition

period, bone development appears to stabilize, and may even accelerate. The effect of diet on bone development, while not significant in this investigation, cannot be ruled out, since as a group, the children were consuming the recommended intakes. This suggests that the maximum benefit of dietary intake on bone development was already attained.

7. Clinical Study: Strengths and Limitations

While many studies have investigated bone mass in healthy children using DXA (89,101-2,106), and many studies have investigated bone mass in children with diabetes using other methods (2,6,34-7), few have investigated bone mass in children with diabetes using DXA. Consistency of the DXA measurements was aided by the fact that all scans were performed by one trained individual. Comparison of bone mass measurements in the control group with published reference data helped ensure that these children were undergoing expected growth and were providing an acceptable reference with which to compare the diabetes group. In spite of these strengths, there were a number of limitations to the study.

The first limitation relates to method of recruitment. Because all subjects were volunteers, the results of the study are prone to selection bias. The small sample size is also a limitation. To ensure adequate power, it was initially proposed to recruit 100 children in each of the diabetes and control groups. However, this proved impossible over the expected time frame for the study, and the goal became to recruit as many children as possible. While a sample of 119 children may seem adequate, the size of each of the four groups (ie control females and males and diabetes females and males) was not large. As a result, differences between groups that may have been significant given a larger sample size were not significant in this study, leading to a possible Type II error. This is particularly true for the analyses of diet and physical activity,

since the number of subjects who completed the 3-day food records and physical activity questionnaires was low. Interpretation of the results of the physical activity questionnaire is further complicated by the finding of significant differences in bone mass between those who responded to the questionnaire and those who did not. Another limitation relates to the whole body scan. While whole body measurements of bone mass are the best measurements for children due to changing bone geometry during growth, they may mask changes that are occurring at regional sites. Whole body measurements do not allow for differentiation of trabecular and cortical bone, which may be important given that most changes in bone mass in diabetic osteopenia have been reported to occur in trabecular bone. There were also limitations to the nutrient analyses program used to analyze nutrient intake; intakes of PUFA could be analyzed only as total PUFA, with no distinction made between n-6 and n-3 PUFA. In addition, grouping children based on intake as a percentage of the RNI's in order to compare bone mass has limitations. This is because the RNI's are not consistently proportionate to body size. One way around this would be to correct intake of individual nutrients to energy intake. Finally, this was an observational study only, with no treatment being applied. As a result, any significant differences or relationships seen in this study cannot be attributed to a treatment per say.

8. Clinical Study: Future Research

The direction of future research is based on the limitations of the study. Although recruitment is difficult, future studies should attempt to recruit a larger sample, allowing for more subjects per group, thereby increasing the possibility of observing significant differences. A longitudinal study would provide more information on the changes in bone development that occur over time in children both with and without Type 1 diabetes, and might allow for measurement of peak bone mass. Future investigations might also differentiate between cortical and trabecular bone through regional, rather than whole body, scans, and investigate changes in each type of bone. As well, differentiating between intakes of n-6 and n-3 PUFA using a different nutrient analysis program would be of benefit. Finally, to further examine the role of diet and physical activity on bone development in children with diabetes, an experimental design is necessary with children randomly assigned to a particular diet and/or exercise treatment group.

9. Basic Study: Methods

9.1 Rationale for Diabetes Induction: The rat model is frequently used to study complications due to diabetes such as diabetic osteopenia (11). In rats, diabetes is generally defined as blood glucose levels of greater than 19 mmol/L (12,18,107). Two models for Type 1 diabetes in rats exist: the spontaneously diabetic or BB rat, and the streptozotocin (stz)-induced diabetic rat. Stz is an antibiotic which, when administered in the appropriate dose, selectively destroys the pancreatic β-cells. Destruction of the pancreatic β-cells is extensive in the BB rat, resulting in a ketotic form of diabetes similar to Type 1 diabetes in humans (50). However, the proportion of these rats that develop diabetes is variable. Although the proportion of animals which develop diabetes following stz injection also varies, ranging from 40% to 100% (11-2,18,107-8), development is more rapid than in the BB rat, and allows the experimenter greater control. Rats injected with stz test positive for diabetes within 1 to 5 days (11-2,109-10). For these reasons, stz-induced diabetic rats were used in this research. A review of the literature (**Table 9.1**), reveals that there is little standardization in terms of injection site, concentration of stz used, or the success rate of the procedure. This may be due at least in part to the varying ages, weights, and type of rat used by different researchers.

9.2 Rationale for Diet: The dietary component of this investigation was designed to examine the effects of n-3 PUFA on bone compared to the effects of n-6 PUFA. The diet was based on the American Institute of Nutrition (AIN)

standard diet for growth which contains 70 g of soy oil per kg diet. The soy oil diet was therefore rich in n-6 PUFA but low in n-3 PUFA, and was used as the control diet. For the experimental diet, menhaden oil, rich in n-3 PUFA, replaced the soy oil. To ensure that the animals received adequate amounts of n-6 fatty acids to prevent essential fatty acid deficiency, the menhaden oil was combined with corn oil in a 1.3:1 ratio.

9.3 Rationale for Bone Measurements: A couple of measures are commonly used to determine bone formation. Osteoblasts are responsible for bone matrix formation, and therefore, proteins synthesized by osteoblasts may be indicators of osteoblastic metabolism and bone formation. Alkaline phosphatase (ALP) is one such protein that has been frequently used as a measure of bone formation. However, serum measurements do not specifically indicate bone formation, since ALP is derived from sources such as liver and kidney in addition to bone (110-1). Osteocalcin (OC), a 1,25-$(OH)_2D_3$ dependent bone matrix protein, is another protein produced by osteoblasts. Determined by radioimmunoassay, it is the standard measure of bone formation. This osteoblastic protein is secreted by mature osteoblasts into the blood, and once in circulation is cleared quickly by the kidney (10,15). Although OC, which has a high affinity for hydroxyapatite, is found primarily in bone, serum OC levels provide a non-invasive biochemical measure of bone mineralization rate and bone formation (10,15, 113).

A number of methods are available for measuring bone resorption, but of these, a relatively new method appears to be the most direct, reproducible, and reliable (114). This assay involves quantitation of type 1 collagen cross-linked N-telopeptides in urine. Although 2 pyridinoline-forming sites exist within bone type I collagen, the N-telopeptide site is used in this assay for several reasons. Fragments from this site are found in abundance in urine, the site contains approximately 60% of the lysylpyridinoline in human bone collagen, and interactions forming pyridinolines at this site allow for the distinction of bone collagen, as opposed to type 1 collagen from other tissues. Another benefit of this method is its suitability for testing of subjects both with and without disordered bone metabolism. As well, the assay requires minimal amounts of urine and a maximum of 2 hours to attain final results. In addition, the peptide epitope used is very stable, with the ability to withstand several freeze-thaw cycles. N-telopeptide is thus a reliable, non-invasive parameter of bone resorption.

In addition to changes in bone formation and resorption, bone mineralization has been shown to be adversely affected by diabetes (8). The hydroxyapatite crystals which form the mineralized portion of bone are composed of calcium and phosphorus. These 2 minerals, which are easily measured in bone samples, are the primary minerals found in bone. Therefore, they were assayed as a measure of bone mineralization.

9.4 Rationale for Liver Fatty Acid Measurement: Alterations in fatty acid status in various tissues results from alterations in fatty acid intake. In order to demonstrate these changes in tissue fatty acids, the fatty acid content and percent fatty acid composition of liver was assessed. Liver tissue was chosen due to the fact that measuring bone fatty acid status is difficult and time consuming. In addition, it has been established that liver fatty acid status provides a reliable indication of bone fatty acids, since the two have been shown to be related (30,82,115).

9.5 Ethical Approval: The study was approved by the University of Manitoba Protocol Management and Review Committee, and all procedures followed acceptable guidelines as outlined in the Guide to the Care and Use of Experimental Animals (116).

9.6 Population: Eighty male weanling Sprague-Dawley rats (21 days of age) were used to study a period of rapid growth representative of pubertal growth in humans. Upon receipt of the animals from Central Animal Care (University of Manitoba), animals were allowed an adaptation period of 5 to 7 days, during which they were maintained ad libitum on standard laboratory chow and given free access to water. All animals were housed individually to allow for more accurate monitoring of food consumption. Two animals from the diabetes-induced group were not included in the analysis due to premature death; one animal was found dead during the adaptation period, while a second animal was euthanized on day 8 of the study due to excessive weight loss.

9.7 Study Design: Animals were randomly assigned to either the control or the diabetes group, and to one of 2 dietary treatments: soy or fish oil. The control group was further divided into control and pair-weigh groups. In total 40 animals were assigned to the diabetes group, and 20 each to the control and pair-weigh groups. Animals were equally divided between the dietary treatment groups. Animals were studied in 10 blocks of 8, with each block containing all of the treatment and diet groups.

9.8 Streptozotocin Treatment: On day 1 of the experiment, following the adaptation period, 39 of the 40 rats in the diabetes group received 2 intraperitoneal injections of stz (Sigma-Aldrich, St. Louis, MO, 60 mg/kg) freshly dissolved in 0.9% NaCl (Health Sciences Centre, Winnipeg, MB) at a concentration of 15 mg/mL. The injections were given approximately 24 hours apart. The 40 rats in the control groups, plus one rat from the diabetes group which experienced growth failure during the adaptation period, were injected with saline buffer only (0.3-0.5 mL). Three days after the second injection, a blood sample to measure blood glucose levels was taken via the saphenous vein from all of the stz-injected rats and 18 of the saline-injected rats. Diabetes was defined as blood glucose levels of 13 mmol/L or greater. Outward signs of diabetes such as increased food and water intake and polyuria were used to confirm the diagnosis. Rats which had received stz-injection, but had blood glucose levels less than 13 mmol/L remained in the diabetes group, and were labelled non-responders.

9.9 Diet: The soy diet consisted of the standard AIN diet for laboratory rodents with 70 g of soybean oil (Vita Health, Winnipeg, MB) per kg diet, but with dextrose (Moonshiners, Winnipeg, MB) replacing sucrose (**tables 9.2-9.4**) (117). The fish oil diet was composed of the standard diet but with menhaden oil (Omega Protein Inc. Reedville, VA, 40 g/kg diet) plus corn oil (Mazola, University of Manitoba Food Services, Winnipeg, MB, 30 g/kg diet) replacing the soybean oil. All other diet ingredients except the cornstarch (Best Foods Canada, Etobicoke, ON) were obtained from Harlan Teklad (Madison, WI). Diets were prepared prior to the start of the experiment and were stored at 4°C. Rats were maintained on the diet for 5 weeks, starting on day 1 of the experiment. Fresh feed was provided daily, along with free access to water throughout the experimental period.

In order to control for the lower weight gain commonly seen in diabetic rats (9), the control group was divided into a control and a pair-weighed group. Using weight gain as a measure, the pair-weighed group was matched with the corresponding stz-injected rats from the same block and diet group. To limit weight gain in the pair-weigh group, feed was provided using the following equation:

Mean weight of stz-injected rats from previous 3 days

x mean feed efficiency of the control rat over the previous 3 days.

Feed efficiency was defined as total amount eaten per day (g) ÷ the weight of the rat (g) on that particular day. Feed restriction was begun when difference

in weight gain between the control and diabetes groups became apparent, generally day 7 or 8.

9.10 Growth: All rats were weighed upon receipt, prior to stz- or saline-injection, and then daily throughout the experimental period using a scale with an animal weighing program (Metler Toledo SB32000, VWR Canlab, Mississauga, ON).

9.11 Termination: On day 34 or 35 of the experiment, all animals within a block were housed individually in metabolic cages for a 12 hour overnight fast beginning at 8:00 p.m. Twelve hour urine volume was recorded and urine was collected and stored at -20° C. After removal from the metabolic cages, rats were anaesthetized with an intraperitoneal injection of Ketamine/Xylazine (Ketamine: 100 mg/mL, 90 mg/kg; Xylazine: 20 mg/mL, 10 mg/kg). Tail length was measured with animals lying on their backs, and the tail extended to the side. The measurement was made using a measuring tape, starting from the spot where the tail extended from the body to the tip of the tail. To improve accuracy, all measurements were taken by one investigator. When rats were no longer responsive, they were exsanguinated via cardiac puncture. An incision was then made into the peritoneal cavity to allow for excision of organs. The heart, liver, pancreas, and both kidneys were removed, weighed to the nearest 0.1 g, flash frozen in liquid nitrogen, and stored at -80° C. The left femur was removed and stored at -20° C. The diaphyseal shaft of the right femur was removed, freed of soft tissue and marrow, placed in 10 mL of 10%

Hank's Balanced Salt Solution (Sigma Diagnostics, Inc., St. Louis, MO), and incubated in a 37° C waterbath for 2 hours. The femur was then removed from the Hank's solution and weighed to the nearest 0.1mg, and both femur and Hank's solution were stored at -20° C. The blood collected by cardiac puncture was placed in vacutainers containing 100 u.s.p. units of sodium heparin (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ), and kept on ice until processed. Processing consisted of centrifuging the whole blood at 2400 rpm for 15 minutes at 4° C (Beckman TJ-6R, Beckman Instruments, Inc., Mississauga, ON), pipetting the plasma into a clean glass tube, centrifuging again at 2400 rpm for 15 minutes at 4° C, and then pipetting the plasma into a glass vial for storage at -20° C.

9.12 Femur Length: The whole femur was scraped clean of all soft tissue, then femur length was measured to the nearest 0.001 inches using calipers. This measurement was then converted to centimetres. To ensure consistency, measurements were always taken with the calipers lying on a flat surface, the femur aligned with the hip joint to the right, and the femur lying as flat as possible against the tabletop. Each femur was measured once, then a random sample of 35 femurs was re-measured to ensure precision of measurements.

9.13 Calcium and Phosphorus: Both the femur shaft and a urine sample were assayed for calcium and phosphorus content, indicators of bone mineral content. For the former, a 40 -60 mg piece of bone was tested, while for the latter, 0.25 mL of urine was analyzed, with selected urine samples assayed in

triplicate. In either case, the sample was placed in a glass test tube to which 0.5 mL concentrated HNO_3 (Fisher Scientific, Nepean, ON) was added. The tube was left in the fume hood overnight to allow the sample to dissolve completely. When the contents of the tube were completed dissolved, 9.5 mL of deionized water was added. The mixture was then transferred to a 20 mL scintillation vile for storage until calcium and phosphorus content were analyzed via emission spectrometry (Varian Liberty 200 ICP, Varian Canada, Mississauga, ON).

9.14 Glucose: Plasma glucose is commonly used to test for diabetes, with elevated levels (> 13 mmol/ L in this experiment) indicating the presence of the disease. Glucose was assayed using an adapted microassay (Procedure number 510-A,Sigma Diagnostics, Inc., St. Louis, MO) as per the clinical study except samples were diluted 0- to 75-fold.

9.15 Creatinine: Creatinine was assayed using a colourimetric assay (Procedure number 555,Sigma Diagnostics, Inc., St. Louis, MO) to control for urinary dilution.

Procedure: Urine samples were diluted 0- to 20-fold with deionized water, and the mixture was vortexed for 5 to 10 seconds to ensure uniformity. A standard curve was developed using 4 concentrations of standard: 2.5 mg/dL (25 μL of 10 mg/dL standard + 75 μL deionized water), 5.0 mg/dL (50 μL of 10 mg/dL standard + 50 μL deionized water), 7.5 mg/dL (75 μL of 10 mg/dL standard + 25 μL deionized water), and 10.0 mg/dL (100 μL of 10 mg/dL)

standard. Deionized water (blank), standard or sample (0.3 mL) were pipetted into a designated cuvet. A 5:1 mixture of creatinine colour reagent and sodium hydroxide formed an alkaline picrate solution; 3 mL of this solution was added to each cuvet. Cuvets were then covered with parafilm, inverted gently 3 times to mix, and incubated at room temperature (18-26°C) for 8 to 12 minutes. After the incubation, the absorbance was read at 500 nm on a Milton Roy Spectronic 3000 Array (Fisher Scientific, Nepean, ON). Acid reagent (0.1 mL) was then added to all cuvets, which were again covered with parafilm and inverted gently 3 times to mix. Cuvets were incubated at room temperature (18-26°C) for 5 minutes, and the absorbance was again read at 500 nm. The sample concentration was calculated from the standard curve, as per the clinical study.

9.16 Osteocalcin: The osteocalcin (OC) content of plasma was assayed using an osteocalcin ^{125}I radioimmunoassay kit (Procedure number 15130, DiaSorin, Stillwater, MN). OC, a $1,25(OH)_2D_3$ dependent bone matrix protein, is produced by osteoblasts, and is the standard measure of bone formation. This osteoblastic protein is secreted by mature osteoblasts into the blood, and once in circulation is cleared quickly by the kidney (10,15). Although OC, which has a high affinity for hydroxyapatite, is found primarily in bone, plasma OC levels provide a non-invasive biochemical measure of bone mineralization rate and bone formation.

Procedure: The 0 standard was reconstituted with 20 mL deionized water, and inverted gently 2 or 3 times to mix. The standard (28 mg/mL) and OC controls were reconstituted with 1.0 mL deionized water, and inverted 2 or 3 times to mix. Once reconstituted and mixed, the standards and controls sat at room temperature for 15 to 20 minutes until contents were completely dissolved. A set of 5 standards was then made by serial dilution, starting with the addition of 250 μL of 28 ng/mL standard to 250 μL of 0 standard.

All standards, OC controls, and undiluted samples (50 μL) were pipetted in duplicate into a designated 12 x 75 mm tube to which 200 μL of OC antiserum was added. ^{125}I osteocalcin (200 μL) was then added to all tubes. To control for nonspecific binding, one tube contained 50 μL of 0 standard and 200 μL ^{125}I osteocalcin, but no antiserum. To determine the total counts expected, one tube contained only ^{125}I osteocalcin. All tubes were vortexed at a low speed for approximately 5 seconds. Tubes were then incubated at 2-8° C for 16 to 24 hours.

Following incubation, the precipitating complex was reconstituted with 35 mL deionized water, inverted 2 or 3 times to mix, and allowed to sit for 30 minutes at room temperature. The complex was then swirled and shaken gently to ensure complete mixing, and 500 μL was added to each tube except the total count tube. All tubes were vortexed at low speed for approximately 5 seconds and incubated for 2 hours at 2-8° C.

Tubes were then centrifuged (Beckman TJ-6R, Beckman Instruments, Inc., Mississauga, ON) for 40 minutes. Being careful not to disturb the precipitate, the supernatant fraction was poured off and discarded from all tubes except the total counts tube. Decanted tubes were then inverted and blotted on paper towel. They were left inverted for 30 to 60 minutes; any remaining drops were then blotted dry with Kim Wipes. The total count tube and the precipitate in all other tubes was then counted using a Cobra^{TM} II Auto-Gamma (Canberra Packard, Mississauga, ON). OC content of the samples was determined by plotting the regression of x from y using the total counts and known OC concentration of the standards using GraphPad Prism 2.0 (GraphPad Software Inc., San Diego, CA).

9.17 Parathyroid Hormone: Plasma PTH was measured using an enzyme-linked immunosorbent assay (ELISA) (Procedure number 60-2500, Immutopics, Inc., San Clemente, CA). PTH is a primary hormone regulating intestinal absorption, renal resorption, and bone turnover of calcium, and therefore is an important factor in bone metabolism.

Procedure: The 0 standard was reconstituted with 2.0 mL of deionized water, and the remaining standards (1 to 2700 pg/mL) and controls were reconstituted with 1.0 mL of deionized water. All vials were mixed by gentle inversion and swirling and allowed to sit for 20 minutes. Wash solution was prepared by diluting wash concentrate with 20 times deionized water and stirring to mix. Working antibody solution was prepared just prior to use by

mixing equal volumes of rat PTH biotinylated antibody and rat PTH HRP (horseradish peroxidase) conjugated antibody. Standards, controls, and undiluted samples (25 μL) were pipetted in duplicate into the designated well in the microplate. Working antibody solution (100 μL) was then pipetted into each well. The plate was covered with a plate sealer and aluminum foil to avoid exposure to light, and incubated at room temperature (18-24° C) for 3 hours, being swirled occasionally to mix. Following incubation, the aluminum foil and plate sealer were removed, and the plate was inverted to discard the contents. The plate was then washed by pipetting 350 μL of wash solution into each well, inverting the plate, and blotting it dry. The wash step was repeated 5 times. ELISA HRP substrate (150 μL) was then pipetted into each well. The plate was again covered with a plate sealer and aluminum foil, incubated at room temperature for 30 minutes, and swirled occasionally to mix contents. After 30 minutes, the aluminum foil and plate sealer were removed, and the absorbance of the plate was read at 595 nm using the Spectra Max 340 Microplate Spectrophotometer System (Molecular Devices, Sunnyvale, CA). Immediately after reading the absorbance, 100 μL of ELISA stop solution was pipetted into each well, and the plate was swirled gently to mix. The absorbance was then read again at 450 and 595 nm.

9.18 Insulin-like Growth Factor-I: Plasma IGF-I was measured using an enzyme immunoassay kit (Procedure number DSL-10-2900, Diagnostic Systems Laboratories, Inc. Webster, TX). IGF-I acts both directly and

indirectly on bone, and its measurement provides an indication of metabolism affecting bone development.

Procedure: Six standards ranging from 0 to 3600 ng/mL rat IGF-I and 2 controls were reconstituted with 1.0 mL of deionized water, swirled and gently inverted 3 to 5 times to mix thoroughly, and allowed to sit at room temperature for 5 to 10 minutes. Sample buffer I (140 μL) was pipetted into a culture tube. Undiluted sample (10 μL) was added to the sample buffer; the mixture was vortexed for 5 to 10 seconds and incubated at room temperature for 30 minutes. Following incubation, 150 μL sample buffer II was added to the tube and the mixture was vortexed again for approximately 5 seconds. Standards, controls, and pretreated samples (50 μL) were pipetted in duplicate into the designated well in the microplate. Rat IGF-I biotin conjugate solution was prepared by diluting IGF-I biotin conjugate concentrate with 2000 times the amount of biotin conjugate diluent. After sitting for 10 to 15 minutes, 100 μL of biotin conjugate solution was added to each well. Rat IGF-I antiserum (100 μL) was then added to each well. The plate was incubated for 1 hour at room temperature (18-24° C), being swirled occasionally to ensure mixing. Wash buffer was prepared by adding 25 parts deionized water to 1 part wash concentrate. The plate was washed by pipetting 350 μL of wash solution into each well, inverting to discard the contents, and blotting dry on paper towel. The wash step was repeated 4 times. Streptavidin-enzyme solution was prepared immediately prior to use by adding 1 part streptavidin-enzyme

conjugate concentrate into 50 parts streptavidin-enzyme conjugate diluent. Streptavidin-enzyme conjugate solution (200 μL) was then pipetted into each well, following which the plate was incubated for 30 minutes at room temperature (18-24° C). The plate was then washed 5 times as before. TMB chromogen solution (100 μL) was added to each well, and again the plate was incubated for 30 minutes at room temperature (18-24° C), being swirled occasionally to ensure mixing. Stop solution (100 μL) was pipetted into each well, and the plate was gently shaken for 5 to 10 seconds. The absorbance was then read at 450 and 600 nm on a Spectra Max 340 Microplate Spectrophotometer System (Molecular Devices, Sunnyvale, CA).

9.19 N-telopeptide: Urinary N-telopeptide (NTx) was assayed using an Osteomark competitive-inhibition ELISA (Ostex International Inc. Seattle, WA). This assay involves quantitation of type 1 collagen cross-linked N-telopeptides which are specific to bone. Osteoclasts regulate the formation of NTx which is eventually degraded and excreted in urine. NTx is therefore a marker of bone resorption.

Procedure: Wash solution was prepared by diluting the required amount of wash concentrate with 30 times deionized water, and mixing gently. Antibody conjugate solution was prepared by diluting antibody conjugate concentrate in a 1:101 ratio with antibody conjugate diluent, and swirling to mix. Blank (deionized water), standards (1 to 3000 nm bone collagen equivalents [BCE]), controls, and undiluted samples (25 μL) were pipetted in

duplicate into the designated well of the microplate. Antibody conjugate solution (200 μL) was then added to each well, the plate was covered with a plate sealer, swirled gently for 5 to 10 seconds, and incubated at room temperature (18-24° C) for 90 minutes.

During the last 10 minutes of incubation, the chromogen/buffered substrate was prepared by diluting chromogen reagent with buffered substrate in a 1:101 ratio, and swirling to mix. At the end of the incubation period, the plate sealer was removed from the plate, and the plate was washed 5 times. Washing consisted of inverting the plate to discard the contents of the wells, blotting the plate dry on paper towel, then pipetting 350 μL of wash solution into each well, and again inverting the plate and blotting dry. After washing, 200 μL of chromogen/buffered solution was added to each well. The plate was covered with a plate sealer and incubated at room temperature (18-24° C) for 15 minutes. Following this incubation, 100 μL of stop reagent was added to each well, and the plate was swirled for 5 to 10 seconds to mix. The plate was allowed to sit for approximately 5 minutes at room temperature (18-24° C) before being read at 450 and 630 nm on the Spectra Max 340 Microplate Spectrophotometer System (Molecular Devices, Sunnyvale, CA).

9.20 Prostaglandin E_2: Prostaglandin E_2 was assayed using a competitive enzyme immunoassay (Procedure number DE0100, R&D Systems, Inc., Minneapolis, MN). PGE_2, which is synthesized indirectly from arachidonic acid, is known to stimulate bone resorption and formation.

Procedure: All samples were diluted 10-fold in assay buffer ED1. Eight standards were made by serial dilution, starting with 100 μL of 50,000 pg/mL standard stock solution being added to 900 μL of assay buffer ED1. Wash solution was made by diluting the required amount of wash buffer concentrate with 10 times the amount of deionized water. Assay buffer, standard or sample (100 μL) was pipetted in duplicate into the designated wells in the microplate. PGE_2 conjugate (50 μL) was then pipetted into each well, followed by 50 μL of PGE_2 antibody solution. The plate was then covered with an adhesive strip and incubated for 2 hours at room temperature, occasionally being shaken gently to ensure complete mixing. Following the incubation, the plate was washed 3 times by decanting the contents, blotting the plate dry, and adding 350 μL wash solution to each well. pNPP substrate (200 μL) was then added to all wells, and the plate was incubated at room temperature for 1 hour. Stop solution (50 μL) was added to each well, and the optical density was determined using a SPECTRAmax 340 Microplate Spectrophotometer System (Molecular Devices, Sunnyvale, CA) with wavelength set at 405 nm and 580 nm.

9.21 Vitamin D: $1,25(OH)_2D_3$ is formed through liver and kidney hydroxylation of vitamin D_3 synthesized in the skin. It is controlled by levels of calcium, phosphorus, and PTH, and as a result, reflects their metabolism. $1,25(OH)_2D_3$ was measured by RIA.

Procedure: Standards ranging from 5 to 200 pg/mL were prepared by reconstituting with 3.0 mL of 0 standard, mixing thoroughly, and allowing to stand at room temperature for 15 to 20 minutes. All other reagents were also used at room temperature. Five hundred μL of each standard and control, and 100 μL (insulin deficient) or 500 μL(insulin sufficient) of sample was pipetted into a 12 x 75 mm tube. Five hundred μL acetonitrile (Fisher Scientific, Nepean, ON) was then added to each tube, and tubes were vortexed 3 times over the next 10 minutes, then centrifuged (Beckman TJ-6R, Beckman Instruments, Inc., Mississauga, ON) for 10 minutes at 20-25° C. Following centrifugation, the supernatant fraction was poured into a clean 12 x 75 mm tube, 500 μL of Pretreatment Solution was added to each tube, and the tube was vortexed. A Vac Elut SPS 24 (Varian Sample Preparation Products, Harbor City, CA) was used for the extraction procedure. With the Vac Elut set to "waste", and prior to applying the samples to the column, the cartridges were cleaned by pouring 2 to 3 mL of methanol (Fisher Scientific, Nepean, ON) onto each one. The sample was then added to the column, followed in turn by 5 mL 70:30 methanol/water, 5 mL hexane/methylene chloride (Omnisolv hexane: Em Science, Gibbstown, NS; methylene chloride: Fisher Scientific, Nepean, ON), and 5 mL 99:1 hexane/isopropanol (Fisher Scientific, Nepean, ON). The Vac Elut was then switched to "collect", and 3 mL 92:8 hexane/isopropanol was added to each cartridge. Samples were then stored at -20° C for 16 to 20 hours.

Sample tubes were evaporated to dryness under nitrogen in a 40° C waterbath, then reconstituted with 75 μL 95% ethanol and vortexed at low speed. 125 μL ^{125}I was then added to each tube, and the tubes were again vortexed. The remainder of the assay was performed in duplicate. 75 μL of standard, control, or sample was added to a 12 x 75 mm tube and 300 μL of antibody was added to each tube. The tubes were then incubated for 2 hours, after which 500 μL of precipitating complex was added. Tubes were incubated for 20 minutes at room temperature, and centrifuged for 40 minutes at 20-25° C. The supernatant was decanted, and tubes inverted then blotted dry as per the osteocalcin assay. To determine total counts and non-specific binding, TC and NSB tubes were prepared by adding 125 μL tracer to 75 μL 95% ethanol. 300 μL of NSB buffer was then added to the NSB tube. The TC tube and the precipitate in all other tubes were then counted using a Cobra™ II Auto-Gamma (Canberra Packard, Mississauga, ON). $1,25(OH)_2D_3$ content was determined using the known concentration of the standards.

9.22 Liver Fatty Acids: Liver fatty acids were analyzed to differences in fatty acid status based on intakes of n-6 and n-3 PUFA.

Extraction Procedure: Liver fatty acids were extracted using chloroform:methanol (2:1) following the method devised by Folch et al. (118). For each animal, a piece of liver weighing 0.9 to 1.1 g was placed in a 50 mL screw top tube containing 10 mL of 2:1 chloroform: methanol (pesticide grade, Fisher Scientific, Nepean, ON) with 0.01% BHT, and 100 μL of C17 standard.

The standard consisted of 16 mg/mL heptadecanoic acid (Sigma, St. Louis, MO) in chloroform. The contents of the tube were homogenized (Vertis Model 23-Macro, Canlab, Mississauga, ON) for 10 to 20 seconds until the liquid had a uniform consistency. Pesticide grade methanol (Fisher Scientific, Nepean, ON, 3 mL) was then added to the tube, and the tube was capped and vortexed for 15 seconds. The rotor of the homogenizer was rinsed clean between each sample. Tubes were centrifuged for 15 minutes at 1500 rpm using a Beckman GS-6 centrifuge (Beckman Instruments, Inc., Mississauga, ON). Following centrifugation, the solvent layer was poured into a clean 50 mL screw top tube and 6 mL of chloroform and 5 mL of 0.73% NaCl (Fisher Scientific, Nepean, ON) were added to each tube. The tubes were capped and vortexed for 30 seconds, then centrifuged for 10 minutes as before. The top layer was pipetted off and discarded. Top upper phase (TUP, 1 to 2 mL) (chloroform: methanol: water 3:48:47) was added to each tube. The top layer was then pipetted off and discarded. Another 1 to 2 mL of TUP was added to each tube, and again the top layer was pipetted off and discarded. The remaining bottom layer was then pipetted into a 15 mL screw top tube and evaporated to dryness under nitrogen (Praxair Products Inc., Mississauga, ON) in a 30° C water bath (OA-Sys Heating System, Organomation Associates, Inc., Berlin, MA). Once dry, 2 mL chloroform was added to the tube, and contents were transferred to a 4 mL vial and stored at -20°C.

Methylation Procedure: Extracted lipid (500 mL) was pipetted into an 8 mL screw top tube, and evaporated to dryness under nitrogen in a 30° C water bath. Once dry, 1 mL toluene (Optima grade, Fisher Scientific, Nepean, ON) and 1.2 mL methanolic HCl (Supelco, St. Louis, MO) were added to each tube, and tubes were capped and vortexed for 30 seconds. Capped tubes were then incubated in an 80° C oven for 1 hour. Tubes were removed from the oven after 1 hour, and allowed to cool for approximately 15 minutes. Once cooled, 1 mL deionized water was added to each tube, and tubes were capped and vortexed for 15 seconds, then centrifuged in either the Beckman TJ-6R or the Beckman GS-6 centrifuge (Beckman Instruments, Inc., Mississauga, ON) at 1800 rpm for 5 minutes. The top layer was then pipetted to a clean 8 mL tube. Petroleum ether (Fisher Scientific, Nepean, ON, 1 mL) was added to the bottom layer, and again the tube was capped, vortexed for 15 seconds and centrifuged for 5 minutes. The top layer was pipetted off and added to the previously removed top layer. Deionized water (2 mL) was added to the combined top layers, and each tube was capped, vortexed for 15 seconds, and again centrifuged for 5 minutes at 1800 rpm. Approximately half of the top layer was pipetted into a gas chromatography vial (GC), and partially dried under nitrogen in a 30° C dry bath. Most of the remainder of the top layer was then added to the GC vial and evaporated to dryness in the dry bath. Once dry, 200 μL hexane (Optima grade, Fisher Scientific, Nepean, ON) was added

to the vial which was capped, flicked lightly to ensure mixing, and stored at -20° C until analyzed by gas chromatography.

9.23 Dietary Fatty Acids: Procedure: Approximately 0.11 g of each oil used in the diet (menhaden, corn, and soy) was poured into a 5 mL vial. Hexane (1 mL) was pipetted into the vial. A portion of the oil-hexane mixture (0.1 mL) was then pipetted into an 8 ml screw top tube and evaporated under nitrogen (Praxair Products Inc., Mississauga, ON) in a 35° C water bath (OA-Sys Heating System, Organomation Associates, Inc., Berlin, MA). Once evaporation was complete, 1 mL of toluene (Optima grade, Fisher Scientific, Nepean, ON) was added to each tube, and the tubes were vortexed for 5 to 10 seconds. Methanolic HCl (Supelco, St. Louis, MO, 1 mL) was then added, and the tubes were capped tightly with Teflon-lined lids, and vortexed for 5 to 10 seconds. The tubes were incubated in an 80° C oven for 1 hour, then removed from the oven and cooled to room temperature. Once cool, 1 mL of deionized water was added to each tube, followed by 1 mL of hexane. The tubes were capped and vortexed for 20 seconds, then centrifuged in a Beckman GS-6 centrifuge (Beckman Instruments, Inc., Mississauga, ON) at approximately 2000 rpm for 4 minutes. The upper layer (hexane) was transferred to a clean 8 mL screw top tube. Deionized water (1 mL) was then added to the hexane layer in each tube. Tubes were again capped, vortexed for 20 seconds, and centrifuged at 2000 rpm for 4 minutes as before. Part of the upper (hexane) layer was transferred to a GC vial for GC analysis. As each oil was analyzed

separately, the fatty acid composition of the fish + corn oil mixture was calculated.

9.24 Statistical Analysis: Statistical analysis was again performed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA) or Sigma Stat (Jandel Scientific, San Rafael, CA). Two-tailed Pearson correlation analysis for parametric nominal data was conducted to determine relationships of femur weight, femur length, OC, NTx, and PGE_2 with other biochemical indices. Two-way ANOVA was used to study the effect of diet and insulin deficiency on bone development, weight gain, organ weights, and indicators of diabetic state (plasma glucose, urinary creatinine, urine volume, growth rate, and feed intake) with Student-Newman-Keul's test used to determine differences between means. T-tests were conducted to determine differences in liver fatty acid composition between insulin deficient and insulin sufficient animals, and between animals fed fish oil compared to those fed soy oil. One-way ANOVA was conducted to determine differences between groups in total n-6 PUFA, total n-3 PUFA, and n-6:n-3 PUFA ratio. Differences between means were determined using Tukey's test. Best subsets regression was conducted to determine which factors most contributed to bone weight and length. For all analyses, differences between groups were considered significant at $p < 0.05$.

Table 9.1: Age and type of rat, injection concentration, site, and vehicle, time until diagnosis of diabetes, and success rate of stz-induction of diabetes in the rat model by various researchers

Author	Age of Rat	Type of Rat	Injection Conc. and Site	Injection Vehicle	Time to Diabetes Attained	Success Rate
Rodgers (109)	adult (120-140 g)	Male Long-Evans	85 mg/kg x 2 days (IP)	0.9% NaCl	3 days	not given
Yang (107)	38 days (125-150 g)	Male Sprague-Dawley	75 mg/kg (IP)	50 mmol/L citrate	not given	Most (blood glucose > 350 mg/dl)
Binz (119)	adult (115-125 g)	Male SIV	90 mg/kg (IV)	0.9 % saline	not given	not given
Takeshita (110)	9 weeks	Female Winstar	60 mg/kg (tail vein)	2 mM citrate	1 day	not given
Shires (18)	adult (290-340 g)	Male Lewis	65 mg/kg (IV)	citrate	measured on day 14	15 of 15 (blood glucose > 350 mg/dl)
Yoshino (11)	9 weeks	Female Winstar	65 mg/kg (tail vein)	2 mM citrate	1 day	35 of 35 (blood glucose > 300 mg/dl)
Goodman (12)	21 days	Male Holtzman	90 mg/kg (tail vein)	citrate	5 days	45 of 50 (blood glucose > 350 mg/dl)
Rosholt (8)	43 days	Male & female Sprague-Dawley	70 mg/kg (tail vein)	5% dextrose in 0.9% saline	not given	not given
Younoszai (108)	weanling	Male albino	60 mg/kg (day 1) 50 mg/kg (day 2) (IP)	citrate	not given	40 %

Table 9.2: AIN-93G diet formulated for growth

Ingredient	g/kg diet
Cornstarch	397.486
Casein (≥ 85% protein)	200.000
Dextrinized cornstarch (90-94% tetrasaccharides)	132.000
Sucrose	100.000
Soybean oil (no additives)	70.000
Fibre	50.000
Mineral Mix	35.000
Vitamin Mix	10.000
L-Cystine	3.000
Choline bitartrate (41.1% choline)	2.500
Tert-butylhydroquinone	0.014

Source: adapted from Reeves et al. (1993)

Table 9.3: Mineral mix that supplies the recommended concentrations of elements for the AIN-93G diet

Ingredient	
	g/kg mix
Essential mineral element	
Calcium carbonate, anhydrous, 40.04% Ca	357.00
Potassium phosphate, monobasic, 22.76% P; 28.73% K	196.00
Potassium citrate, tri-potassium, monohydrate, 36.16%K	70.78
Sodium chloride, 39.34% Na; 60.66% Cl	74.00
Potassium sulfate, 44.87% K; 18.39% S	46.60
Magnesium oxide, 60.32% Mg	24.00
Ferric citrate, 16.5% Fe	6.06
Zinc carbonate, 52.14% Zn	1.65
Manganous carbonate, 47.79% Mn	0.63
Cupric carbonate, 57.47% Cu	0.30
Potassium iodate, 59.3% I	0.01
Sodium selenate, anhydrous, 41.79% Se	0.01025
Ammonium paramolybdate, 4 hydrate, 54.34% Mo	0.00795
Potentially beneficial mineral element	
Sodium meta-silicate, 9 hydrate, 9.88% Si	1.45
Chromium potassium sulfate, 12 hydrate, 10.42% Cr	0.275
Lithium chloride, 16.38% Li	0.0174
Boric acid, 17.5% B	0.0815
Sodium fluoride, 45.24% F	0.0635
Nickel carbonate, 45% Ni	0.0318
Ammonium vanadate, 43.55% V	0.0066
Powdered sucrose	221.026

Source: adapted from Reeves et al. (1993)

Table 9.4: Vitamin mix for the AIN-93G diet

Vitamin	
	g/kg mix
Nicotinic acid	3.000
Ca Pantothenate	1.600
Pyridoxine-HCl	0.700
Thiamin-HCl	0.600
Riboflavin	0.600
Folic acid	0.200
D-Biotin	0.020
Vitamin B12 (cyanocobalamin) (0.1% in mannitol)	2.500
Vitamin E (all-rac-a-tocopheryl acetate) (500 IU/g)	15.00
Vitamin A (all-trans retinyl palmitate) (500,000 IU/g)	0.800
Vitamin D3 (cholecalciferol) (400,000 IU/g)	0.250
Vitamin K (phylloquinone)	0.075
Powdered sucrose	974.655

Source: adapted from Reeves et al. (1993)

10. Basic Study: Results

The fatty acid composition of the oils used in the soy oil and fish oil diets is shown in **Table 10.1**. The combination of fish oil and corn oil was calculated to have a higher n-3 fatty acid composition compared to the soy oil, while the soy oil had a higher n-6 fatty acid composition and a higher n-6:n-3 fatty acid ratio.

The success rate of stz-injection in producing a diabetic state in weanling rats has been reported to be anywhere from 40 - 100%, with success defined as a blood glucose level of 19 mmol/L or higher (11-2,18,107-8). Based on a blood glucose level of 13 mmol/L or higher, stz-injections were successful in 32 of the 37 rats tested. Twenty-five of these rats had a blood glucose value of 19 mmol/L or higher on day 5 of the study. When other indicators of diabetes were taken into account such as measures of growth (growth rate and tail length), 12-hour urine volume, and feed intake both as a total amount and corrected to body weight, (**Table 10.2**), this success rate was confirmed; only 4 of the stz-injected rats appeared to be unaffected. For the purposes of analysis, all stz-injected animals were included. Using 2-way ANOVA, plasma glucose levels (mmol/L), 12-hour urine volume (mL), and amount eaten (total g and g/g body weight) were significantly higher in the stz-injected rats compared to the pair-weigh and control rats, while urinary creatinine excretion (mmol/L), growth rate (g/day) and tail length (cm) were significantly lower (**Table 10.2, Appendix D**). Within treatment groups, there were no significant differences based on diet.

Although there were no significant differences between any of the groups in terms of initial body weight as analyzed by 1-way ANOVA, total weight gain and therefore final body weight were significantly lower in the stz-injected rats compared to both the control and pair-weigh rats as analyzed by 2-way ANOVA (**Table 10.3, Appendix D**). Animals in the pair-weigh groups also had significantly lower weight gain and final body weight compared to the control groups. Organ weights and femur weights and lengths were also analyzed by 2-way ANOVA to determine differences between groups (**Table 10.4, Appendix E**). Heart weights were significantly higher in the control group compared to both the pair-weigh and diabetes groups, and were significantly lower in the diabetes groups compared to the other 2 groups; however, heart weights corrected to body weight were significantly higher in the diabetes groups compared to the pair-weigh and control groups. Kidney weights and liver and kidney weights corrected to body weight were significantly higher in the stz-injected rats. Liver weights were not significantly different in any of the groups. Whole femur weights were significantly lower in the diabetes group compared to the pair-weigh and control groups, and were significantly lower in the pair-weigh group compared to the control group, but whole femur weights corrected to body weight were significantly higher in the stz-injected animals, with no significant differences between the pair-weigh and control groups. Femur lengths were significantly higher in the control group compared to the pair-weigh and diabetes groups, and significantly lower in the diabetes group

compared to the pair-weigh and control groups. Again, there were no significant differences within treatment groups between the rats fed fish oil and the rats fed soy oil.

Femur, plasma, and urine biochemistry was analyzed by 2-way ANOVA to determine difference between groups based on diet and treatment (**Table 10.5, Appendix F**). There were no treatment or diet effects for femur diaphysis calcium or femur diaphysis phosphorus. Total urinary calcium excretion (mmol/L) showed a significant treatment effect, with pair-weigh and diabetes groups having higher excretion compared to the control groups. There was no effect of diet for total urinary calcium excretion. When corrected to creatinine and when corrected to body weight and urine volume, urinary calcium excretion was significantly higher in the diabetes groups compared to the pair-weigh and control groups, with no significant differences between the latter groups, and again no differences between dietary groups. Total urinary phosphorus excretion also had a significant treatment effect and no diet effect. Excretion was significantly higher in the control groups compared to the pair-weigh and diabetes groups, and was significantly higher in the diabetes groups compared to the pair-weigh groups. When corrected to creatinine, urinary phosphorus excretion was significantly higher in the diabetes groups compared to the pair-weigh and control groups. There were no significant differences between the pair-weigh and control groups, and none between dietary groups within each treatment. When urinary phosphorus was corrected

to body weight and urine volume, excretion was significantly lower in the pair-weigh group compared to the control and diabetes groups, with no significant differences between the latter two groups. Again, there was no significant effect of diet. Plasma $1,25(OH)_2D_3$ was significantly higher in the control groups compared to the diabetes groups, with no significant difference between the pair-weigh and diabetes groups, and no significant diet effect. There were no significant differences between any of the groups in PTH. For OC, there was a significant diet effect, with animals fed soy oil having significantly higher OC compared to those fed fish oil. For NTx and IGF-I, there was a significant treatment effect, with stz-injected rats having significantly higher amounts of NTx and significantly lower amount of IGF-I compared to pair-weigh and control rats. PGE_2 showed both a diet and a treatment effect, with rats fed soy oil having significantly higher amounts compared to rats fed fish oil, and stz-injected rats having significantly higher amounts compared to saline-injected rats.

Correlation analysis was conducted to examine the relationship between femur weight and factors involved in, or known to affect, bone growth and development (**Table 10.6**). In the PS group, there were no significant correlations between femur weight and any of the parameters tested. In the CS group, there was a significant positive relationship between PTH and femur weight. In the DS group, there were significant positive relationships between femur weight and OC, femur weight and IGF-I, femur weight and femur

diaphysis phosphorus, and femur weight and n-6:n-3 PUFA, and significant negative relationships between femur weight and both day 5 and necropsy plasma glucose. In the PF group, there were no significant relationships between femur weight and any of the parameters. In the CF group, there was a significant negative correlation between plasma $1,25(OH)_2D_3$ and femur weight. In the DF group, there were significant negative correlations between NTx and femur weight and necropsy plasma glucose and femur weight.

Correlation analysis was also conducted to examine the relationship between femur length and factors involved in bone development (**Table 10.7**). In the PS, CS, and CF groups, no significant relationships were found between femur length and any of the parameters. In the DS group, significant positive relationships were found between femur length and OC, femur length and IGF-I, femur length and femur diaphysis phosphorus, and femur length and liver n-6:n-3 PUFA ratio. Significant negative relationships were found between femur length and both day 5 and necropsy plasma glucose in this group. In the PF group, significant positive relationships were found between femur length and IGF-I, and femur length and plasma $1,25(OH)_2D_3$. In the DF group, there were negative correlations between femur length and NTx, and femur length and necropsy plasma glucose.

To study the relationship between bone formation, as measured by OC, and other factors involved in bone development, correlation analysis was conducted (**Table 10.8**). In the DS group, there was a significant negative

correlation between OC and PGE_2, and significant positive correlations between OC and femur diaphysis phosphorus and OC and urinary phosphorus excretion corrected to creatinine. In the DF group, significant positive correlations were found between OC and urinary phosphorus excretion corrected to creatinine and OC and necropsy plasma glucose. A significant positive correlation was found between OC and IGF-I in the CF group. There were no significant correlations found in any of the other groups.

Correlation analysis was also conducted to examine the relationship between bone resorption, represented by NTx, and other factors involved in bone development (**Table 10.9**). A significant negative correlation was found between NTx and PTH in the PS group. A significant negative correlation was found in the PF group between NTx and $1,25(OH)_2D_3$. In the DF group, significant positive relationships were found between NTx and urinary phosphorus excretion and NTx and necropsy plasma glucose. No significant reiationships were found between NTx and other factors in the CS, DS, or CF groups.

The relationship of PGE_2 with other indicators of bone development was examined through correlation analysis (**Table 10.10**). In addition to the significant negative relationship between OC and PGE_2 in the DS group, there was a significant negative relationship of PGE_2 with PTH in the DS group, a significant positive relationship of PGE_2 with vitamin D in the PS group, and

significant negative relationships of PGE_2 with IGF-I and with femur diaphysis calcium and phosphorus in the PF group.

The liver fatty acid composition of rats fed soy oil was compared to that of rats fed fish oil by 1-way ANOVA (**Table 10.11, Appendix C**). For most fatty acids, percent contribution to total fatty acids was higher in rats fed soy oil; however, rats fed fish oil had a higher proportion of n-3 PUFA than rats fed soy oil. In particular, C16:0, C20:0, C20:3 n-6, C20:5 n-3, C22:5 n-3, C22:6 n-3, and C24:1 n-9 contributed a larger proportion to the total fatty acids than they did in the soy-fed animals.

There were also significant differences seen in liver fatty acid composition when stz-injected rats were compared to saline-injected rats (**Table 10.12, Appendix C**). Liver fatty acid content tended to be higher in the stz-injected animals (data not shown), although the proportion of fatty acids at the beginning of the n-6 fatty acid metabolic pathway (C18:2 n-6 and C18:3 n-6) was lower in the diabetes groups compared to the pair-weigh and control groups (**Appendix C**). Proportion of some fatty acids in the n-3 metabolic pathway (C18: n-3 and C20:5 n-3) also tended to be lower in the stz-injected animals compared to the saline-injected animals.

Total liver n-6 PUFA, total liver n-3 PUFA, and the liver n-6:n-3 ratio in all animals were compared by 1-way ANOVA (**Figures 10.1-10.3**). Total n-6 PUFA were significantly higher in the DS group compared to all other groups and were significantly higher in the DF group compared to the PF and CF

groups (**Figure 10.1**). They were also significantly higher in the PS and CS groups compared to the PF and CF groups, with no significant differences between PS and CS or between PF and CF. There was also no significant difference between the CS and DF group in terms of total n-6 PUFA. Total n-3 PUFA were significantly lower in the PS, CS, and DS groups compared to the PF, CF, and DF groups (**Figure 10.2**). There were no significant differences among the PF, CF, and DF groups. The PS and CS groups had a significantly lower proportion of n-3 PUFA compared to the DS group. The n-6:n-3 PUFA ratio was significantly higher in the PS, CS, and DS groups compared to the PF, CF, and DF groups (**Figure 10.3**). The PS group also had a significantly higher ratio compared to the CS and DS groups and the CS group had a significantly higher n-6:n-3 ratio compared to the DS group. There were no significant differences in n-6:n-3 ratio among the PF, CF and DF groups.

To determine which factors most contribute to bone weight in all animals grouped together, best subsets regression was conducted (**Table 10.13**). Independent variables included in the analysis were IGF-I (pmol/L), NTx (mM BCE/mM creatinine), OC (pmol/L), PGE_2 (mmol/L), PTH (nmol/L), $1,25(OH)_2D_3$ (nmol/L), urinary calcium excretion (mmol/mmol creatinine), urinary phosphorus excretion (mmol/mmol creatinine), necropsy plasma glucose (mmol/L), and the n-6:n-3 PUFA ratio. Analysis revealed the following equation: Whole femur weight = 0.823 + 0.001(IGF-I) - 0.002(PGE_2) + 0.001(PTH) - 0.014(urinary calcium/creatinine excretion) - 0.004(necropsy

plasma glucose). R^2 for the equation was 0.529, with R^2 adjusted equal to 0.475.

In order to examine whether factors contributing to bone weight differed between stz-injected animals and saline-injected animals, best subsets regression was also conducted for each of these groups separately (**Tables 10.14-10.15**). For the stz-injected animals, independent variables were IGF-I (pmol/L), NTx (mM BCE/mM creatinine), OC (pmol/L), PGE_2 (mmol/L), $1,25(OH)_2D_3$ (nmol/L), urinary calcium excretion (mmol/mmol creatinine), urinary phosphorus excretion (mmol/mmol creatinine), necropsy plasma glucose (mmol/L), and the n-6:n-3 PUFA ratio. The equation for this analysis (**Table 10.14**) was whole femur weight = 0.610 - 0.0004(NTx) + 1.04(OC) + 0.002(PGE_2), + 1.46($1,25(OH)_2D_3$), - 0.009(necropsy plasma glucose) - 0.151(n-6:n-3 ratio). R^2 for the equation was 0.945, with R^2 adjusted equal to 0.879. For the saline-injected animals, the independent variables were the same, with the exception of necropsy plasma glucose, which was not included. Analysis resulted in the equation (**Table 10.15**): whole femur weight = 0.804 - 0.001(PGE_2) - 0.052($1,25(OH)_2D_3$) + 0.297(urinary phosphorus/creatinine excretion). R^2 for this equation was 0.264, and R^2 adjusted was 0.199.

The factors which contribute most to femur length were also determined using best subsets regression (**Tables 10.16-10.18**). Again, animals were analyzed as one group, and separately as stz-injected and saline-injected. For each analysis, independent variables were the same as those used in the

analysis for femur weight: IGF-I (pmol/L), NTx (mM BCE/mM creatinine), OC (pmol/L), PGE_2 (mmol/g), PTH (nmol/L), $1,25(OH)_2D_3$ (nmol/L), urinary calcium excretion (mmol/mmol creatinine), urinary phosphorus excretion (mmol/mmol creatinine), and the n-6:n-3 PUFA ratio. Necropsy plasma glucose (mmol/L) was included in the analysis for the entire group and the stz-injected animals only. For the entire group, factors contributing to femur length were the same as those contributing to femur weight (**Table 10.16**), with the equation being: femur length = 3.404 + 0.002(IGF-I) - 0.002(PGE_2) + 0.002(PTH) - 0.022(urinary calcium/creatinine excretion) - 0.010(necropsy plasma glucose). R^2 for the equation was 0.557, with an R^2 adjusted of 0.508. For stz-injected animals, factors contributing to femur length were also the same as those contributing to femur length, except that IGF-I was included in the equation (**Table 10.17**): femur length = 2.791 +0.003(IGF-I) - 0.001(NTx) + 1.528(OC) + 0.002(PGE_2) + 1.907($1,25(OH)_2D_3$) - 0.071(urinary phosphorus excretion) - 0.008(necropsy plasma glucose) - 0.166(n-6:n-3 ratio). R^2 and R^2 adjusted were 0.981 and 0.931 respectively. For the saline-injected rats, the factors contributing to femur length were different than those contributing to femur weight, with the exception of urinary phosphorus excretion, which was found in both equations (**Table 10.18**). In saline-injected animals, femur length = 3.49 + 0.554(urinary phosphorus excretion) - 0.459(OC) + 0.001(IGF-I). For this equation, R^2 was 0.327 and R^2 adjusted was 0.270.

Table 10.1: Fatty acid composition of fish, corn, and soy oil

Fatty acid	Fish oil	Corn oil	Fish + corn oil	Soy oil
C14:0	9.13	10.92	9.90	---
C15:0	0.76	---	0.43	---
C16:0	17.81	---	10.18	10.34
C16:1	11.40	---	6.51	---
C17:0	1.51	---	0.86	---
C17:1	1.49	---	0.28	---
C18:0	3.02	2.03	2.74	---
C18:1 n-9	8.00	28.32	16.71	20.61
C18:1 n-7	3.28	0.54	2.11	1.27
C18:2 n-6	1.54	57.04	25.33	54.01
C18:3 n-6	0.71	0.90	0.79	---
C18:3 n-3	1.36	---	0.78	7.64
C18:4 n-3	3.84	---	2.19	---
C20:0	0.00	0.25	0.11	0.35
C20:1 n-9	1.00	---	0.57	0.20
C20:1 n-7	0.27	---	0.15	---
C20:3 n-6	0.22	---	0.13	---
C20:4 n-6	0.65	---	0.37	---
C20:4 n-3	1.53	---	0.87	---
C20:5 n-3	14.00	---	8.00	---
C21:5 n-3	0.63	---	0.36	---
C22:0	---	---	---	0.34
C22:5 n-3	2.00	---	1.14	---
C22:6 n-3	11.22	---	6.41	---
C24:0	---	---	---	0.09
C24:1	0.31	---	0.18	---
Total n-6	3.12	57.94	26.62	54.01
Total n-3	34.58	---	19.75	7.64
Total n-6/n-3	0.1:1.0	---	1.4:1.0	7.1:1.0
Total PUFA	37.70	57.94	46.37	61.65

Data shown as percent wt/wt of total lipid

Table 10.2: Indicators of diabetic state in stz-injected rats compared to saline-injected rats

	PS (n=10)*	CS (n=11)*	DS (n=18)*	PF (n=10)*	CF (n=10)*	DF (n=19)*
Day 5 plasma glucose (mmol/L)	7.6[a] (0.2) (n=6)	6.5[a] (0.8) (n=3)	23.8[b] (2.4) (n=17)	6.9[a] (0.5) (n=3)	7.1[a] (0.2) (n=6)	23.5[b] (2.4)
Necropsy plasma glucose (mmol/L)	16.6[a] (4.3)	9.2[b] (3.1)	20.1[c] (6.8)	17.8[a] (7.6)	11.4[b] (5.5)	24.1[c] (12.4)
Urinary creatinine (mmol/L)	6.6[a] (3.2)	5.6[a] (3.9)	1.6[b] (1.3)	4.8[c] (1.5)	4.0[c] (2.0)	1.3[d] (1.0)
12 hour urine volume (mL)	7.4[a] (3.5)	11.0[a] (7.1)	21.9[b] (9.0)	8.8[a] (3.2)	12.8[a] (4.5)	24.4[b] (12.1)
Growth rate (g/day)	6.2[a] (0.8)	7.9[b] (1.3)	3.8[c] (1.5)	5.6[a] (2.2)	8.2[b] (0.7)	3.9[c] (2.0)
Feed Intake (g/day)	17.0[a] (1.6)	21.3[b] (2.8)	31.2[c] (5.2)	17.2[a] (1.7)	21.4[b] (1.3)	33.4[c] (6.2)
Feed intake (g/g body weight)	0.085[a] (0.005)	0.100[a] (0.010)	0.174[b] (0.031)	0.087[a] (0.009)	0.097[a] (0.006)	0.182[b] (0.035)
Tail length (cm)	16.1[a] (0.7)	17.7[b] (1.2)	15.5[c] (1.8)	16.2[a] (1.0)	16.9[b] (0.6)	15.0[c] (1.1)

Data shown as mean (SD)
Within rows, data with different superscripts are significantly different at $p < 0.05$ as analyzed by 2-way ANOVA, post-hoc analysis by Student-Newman-Keuls.
PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet
* Values for n as shown at top of table except where otherwise indicated

Table 10.3: Initial and final body weights and total weight gain in stz- and saline-injected rats fed soy oil or fish oil

	PS (n=10)	CS (n=11)	DS (n=18)	PF (n=10)	CF (n=10)	DF (n=19)
Baseline weight (g)	52.5 (6.7)	49.8 (5.1)	52.7 (4.8)	52.6 (6.8)	48.9 (5.6)	52.6 (6.2)
Final weight (g)	310.7[a] (25.1)	348.9[b] (55.4)	231.4[c] (56.2)	312.1[a] (22.3)	367.8[b] (33.1)	230.2[c] (51.1)
Total weight gain (g)	258.2[a] (20.5)	299.1[b] (51.8)	178.7[c] (56.5)	259.5[a] (25.7)	318.9[b] (31.7)	177.6[c] (53.3)

Data shown as mean (SD)

Within rows, data with different superscripts are significantly different at $p < 0.05$ as analyzed by 2-way ANOVA, post-hoc analysis by Student-Newman-Keuls

PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet

Table 10.4 : Organ and femur weights and femur lengths in stz- and saline-injected rats fed soy or fish oil

	PS (n=10)	CS (n=11)	DS (n=18)	PF (n=10)	CF (n=10)	DF (n=19)
Heart (g)	1.1^{a} (0.1)	1.3^{b} (0.2)	0.9^{c} (0.2)	1.1^{a} (0.2)	1.3^{b} (0.2)	0.9^{c} (0.2)
Heart (mg/g body weight)	3.4^{a} (0.4)	3.6^{a} (0.3)	3.9^{b} (0.4)	3.6^{a} (0.3)	3.5^{a} (0.4)	3.9^{b} (0.4)
Liver (g)	11.3 (1.4)	12.1 (1.8)	10.8 (1.5)	11.5 (1.5)	12.0 (1.7)	12.4 (1.6)
Liver (mg/g body weight)	36.9^{a} (4.3)	33.8^{a} (1.1)	46.9^{b} (8.5)	36.7^{a} (4.3)	34.3^{a} (2.2)	55.2^{b} (11.4)
Left kidney (g)	1.2^{a} (0.3)	1.3^{a} (0.2)	1.5^{b} (0.2)	1.1^{a} (0.1)	1.3^{a} (0.2)	1.7^{b} (0.2)
Kidney (mg/g body weight)	3.9^{a} (0.6)	3.7^{a} (0.2)	6.1^{b} (1.4)	3.7^{a} (0.3)	3.8^{a} (0.4)	7.1^{b} (1.4)
Whole femur (g)	0.736^{a} (0.059)	0.818^{b} (0.077)	0.661^{c} (0.098)	0.767^{a} (0.057)	0.815^{b} (0.070)	0.650^{c} (0.105)
Whole femur (mg/g body weight)	0.237^{a} (0.015)	0.228^{a} (0.022)	0.283^{b} (0.032)	0.233^{a} (0.013)	0.233^{a} (0.025)	0.288^{b} (0.031)
Femur length (cm)	3.29^{a} (0.07)	3.39^{b} (0.14)	3.11^{c} (0.18)	3.29^{a} (0.09)	3.40^{b} (0.08)	3.14^{c} (0.19)
Femur weight/length (g/cm)	0.22^{a} (0.01)	0.23^{a} (0.02)	0.21^{b} (0.02)	0.23^{a} (0.02)	0.25^{a} (0.02)	0.21^{b} (0.02)

Data shown as mean (SD)

Within rows, data with different superscripts are significantly different at $p < 0.05$ as analyzed by 2-way ANOVA, post-hoc analysis by Student-Newman-Keuls

PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet

Table 10.5: Plasma, urine, and femur biochemistry of stz- and saline-injected rats fed soy oil or fish oil

	PS (n=10)*	CS (n=11)*	DS (n=18)*	PF (n=10)*	CF (n=10)*	DF (n=19)*
Diaphysis Ca (mg/g)	222.8 (14.7)	233.3 (8.9)	228.2 (7.3)	220.7 (18.3)	224.7 (13.0)	229.6 (11.2)
Diaphysis P (mg/g)	110.8 (7.4)	116.6 (4.5)	113.3 (4.2)	109.6 (9.8)	111.4 (6.3)	114.2 (6.9)
Urinary Ca (mmol/L)	1.7^{a} (0.8)	0.6^{b} (0.4)	1.6^{a} (1.3)	2.2^{a} (1.6)	0.5^{b} (0.3)	1.4^{a} (2.2) (n=18)
Urinary P (mmol/L)	0.15^{a} (0.26)	0.73^{b} (0.26)	0.34^{c} (0.36)	0.12^{a} (0.14)	0.60^{b} (0.26)	0.30^{c} (0.18) (n=18)
Urinary Ca/creatinine (mmol/mmol)	0.29^{a} (0.17)	0.14^{a} (0.09)	2.36^{b} (3.06)	0.47^{a} (0.37)	0.14^{a} (0.07)	1.79^{b} (2.45) (n=18)
Urinary P/creatinine (mmol/mmol)	0.03^{a} (0.05)	0.17^{a} (0.08)	0.31^{b} (0.32)	0.02^{a} (0.02)	0.16^{a} (0.06)	0.43^{b} (0.41) (n=18)
Urinary Ca/body weight (nmol/g/h)	3.15^{a} (2.04)	1.26^{a} (0.63)	13.05^{b} (9.95)	4.76^{a} (3.49)	1.38^{a} (0.72)	13.07^{b} (13.45) (n=18)
Urinary P/body weight (nmol/g/h)	0.26^{a} (0.49)	1.59^{b} (0.49)	1.46^{b} (1.12)	0.23^{a} (0.21)	1.64^{b} (0.77)	2.19^{b} (1.73) (n=18)
$1,25(OH)_2D_3$ (nmol/L)	0.22^{ab} (0.05)	0.37^{a} (0.38)	0.06^{b} (0.06)	0.16^{ab} (0.11)	0.27^{a} (0.16)	0.15^{b} (0.36) (n=18)
Osteocalcin (pmol/L)	0.580^{a} (0.091)	0.576^{a} (0.083)	0.566^{a} (0.099)	0.549^{b} (0.081)	0.562^{b} (0.080)	0.466^{b} (0.100) (n=16)
IGF-I (pmol/L)	67.59^{a} (30.86)	63.61^{a} (34.13) (n=10)	21.95^{b} (17.03) (n=9)	52.74^{a} (22.22)	67.24^{a} (25.75)	30.48^{b} (31.53) (n=10)
PTH (nmol/L)	22.32 (12.35)	13.56 (5.42)	19.36 (14.18)	13.58 (8.06)	13.48 (5.22)	18.35 (12.87)
NTx (nM BCE/mM)	22.74^{a} (2.09)	20.61^{a} (2.98) (n=9)	120.60^{b} (36.54) (n=17)	20.80^{a} (3.09)	25.46^{a} (4.52)	65.76^{b} (9.39) (n=15)
PGE_2 (mmol/g)	37.13^{a} (12.88)	38.33^{a} (13.13)	51.22^{b} (27.08)	16.77^{c} (6.99)	19.82^{c} (7.09)	34.04^{d} (22.71) (n=17)

Data shown as mean (SD)
Within rows, data with different superscripts are significantly different at $p < 0.05$ as analyzed by 2-way ANOVA, post-hoc analysis by Student-Newman-Keuls
PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet;
DF: Diabetes group, fish oil diet
* Values for n are as shown at top of table, except where otherwise indicated

Table 10.6: Relationship of femur weight with plasma, urine, femur, or liver biochemistry

	PS (n=10)*	CS (n=11)*	DS (n=18)*	PF (n=10)*	CF (n=10)*	DF (n=19)*
OC (pmol/L)	r= 0.10 p= 0.79	r= -0.01 p= 0.98	**r= 0.52 p =0.03**	r= 0.26 p= 0.46	r= -0.38 p= 0.27	r= -0.27 p= 0.31 (n=16)
NTx (nM BCE/mM)	r= -0.25 p= 0.49	r= 0.21 p= 0.62 (n=9)	r= -0.17 p= 0.51 (n=17)	r= -0.45 p=0.19	r= 0.35 p= 0.32	**r= -0.54 p= 0.04** (n=15)
IGF-I (pmol/L)	r= -0.23 p= 0.51	r= 0.38 p= 0.30 (n=10)	**r= 0.72 p= 0.03** (n=9)	r=0.58 p=0.08	r= -0.18 p= 0.61	r= 0.45 p= 0.19 (n=10)
PGE_2 (mmol/g)	r= -0.38 p= 0.28	r= -0.29 p= 0.42	r= -0.34 p= 0.16	r= -0.31 p= 0.38	r= -0.41 p= 0.24	r= -0.17 p= 0.51 (n=17)
$1,25(OH)_2D_3$ (nmol/L)	r= 0.03 p= 0.93	r= -0.25 p= 0.49	r= 0.27 p= 0.29	r= 0.57 p= 0.08	**r= -0.81 p= 0.004**	r= 0.12 p= 0.63 (n=18)
PTH (nmol/L)	r= 0.53 p= 0.11	**r= 0.63 p= 0.05**	r= 0.12 p= 0.62	r= -0.07 p= 0.84	r= -0.0003 p= 1.0	r= -0.06 p= 0.82
Diaphysis Ca (mg/g)	r= -0.16 p= 0.67	r= -0.34 p= 0.33	r= 0.37 p= 0.13	r= -0.01 p= 0.99	r= -0.01 p= 0.99	r= 0.33 p= 0.17
Diaphysis P (mg/g)	r= -0.08 p= 0.82	r= -0.40 p= 0.26	**r= 0.50 p= 0.03**	r= 0.04 p= 0.91	r= -0.11 p= 0.76	r= 0.35 p= 0.15
Urine Ca/creatinine (mmol/mmol)	r= -0.11 p= 0.76	r= -0.45 p= 0.19	r= -0.38 p= 0.12	r= 0.22 p= 0.54	r= -0.38 p= 0.28	r= -0.33 p= 0.19 (n=18)
Urine P/creatinine (mmol/mmol)	r= 0.58 p= 0.08	r= -0.46 p= 0.18	r= 0.02 p= 0.93	r= -0.04 p= 0.92	r= -0.56 p= 0.09	r= -0.35 p= 0.16 (n=18)
Day 5 Glucose (mmol/L)	r= -0.56 p= 0.23 (n=6)	— ** (n=3)	**r= -0.52 p= 0.03** (n=17)	— ** (n=3)	r= -0.39 p= 0.45 (n=6)	r= -0.30 p= 0.22
Necropsy Glucose (mmol/L)	r= 0.16 p= 0.66	r= 0.20 p= 0.58	**r= -0.57 p= 0.013**	r= 0.02 p= 0.98	r= 0.28 p= 0.43	**r= -0.72 p= 0.0005**
Liver n-6:n:3 PUFA ratio	r= -0.37 p= 0.29	r= -0.31 p= 0.38	**r= 0.78 p= 0.0002**	r= -0.15 p= 0.71	r= -0.03 p= 0.94	r= 0.11 p= 0.66 (n=18)

r = Pearson correlation coefficient
Significant correlations are shown in bold
PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet;
DF: Diabetes group, fish oil diet
* Values for n as shown at top of table, except where otherwise indicated
** Insufficient data for analysis

Table 10.7: Relationship of femur length with femur, plasma, or urine biochemistry

	PS (n=10)*	CS (n=11)*	DS (n=18)*	PF (n=10)*	CF (n=10)*	DF (n=19)*
OC (pmol/L)	r= -0.20 p= 0.58	r= -0.28 p= 0.40	**r= 0.59 p= 0.01**	r= 0.30 p= 0.40	r= -0.35 p= 0.32	r= -0.30 p= 0.26 (n=16)
NTx (nM BCE/mM)	r= 0.004 p= 0.99	r= 0.18 p= 0.64 (n=9)	r= -0.06 p= 0.83 (n=17)	r= -0.54 p= 0.11	r= 0.52 p= 0.12	**r= -0.53 p= 0.04** (n=15)
IGF-I (pmol/L)	r= -0.55 p= 0.10	r= 0.35 p= 0.33 (n=10)	**r= 0.73 p= 0.03** (n=9)	**r= 0.69 p= 0.03**	r= -0.19 p= 0.60	r= 0.45 p= 0.20 (n=10)
PGE_2 (mmol/g)	r= -0.03 p= 0.94	r= -0.52 p= 0.10	r= -0.40 p= 0.10	r= -0.20 p= 0.58	r= -0.07 p= 0.84	r= -0.08 p= 0.76 (n=17)
$1,25(OH)_2D_3$ (nmol/L)	r= 0.26 p= 0.46	r= -0.08 p= 0.82	r= 0.34 p= 0.17	**r= 0.75 p= 0.012**	r= -0.62 p= 0.06	r= 0.10 p= 0.69 (n=18)
PTH (nmol/L)	r= 0.35 p= 0.33	r= 0.59 p= 0.06	r= 0.15 p= 0.56	r= -0.29 p= 0.42	r= 0.20 p= 0.57	r= -0.03 p= 0.90
Diaphysis Ca (mg/g)	r= 0.07 p= 0.85	r= -0.19 p= 0.58	r= 0.35 p= 0.16	r= -0.02 p= 0.96	r= -0.26 p= 0.44	r= 0.24 p= 0.33
Diaphysis P (mg/g)	r= 0.10 p= 0.78	r= -0.20 p= 0.56	**r= 0.48 p= 0.05**	r= 0.02 p= 0.96	r= -0.33 p= 0.35	r= 0.26 p= 0.29
Urine Ca/creatinine (mmol/mmol)	r= 0.06 p= 0.86	r= -0.37 p= 0.26	r= -0.28 p= 0.26	r= 0.17 p= 0.63	r= -0.32 p= 0.36	r= -0.18 p= 0.47 (n=18)
Urine P/creatinine (mmol/mmol)	r= 0.37 p= 0.30	r= -0.43 p= 0.18	r= 0.14 p= 0.58	r= -0.18 p= 0.63	r= -0.17 p= 0.65	r= -0.23 p= 0.36 (n=18)
Day 5 Glucose (mmol/L)	r= -0.60 p= 0.20 (n=6)	— ** (n=3)	**r= -0.50 p= 0.04** (n=17)	— ** (n=3)	r= -0.53 p= 0.28 (n=6)	r= -0.34 p= 0.16
Necropsy Glucose (mmol/L)	r= 0.26 p= 0.46	r= -0.03 p= 0.93	**r= -0.54 p= 0.02**	r= -0.14 p= 0.70	r= 0.17 p= 0.65	**r= -0.75 p= 0.0002**
Liver n-6:n:3 PUFA ratio	r= -0. 20 p= 0.58	r= -0.23 p= 0.49	**r= 0.78 p= 0.0001**	r= -0.07 p= 0.86	r= 0.42 p= 0.23	r= 0.25 p= 0.29 (n=18)

r = Pearson Correlation Coefficient
Significant correlations are shown in bold
PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet;
DF: Diabetes group, fish oil diet
* Values for n as shown at top of table, except where otherwise indicated
** Insufficient data for analysis

Table 10.8: Relationship of osteocalcin with femur, plasma, or urine biochemistry

	PS (n=10)*	CS (n=11)*	DS (n=18)*	PF (n=10)*	CF (n=10)*	DF (n=19)*
NTx (nM BCE/mM)	r= -0.52 p= 0.12	r= 0.12 p= 0.75 (n=9)	r= 0.29 p= 0.27 (n=17)	r= -0.50 p= 0.14	r= -0.51 p= 0.14	r= 0.17 p= 0.59 (n=15)
IGF-I (pmol/L)	r= 0.52 p= 0.13	r= 0.24 p= 0.51 (n=10)	r= 0.33 p= 0.38 (n=9)	r= 0.52 p= 0.12	**r= 0.76 p= 0.01**	r= -0.19 p= 0.65 (n=10)
PGE_2 (mmol/g)	r= -0.09 p= 0.80	r= 0.46 p= 0.16	**r= -0.67 p= 0.002**	r= -0.47 p= 0.17	r= 0.27 p= 0.45	r= 0.007 p= 0.98 (n=17)
$1,25(OH)_2D_3$ (nmol/L)	r= 0.20 p= 0.57	r= -0.04 p= 0.91	r= 0.16 p= 0.53	r= 0.45 p= 0.20	r= 0.25 p= 0.49	r= 0.42 p= 0.12 (n=18)
PTH (nmol/L)	r= 0.36 p= 0.31	r= -0.06 p= 0.87	r= 0.29 p= 0.24	r= -0.02 p= 0.96	r= 0.39 p= 0.26	r= 0.45 p= 0.08
Diaphysis Ca (mg/g)	r= -0.36 p= 0.30	r= 0.03 p= 0.93	r= 0.39 p= 0.11	r= - 0.004 p= 0.99	r= 0.11 p= 0.76	r= -0.31 p= 0.24
Diaphysis P (mg/g)	r= -0.30 p= 0.41	r= 0.01 p= 0.97	**r= 0.48 p= 0.0455**	r= 0.04 p= 0.91	r= 0.19 p= 0.59	r= 0.25 p= 0.37
Urine Ca/creatinine (mmol/mmol)	r= -0.32 p= 0.37	r= -0.36 p= 0.28	r= 0.17 p= 0.49	r= 0.36 p= 0.30	r= 0.05 p= 0.90	r= 0.25 p= 0.37 (n=18)
Urine P/creatinine (mmol/mmol)	r= -0.12 p= 0.75	r= -0.44 p= 0.18	**r= 0.50 p= 0.03**	r= 0.13 p= 0.72	r= 0.29 p= 0.41	**r= 0.57 p= 0.03** (n=18)
Day 5 Glucose (mmol/L)	r= 0.65 p= 0.16 (n=6)	— ** (n=3)	r= -0.09 p= 0.73 (n=17)	— ** (n=3)	r= 0.50 p= 0.31 (n=6)	r= 0.40 p= 0.13
Necropsy Glucose (mmol/L)	r= 0.52 p= 0.13	r= 0.07 p= 0.85	r= -0.06 p= 0.81	r= 0.44 p= 0.21	r= 0.62 p= 0.06	**r= 0.52 p= 0.04**

r = Pearson correlation coefficient
Significant correlations are shown in bold
PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet
* Values for n as shown at top of table, except where otherwise indicated
** Insufficient data for analysis

Table 10.9: Relationship of NTx with femur, plasma, or urine biochemistry

	PS (n=10)*	CS (n=11)*	DS (n=18)*	PF (n=10)*	CF (n=10)*	DF (n=19)*
OC (pmol/L)	r= -0.52 p= 0.12	r= 0.12 p= 0.75	r= 0.26 p= 0.30	r= -0.50 p= 0.14	r= -0.51 p= 0.14	r= 0.17 p= 0.59 (n=16)
IGF-I (pmol/L)	r= -0.05 p= 0.90	r= 0.65 p= 0.06 (n=10)	r= 0.07 p= 0.86 (n=9)	r= -0.43 p= 0.22	r= -0.45 p= 0.20	r= -0.50 p= 0.25 (n=10)
PGE_2 (mmol/g)	r= -0.08 p= 0.82	r= 0.05 p= 0.91	r= -0.08 p= 0.75	r= -0.05 p= 0.89	r= 0.15 p= 0.69	r= 0.35 p= 0.22 (n=17)
$1,25(OH)_2D_3$ (nmol/L)	r= -0.19 p= 0.60	r= -0.58 p= 0.10	r= -0.21 p= 0.41	**r= -0.64 p= 0.04**	r= -0.36 p= 0.30	r= -0.36 p= 0.21 (n=18)
PTH (nmol/L)	**r= -0.75 p= 0.01**	r= 0.57 p= 0.11	r= -0.15 p= 0.54	r= 0.20 p= 0.59	r= -0.21 p= 0.57	r= 0.44 p= 0.10
Diaphysis Ca (mg/g)	r= -0.36 p= 0.31	r= 0.46 p= 0.21	r= 0.21 p= 0.40	r= 0.34 p= 0.34	r= -0.58 p= 0.08	r= -0.24 p= 0.39
Diaphysis P (mg/g)	r= -0.36 p= 0.30	r= 0.32 p= 0.40	r= 0.21 p= 0.40	r= 0.31 p= 0.38	r= -0.61 p= 0.06	r= -0.14 p= 0.61
Urine Ca/creatinine (mmol/mmol)	r= 0.43 p= 0.21	r= -0.52 p= 0.15	r= 0.92 p <0.0001	r= 0.09 p= 0.81	r= 0.04 p= 0.92	r= 0.29 p= 0.29 (n=18)
Urine P/creatinine (mmol/mmol)	r= -0.45 p= 0.19	r= -0.24 p= 0.54	r= 0.80 p <0.0001	r= -0.21 p= 0.56	r= -0.06 p= 0.87	**r= 0.61 p= 0.02** (n=18)
Day 5 Glucose (mmol/L)	r= -0.51 p= 0.30 (n=6)	— ** (n=3)	r= 0.27 p= 0.29 (n=17)	— ** (n=3)	r= 0.02 p= 0.97 (n=6)	r= 0.20 p= 0.48
Necropsy Glucose (mmol/L)	r= -0.44 p= 0.20	r= -0.36 p= 0.34	r= 0.31 p= 0.21	r= 0.30 p= 0.39	r= -0.22 p= 0.55	**r= 0.76 p= 0.001**

r = Pearson Correlation Coefficient
Significant correlations are shown in bold
PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet
* Values for n as shown at top of table, except where otherwise indicated
** Insufficient data for analysis

Table 10.10: Relationship between PGE_2 and femur, plasma, and urine biochemistry

	PS (n=10)*	CS (n=11)*	DS (n=18)*	PF (n=10)*	CF (n=10)*	DF (n=19)*
OC (pmol/L)	r= -0.09 p= 0.80	r= 0.46 p= 0.16	**r= -0.67 p= 0.002**	r= -0.47 p= 0.17	r= 0.27 p= 0.45	r= 0.01 p= 0.98 (n=16)
NTx (nM BCE/mM)	r= -0.08 p= 0.82	r= 0.05 p= 0.91 (n=9)	r= -0.11 p= 0.68 (n=17)	r= -0.05 p= 0.89	r= 0.15 p= 0.69	r= 0.35 p= 0.22 (n=15)
IGF-I (pmol/L)	r= -0.39 p= 0.26	r= -0.34 p= 0.33 (n=10)	r= -0.37 p= 0.33 (n=9)	**r= -0.64 p= 0.048**	r= 0.16 p= 0.65	r= -0.17 p= 0.63 (n=10)
Vitamin D (nmol/L)	**r= 0.68 p= 0.03**	r= -0.37 p= 0.27	r= 0.02 p= 0.95	r= -0.17 p= 0.64	r= 0.41 p= 0.24	r= -0.26 p= 0.32 (n=18)
PTH (nmol/L)	r= -0.25 p= 0.49	r= -0.28 p= 0.40	**r= -0.48 p= 0.04**	r= 0.20 p= 0.58	r= 0.03 p= 0.93	r= 0.47 p= 0.05
Diaphysis Ca (mg/g)	r= 0.59 p= 0.07	r= -0.10 p= 0.76	r= -0.25 p= 0.31	**r= -0.70 p= 0.02**	r= -0.42 p= 0.23	r= -0.54 p= 0.02
Diaphysis P (mg/g)	r= 0.55 p= 0.10	r= -0.10 p= 0.76	r= -0.24 p= 0.34	**r= -0.75 p= 0.01**	r= -0.56 p= 0.09	r= -0.38 p= 0.12
Urine Ca/creatinine (mmol/mmol)	r= -0.13 p= 0.73	r= 0.38 p= 0.24	r= -0.13 p= 0.59	r= -0.54 p= 0.11	r= 0.16 p= 0.66	r= 0.15 p= 0.56 (n=18)
Urine P/creatinine (mmol/mmol)	r= 0.03 p= 0.93	r= 0.07 p= 0.84	r= -0.38 p= 0.12	r= -0.07 p= 0.85	r= 0.30 p= 0.40	r= 0.02 p= 0.94 (n=18)
Day 5 Glucose (mmol/L)	r= 0.75 p= 0.09 (n=6)	--- ** (n=3)	r= -0.10 p= 0.70 (n=17)	— ** (n=3)	r= 0.59 p= 0.21 (n=6)	r= 0.29 p= 0.24
Necropsy Glucose (mmol/L)	r= 0.26 p= 0.47	r= 0.49 p= 0.13	r= 0.08 p= 0.74	r= -0.38 p= 0.28	r= 0.12 p= 0.75	r= 0.20 p= 0.42

r = Pearson Correlation Coefficient
Significant correlations are shown in bold
PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet
* Values for n as shown at top of table, except as otherwise indicated
** Insufficient data for analysis

Table 10.11: Liver fatty acid composition affected by diet in stz- and saline-induced rats

	Soy Oil Diet (n=39)	Fish Oil Diet (n=38)
C16:0	19.366 (4.512)*	20.805 (3.784)
C16:1 n-9	0.243 (0.123)*	0.162 (0.081)
C18:2 n-6	17.901 (3.659)*	15.222 (3.122)
C18:3 n-6	0.263 (0.121)**	0.105 (0.067)
C18:3 n-3	0.925 (0.570)**	0.238 (0204)
C20:0	0.068 (0.036)*	0.098 (0.036)
C20:2 n-6	0.346 (0.186)**	0.184 (0.060)
C20:3 n-6	0.673 (0.408)**	1.282 (0.566)
C20:4 n-6	17.989 (4.937)**	9.983 (4.071)
C20:3 n-3	0.050 (0.039)*	0.033 (0.019)
C20:5 n-3	0.330 (0.626)**	3.649 (2.074)
C22:0	0.145 (0.070)**	0.094 (0.035)
C22:4 n-6	0.506 (0249)**	0.144 (0.064)
C22:5 n-3	0.754 (0.364)**	2.217 (0.730)
C22:6 n-3	6.170 (3.122)**	11.971 (2.571)
C24:1 n-9	0.075 (0.270)**	0.159 (0.056)

Data shown as mean (SD) % wt/wt

*Differences significant by t-test at $p < 0.05$.

**Differences significant by t-test at $p < 0.0001$.

Table 10.12: Liver fatty acid composition affected by stz injection compared to saline injection in rats fed soy oil or fish oil

	Saline-injected (n=40)	**Stz-injected** (n=37)
C16:0	23.230 (2.267)**	16.582 (2.910)
C16:1 n-9	0.256 (0.104)**	0.148 (0.092)
C16:1 n-7	3.676 (1.252)**	0.849 (0.997)
C18:0	11.288 (1.785)**	19.399 (5.410)
C18:1 n-9	14.901 (2.972)**	7.663 (4.229)
C18:1 n-7	3.105 (0.512)**	2.145 (0.668)
C18:3 n- 3	0.737 (0.521)*	0.443 (0.553)
C20:0	0.063 (0.026)**	0.103 (0.039)
C20:1 n-9	0.114 (0.039)*	0.139 (0.060)
C20:2 n-6	0.205 (0.117)*	0.338 (0.176)
C20:3 n-6	0.668 (0.301)**	1.286 (0.631)
C20:4 n-6	11.555 (4.941)**	17.015 (5.905)
C20:3 n-3	0.028 (0.020)**	0.058 (0.036)
C20:5 n-3	2.643 (2.689)*	1.121 (1.210)
C22:0	0.089 (0.025)**	0.155 (0.070)
C22:4 n-6	0.232 (0.131)**	0.443 (0.315)
C22:6 n-3	7.162 (3.573)**	10.887 (3.725)
C24:0	0.213 (0.057)**	0.341 (0.124)
C24:1 n-9	0.102 (0.029)*	0.130 (0.080)

Data shown as mean (SD) % wt/wt

*Differences significant by t-test at $p < 0.05$

**Differences significant by t-test at $p \leq 0.0001$

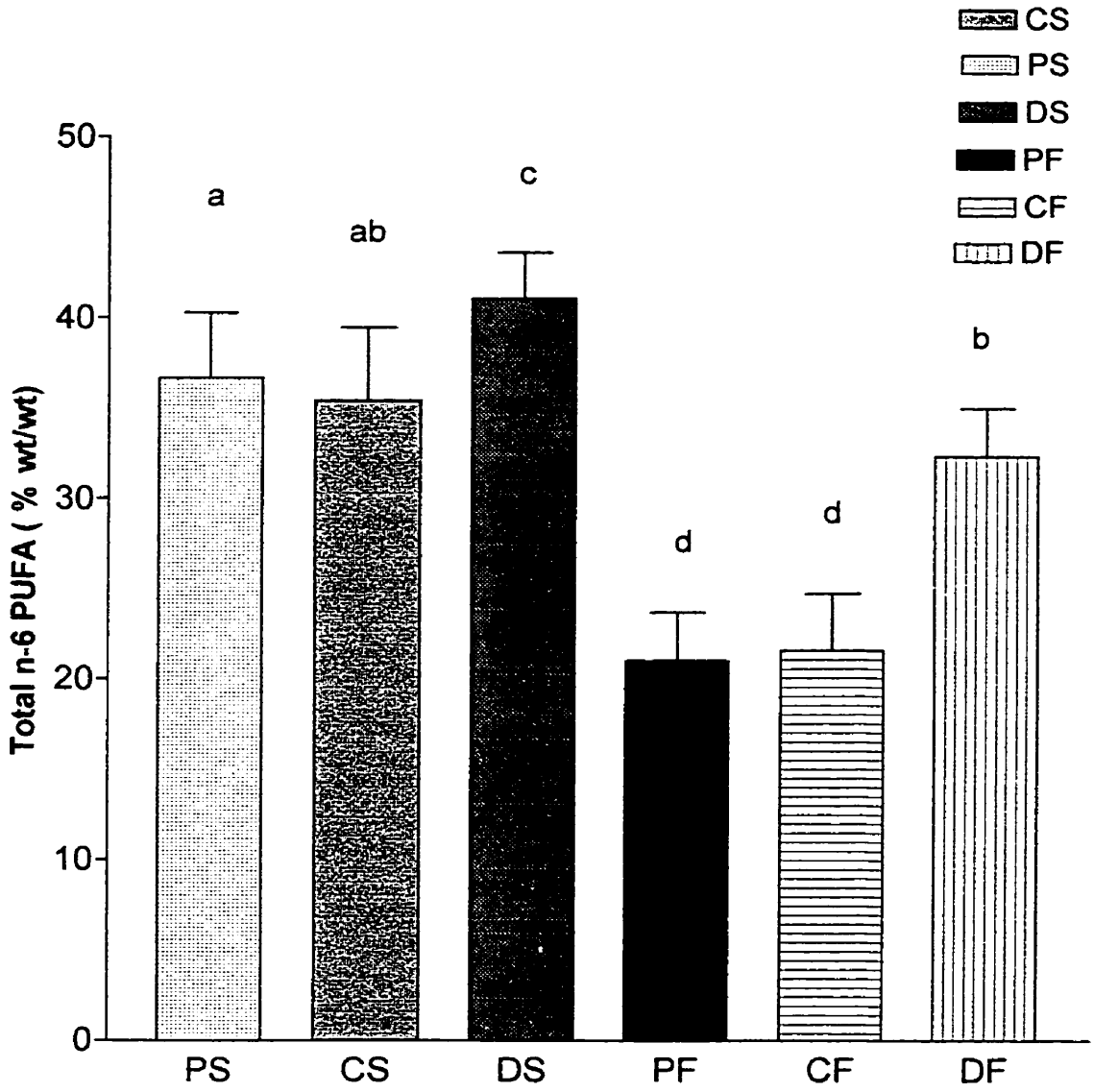

Figure 10.1: Total n-6 PUFA in the liver of stz- and saline-injected rats fed soy oil or fish oil

Columns with different superscripts are significantly different at $p < 0.05$ as analyzed by 1-way ANOVA, post-hoc analysis by Tukey's test

PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet

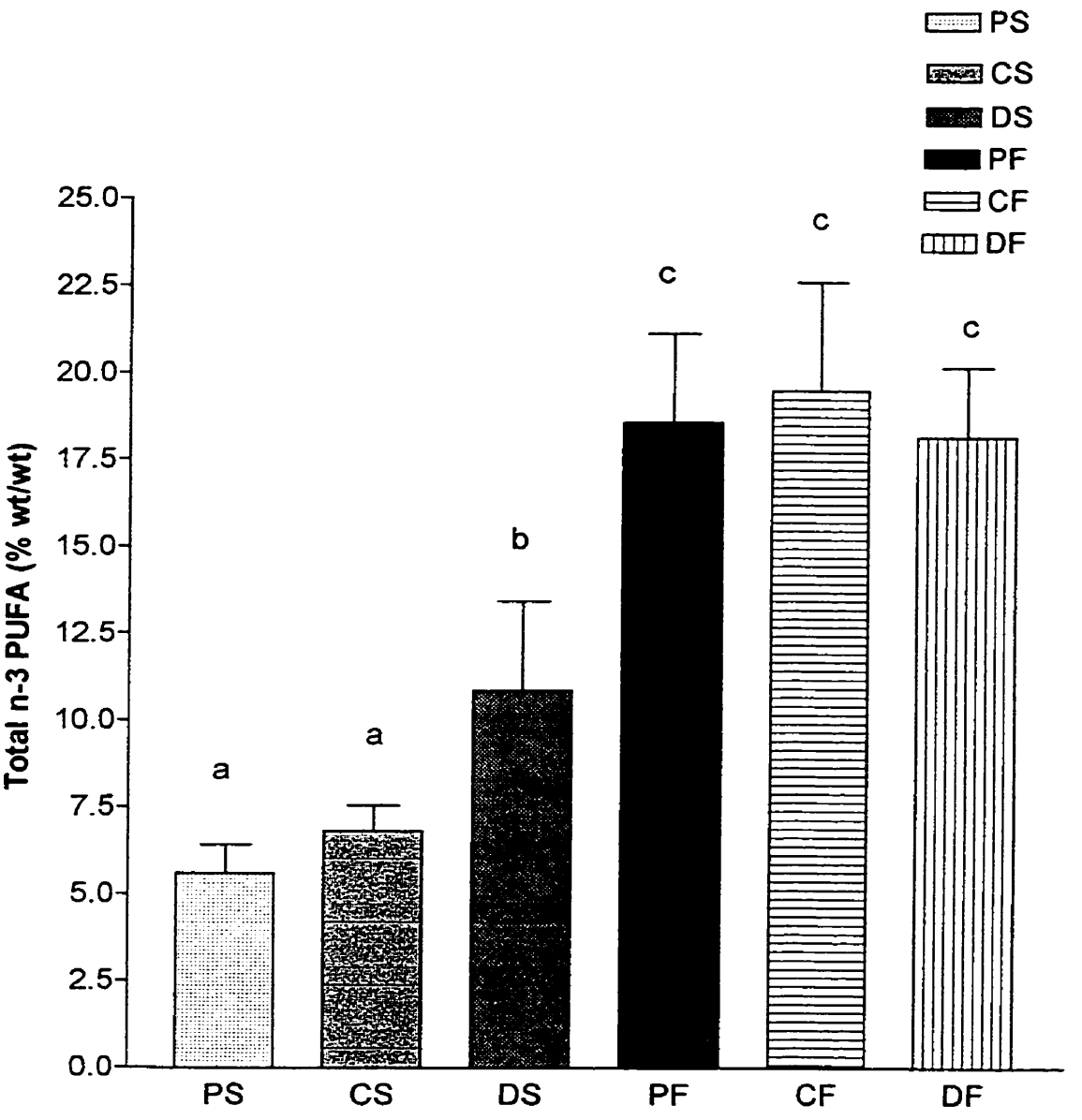

Figure 10.2: Total n-3 PUFA in the liver of stz- and saline-injected rats fed soy oil or fish oil

Columns with different superscripts are significantly different at $p < 0.05$ as analyzed by 1-way ANOVA, post-hoc analysis by Tukey's test

PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet

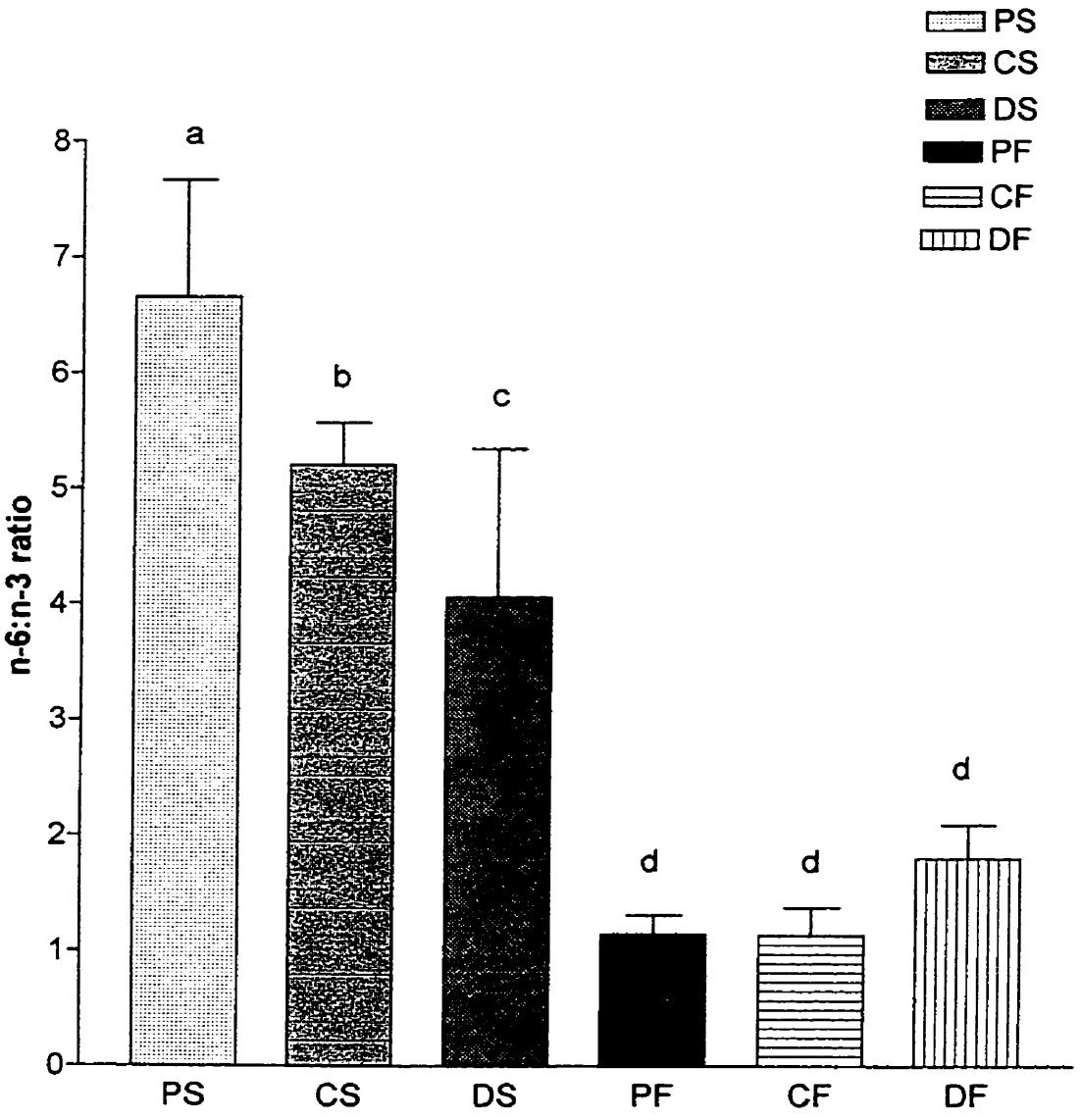

Figure 10.3: n-6: n-3 PUFA ratio in the liver of stz- and saline-injected rats fed soy oil or fish oil

Columns with different superscripts are significantly different at $p < 0.05$ as analyzed by 1-way ANOVA, post-hoc analysis by Tukey's test

PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet

Table 10.13: Best subsets regression of factors related to whole femur weight in stz- and saline-injected rats fed soy oil or fish oil

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent Whole femur weight (g) R^2 = 0.529, R^2 adj. = 0.475				
Constant	0.823	0.045	18.135	<0.001
IGF-I (pmol/L)	0.001	0.0004	2.179	0.035
PGE_2 (mmol/g)	-0.002	0.0007	-2.550	0.014
PTH (nmol/L)	0.001	0.001	0.886	0.381
Urinary Ca/creatinine (mmol/mmol)	-0.014	0.007	-2.172	0.035
Necropsy plasma glucose (mmol/L)	-0.004	0.002	-2.302	0.026

Whole femur weight = constant + IGF-I (mean) - PGE_2 (mean) + PTH (mean) - urinary Ca/creatinine (mean) - necropsy plasma glucose (mean) = 0.823 + 0.001(51.38) - 0.002(34.94) + 0.001(16.97) - 0.014(1.12) - 0.004(17.72)

Table 10.14: Best subsets regression of factors related to whole femur weight in stz- injected rats fed soy oil or fish oil

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent Whole femur weight (g) R^2 = 0.945, R^2 adj. = 0.879				
Constant	0.610	0.048	12.655	<0.001
NTx (mM BCE/mM creatinine)	-0.0004	0.001	-3.328	0.021
OC (pmol/L)	1.04	0.143	7.277	<0.001
PGE_2 (mmol/g)	0.002	0.001	2.677	0.044
$1,25(OH)_2D_3$ (nmol/L)	1.46	0.206	7.060	<0.001
Necropsy plasma glucose (mmol/L)	-0.009	0.001	-11.209	<0.001
Liver n-6:n-3 PUFA ratio	-0.151	0.017	-8.692	<0.001

Whole femur weight = constant - NTx(mean) + OC(mean) + PGE_2(mean) + $1,25(OH)_2D_3$(mean) - necropsy plasma glucose(mean) - liver n-6:n-3 PUFA ratio(mean) = 0.610 - 0.0004(94.88) + 1.04(0.52) + 0.002(42.63) + 1.46(0.11) - 0.009(22.13) - 0.151(2.90)

Table 10.15: Best subsets regression of factors related to whole femur weight in saline- injected rats fed soy oil or fish oil

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent Whole femur weight (g) R^2 = 0.264, R^2 adj. = 0.199				
Constant	0.804	0.026	30.910	<0.001
PGE_2 (mmol/g)	-0.001	0.001	-1.972	0.057
$1,25(OH)_2D_3$ (nmol/L)	-0.052	0.046	-1.125	0.268
Urinary P/creatinine (mmol/mmol)	0.297	0.102	2.904	0.006

Whole femur weight = constant - PGE_2 (mean) - $1,25(OH)_2D_3$(mean) + Urinary P/creatinine(mean) = 0.804 - 0.001(28.26) - 0.052(0.26) + 0.297(0.10)

Table 10.16: Best subsets regression of factors related to femur length in stz- and saline-injected rats fed soy oil or fish oil

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent Femur length (cm) R^2 = 0.557, R^2 adj. = 0.508				
Constant	3.404	0.070	48.854	<0.001
IGF-I (pmol/L)	0.002	0.006	2.626	0.012
PGE_2 (mmol/g)	-0.002	0.001	-1.723	0.092
PTH (nmol/L)	0.002	0.002	1.501	0.140
Urinary Ca/creatinine (mmol/mmol)	-0.022	0.011	-2.049	0.046
Necropsy plasma glucose (mmol/L)	-0.010	0.003	-3.169	0.003

Femur length = constant + IGF-I(mean) - PGE_2 (mean) + PTH(mean) - Urinary Ca/creatinine(mean) - Necropsy plasma glucose(mean) = 3.404 + 0.002(51.38) - 0.002(34.94) + 0.002(16.97) - 0.022(1.12) - 0.010(17.72)

Table 10.17: Best subsets regression of factors related to femur length in stz- -injected rats fed soy oil or fish oil

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent Femur length (cm) R^2 = 0.981, R^2 adj. = 0.931				
Constant	2.791	0.092	30.344	<0.001
IGF-I (pmol/L)	0.003	0.001	4.399	0.022
NTx (mM BCE/mM creatinine)	-0.001	0.0002	-4.696	0.018
OC (pmol/L)	1.528	0.205	7.464	0.005
PGE_2 (mmol/g)	0.002	0.001	2.471	0.090
$1,25(OH)_2D_3$ (nmol/L)	1.907	0.496	3.845	0.031
Urinary P/creatinine (mmol/mmol)	-0.071	0.030	-2.390	0.097
Necropsy plasma glucose (mmol/L)	-0.008	0.002	-3.825	0.031
Liver n-6:n-3 PUFA ratio	-0.166	0.029	-5.834	0.010

Femur length = constant + IGF-I(mean) - NTx(mean) + OC(mean) + PGE_2(mean) + $1,25(OH)_2D_3$(mean) -Urinary P/creatinine(mean)- Necropsy plasma glucose(mean) - Liver n-6:n-3 PUFA ratio(mean) = 2.791 + 0.003(26.44) - 0.001(94.88) + 1.528(0.52) + 0.002(42.63) + 1.907(0.11) - 0.071(0.37) - 0.008(22.13) - 0.166(2.90)

Table 10.18: Best subsets regression of factors related to femur length in saline-injected rats fed soy oil or fish oil

Variable	Coefficient	Standard Error	t-ratio	P-value
Dependent Femur length (cm) R^2 = 0.327, R^2 adj. = 0.270				
Constant	3.49	0.106	32.945	<0.001
Urinary P/creatinine (mmol/mmol)	0.554	0.152	3.642	<0.001
OC (pmol/L)	-0.459	0.211	-2.182	0.036
IGF-I (pmol/L)	0.001	0.001	1.747	0.089

Femur length = constant + Urinary P/creatinine(mean) - OC(mean) + IGF-I(mean) = 3.49 + 0.554(0.10) - 0.459(0.57) + 0.001(62.79)

11. Basic Study: Discussion

Numerous factors have been implicated as causes of the reduced bone mass seen in individuals with Type 1 diabetes. Animal studies have investigated the metabolism of calcium, phosphorus, PTH, and $1,25(OH)_2D_3$, and the role of both insulin and IGF-I in diabetic osteopenia (7-9,18,119-20). The relative actions of osteoblasts and osteoclasts have also been studied. Stz-injected and spontaneously insulin deficient rats have been shown to have greatly reduced numbers of osteoblasts and reduced osteoblast activity, but normal or only slightly reduced osteoclast activity (10,13,37,50). Recently, the role of dietary fat in bone resorption and formation has been investigated. PGE_2, which is formed through the metabolism of n-6 PUFA, is a potent stimulator of both bone formation and bone resorption (30,80-2). The extent of stimulation appears to be affected in part by PGE_2 concentration. Moderate concentrations have been shown to result in elevated bone formation, while high concentrations reduce bone formation (80). The concentration of PGE_2 may be altered by a number of factors, among them PGE_3. This prostaglandin, a metabolite of the n-3 PUFA α-linolenic acid, has been shown to inhibit bone resorption, and may also play a role in bone formation (85,121). Animals fed diets high in n-3 PUFA had higher bone formation rates than animals fed diets high in n-6 PUFA (30). Since both n-3 and n-6 PUFA are synthesized by the same converting enzyme system, there is competition between the two for synthesis of their respective prostaglandins. PGE_3 formation is more limited

than that of PGE_2; however, synthesis of PGE_3 takes precedence over synthesis of PGE_2 by the converting enzyme system. As a result, less total prostaglandin is produced, possibly reducing the concentration of PGE to a level at which it promotes bone formation (85,87). A reduction in the concentration of PGE_2 may also result in reduced bone resorption, although resorption appears to be less affected than formation (30). While a number of studies have found similar results in insulin sufficient animals, little work has been done in insulin deficient animals. Thus the hypothesis of this portion of the research was: **in insulin deficiency, dietary fish oil elevates bone formation in relation to bone resorption through a PGE_2-dependent pathway.** The objective in diabetes-induced growing rodents was to determine the effect of dietary fish oil on mineral content and markers of bone formation and resorption.

Induction of diabetes was successfully achieved using stz as evidenced by elevated blood glucose levels (**Table 10.2, Appendix D**), and also significantly higher intake of both feed and water, larger urine volume, and lower growth rate and final body weight (**Table 10.3, Appendix D**). Organ weights, when corrected to body weight, were significantly higher in the diabetes groups compared to the pair-weigh and control groups (**Table 10.4, Appendix E**). This finding of higher organ weights is common in induced diabetes, although some researchers have found that only the kidneys and intestines, and not the heart or liver, undergo hypertrophy (122). Also similar to the findings of others

(7-8,10), bone weight (whole femur weight in this study) was significantly lower in the diabetes groups compared to the pair-weigh and control groups (**Table 10.4, Appendix E**). However, this difference appears to have been due primarily to body size, since whole femur weight corrected to body weight was actually higher in stz-injected animals. Again similar to the findings of Rosholt and Hegarty who studied 122 day old stz-injected rats (8), femur lengths were significantly lower in the diabetes groups (**Table 10.4, Appendix E**), suggesting that bone size may be more affected than bone mineralization, although the measurements for femur length were not corrected for body size as were the measurements for femur weight. In insulin deficient animals compared to insulin sufficient animals, the overall reduction in size of the femur resulted in a lower weight to length ratio in the femur (**Table 10.4, Appendix E**).

Femur diaphysis concentrations of calcium and phosphorus were not different in the diabetes groups compared to the pair-weigh and control groups (**Table 10.5, Appendix F**), in spite of higher total urinary excretion of calcium in the diabetes groups compared to the control groups, and higher urinary excretion of phosphorus in the diabetes group compared to both the pair-weigh and the control groups (**Table 10.5, Appendix F**). Similar bone calcium and phosphorus content between diabetic and control animals were also found in other studies (8,18), as were reports of higher urinary calcium and phosphorus excretion (9,123). This discrepancy between normal femur diaphysis calcium

and phosphorus concentration and elevated urinary excretion may be explained by the high feed intake seen in the insulin deficient animals. A higher feed intake would result in higher calcium and phosphorus intakes, potentially maintaining calcium and phosphorus balance (9). In contrast to the findings of Yamada et al. (123), dietary fish oil did not reduce urinary phosphorus excretion in the stz-injected animals (**Table 10.5, Appendix F**). However, in support of the findings of Buck et al. (88) and of Claassen et al. (87), insulin deficient animals fed fish oil did have lower urinary calcium excretion (**Table 10.5, Appendix F**) than insulin deficient animals fed soy oil, although the difference was not significant. Buck et al. (88) investigated rats given an IP injection of calcium gluconate designed to induce nephrocalcinosis. They compared rats given Mexepa oil containing 180 mg EPA 4 days prior to the injection with rats who received only the injection, and found that urinary calcium excretion was significantly lower in the former group. Claassen et al. (87) supplemented the diet of 5 to12 week old rats with varying ratios of n-6:n-3 PUFA, and found a significant negative correlation between dietary n-3 PUFA and urinary calcium excretion.

The speculation that calcium balance was maintained in stz-injected animals is supported by the lack of significant difference in plasma PTH levels between the groups (**Table 10.5, Appendix F**). While PTH levels were elevated in the insulin deficient rats compared to the control rats, the difference was not significant. Normal PTH levels have been reported in some studies

(110), while others have reported reduced levels (18). However, while the differences between the groups were not significant, this may be the result of the timing of the sample. Since PTH levels were measured in the fasting state, they would tend to be elevated in all animals, potentially masking any differences that may have existed between groups. This may also apply to the measurements of calcium and phosphorus, and might help to explain the significant differences observed in $1,25(OH)_2D_3$ (**Table 10.5, Appendix F**).

Reduced concentrations of plasma vitamin D in stz-injected and spontaneously diabetic rats has been well established (7,18,23,73,110), and similar results were found in this study. While there was no significant effect of diet on plasma $1,25(OH)_2D_3$, levels in the insulin deficient animals were considerably higher in rats fed fish oil compared to those fed soy oil, and were considerably lower among the insulin sufficient animals fed fish oil compared to those fed soy oil (**Table 10.5, Appendix F**). Within diet groups, levels of $1,25(OH)_2D_3$ were significantly lower in the stz-injected animals compared to the control animals. In rats fed soy oil, those in the diabetes group also tended to have lower plasma vitamin D than those in the pair fed group, although the difference was not significant. This reduced concentration of $1,25(OH)_2D_3$ in spite of normal, or in the case of this study, slightly elevated levels of PTH has been reported elsewhere, and is likely due to impaired renal synthesis and the action of hyperphosphatemia on hydroxylation of calcidiol (23). Measurement of magnesium might offer an explanation for the low $1,25(OH)_2D_3$ seen in the

stz-injected animals since magnesium wasting may lead to reduced α-1-hydroxylase activity, and subsequently to reduced $1,25(OH)_2D_3$ (124). Another mechanism may relate to synthesis of PGE_2, as the prostaglandin has been shown to stimulate production of $1,25(OH)_2D_3$. It has been suggested that decreased renal synthesis is, at least in part, the result of an impaired renal response to PGE_2-like prostaglandins (125).

The reduced levels of $1,25(OH)_2D_3$ seen in the insulin deficient animals might also be linked to reduced levels of IGF-I. Levels of IGF-I have been reported to be reduced in diabetes (119-20). This is not unexpected, since insulin levels are positively correlated with levels of IGF-I. In turn, IGF-I may contribute to renal synthesis of $1,25(OH)_2D_3$ and regulate its actions in bone remodelling (64,71). Using an in vitro model of mouse proximal tubular cells, Menaa et al. showed that application of 10 - 100 ng/mL IGF-I to cultured cells elevated the detectable concentration of $1,25(OH)_2D_3$ after 18 hours of incubation (71). Bianda et al. (64) observed significantly higher serum $1,25(OH)_2D_3$ concentrations in healthy males infused subcutaneously with IGF-I compared to those infused with 0.9% saline. Since PTH, phosphorus, and calcium levels were similar in both groups, the authors concluded that the increase in $1,25(OH)_2D_3$ was due to a direct stimulatory effect of IGF-I . In this thesis research, levels of IGF-I were significantly reduced in the stz-injected animals (**Table 10.5, Appendix F**). Although IGFBP were not measured, it is likely that the alterations in IGF-I observed in this study were related to

alterations in IGFBP. IGFBP regulate the both bioavailability and the activity of IGF-I, either stimulating it (as in the case of IGFBP-3) or inhibiting it (IGFBP-1) (20).

Although IGF-I was not significantly affected by diet in this study, dietary lipids have been shown to affect IGF-I levels (82). Watkins et al. found that animals fed diets high in n-3 PUFA had significantly higher concentrations of plasma IGF-I at 14 days of age than animals fed diets low in n-3 PUFA. The difference may be in the timing of sampling; Watkins found no significant differences between dietary groups at 28, 42, or 56 days of age. In the current study, animals were approximately 8 weeks old, and may have been past their peak growth spurt when IGF-I concentrations are highest (17). In another study, Watkins showed that bone concentrations of IGF-I were not affected by dietary fat (30). On the other hand, osteoblastic production of IGF-I has been shown to be stimulated by locally synthesized PGE_2 (80,126-8). Osteoblast-enriched cultures exposed to PGE_2 a metabolite of n-6 PUFA, showed significantly elevated IGF-I polypeptide accumulation (80). This occurs through stimulation of intracellular cAMP in osteoblast cells, which in turn results in elevated IGF-I (80,126). In addition to its direct stimulatory effects on IGF-I, PGE_2 may elevate plasma IGF-I concentrations through its bone resorbing actions which result in skeletal release of IGF-I (128).

As expected, rats fed soy oil had significantly higher levels of diaphyseal PGE_2 than those fed fish oil (**Table 10.5, Appendix F**). The higher amounts of

n-3 PUFA in the fish oil diet may have tipped the balance in favor of the n-3 metabolic pathway over the n-6 metabolic pathway, resulting in less PGE_2 being produced. In addition, insulin deficient animals had significantly higher levels of PGE_2 than insulin sufficient animals. Similar results have been found in studies of children (129). Although the mechanism behind the increase is not known, it may be related to the alterations in PTH and vitamin D seen in insulin deficiency.

An examination of the relationship of PGE_2 with other parameters (**Table 10.10**) does not help to shed any light on this altered metabolism. The inverse relationship of PGE_2 with bone formation, measured as OC, in the DS group is reasonable since PGE_2 might be elevated to levels at which it inhibits bone formation. The positive relationship between $1,25(OH)_2D_3$ and PGE_2 in the PS group also might be expected, given the stimulatory effect of PGE_2 on vitamin D, although a relationship between bone PGE_2 and renal $1,25(OH)_2D_3$ synthesis has not been reported. However, the lack of relationship between PGE_2 and bone resorption and, except in the DS group, between PGE_2 and bone formation is unexpected since PGE_2 is known to stimulate both (30,80-2).

While PTH, vitamin D, and PGE_2 all regulate bone development, they may do so through mediation of osteoprotegerin (OPG) and its ligand (130). These cytokines respectively inhibit and stimulate osteoclastogenesis, following binding to preosteoclasts - the precursors to osteoblastic cells (131). OPG ligand promotes bone resorption by stimulating differentiation of preosteoclasts

into osteoclasts. In contrast, OPG inhibits osteoclastogenesis, thereby inhibiting bone resorption. OPG deficiency, in both humans and animals has been linked with the development of osteoporosis (130-1). Although OPG was not measured in this study, the changes seen in $1,25(OH)_2D_3$ and PGE_2, both of which have been shown to stimulate OPG ligand and inhibit OPG, might offer a further explanation of the observed changes in bone formation and resorption.

In insulin deficiency, bone formation, as indicated by OC, was limited (**Table 10.5, Appendix F**), although the difference between insulin deficient and insulin sufficient animals was not significant. Verhaeghe et al. also found bone formation to be reduced in spontaneously diabetic rodents compared to control and semi-starved rats (7,10). In the current study, diet had a significant effect on bone formation, with animals fed fish oil having lower levels than those fed soy oil. The lowest levels of formation were seen in the insulin deficient rats fed fish oil, suggesting that lack of normal amounts of insulin and reduced PGE_2 are key to understanding bone metabolism.

While the differences in bone formation between the stz- and saline-injected animals were not significant, the differences between these two groups were significant for bone resorption (**Table 10.5, Appendix F**). Urinary NTx, the measure of bone resorption, was significantly higher in insulin deficient rats compared to control and pair-weigh animals, suggesting higher bone resorption in the former group. This is in contrast to the findings of

Goodman and Hori, who observed no difference in bone resorption between diabetic and control rats (12). This discrepancy may be due to the difference in method, as these researchers measured bone resorption at the endosteum using a tetracycline label. Again, diet appeared to have an effect, with bone resorption considerably, although not significantly, lower in the diabetic rats fed fish oil compared to those fed soy oil. However, this effect of diet was apparent only in the diabetic rats. Thus in contrast to the findings of other researchers (7,9), insulin deficient animals had greatly elevated bone resorption, while bone formation was relatively unaffected unless fish oil was fed.

Since OC synthesis is stimulated primarily through the actions of $1,25(OH)_2D_3$ (10), a significant correlation between bone formation and plasma vitamin D might be expected. However, there was no significant relationship between plasma concentrations of $1,25(OH)_2D_3$ and bone formation in any of the diet or treatment groups (**Table 10.8**). In fact, there were few significant relationships of bone formation with any of the biochemical indicators measured in plasma, urine, or bone. While a correlation of IGF-I with bone formation has been reported (120,132), a significant positive relationship was found only in the CF group. The significant negative relationship between formation and PGE_2 found in the DS group is expected, since as mentioned earlier, high concentrations of PGE_2 may inhibit bone formation (80). Although no studies have reported a correlation of femur phosphorus levels with bone

formation, this finding in the DS group is reasonable. Higher femur diaphysis phosphorus content may be indicative of healthier bone, which would undergo elevated formation compared to less healthy bone. In addition, urinary phosphorus excretion may be an indicator that plasma phosphorus is adequate, allowing mineral to be adsorbed in bone. Thus the correlation of urinary phosphorus excretion with bone formation in the DS and DF groups might have been expected in the other groups also. However, a positive correlation of plasma glucose with bone formation in the DF group is more difficult to explain. Plasma glucose level, an indicator of metabolic control, is generally known to be negatively correlated with bone formation (4,6,21,33).

As with bone formation, there were few significant correlations of bone resorption with plasma, urine, or bone biochemical indicators (**Table 10.9**). In the PS group, PTH was significantly negatively correlated with bone resorption, while in the PF group, $1,25(OH)_2D_3$ was significantly negatively correlated with resorption. The only other significant correlations were found in the insulin deficient groups. The highly significant positive relationships between urinary calcium excretion and bone resorption in the DS group and urinary phosphorus excretion in the DF and DS groups may be the result of elevated intake. Calcium and phosphorus are released from bone as it is resorbed, and would eventually be excreted in the urine. The levels of calcium and phosphorus being excreted were not elevated in the insulin sufficient groups which might explain why no significant correlation was found between

calcium or phosphorus excretion and bone resorption in these groups; however this does not explain the lack of significant relationship found between calcium excretion and bone resorption in the DF group, since these animals did have elevated levels of excretion compared to the insulin sufficient animals. The strong positive correlation of plasma glucose with bone resorption in the DF group is also reasonable, since high blood glucose might be expected to elevate bone resorption. In fact, Krakauer et al. proposed that poor metabolic control indirectly elevates bone resorption by increasing urinary calcium excretion and acidosis (37). This makes it difficult to determine whether it is lack of insulin or elevated plasma glucose, or some combination of the two, which caused the elevated bone resorption seen in the insulin deficient animals.

One way to determine the end effect of bone formation and resorption is to measure bone size. In this study, bone size was measured as bone weight and bone length. Bone formation was positively correlated with femur weight in the DS group only, while bone resorption was negatively correlated with femur weight in the DF group (**Table 10.6**). Besides formation and resorption, a number of factors contribute to the size of the bone. Mineral content is an obvious contributor to bone weight, although femur diaphysis phosphorus was significantly correlated with femur weight only in the DS group, and femur diaphysis calcium was not significantly related to femur weight in any of the groups. Since high $1,25(OH)_2D_3$ precedes bone resorption, and therefore loss

of calcium from bone, it might be expected to be negatively correlated with femur weight; however, this negative correlation was found only in the CF group. Urinary calcium and phosphorus excretion might also be expected to be negatively correlated with femur weight for similar reasons; however, no significant relationships were found between either urinary calcium excretion or urinary phosphorus excretion and femur weight. As with bone formation and resorption, there was a significant negative correlation of plasma glucose with femur weight in the insulin deficient animals. The ratio of n-6:n-3 was highly positively correlated with bone weight in the DS group, suggesting perhaps that in this group, PGE_2 concentration was at a level at which bone formation was being promoted.

Relationships of plasma, urine, and femur biochemical indicators with femur length were similar to those with femur weight (**Table 10.7**). Again there was a significant positive correlation of bone formation with femur length in the DS group only, and a significant negative correlation of bone resorption with femur length in the DF group only, although these relationships might have been expected in all groups, since bone formation would tend to increase bone size, while resorption would decrease it. As IGF-I is a growth factor, it would be expected to contribute to growth of bone length, and in fact was positively correlated with femur length in the DS and PF groups.

Given the lack of consistent relationships of femur weight and length with biochemical indicators, what contributed to bone size? Looking at all the

animals combined, the factors that most contributed to both femur weight and femur length were IGF-I, PGE_2, PTH, urinary calcium excretion corrected to creatinine, and necropsy plasma glucose (**Tables 10.13 and 10.16**). However, the fact that these variables account for only 47.5% of the variability in femur weight (**Table 10.13**) and only 50.8% of the variability in femur length (**Table 10.16**) suggests that there are other factors, besides those analyzed, which contribute to weight and length of the bone. This is supported by the fact that the coefficient for each of these values is very small, indicating their effect on bone weight and length is also very small. Using mean values for each of these factors in the equation, for every gram increase in femur weight, IGF-I contributes by 0.051 and PTH by 0.017, while PGE_2, plasma glucose and urinary calcium excretion decrease femur weight by 0.070, 0.071, and 0.016 respectively (**Table 10.13**). The numbers are slightly different for femur length: for every centimetre increase in femur length, IGF-I contributes by 0.103 and PTH by 0.034, while PGE_2 decreases it by 0.070, urinary calcium excretion by 0.025, and plasma glucose by 0.177 (**Table 10.16**).

Similar to the whole group, factors contributing to femur length in insulin deficient animals (**Table 10.17**) are the same as those contributing to femur weight (**Table 10.14**), with the exception of IGF-I which contributes to length, but not to weight. In this case, 87.9% of the variability in femur weight (**Table 10.14**) and 93.1% of the variability in femur length (**Table 10.17**) is explained by these factors. However, except for OC, the contribution of each factor is

still small. For each gram increase in femur weight in insulin deficient animals, OC contributes 0.541, PGE_2 contributes 0.085, and $1,25(OH)_2D_3$ contributes 0.161, while femur weight is decreased by NTx (0.038), plasma glucose (0.199), and liver n-6:n-3 PUFA (0.438) (**Table 10.14**). Each centimetre increase in femur length is contributed to by IGF-I (0.079), OC (0.795), PGE_2 (0.085), and $1,25(OH)_2D_3$ (0.210), and decreased by NTx (0.095), urinary phosphorus excretion (0.026), plasma glucose (0.177), and liver n-6:n-3 ratio (0.481) (**Table 10.17**). Thus OC is the primary contributor to both femur weight and femur length in stz-injected animals, while the liver n-6:n-3 PUFA ratio decreases both by a considerable amount. It is not surprising that OC, a measure of bone formation, contributes a large portion to femur weight and length. That a higher n-6:n-3 PUFA ratio decreases bone size, supports the findings of other researchers. Watkins et al. found that bone formation was negatively correlated with PGE_2 (121). A higher n-6:n-3 PUFA ratio resulted in significantly higher PGE_2 levels in the current study. Among insulin deficient animals, those fed the soy oil diet (the higher n-6:n-3 ratio) also had higher levels of OC, although among insulin deficient animals fed soy oil, PGE_2 was negatively correlated with OC.

Interestingly, factors contributing to femur weight in insulin sufficient animals (**Table 10.15**) are for the most part different from those that contribute to femur length (**Table 10.16**). For each gram increase in femur weight, urinary phosphorus/creatinine contributes 0.030, while PGE_2 and $1,25(OH)_2D_3$

decrease femur weight by 0.028 and 0.014 respectively (**Table 10.15**). Once again, there are evidently other factors involved than those analyzed since urinary phosphorus, PGE_2, and $1,25(OH)_2D_3$ account for only 19.9% of the variability in femur weight. This is also true of the factors contributing to femur length, which account for only 27.0% of the variability (**Table 10.18**). For each centimetre increase in femur length, urinary phosphorus contributes 0.055 and IGF-I contributes 0.063, while OC decreases femur length by 0.262. These findings are rather difficult to explain, since urinary phosphorus excretion might be expected to decrease bone length, while OC would be expected to contribute to increases in bone length.

Investigations into the effect of insulin deficiency on liver fatty acid composition have been conducted previously (133-5). In this study, insulin deficiency clearly altered the fatty acid composition of the liver (**Tables 10.11 and 10.12, Appendix C**). Of particular interest is the effect of insulin deficiency on the n-3 and n-6 PUFA. While levels of some n-3 PUFA (C20:3 n-3 and C22:6 n-3) were elevated in insulin deficient animals compared to levels in insulin sufficient animals (**Table 10.12, Appendix C**), levels of others were reduced (C18:3 n-3, C20:5 n-3, and C22:5 n-3). On the other hand, levels of almost all n-6 PUFA were elevated in insulin deficient animals compared to insulin sufficient animals, with the only exception being C18:3 n-6 (**Table 10.12, Appendix C**). This is partially in contrast to the findings of Giron et al. (133) who found that insulin deficient animals had significantly lower

levels of arachidonic acid (C20:4 n-6) compared to insulin sufficient animals. When the treatment groups were combined and studied based on dietary group, liver fatty acid composition closely reflected the fatty acid composition of the oil or oil mixture used in the diet (**Table 10.11, Appendix C**). It is interesting to note that compared to those fed soy oil, animals fed fish oil had significantly lower levels of C20:4 n-6, the precursor to PGE_2, and significantly higher levels of C20:5 n-3 (**Table 10.11, Appendix C**), the precursor to PGE_3, while the opposite was seen in insulin deficient animals compared to insulin sufficient animals (**Table 10.12, Appendix C**). That is, within each diet group, stz-injected animals had significantly higher levels of C20:4 n-6 and significantly lower levels of C20:5 n-3 compared to saline-injected animals.

Total n-6 PUFA were higher in the DS group compared to the PS and CS groups, and in the DF group compared to the PF and CF group. On the other hand total n-3 PUFA were higher in the DS group compared to the PS and CS groups, but not in the DF group compared to the PF and CF groups. As a result, the n-6:n-3 ratio was lower in the DS group compared to the PS and CS groups, but was not different in the DF group compared to the PF and CF groups.

This study confirms the finding of other researchers that diabetes does affect bone formation and resorption (7-9,18,119-20). In this study, insulin deficient animals had lower bone formation, but considerably higher bone resorption than insulin sufficient animals (**Table 10.5, Appendix F**).

Interestingly, these changes did not appear to affect bone mineralization, but did affect bone growth; femur diaphysis calcium and phosphorus content was not different in insulin deficient animals compared to insulin sufficient animals (**Table 10.5, Appendix F**), but femur lengths in the former group were lower than in the latter group (**Table 10.4, Appendix E**). This effect on bone growth may be explained by the fact that insulin is known to stimulate collagen synthesis, which contributes to growth in length (1). The changes to formation and resorption may be the result of any of a number of metabolic changes including elevated levels of PTH, reduced levels of $1,25(OH)_2D_3$, reduced levels of IGF-I, or higher levels of PGE_2. This elevation in PGE_2 levels is most likely due to the higher total n-6 PUFA found in the liver of the insulin deficient animals. In particular, arachidonic acid, the precursor of PGE_2 was significantly elevated in insulin deficiency (**Table 10.5, Appendix F**). Following from this observed elevation in arachidonic acid, a proposed pathway of changes seen in insulin deficient animals is as follows (**Figure 11.1**). Elevated arachidonic acid results in elevated levels of PGE_2 and reduced hepatic production of IGF-I. Reduced liver IGF-I results in reduced plasma IGF-I which in turn contributes to a reduction in both bone formation and renal synthesis of $1,25(OH)_2D_3$. The elevation in PGE_2 also contributes to reduced bone formation, as well as to elevated bone resorption. Elevated bone resorption leads to higher urinary calcium and phosphorus excretion. As well, the combination of lower bone formation and higher bone resorption

results in higher bone turnover. This may lead to hypercalcemia and hyperphosphatemia which themselves result in both reduced PTH and reduced $1,25(OH)_2D_3$.

The effects of insulin deficiency on bone metabolism are moderated by dietary n-3 PUFA (**Figure 11.2**). Along with a higher n-3 fatty acid intake is a concomitant reduction in n-6 fatty acid intake. This may lead to elevated hepatic production of IGF-I, and subsequently to elevated plasma IGF-I. Although in this study plasma IGF-I was not significantly elevated in animals fed fish oil, others have observed significant differences in plasma IGF-I in animals fed fish oil compared to animals fed soy oil (82). Also stemming from the change in n-6:n-3 ratio, enzyme activity is diverted from the n-6 metabolic pathway to the n-3 metabolic pathway, and less PGE_2 is produced. In addition, while the n-3 PUFA are precursors to PGE_3, rate of PGE_3 synthesis is lower than rate of PGE_2 synthesis, resulting in a net reduction of total PGE. This reduction in PGE_2 reduces bone resorption, which in turn reduces calcium, and possibly phosphorus, excretion. Lower PGE_2 concentrations also contribute to reduced bone formation. The reductions in both formation and resorption combine to reduce bone turnover. Consequently, plasma calcium and phosphorus are reduced, leading to elevated PTH and then to elevated $1,25(OH)_2D_3$.

In conclusion, insulin deficiency resulted in interrelated changes in the synthesis and/or metabolism of PGE_2, IGF-I, PTH, calcium, phosphorus, and

$1,25(OH)_2D_3$, which together contributed to a state of high bone turnover. In contrast to the hypothesis of this study, dietary fish oil did not elevate bone formation in relation to bone resorption. However, it did moderate the high bone turnover observed in the insulin-deficient animals, as indicated by reductions in both bone formation and bone resorption.

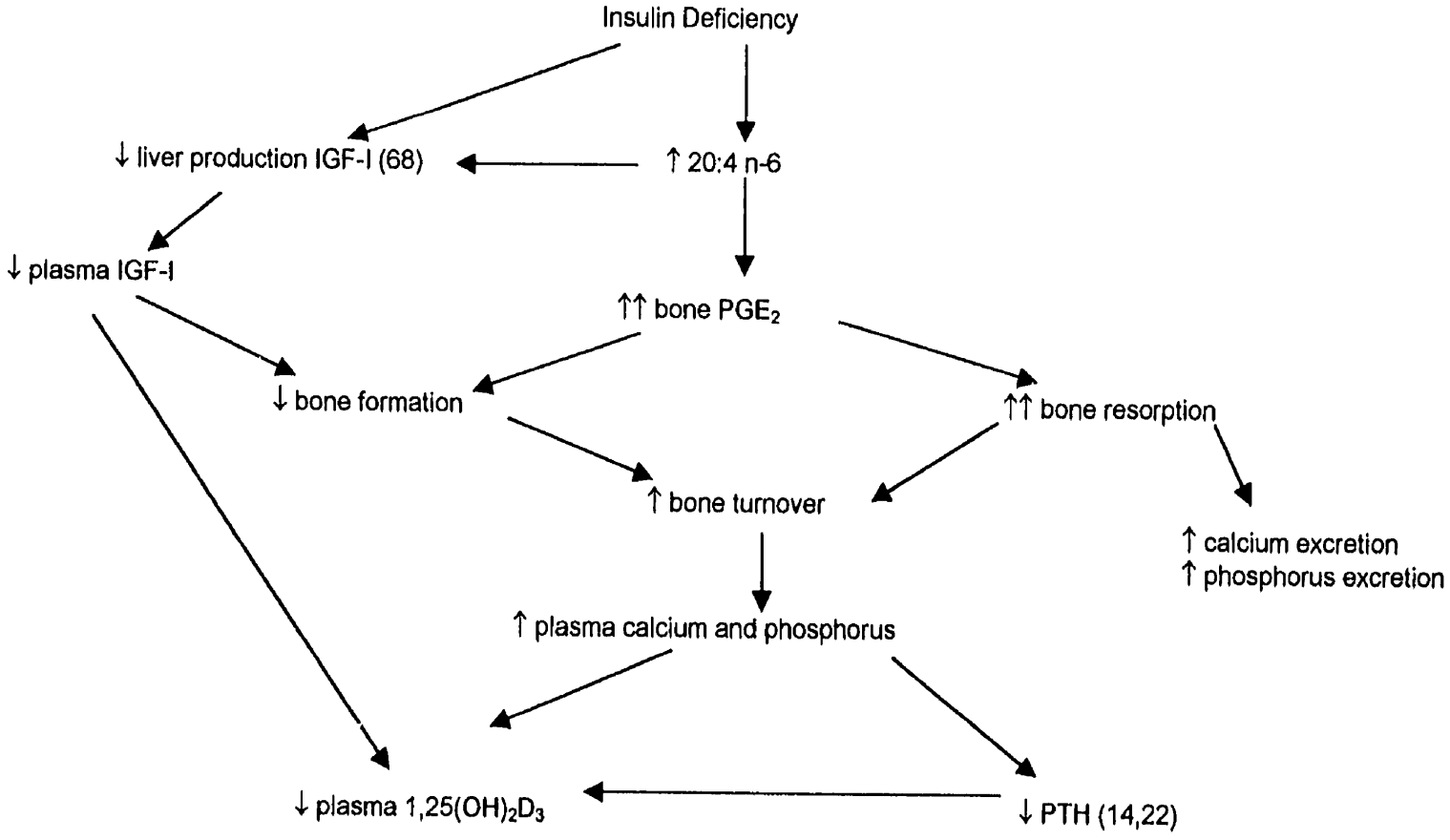

Figure 11.1: Proposed pathway of changes in bone metabolism in insulin deficiency

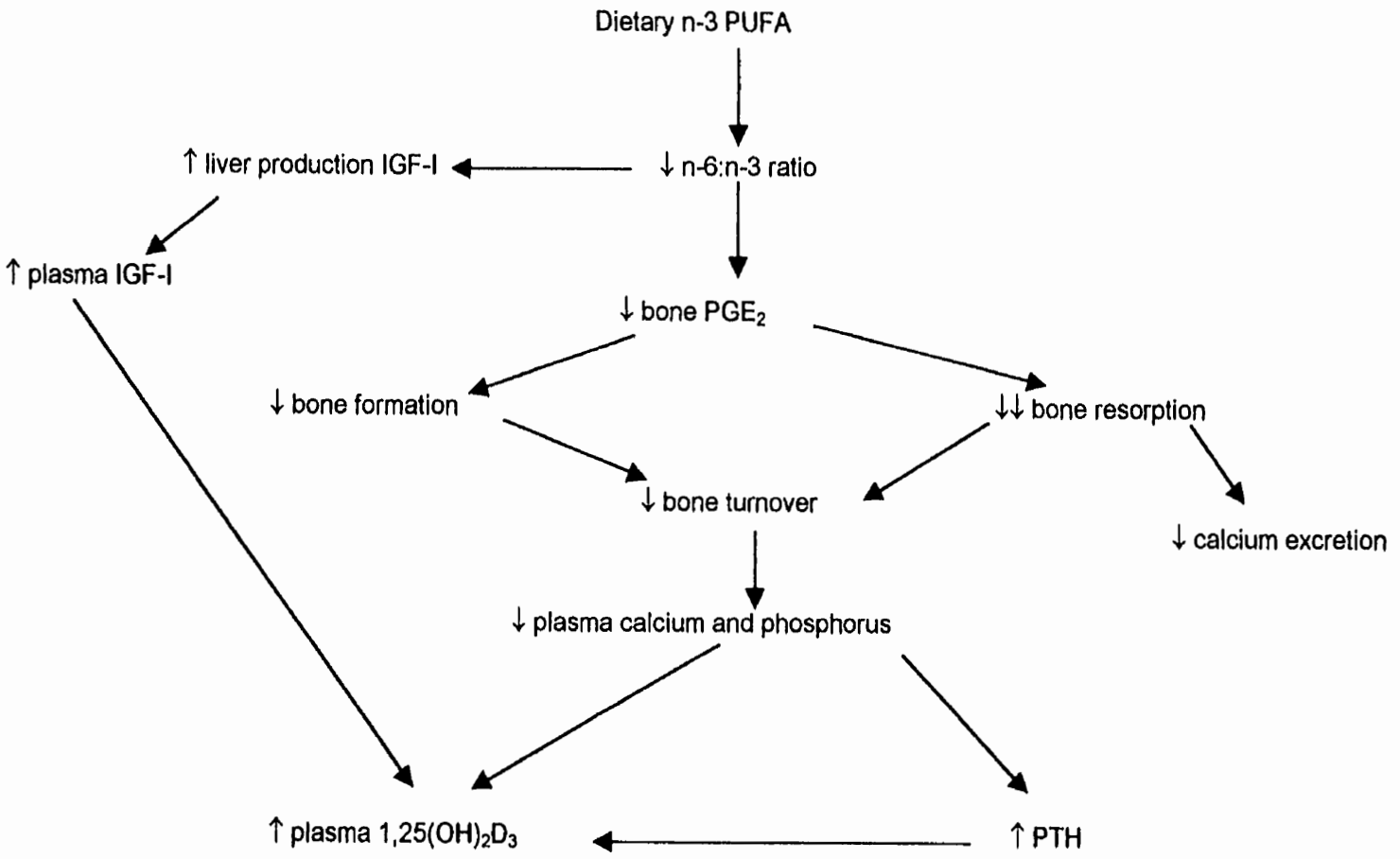

Figure 11.2: Proposed effect of n-3 PUFA on bone metabolism in insulin deficiency

12. Basic Study: Strengths and Limitations

Similar to the clinical study, this study had both strengths and limitations. The stz-induced diabetes model is a well-investigated model representative of Type 1 diabetes in humans (11-1,107,110,119). The success rate of the stz-injections was based on a number of indicators: plasma glucose, growth rate, food and water consumption, and volume of urine excreted. This allowed for a clear determination of success, and therefore a clear distinction between insulin sufficient and insulin deficient animals. Based on these indicators, the success rate of diabetes induction was high, providing an adequate sample of insulin deficient animals. Another strength of the study was that animals were housed individually, aiding determination of food and water consumption. However, there were also limitations. The primary limitation revolves around the use of an animal model. Although stz-induced rodents are a good model of diabetes, differences between rodent metabolism and human metabolism limit the ability to generalize the results of this study to humans. As with the clinical study, another main limitation was sample size. Although 80 animals is a relatively large sample, when the animals were divided into groups based on diet and treatment, some of the insulin sufficient groups contained fewer than 10 animals. In addition, given the amount of blood sample collected, some laboratory tests were not conducted on all animals, reducing the sample size further. This reduced sample size would limit the power of the statistical analysis, again potentially resulting in a Type II error. On the other hand,

considering the number of statistical analyses that were conducted, the likelihood of finding significant differences, when in fact no true difference exists, was increased, thereby increasing the possibility of a Type 1 error. To avoid this , Bonferroni correction could be used; however, in an exploratory study such as this, Bonferroni may overcorrect, resulting in reduced power and a possible Type II error (136). Another limitation relates to the pair-weigh group. While the pair-weigh group was included in the study to control for the lower growth rate expected in the stz-injected animals, the growth rate in the former group was significantly higher than that in the latter, in spite of the feed restrictions. This limits the usefulness of the pair-weigh group as a weight control. As well, there were limitations with the assay conducted to measure PGE_2. This assay cross reacts with PGE_3 at a cross reactivity of 16.3%, not allowing for a clear distinction to be made between PGE_2 and PGE_3.

13. Basic Study: Future Research

Given the effect of insulin deficiency on bone development and related metabolism observed in this study, a logical next step would be to compare insulin deficient animals given insulin injections with insulin deficient animals not given insulin. As with the clinical study, an investigation comparing changes in trabecular bone with those in cortical bone would be beneficial. In terms of diet, experimentation with different ratios of n-6:n-3 would help to determine the optimum levels of dietary n-3 and n-6 PUFA for maximal bone development in insulin deficiency. Finally, this study investigated male rodents only. Future studies should include female rodents in order to examine the effects of insulin deficiency, dietary n-3 PUFA and interactions between the two compared to males.

14. Conclusions

Numerous investigations have shown lower bone mass in children with Type 1 diabetes; however, few researchers have measured bone mass in children with diabetes using DXA (1-5). In the first thesis project, BMC, BA, and BMD were measured in children 8 to 18 years of age using a whole body DXA scan. As a group, children with Type 1 diabetes did not have low bone mass when compared to age- and sex-matched controls. However, BMC and BMD in boys with diabetes did tend to be lower than in boys without the disease, although the differences were not significant. More importantly, when children were grouped based on duration of diabetes, those who had had the disease for one year or less had significantly lower bone mass than children who had had diabetes for more than five years. Both BMC and BA were affected, although more severe deficits were seen in BA. These results suggest that from onset of the disease, or perhaps even prior to onset, until approximately one year following diagnosis, there is a period of transition during which bone mass is adversely affected. Following this transition period, bone development stabilizes, and may even accelerate. It might be speculated that this stabilization is the result of adaptation to insulin therapy.

The existence of the transition period, and its effects on bone and related metabolism, was confirmed in the second thesis project. Bone formation and resorption, along with other biochemical indicators were measured in diabetes-induced growing rats and compared to control animals. Since the diabetes-

induced animals were not given insulin injections, their altered metabolism during the five-week trial provides a picture of the altered metabolism that occurs in children during the transition period before they are diagnosed and treatment is initiated. These animals appeared to have a high turnover bone disease due to reduced bone formation but greatly elevated bone resorption. This in turn resulted in lower bone weight and length, although BMC, measured as femur diaphysis calcium and phosphorus, did not appear to be affected. Insulin is known to stimulate collagen synthesis (1-2); therefore, it is possible that the poor bone growth was due to the lack of insulin stimulus. The alteration in rate of bone turnover - likely the result of a number of interrelated metabolic changes known to affect bone development, such as reduced plasma IGF-I and vitamin D and elevated calcium and phosphorus excretion, was moderated by diet.

Intakes of certain nutrients, namely calcium, phosphorus, and vitamin D, are known to be related to bone health (43,69,72-3,76-7). More recently, a link has also been made between PUFA and bone development (80-2). While no relationship was found between PUFA intake and any of the indicators of bone development in control children or girls with diabetes in the first project, there was a significant positive correlation of dietary PUFA intake with both BMC z-score and BA z-score in the boys with diabetes. This is consistent with the observation that both n-6 and n-3 PUFA influence bone formation and bone

resorption through the production of PGE_2 and PGE_3 (85,121). The individual roles of n-6, and in particular n-3, PUFA were clarified in the second project.

Few researchers have examined the effect of n-3 PUFA on bone development in insulin deficiency (123). Thus the observation that high bone turnover in insulin deficient animals was moderated by n-3 PUFA intake is unique. Fish oil, high in n-3 PUFA reduced both bone formation and resorption in the insulin deficient animals. In addition, higher dietary n-3 PUFA in relation to n-6 PUFA contributed to higher bone weight and bone length in insulin deficient animals.

This research confirms that prior to diagnosis and treatment of Type 1 diabetes, various interrelated metabolic changes occur that adversely affect bone development. These adverse effects are diminished by higher consumption of PUFA and especially n-3 PUFA in the form of fish oil.

15. References

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Appendix A

Dear participant and parent(s): January 30, 1999

Re: Study: Body Composition in Children

University of Manitoba and the Manitoba Clinic

Thank you for your past participation in this project. Instead of asking you to return to the clinic for a six month and one year visit, we would like you to complete the enclosed three day food record and physical activity questionnaire, and return them to us in the enclosed addressed and stamped envelope. We have also enclosed a 5$ gift certificate for your important contribution to this research.

We are asking parents to assist their children with completing and mailing the enclosed forms. If you have any questions or concerns, please do not hesitate to call one of the investigators listed below. To make the information useful to us, the accuracy of the recorded information we receive from you is essential. In order to complete the food record properly, please follow the instructions outlined below.

III. Choose 2 week days and 1 weekend day to keep records of everything you eat or drink on those days. Please try to choose non-consecutive days (e.g. Tuesday, Thursday and Saturday would be good choices). Do not use a day when you are feeling sick.

IV. For reasons of confidentiality, we have written your subject number on each form, therefore, please do not write your name on any of the forms.

V. Record all foods/liquids consumed each day starting when you wake-up, making sure to write down the time when any food /liquid is consumed. Start a new line for each food/liquid recorded. Try to list foods/liquids immediately after eating/drinking.

VI. Indicate the food/liquid type in detail, including brand names if appropriate and any toppings or spreads. If you are eating a mixed dish such as a stir fry, please write the recipe on the back of the food record.

VII. If you run out of space please write on the back or attach another piece of paper.

VIII. Indicate the amount of food/liquid consumed in one of the following ways: 1)using standard household measuring cups/spoons, 2)recording the weight of the food eaten in grams or ounces, or 3)by drawing a picture of the food eaten on the back of the food record, or on a separate piece of paper. This picture should also indicate how thick the food is (see the back of the example food record).

IX. **For children who have diabetes**, please indicate your blood sugar readings and insulin type and amount used in the column marked **Sugar/Insulin.**

X. If you take any vitamins/supplements/medications on a regular basis, please write them down at the bottom of the food record and indicate the amount taken.

XI. Please complete the physical activity questionnaire by following the directions printed on the questionnaire.

When you are finished the three day food record and the physical activity questionnaire, please return it to us by mail, in the addressed and stamped envelope. Thank you for your continued support. If you have any questions, please call one of the following investigators:

Laela Janzen (204) 452-9480
Kathy Green (204) 269-0932
Dr. Hope Weiler (204) 474-6798

EXAMPLE **Food Record - Day 1**

Subject:_____ **Date and day of week:** Tuesday, Feb 1, 1999

Time	Food	Brand	Amount	Sugar/ Insulin
0.3125	TOAST	COUNTRY HARVEST CRACKED OAT	2 SLICES	6.4/ 8R + 17NPH
	MARGARINE	REGULAR BECEL	2 TSP.	
	JAM	DIET RASPBERRY	1 TBSP.	
	PEANUT BUTTER	KRAFT LIGHT SMOOTH	2 TBSP.	
	BANANA		1 SMALL	
	MILK	SKIM	½ CUP	
0.41667	CHEESE	KRAFT 27% M.F. CHEDDAR	2 OZ.	
	CRACKERS	SODA	4	
0.5	SOUP	CAMPBELL'S CREAM OF MUSHROOM - HALF THE FAT, MADE WITH SKIM MILK	1 CUP	8.9/ None
	½ HAM SANDWICH			
	BREAD		1 SLICE	
	HAM	DELI SHAVED	1 OZ	
	MUSTARD	YELLOW	1 TSP	
	MAYONNAISE	KRAFT LIGHT MIRACLE WHIP	1 TSP	
	APPLE		1 MED.	
0.16667	BANANA BREAD WITH NUTS (NO ICING)	HOMEMADE	SEE PICTURE	
	MARGARINE	REGULAR BECEL	1 TSP	

Vitamins/Supplements/Medications: Flinstones chewable multivitamin/mineral - 250mg tablet daily

Food Record - Day 1

Subject:_____ **Date and day of week:**_____

Time	Food	Brand	Amount	Sugar/Insulin

Vitamins/Supplements/Medications:_____

Food Record - Day 2

Subject:_____ **Date and day of week:**_____

Time	Food	Brand	Amount	Sugar/Insulin

Vitamins/Supplements/Medications:_____

Food Record - Day 3

Subject:_____ **Date and day of week:**_____

Time	Food	Brand	Amount	Sugar/Insulin

Vitamins/Supplements/Medications:_____

Appendix B

Modifiable Activity Questionnaire for Adolescents

1. How many times in the past 14 days have you done at least 20 minutes of exercise hard enough to make you breathe heavily and make your heart beat fast? (Hard exercise includes, for example, playing basketball, jogging, or fast bicycling; include time in physical education class)

   ( ) None
   ( ) 1 to 2 days
   ( ) 3 to 5 days
   ( ) 6 to 8 days
   ( ) 9 or more days

2. How many times in the past 14 days have you done at least 20 minutes of light exercise that was not hard enough to make you breathe heavily and make your heart beat fast? (Light exercise includes playing basketball, walking or slow bicycling; include time in physical education class)

   ( ) None
   ( ) 1 to 2 days
   ( ) 3 to 5 days
   ( ) 6 to 8 days
   ( ) 9 or more days

3. During a normal week how many hours a day do you watch television and videos, or play computer or video games before or after school?

   ( ) None
   ( ) 1 hour or less
   ( ) 2 to 3 hours
   ( ) 4 to 5 hours
   ( ) 6 or more hours

4. During the past 12 months, how many team or individual sports or activities did you participate in on a competitive level, such as varsity or junior varsity sports, intramurals, or out-of-school programs.

   ( ) None
   ( ) 1 activity
   ( ) 2 activities
   ( ) 3 activities
   ( ) 4 or more activities

   What activities did you compete in?

   _____
   _____
   _____
   _____
   _____
   _____

From Aaron et al, 1993

PAST YEAR LEISURE-TIME PHYSICAL ACTIVITY

Check all activities that you did at least 10 times in the **PAST YEAR**. Do not include time spent in school physical education classes. Make sure you include all sport teams that you participated in during the Last year.

Aerobics	Gymnastics	Swimming (Laps)
Band/Drill Team	Hiking	Tennis
Baseball	Ice Skating	Volleyball
Basketball	Roller Skating	Water Skiing
Bicycling	Running for Exercise	Weight Training (Competitive)
Bowling	Skateboarding	Wrestling
Cheerleading	Snow Skiing	Others:
Dance Class	Soccer	_____
Football	Softball	_____
Garden/Yard Work	Street Hockey	_____

List each activity that you checked above in the "Activity" box below.
Check the months you did each activity and then estimate the amount of time spent in each activity.

Activity	Jan	Feb	Mar	Apr	May	Jun	Jly	Aug	Sep	Oct	Nov	Dec	Months per Year	Days Per Week	Minutes Per Day

Appendix C: Liver fatty acid content of rats fed soy oil or fish oil

	Soy oil diet			Fish oil diet		
Fatty acid	**PS** n = 10	**CS** n = 11	**DS** n = 18	**PF** n = 10	**CF** n = 10	**DF** n = 19
C10:0	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)
C12:0	0.01 (0.01)	0.01 (0.01)	0.00 (0.01)	0.01 (0.01)	0.01 (0.01)	0.00 (0.02)
c12:1	0.01 (0.02)	0.04 (0.05)	0.12 (0.18)	0.05 (0.09)	0.02 (0.03)	0.11 (0.18)
C14:0	0.46 (0.13)	0.49 (0.12)	0.13 (0.13)	0.56 (0.18)	0.54 (0.12)	0.19 (0.12)
c14:1	0.03 (0.02)	0.02 (0.02)	0.00 (0.01)	0.03 (0.03)	0.03 (0.02)	0.01 (0.03)
C15:0	0.13 (0.02)	0.10 (0.02)	0.11 (0.02)	0.12 (0.02)	0.11 (0.02)	0.14 (0.03)
C16:0	21.45 (1.63)	22.92 (2.47)	14.72 (2.82)	23.56 (1.69)	24.05 (1.52)	17.48 (2.14)
C16:1 n-9	0.29 (0.10)	0.31 (0.09)	0.14 (0.09)	0.19 (0.09)	0.19 (0.08)	0.12 (0.05)
C16:1 n-7	3.04 (1.08)	3.01 (0.88)	0.48 (0.95)	4.46 (1.50)	4.02 (0.86)	0.86 (0.79)
C17:1	0.09 (0.03)	0.09 (0.02)	0.02 (0.04)	0.19 (0.05)	0.17 (0.03)	0.09 (0.05)
C18:0	12.50 (1.93)	11.50 (1.83)	22.31 (5.23)	11.06 (1.80)	10.78 (1.42)	18.59 (3.57)
C18:1 n-9	14.21 (2.59)	14.77 (3.06)	6.20 (4.17)	15.04 (2.53)	14.29 (2.63)	7.47 (3.03)
C18:1 n-7	3.58 (0.62)	3.03 (0.35)	1.84 (0.63)	3.15 (0.55)	2.88 (0.23)	2.19 (0.54)
C18:2 n-6	17.81 (2.61)	18.98 (3.10)	16.28 (3.61)	13.13 (1.63)	13.22 (2.75)	17.10 (2.42)
C18:3 n-6	0.25 (0.04)	0.35 (0.13)	0.19 (0.11)	0.08 (0.01)	0.08 (0.01)	0.12 (0.05)
C18:3 n-3	0.80 (0.22)	1.38 (0.34)	0.53 (0.53)	0.25 (0.05)	0.30 (0.08)	0.13 (0.10)
C20:0	0.06 (0.01)	0.04 (0.01)	0.10 (0.03)	0.09 (0.03)	0.08 (0.02)	0.12 (0.03)
C20:1 n-9	0.16 (0.04)	0.08 (0.02)	0.11 (0.03)	0.14 (0.02)	0.10 (0.02)	0.17 (0.06)
C20:2 n-6	0.37 (0.14)	0.20 (0.05)	0.47 (0.19)	0.16 (0.03)	0.13 (0.03)	0.23 (0.05)
C20:3 n-6	0.83 (0.27)	0.31 (0.05)	0.93 (0.44)	0.94 (0.14)	0.81 (0.12)	1.79 (0.41)
C20:4 n-6	16.99 (2.63)	15.19 (2.92)	22.37 (4.08)	6.57 (1.38)	7.23 (1.21)	12.90 (3.49)
C20:3 n-3	0.05 (0.02)	0.02 (0.02)	0.08 (0.03)	0.03 (0.01)	0.03 (0.01)	0.04 (0.02)
C20:5 n-3	0.35 (0.09)	0.27 (0.08)	0.14 (0.07)	5.92 (1.12)	5.11 (1.43)	2.11 (1.04)
C22:0	0.12 (0.03)	0.09 (0.02)	0.21 (0.06)	0.08 (0.01)	0.07 (0.01)	0.12 (0.03)
C22:4 n-6	0.38 (0.07)	0.33 (0.04)	0.76 (0.21)	0.11 (0.02)	0.10 (0.01)	0.18 (0.07)
C22:5 n-3	0.70 (0.17)	0.48 (0.10)	0.98 (0.31)	2.67 (0.55)	2.44 (0.68)	2.05 (0.62)
C22:6 n-3	3.70 (0.71)	4.50 (0.83)	9.12 (2.78)	9.69 (1.48)	11.60 (1.65)	13.80 (1.55)
C24:0	0.28 (0.06)	0.21 (0.06)	0.39 (0.15)	0.21 (0.04)	0.19 (0.03)	0.34 (0.09)
C24:1 n-9	0.09 (0.02)	0.08 (0.02)	0.06 (0.03)	0.12 (0.02)	0.13 (0.03)	0.21 (0.05)

Data shown as mean (SD) percent wt/wt of total lipid

PS: Pair-weigh group, soy oil diet; CS: Control group, soy oil diet; DS: Diabetes group, soy oil diet; PF: Pair-weigh group, fish oil diet; CF: Control group, fish oil diet; DF: Diabetes group, fish oil diet

Appendix D: 2-way ANOVA main effects for indicators of diabetic state

	p-value		
	Diet	Treatment	Interaction
Day 5 plasma glucose (mmol/L)	0.965	<0.001	0.986
Necropsy plasma glucose (mmol/L)	0.198	<0.001	0.813
Urinary creatinine (mmol/L)	0.020	<0.001	0.345
12 hour urine volume (mL)	0.329	<0.001	0.969
Growth rate (g/day)	0.860	<0.001	0.672
Feed Intake (g/day)	0.395	<0.001	0.564
Feed intake (g/g body weight)	0.696	<0.001	0.672
Tail length (cm)	0.444	<0.001	0.800
Final weight (g)	0.647	<0.001	0.868
Total weight gain (g)	0.662	<0.001	0.877

Diet = Soy oil versus fish oil
Treatment = Pair-weigh versus control versus diabetes

Appendix E: 2-way ANOVA main effects for organ weights and femur weights and lengths

	p-value		
	Diet	Treatment	Interaction
Heart (g)	0.513	<0.001	0.986
Heart (mg/g body weight)	0.593	<0.001	0.834
Liver (g)	0.200	0.185	0.118
Liver (mg/g body weight)	0.538	<0.001	0.220
Left kidney (g)	0.491	<0.001	0.075
Corrected kidney (mg/g body weight)	0.933	<0.001	0.261
Whole femur (g)	0.255	<0.001	0.668
Corrected whole femur (mg/g body weight)	0.757	<0.001	0.783
Femur length (cm)	0.793	<0.001	0.969
Femur weight/length (g/cm)	0.084	0.020	0.173

Diet = Soy oil versus fish oil
Treatment = Pair-weigh versus control versus diabetes

Appendix F: 2-way ANOVA main effects for plasma, urine, and femur biochemistry

	p-value		
	Diet	Treatment	Interaction
Diaphysis Ca (mg/g)	0.275	0.078	0.321
Diaphysis P (mg/g)	0.242	0.099	0.239
Urinary Ca (mmol/L)	0.821	0.008	0.657
Urinary P (mmol/L)	0.294	<0.001	0.781
Urinary Ca/creatinine (mmol/mmol)	0.776	<0.001	0.749
Urinary P/creatinine (mmol/mmol)	0.541	<0.001	0.584
Urinary Ca/body weight (nmol/g/h)	0.970	<0.001	0.646
Urinary P/body weight (nmol/g/h)	0.561	<0.001	0.680
$1,25(OH)_2D_3$ (nmol/L)	0.688	0.008	0.296
Osteocalcin (pmol/L)	0.030	0.067	0.188
IGF-I (pmol/L)	0.902	<0.001	0.384
PTH (nmol/L)	0.215	0.208	0.377
NTx (nM BCE/mM)	0.361	0.001	0.315
PGE_2 (mmol/g)	<0.001	0.005	0.955

Diet = Soy oil versus fish oil
Treatment = Pair-weigh versus control versus diabetes