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RISK FACTORS FOR FRAGILITY FRACTURES AND ROLE OF WNT SIGNALING IN TYPE 1 DIABETES RELATED BONE FRAGILITY: CLINICAL AND PRECLINICAL STUDIES

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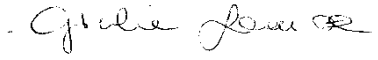

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ABSTRACT

BACKGROUND

Patients with Type 1 diabetes (T1D) have low bone mass and up to five times higher risk of hip fracture than normal peers, leading to an increased morbidity and mortality. However, risk factors for fractures in T1D have not been clearly identified and fractures are still a poorly screened diabetic complication. In a preliminary clinical study, we found high circulating levels of the Wnt inhibitor sclerostin in patients with T1D relative to normal controls. This result would be consistent with findings of decreased serum markers of bone formation in humans and preclinical models of T1D, suggesting reduced bone turnover in T1D. However, diabetes-related mechanisms underlying a lower bone quality and increased risk of fractures in diabetics are, similarly to clinical factors, still not well established.

The overall objectives of the study were to evaluate risk factors for fractures in T1D and mechanisms underlying bone fragility in an experimental model of T1D.

STUDY 1: CLINICAL STUDY

OBJECTIVE: To determine clinical diabetes-related risk factors for fractures in type 1 diabetes.

RESEARCH DESIGN AND METHODS: History of bone fragility fractures occurring after T1D diagnosis was assessed by questionnaire in this cross-sectional study in 600 T1D subjects. Glycated hemoglobin A1c (HbA1c) over the previous 5 years was used as an index of long-term glycemic control;

complications were adjudicated by physician assessment. Multinomial logistic regression models were used to assess the associations between diabetes-related risk factors and fracture history.

RESULTS: One-hundred-eleven patients (18,5%) reported at least one fracture; of these 73.8% had only one and 26.2% had more than one fracture. Average age was 41.9±12.8 years, with even gender distribution; disease duration was 19.9±12.0 years; and BMI was 24.4±3.7 kg/m^2. The 5-year average HbA1c was 7.6±1.0% (60 mmol/mol). In adjusted models, reduced risk for 1 fracture was found in those with higher eGFR (RRR 0.22 [95%CI: 0.06-0.83] for 1 unit increase in lneGFR, p=0.03) and increased risk in those with neuropathy (RRR 2.57 [1.21-5.46], p=0.01).

Increased risk for ≥2 fractures was found in subjects in the highest tertile of HbA1c (≥7.9%) compared with the lowest tertile (≤7.17%) (RRR 3.50 [1.04-11.7], p=0.04) and of disease duration (≥ 26 years versus < 14 years) (RRR 7.59 [1.60-36.0], p=0.01).

Summary study 1: Poor glycemic control and long exposure to the disease are independent diabetes-related risk factors for multiple bone fractures in type 1 diabetes.

STUDY 2: PRECLINICAL STUDY

OBJECTIVE: To test the potential role of sclerostin and Wnt signaling in diabetes induced bone disease.

RESEARCH DESIGN AND METHODS: We introduced the sclerostin-resistant *Lrp5*^{A214V} mutation, associated with high bone mass (HBM), in mice carrying the *Ins2*^{Akita} mutation (Akita), which causes hyperglycemia and

hypoinsulinemia within 5 weeks after birth. Bone microarchitecture and body composition were longitudinally evaluated respectively by in vivo µ-CT and DXA. Glucose metabolism was evaluated by random blood capillary measurements and intraperitoneal glucose and insulin tolerance tests.

RESULTS: Bone mass by DXA was significantly higher in Akita/HBM relative to Akita littermates at 12 weeks (88.2±5.2 vs 67.9±4 mg/cm^2; $p<0.001$; n=7-11), persisting higher for up to 26 weeks (90.2±3.0 vs 70.4±1.7mg/cm^2; $p<0.001$ n=5) despite overt diabetes. Further analysis by µCT at age 20 weeks revealed lower trabecular bone volume/total volume (BV/TV) in Akita compared to wild type (WT) mice (0.2±0.02 vs 0.35±0.05; $p<0.05$; n=3-5). Conversely, both trabecular (Tb) and cortical (Ct) parameters were significantly higher in Akita/HBM mutants compared to Akita littermates, including total Ct area (1.6±0.06 vs 1.2 ±0.07mm^2), bone area (0.9±0.1 vs 0.6±0.05mm^2), and Ct thickness (0.2±0.02 vs 0.1±0.01 mm, $p<0.001$, n=5-7). Tb BV/TV and Tb thickness were also higher in Akita/HBM mutants relative to Akita littermates (0.4±0.05 vs 0.2±0.02; and 0.1±0.02 vs 0.09±0.03mm, respectively, $p<0.001$, n=3-5). We found no significant differences in total Ct area between Akita and WT mice, consistent with observations in humans with T1D. As expected, both Akita and Akita/HBM mutants developed diabetes (non-fasting blood glucose >300 mg/dl), albeit with different onset timing. At 8 weeks, only 40 % of Akita/HBM mice had developed hyperglycemia, compared to 90% of Akita mice (n=10). Only at 12 weeks were most Akita/HBM mice hyperglycemic. Intriguingly at age 6 and 8 weeks, glucose tolerance was significantly better in Akita/HBM relative to Akita mice ($p<0.05$ for difference in areas under

the curve, AUC; n=3-6). Likewise, insulin sensitivity (by intraperitoneal insulin tolerance test) was higher in the Akita/HBM compared to the Akita group ($p<0.01$ for difference in AUC; n=4-8) at age 7 weeks.

Summary study 2: The metabolic changes caused by hypoinsulinemia (chronic hyperglycemia) do not alter the consequence of sclerostin resistance and Wnt hyperactivation on bone. Furthermore, Wnt activation retards the onset of metabolic abnormalities in T1D.

CONCLUSIONS

Our data clearly show that T1D patients should be carefully screened for fragility fractures and we propose risk factors for any and multiple fragility fractures to use for a model of prediction for fractures. We have proved that HbA1c, disease duration, presence of neuropathy and eGFR values could be targeted for prevention of fractures in diabetes. With our preclinical study we explored for the first time in vivo the effect of T1D and sclerostin resistance on bone, demonstrating that targeting Wnt signaling protects bone mass in T1D. We also potentially target a role of sclerostin resistance to improve not only bone fragility but also glucose metabolism. This study provides novel scientific inputs in the relationship between glucose homeostasis and bone health and may offer new avenues for assessment and treatment of bone alterations in diabetes.

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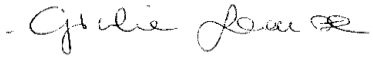

1. Type 1 diabetes mellitus

1.1 Classification

Diabetes is a group of metabolic chronic disorders characterized by high blood glucose levels resulting from defects in insulin secretion that may be absolute or relative in the context of coexistence insulin resistance [1]. Accordingly to the American Diabetes Association (ADA), clinical diabetes is divided into four general subclasses: type 1 diabetes (T1D), characterized by absolute insulin deficiency, type 2 diabetes (T2D) characterized by insulin resistance and relative insulin deficiency, other specific type of diabetes associated with identifiable clinical conditions or syndromes and gestational diabetes [2]. An additional category defines prediabetes as a condition characterized by impaired glucose tolerance, impaired fasting glucose and high glycohemoglobin (hemoglobin A1c [HbA1c] 5.7 to 6.4%) describing an impaired metabolic state that is often part of the disease's natural history [3]. T1D, previously defined insulin-dependent or juvenile-onset diabetes [2], is a chronic disease characterized by a cellular-mediated autoimmune destruction of the insulin-producing pancreatic beta cells which results in insulin deficiency and high blood glucose levels and it' s the most common

chronic disease in childhood [4]. Although it's also called juvenile diabetes, nearly 30% of patients receive the diagnosis after the age of 20 years too [2]. In T1D, the effect of insulin deficiency plays a key role in the metabolic imbalance linked to diabetes; chronic hyperglycemia is associated with the long-term microvascular and macrovascular complications causing retinopathy, neuropathy, nephropathy and acute coronary syndrome and this leads to an increased associated morbidity and mortality [4].

1.2 Epidemiology

Worldwide, T1D represents 5-10% of all diabetes cases and the incidence is increasing at 3% to 5% per year, accordingly to the last reports (WHO, 2011Archived). The annual incidence appears to have risen in the last half century, which could imply the introduction of unidentified environmental factors. Incidence rates vary widely by geography, age, gender, family history, and ethnicity. The highest reported incidences rates are in Finland, Scandinavia, Sardinia and United Kingdom (35 to 65 per 100.000 children ≤ 15 years of age) [5], in Japan and China is very low (0.1 to 1.9 per 100.000) [6, 7]. In Northern Europe and the U.S. is an intermediate level (19 per 100.000) (WHO, 2011Archived). Prevalence rates also vary largely, even among ethnicities living the same country, suggesting genetic differences in susceptibility to the disease. Overall, In the United States approximatively 1.25 million of children and adults have T1D (WHO, 2011Archived).

1.3 Clinical manifestations and diagnosis

Symptoms and disease manifestation are different and variable in T1D population, depending on the age of onset; in children and young adults they appear faster and with severe symptoms of polyuria, polydipsia, ketonemia, fatigue, weight loss and blurred vision lasting for up to weeks compared to adults where the onset is more gradual and the clinical manifestations may appear similar to T2D [8]. Classically, patients with T1D have very little or no capacity to produce insulin and they depend on exogenous insulin to compensate metabolic alterations throughout their life [8]. T1D is characterized by two peaks of presentation, one in the childhood around 5-7 years of age and the other in the adolescence around the puberty [9]. Many T1D diagnosis are associated with diabetes ketoacidosis that causes dry skin, rapid deep breathing, drowsiness, increased thirst, frequent urination, abdominal pain, vomiting or they also may appear with the nonketotic hyperosmolar coma [8]. T1D is characterized by a chronic hyperglycemia. Accordingly to ADA and WHO (http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2), diagnostic criteria guidelines include any one of the following: elevated fasting (fasting is defined as no caloric intake for at least 8 hours) blood glucose level (≥126

mg/dl or 7 mmol/l); any random blood glucose levels of ≥200 mg/dl or 11 mmol/l associated with symptoms of hyperglycemia as polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision; impaired blood glucose level (range between 140 to 200 mg/dl) during a 2-hour oral glucose tolerance test (OGTT) [10]. A positive result to one of the above mentioned criteria, in the absence of unequivocal hyperglycemia, should be confirmed by repeating of any of the above-listed methods on a different day. Most physicians prefer to measure a fasting glucose level because of the ease of measurement and the considerable time commitment of formal glucose tolerance testing, which takes two hours to complete and offers no prognostic advantage over the fasting test [11]. Furthermore, at the moment of the diagnosis, more than 90% of T1D patients express at least one of the following autoantibodies disease related as the anti–insulinoma-associated antigen-2 [IA-2A, IA-2β] or zinc-transporter 8 [ZnT8A], autoantibodies to insulin (IAA), autoantibodies to anti–glutamic acid decarboxylase (GAD) [10]. In addition to these markers, T1D as well as many other autoimmune disorders is strongly associated with the major histocompatibility complex (MHC) polymorphisms on chromosome 6, specifically with human leukocyte antigen (HLA) alleles [12-14].

1.4 Pathobiology

T1D is a multifactorial disease in which environmental factors and genetic predisposition promote the triggering of autoimmune T-cells responses against pancreatic beta cells and inflammation, which both lead to beta cell destruction and severe impairment of insulin secretion [15]. This T-cell mediated autoreactive response can occur months up to years before the disease recognition and this contributes to the severity of clinical manifestation at the diagnosis, especially at a younger age [16]. At the time of diagnosis, 60-90% of the β-cells are already destroyed or dysfunctional [17]. Albeit the pancreatic islet cells inflammation (insulitis) has been described for the first time in T1D patients a century ago by Gepts [17], there are few studies describing this phenomenon during the disease or the prediabetes phases due to the difficulties of in studying human islets/β-cells in vivo [18]. The term insulitis indicates the presence of immune and inflammatory cells within and around the pancreatic islets [19]. A consensus definition of the insulitis lesion has been recently reported and include the following characteristics [20]; key elements of this definition are the presence of a predominantly lymphocytic infiltration of the islets of Langerhans, consisting of CD45+ cells/islet and present in a minimum of three islets.

Moreover, insulitis can be found in the islet periphery (peri-insulitis, often showing focal aggregation at one pole of the islet and in contact with the islet periphery) or within the islet parenchyma (intra-insulitis). Several studies indicate that the predominant form of insulitis in the human pancreas is peri-insulitis, and it is much less severe than in experimental mouse models [18, 21, 22]. Insulitis is most often detected in islets containing insulin-positive beta cells, but critically the same pancreas will also show pseudo-atrophic islets devoid of insulin-positive cells.

Investigators have examined the relative proportions of various infiltrating cell types in the insulitis lesion and suggest that heterogeneous profiles may underlie disease severity and progression. While both T and B lymphocytes are reported in insulitis lesions, cytotoxic CD8 T-cells appear to be the predominant population and could target beta cells expressing elevated levels of HLA class I molecules [23]; of note, hyper-expression of class I molecules (and class II molecules) may be associated with viral infections postulated to play a key role in T1D pathogenesis [24]. Hyper-expression of HLA molecules represents another key feature in the pathology of the T1D that highlights a chronic inflammatory state; this is often associated with insulitis [25]. It is presently unknown whether islet-infiltrating CD8 T-cells can target

viral epitopes presented by infected beta cells on their HLA class I molecules. However, studies of nPOD pancreata have demonstrated autoantigen-specific T-cells in the insulitis lesion [26]. These observations directly connect those autoreactive CD8 T-cells to the insulitis and to disease pathogenesis. These studies also compared the diversity of the islet-infiltrating T-cell populations in relation to T1D duration and reported increased diversity in the antigen specificity of the infiltrating CD8 T-cells in patients with longer disease duration. Thus, the autoimmune response appears to evolve with time, even after diagnosis; these results also provide evidence for the chronicity of the process.

1.5 Insulin secretion and action

The main hormone imbalance involved in T1D is insulin, which is the main regulator of homeostatic glycemic control. Insulin regulates body metabolism of carbohydrates, fats and protein through the action on its target tissues that are primarily liver, muscle and fat. The gene coding for human insulin is located on the short arm of chromosome 11.

Insulin is a peptide hormone secreted in mammals by the β-cells of the pancreatic islets, in the endocrine part of the pancreas, which is mainly an exocrine gland [27]. The pancreas is indeed an organ histologically divided in 2 parts, the exocrine and the endocrine section and the endocrine portion accounts for only 2% of the total mass of the pancreas [27]. Within the pancreatic islets, beta cells constitute 65–80% of all the cells. Insulin action is fundamental for the body anabolism at the point that is considered the main anabolic hormone of the body [28].

Insulin synthesis starts in pancreatic β cells as proinsulin which is a single-chain polypeptide of 86–amino acid. The following step in the process to the synthesis of mature insulin is a cleavage of a connecting strand (C-peptide) from the proinsulin to form the smaller, double-chain insulin molecule, which contains 51 amino acid residues [28]. Thus, both insulin and the C-peptide

are packaged in membrane-bound storage granules and the insulin secretion stimulation results in the discharge of equimolar amounts of insulin and C-peptide (and a small amount of proinsulin) into the portal circulation [29]. Whereas a large proportion of insulin is bound to its receptor and subsequently metabolized during its first pass through the liver, the C-peptide fragment largely escapes hepatic metabolism; as a result, peripheral C-peptide levels provide a precise marker of endogenous insulin secretion [29]. Insulin secretion is regulated by mechanisms regulating intra and extracellular osmosis homeostasis and using glucose transporters.

Glucose concentration is the key regulator of insulin secretion that consists in two phases: the first phase is a fast release triggered by a rapid increased in blood glucose levels (i.e. after a meal) and it lasts about 10 minutes, the second phase is a more slow and sustained release of new vesicles that is independent from glucose concentration [30].

In the first insulin phase release, glucose enters the β-cells through the glucose transporter GLUT-2 [31]. This transporter has a relatively low affinity for glucose ensuring physiological range of glucose concentration within the β-cell and in the extracellular department [31]. Upon glucose entered the cells, this starts a cascade of glucose phosphorylation and

biochemistry pathway activation that lead to a rise in the ATP:ADP ratio in the cells. Furthermore, the rising in the ATP:ADP ratio triggers a membrane depolarization (calcium and potassium mediated) that increases finally insulin release.

The above mentioned mechanism is the primary for the insulin release but there are other substances known to stimulate insulin release including some amino acids (arginine and leucine), parasympathetic release of acetylcholine, sulfonylurea, cholecystokinin, and the gastrointestinal derived incretins, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) [32].

1.6 Insulin activation pathways

The effects of insulin on its target tissues start by its binding to specific insulin receptors present in the cell membrane, which are also linked to IGF-1 and IGF-2 insulin-like growth factors. These receptors belong to the tyrosine kinases receptors family that respond to phosphorylation of tyrosine residues of a second messenger or through autophosphorylation [33].

The insulin receptor molecule is a heterodimer containing α- and β subunits linked by disulfide bridges. Insulin binds to the α-subunits of the heterodimer, which faces the extracellular side of the cells. The β subunits pass through the membrane and can be phosphorylated on serine, threonine and tyrosine residues on the cytoplasmic side [33]. The binding of insulin with the receptor provokes the autophosphorylation of the β subunits and subsequently the phosphorylation of proteins inside the cell known as insulin receptor substrates (IRS-1 and IRS-2). The phosphorylation of the IRS activates a variety of signal transduction pathways that leads to the activation of other kinases as well as transcription factors that mediate the intracellular effects of insulin. The cascade leads to the insertion of GLUT4 glucose transporters into the cell membranes of muscle and fat cells, and to the synthesis of glycogen in liver and muscle tissue, as well as the conversion of glucose into

triglycerides in liver, adipose, and lactating mammary gland tissue, operates via the activation, by IRS-1, of phosphoinositol 3 kinase (PI3K). This enzyme converts a phospholipid in the cell membrane by the name of phosphatidylinositol 4, 5-bisphosphate (PIP2), into phosphatidylinositol 3,4,5-triphosphate (PIP3), which, in turn, activates protein kinase B (PKB). Activated PKB facilitates the fusion of GLUT4 containing endosomes with the cell membrane, resulting in an increase in GLUT4 transporters in the plasma membrane [34]. PKB also phosphorylates glycogen synthase kinase (GSK), thereby inactivating this enzyme [35]. This means that its substrate, glycogen synthase (GS), cannot be phosphorylated, and remains dephosphorylated, and therefore active. The active enzyme, glycogen synthase (GS), catalyzes the rate limiting step in the synthesis of glycogen from glucose. Similar dephosphorylations affect the enzymes controlling the rate of glycolysis leading to the synthesis of fats via malonyl-CoA in the tissues that can generate triglycerides, and the enzymes that control the rate of gluconeogenesis in the liver. The overall effect of these final enzyme dephosphorylations is that, in the tissues that can carry out these reactions, glycogen and fat synthesis from glucose are stimulated, and glucose production by the liver through glycogenolysis and gluconeogenesis are

inhibited. The breakdown of triglycerides by adipose tissue into free fatty acids and glycerol is also inhibited.

1.7 Acute diabetic complications

The complications of diabetes are generally divided into two categories: acute and chronic. Risk factors for developing these complications can be modifiable or not modifiable but it has been well established during the past decades studies that overall complications are less severe and less common in patients with well-controlled blood sugar levels [36]. Diabetic ketoacidosis (DKA) is one of the most common acute complications in T1D. DKA shows up frequently at the onset of T1D but can also occur in established diabetic patients as a result of intercurrent illness (i.e. infection), inappropriate reduction in insulin dosage, or missed insulin injections (especially in adolescents). A common scenario is a patient who fails to adjust insulin therapy or to maintain adequate volume status during an illness, typically a viral gastroenteritis or an influenza-like syndrome [37]. The three cardinal biochemical characteristics of DKA are hyperglycemia, ketosis and acidosis resulting from the combined effects of a deficiency in insulin levels and the

excessive secretion of counter-regulatory hormones [37]. The low insulin levels induce the production of ketones from fatty acids and this results in an increased blood pH levels (acidosis) that are very dangerous for the body health. Patients with DKA are typically dehydrated, lethargic and they can have abdominal pain but if the medical intervention is not prompt, ketoacidosis can easily become severe enough to induce coma and death.

Hyperglycemia hyperosmolar state (HHS) is another acute complication in T1D sharing many symptoms with the DKA but it has different origin and treatment. It's defined as serum glucose >600 mg/mL (33 mmol/L), serum osmolality >320 mOsm/L, and minimal ketonemia/ketonuria is being seen with increasing frequency as the presenting indication of T2D and may be associated with mild-to moderate acidosis from severe dehydration, leading to confusion with DKA [37]. In HHS, water is osmotically drawn out of cells into the blood and the kidneys eventually begin to dump glucose into the urine. This results in loss of water and an increase in blood osmolarity. If fluid is not replaced (by mouth or intravenously), the osmotic effect of high glucose levels, combined with the loss of water, will eventually lead to dehydration. The body's cells become progressively dehydrated as water

is taken from them and excreted. Electrolyte imbalances are also common and are always dangerous [37].

The last acute complication highly frequent in T1D because related with insulin therapy is hypoglycemia. Nearly all patients are symptomatically affected at least once per year, and a significant percentage have severe hypoglycemic episodes requiring medical assistance. Studies using continuous glucose monitoring of type 1 diabetics have shown alarmingly high rates of hypoglycemia, especially at night, when sleeping patients are unaware [38]. Symptoms of low or very low blood glucose levels result from the sympathetic activation of the autonomic nervous system, including sweating, tremor, and palpitations are often the earliest subjective warning signs of hypoglycemia. Patients with hypoglycemia may have altered consciousness and in extreme cases, very low blood glucose can lead to coma, seizures, brain damages and finally death [38].

1.8 Chronic diabetic complications

Chronic diabetic complications are classified in two groups: microvascular (neuropathy, nephropathy and retinopathy) and macrovascular complications (atherosclerosis, heart attacks and strokes). Among those with diabetes, chronic complications are the major cause of morbidity and mortality [39].

In the early twentieth century diabetes mellitus was a disease with a poor prognosis and a very short life expectancy. Consequences like hyperosmolarity, ketoacidosis and even coma and death were unavoidable at the very first months or year from the diagnosis. Nowadays, the longer life expectancy of patients with both type of diabetes resulting from improvements in glucose management has allowed the chronic complications to manifest with the age [40].

The pathogenesis of the microvascular and neuropathic complications of diabetes is complex and poorly understood. However, these complications are undoubtedly mediated in large part by the metabolic derangements associated with diabetes, especially hyperglycemia.

Furthermore, recent studies suggested that chronic complications persist and progress even when glycemic control is pharmaceutically achieved via the phenomenon of metabolic memory [40].

It has been also suggested that glucose-induced cell injury is particularly pronounced in those cell types that are unable to regulate their intracellular glucose concentration (e.g., endothelial cells and neurons) and this leads to the increased production of reactive oxygen species (ROS) (superoxide) as well as advanced glycation end products (AGEs), accelerated polyol and hexosamine pathways, and protein kinase C activation [41].

Moreover, other studies suggest that hyperglycemia-mediated intracellular overproduction of ROS may be the common mechanism triggering a variety of pathways contributing to cell injury in diabetes [42].

In addition, a variety of proteins undergo a non-enzymatic glycosylation in vivo, including hemoglobin, plasma protein, low-density lipoprotein, nerve proteins, lens proteins and extra-cellular matrix proteins for prevailing levels of glucose [43]. The capabilities of AGEs to form cross-links render them resistant to natural degradation and leads to their accumulation in different organs and tissues (i.e. kidneys and blood vessels), where they bind to a specific AGEs receptor (RAGE).

The binding of AGEs to RAGE activate signaling mechanisms that lead to diabetic complications causing cell stress, cellular dysfunction and damages in target organs [44]. Interestingly, recent findings indicate that RAGE is not

only an AGEs receptor but it also bind to other ligand such as S100/calgranulins and high mobility group box 1 (HMGB1) linking RAGE to both the consequences and causes of types 1 and 2 diabetes [44].

When large amount of glucose are present in the blood as in diabetes, cells start to use another pathway to use glucose for energy beside the glycolysis via hexokinase enzyme. The polyol pathway is a metabolic pathway in which glucose is reduced to sorbitol through the activity of intracellular aldose reductase and then it's converted to fructose, resulting in decreased glutathione antioxidant activity (through decreased NAD+) and enhanced formation of diacylglycerol (DAG) [45].

DAG formation, in turn, is a physiological activator of specific isoforms of protein kinase C (PKC), which stimulate transforming growth factor-β release and phosphorylates the subunits of NADPH-oxidase play an important role in cell proliferation and vascular permeability and in the increasing of ROS production [42].

In experimental diabetic animals, prevention of superoxide accumulation, inhibition of AGE formation, and specific protein kinase C inhibitors reduce diabetic complications but more studies are needed in patients [42].

2. Diabetes and bone

Bone fragility and fractures are new emerging related complications for both type of diabetes and this leads to an increased associated morbidity and mortality as well as heavy global health expenditure.

Improvements in life expectancy, especially for T1D, are increasing the number of older patients worldwide. In this group of older patients, fractures are a common event. T1D subjects have double the risk of any fractures and four to five times higher the risk of hip fractures compared to the non-diabetic population [46].

Low bone mineral density (BMD) and a higher fracture risk has been reported in childhood and extends throughout the life span, affecting male and female similarly [46]. In type 1 diabetic adults, most studies report a BMD approximately 0.5-1.0 below aged matched subjects without diabetes [47].

Conversely, BMD in T2D is normal or higher than in matched controls, even when normalized for larger body size [48].

This may contribute to explain why the incidence of hip fracture is even stronger for T1D than for T2D [49, 50].

Thus, assessment of skeletal mass in diabetes by BMD is not fully accountable for the increased fracture risk suggesting diabetic patients have

an altered bone quality and a reduced strength compared to healthy subjects. While a few of molecular mechanisms as well as other risk factors contributing to low bone mass in T1D have been identified, most of the determinants of bone fragility in T1D remain largely unclear [49, 51], hindering the development of a conclusive model of bone fragility in diabetes.

The following paragraphs will address the epidemiology, known risk factors, and pathogenesis at the tissue, cellular and molecular levels.

2.1 Epidemiology of bone fragility in diabetes

One of the earliest research on the increased fractures risk in T1D was the Iowa Women's Health Study, an 11-year follow-up of 32,089 postmenopausal women, conducted in 2001. This study shows that women with T1D have up to 12-times incidence to experience hip fractures compared to matched controls [49]. Moreover, women with T2D have a 1.70-fold higher risk of incident hip fracture than women without diabetes, showing that hip fracture risk is higher in T1D compared to T2D. This stronger association between type of diabetes and hip fracture is also confirmed by other studies reporting a hip fractures risk increase of 6.3 in T1D compared to 1.7 for T2D [50, 51]. Results are also consistent between studies of men and women and between studies conducted in the United States and Europe [52, 53]. In another subsequent study, Vestergaard et al. investigated the risk of any fracture in both type of diabetes and they show that it's increase by 1.3 in T1D and 1.2 in T2D. Specifically for T1D, other studies [51] report a higher prevalence of morphometric vertebral fractures (VFx) in T1D patients (24%) compared to controls (6.1%).

2.2 Risk factors for fractures in diabetes

Some factors like hyperglycemia, hypoinsulinemia, low levels of IGF-1 and vitamin D have been mentioned as possible causes but risk factors for fractures in diabetic patients are still not well established. As mentioned before, a lower BMD may be accountable for an increased bone fragility in these patients but there are other mechanisms and factors that may be underlying an altered bone quality and a reduced strength.

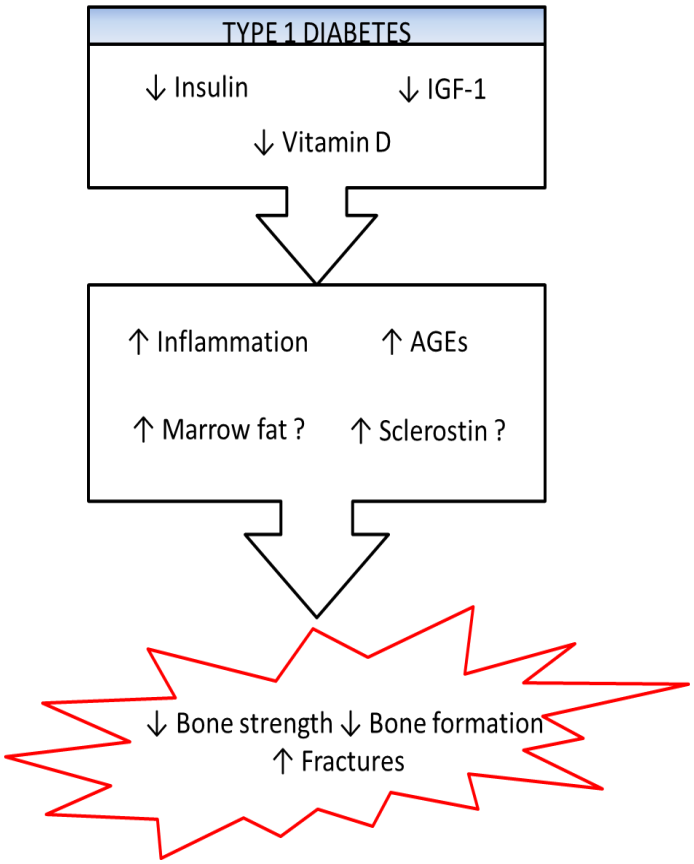

Figure 1 Proposed model of bone fragility in type 1 diabetes

Among those with diabetes, there is still not an established relationship between HbA1c and fracture risk. Most observational studies have found no effect but more data are needed [54].

The Vestergaard meta-analysis reported an average decrease in spine BMD of -22% and a hip Z-score of -37% compared to the age-and gender matched controls and also documented an association between the decreased BMD observed in T1D patients and the presence of a microvascular complication (retinopathy, neuropathy and nephropathy), but failed to document an association between BMD and HbA1c [55]. Some studies suggested that, a decreased BMD, occurred more frequently in a long-term disease duration T1D patients [56, 57], others documented osteopenia at diagnosis of diabetes [58].

The risk of fractures in diabetes is also affected by the incidence of hypoglycemic episodes, especially if are not preceded by prodromal symptoms. Although hypoglycemia can occur with sulfonylurea use, an increased risk of falls with low HbA1c levels is associated mainly with insulin use [59]. Hypoglycemic treatments could modulate the risk of fractures in many ways. Insulin-sensitizing treatment with metformin is not associated with a higher incidence of bone fractures [60].

Recently, data from Napoli et al. (2014), the Osteoporotic Fractures in Men (MrOS) study, has shown that sulfonylurea use may represent a risk factor for non-vertebral fractures in older diabetic men HR 95% CI 1.66 (1.09, 2.51)

[61]. Fracture rates are higher among all patients taking glitazones (TZDs) [62].

Overall, more studies are needed for the prediction and outcomes of fractures in individuals with T1D and T2D.

2.3 Bone strength and material properties in diabetes

There are growing evidences that the higher fracture rate in diabetes observed in diabetes is not well defined by BMD. Patients with T2D have normal or even higher BMD relative to non-diabetic population [63]. T1D is characterized by a reduced BMD but still it does not fully explain to have double higher the risk of any fracture [64].

This has led to the hypothesis that patients with diabetes may have abnormalities in bone microarchitecture and/or material composition, which are both key determinants of bone quality [63].

In T2D, bone strength may be reduced even in absence of changes in areal BMD because of geometric changes.

In a case-control study, Petit et al. [65] have examined the association between T2D and bone volumetric density, geometry, and estimates of bone

strength at both tibia and radius. They used a pQCT in a subset (n=1171) of men who participated in the MrOS study, comparing data between patients affected by T2D (n=190) and controls. At both the distal tibia and radius, patients with T2DM had greater bone vBMD (+2% to +4%, model 1, $p < .05$) and a smaller bone area (ToA −1% to −4%, model 2, $p < .05$), but they showed no differences in estimated compressive bone strength at the distal trabecular bone regions. On the other hand, total bone area was smaller at the cortical bone midshaft sites resulting in lower bone bending strength despite a similar vBMD at these sites, suggesting that bone strength may be impaired in absence of vBMD changes.

Moreover, Farr et al [63] performed *in vivo* microindentation testing of the tibia to directly measure bone mineral strength (BMS) in 60 postmenopausal women including 30 patients diagnosed with T2D for >10 yrs. BMS was significantly lower in T2D patients than controls and porosity tended to be increased in this patients despite no significant changes in other bone microarchitecture parameters. Glucose control was inversely related with strength and bone turnover markers (BTM) were reduces.

Little is known about decreased strength and lowered resistance in T1D. In preclinical studies, Botolin and Mc Cabe in 2007 comparing streptozotocin

(STZ)-induced diabetic mice and NOD mice to controls showed a similar degree of bone loss [66]. To address the effect of T1D on bone mass they used a μCT. Representative images of proximal tibias from control and diabetic NOD and STZ models have demonstrated a reduced trabecular bone density under the growth plate region of the bones. Moreover, quantitation of bone mineral content (BMC), BMD, and bone volume fraction (BVF), expressed as a percentage relative to age-matched controls, has revealed a significant reduction in all trabecular bone parameters (BMC and BMD, 27%; BVF, 50%). They have also measured cortical parameters but the reduction was not significant [66].

Furthermore, Silva et al. in 2009 have demonstrated trabecular bone loss (~20%) and a reduction in diaphyseal growth in STZ-induced diabetic rats using a pQCT in vivo and post mortem [67]. There are very few studies that perform hystomorphometry and μCT to measure trabecular and cortical bone loss in T1D patients. Although it is not known whether patients with T1DM have altered bone material properties, a recent study using high-resolution peripheral quantitative computed tomography (HRpQCT) found that patients with T1D and microvascular complications had significant deficits in both

cortical and trabecular bone microarchitectural parameters at the distal radius and tibia as compared to matched non-diabetic control subjects [68].

Thus, although bone quality appears to be impaired, the pathogenesis of these abnormalities and their relationship to the increased fracture risk observed in these patients needs further study.

2.4 Cellular mechanisms of bone fragility in diabetes

The cellular and molecular mechanism underlying bone fragility in diabetes are complex and they imply several interactions that are only in part common between both types of diabetes.

In T1D, absolute deficiency of insulin and insulin-like growth factor-1 (IGF-1) [69], and low serum levels of Vitamin D as well as the autoimmunity found in patients at the time of diagnosis can contribute to a reduced bone formation, altered mineralization and microarchitecture and to a reduced peak bone mass [69]. Conversely, T2D affects bone health in a later stages of the disease affecting bone by lack of insulin and prolonged hyperglycemia [70].

Pre-clinical and clinical studies have also shown that in diabetes there are high levels of pro inflammatory cytokines, AGEs and increased levels of reactive oxygen species (ROS), capable of affecting differentiation of osteogenic cells [70]. Furthermore, oxidative stress and hyperglycaemia induce the reduction in enzymatic beneficial cross-links and the accumulation in the bone of AGEs, the products of non-enzymatic glycosylation of proteins [42].

A direct effect on osteogenic cells of hyperglycemia, alterations of WNT signaling, and of AGEs may be also be key drivers of impaired bone health in T1D patients, but their effect has been only marginally explored [70].

Verhaeghe et al. performed two studies on diabetic mice after 3 or 4 weeks after the onset of the disease. Serum OCN levels, osteoblast/osteoclast and osteoid surface percentages and the daily mineral apposition rate were reduced in diabetic mice (mineral apposition rate in the tibia 1.0±0.4 vs. 5.6±0.6 μ/day and vertebra 0.2±0.1 vs. 2.3±0.2 μ/day), proving that osteoblast function is compromised with a consequent low bone turnover [71]. In the second study diabetic mice presented 25% less stiffness and strength in the femur than no-diabetic mice and a lower resistance to physical exercise [72].

Some studies have demonstrated that bone metabolism in T1D is characterized by a low bone turnover, specifically a low bone formation [73]. In T1D the osteoblast impairment is featured by several causes as a decreased osteoblastogenesis, a low osteoblast differentiation and a low osteoblast activity (low levels of osteocalcin and reduced mineral apposition rate) [74, 75]. Indeed, osteoclast metabolism is impaired too [74, 76]. In 2013, Napoli et al. have investigated the effect of calcitriol on bone turnover and osteocalcin (OC) in recent-onset T1D patients. At diagnosis, they have found OC levels significantly lower in female than in male patients (17.8±3.1 ng/ml vs. 43.9±6.9 ng/ml; $p<0.01$) and a negative correlation between OC levels and age ($r^2=-0.59$, $p=0.02$) [77].

Dobnig et al. [78] have investigate the effect of T2D on bone turnover in 583 T2D patients and 1,081 control subjects, while hip and other non-vertebral fractures were monitored over 2 years. Diabetic patients had serum PTH (-20.7%) and OCN levels (-22.3%) significantly lower (both P<0.0001) in T2D patients despite similar low serum 25OHD levels. However, a total of 110 hip fractures occurred during the observation period, corresponding to a hip fracture rate of 3.1% (in controls) and 3.4% (in T2D) per 100 patient years, with a not significant difference between the two groups. Recently, Rubin's

group [48] has correlated COP with bone histomorphometric structure and bone markers in T2D patients. The results of the study showed reduced circulating osteogenic precursor cells (COP) in T2D patients in comparison with control subjects. The bone formation markers P1NP and OCN were significantly lower in T2D (P1NP $p<0.01$, OCN $p<0.03$), as the bone resorption serum CTX ($p<0.01$). However, the role of bone markers in diabetes is controversial, and further studies are needed to clarify the results in these patients [48].

2.5 Molecular mechanisms of bone fragility in diabetes

Impaired osteoblastogenesis, alterations of WNT signaling, oxidative stress may be the responsible molecular mechanisms of impaired bone health in T1D patients but their effect has been only marginally explored.

Other parameters that were addressed, concerning bone impairment, are oxidative stress and advanced glycation end products (AGEs) formation. High blood glucose induces formation of advanced glycation end-products AGEs, with negative effects on structural proteins such as type I collagen, the main bone matrix protein. Not only hyperglycaemia but also oxidative

stress induces the reduction in enzymatic beneficial cross-links and the accumulation of AGEs in bone. AGEs may have damaging effects on collagens by forming irreversible cross-links between the fibers in the triple helix [79]. Collagen cross-linking plays an important role in bone strength [80]. AGEs, and in particular pentosidine, being one of the important products of non-enzymatic glycosylation, are widely studied. In a 2015 work, Farley et al. purposed to determine whether AGEs are higher in fracturing and in non-fracturing T1D patients compared to controls and the degree of mineralization of bone (DMB) and hardness comparing the same patients. They have found higher levels of pentosidine in fracturing patients than controls (p 0.04) and a positive correlations between HbA1c and pentosidine (r^2 0.79, $p < 0.003$) and between HbA1c and DMB (r^2 0.64, $p < 0.02$). Not significant differences were found between non fracturing T1D patients and controls [81].

A growing number of studies have evaluated the role of the Wnt pathway components as markers of bone metabolism. WNT canonical pathway controls MSC differentiation to three specific lineages, adipocytes, osteoblasts and chondrocytes. The activation of the Wnt/β-catenin (primarily Wnt10b) promotes osteoblast differentiation and proliferation from

mesenchymal stem cells (MSC), trough stimulation of osteogenic transcription factors, such as Runx2 and osterix. This process activates a negative feedback control with dkk1 and sclerostin production by osteocytes [41]. The relevance of WNT is also proved by the fact that mutations in the LRP-5 Wnt co-receptor are associated with changes in BMD. For example, loss-of-function LRP-5 knock-out mice present reduced bone mass [41].

Clinical studies have also shown that levels of sclerostin are higher in patients with T2D compared with control subjects, inversely related with bone turnover markers and positively associated with spine and hip BMD [82, 83]. Available studies are based on small number of studied subjects or on epidemiological data with limited clinical information. Therefore, further studies are needed to clarify the results in diabetic patients.

3. Study aims

The overall objective of the study was to evaluate features of impaired bone fragility in T1D.

Study 1: we hypothesized that glycemic control and diabetic related complications underlies bone fragility in T1D.

To test our hypothesis, we propose to assess prevalence and risk factors for fragility fractures in T1D patients.

Study 2: we hypothesized that a chronic hyperglycemia induces Wnt signaling downregulation due to increased sclerostin levels and this underlies bone fragility in T1D.

To test our hypothesis, we propose three specific aims:

- To determine in vivo the role of Wnt pathway as a mediator of low bone formation in T1D
- To explore in vitro the effect of sclerostin and wnt signaling co-receptor Lrp5 on adipogenic differentiation of Mesenchymal Stem Cells

4. Clinical study: Glycemic control and disease duration are independent risk factors for multiple fragility fractures in type 1 diabetes

4.1 Rationale

Type 1 diabetes (T1D) is an autoimmune disease characterized by absolute insulin deficiency due to pancreatic beta-cell destruction and consequent hyperglycaemia. Diabetes-related complications are the main causes of morbidity and mortality in subjects with T1D and bone fragility is being recognized as a new complication of both type 1 and type 2 diabetes. The association between diabetes and hip fracture is however stronger for T1D than for Type 2 diabetes (HR 6.3 v 1.4) [50] and Weber et al. showed that the increased risk of hip fractures in subjects with T1D starts already in young adulthood [64]. Despite the recognized increased incidence, diabetes-related risk factors for fractures have not yet been fully elucidated. Hypoinsulinemia, low levels of IGF-1 and vitamin D, poor metabolic control, vascular complications [84, 85] have all been studied as possible contributors to poor bone health in type 1 diabetes, with controversial results [86, 87]. The majority of available clinical studies in this regard are limited in the number of subjects or by the lack of information about crucial data such as metabolic control, insulin exposure or hypoglycemic events [88, 89]. The incidence of T1D is increasing worldwide. Together with an increased life expectancy as a consequence of the improving quality of care, this is causing an exponential

increase in the overall number of subjects with T1D in the age range at increased risk of fragility fractures. As bone fractures are associated with increased morbidity and mortality, a better understanding of factors related to bone fragility in T1D is crucial to identify risk factors to be tackled to decrease the incidence of fractures in this population. The Immunotherapy DIABetes (IMDIAB) study group has conducted several landmark studies in T1D for more than 20 years [90, 91]. This has allowed the enrollment of a large cohort of well-characterized subjects with T1D. Taking advantage of this population, our study aims to investigate diabetes-related clinical factors associated with non-vertebral fragility fractures in T1D.

4.2 Research design and methods

Patients with T1D involved in at least one study conducted by the IMDIAB study group (Rome-Italy) have been screened for participation in this cross-sectional study. Patients attended an outpatient clinic at one of three participating institutions and had been followed for at least 5 years. Diabetes diagnosis was based on the American Diabetes Association criteria (www.who.int). Inclusion criteria were: age ≥ 18 years, duration of the disease ≥ 1 year and eugonadal status. The exclusion criteria were: 1) history of secondary causes of osteoporosis (i.e., non-compensated hypothyroidism, hyperthyroidism, hyperparathyroidism, inflammatory bowel disease, or malignancy), 2) use of drugs that can impair bone metabolism (bisphosphonates, glucocorticoids, anticonvulsants, HRT). A total of 600 type 1 diabetes patients were enrolled (300 males and 300 females) excluding 107 subjects according to the above mentioned criteria. All recruited participants gave their witnessed, informed consent before entering the study, which was approved by local ethical committees and conducted in accordance with Helsinki Declaration II.

Enrolled participants attended a study visit. The visit included measurement of height and weight and completion of a questionnaire. Fractures and hypoglycemic episodes were evaluated by a previously used questionnaire [61] during a dedicated interview. Participants were asked to report the occurrence and circumstances of any fractures after the diagnosis of type 1 diabetes. Also, having defined fragility fractures as occurring with forces equivalent to a fall from a standing height or less [61], the interview explored the circumstances of the fracture, in order to exclude fractures resulting from major trauma. The same questionnaire investigated family history of fragility fractures in a blood relative (first or second degree, e.g., aunts and grandmothers).

Medical history of the enrolled participants was obtained from clinical electronic records from the outpatient services of the three collaborating institutions between 2010 and 2016. To assess long-term glucose control we considered the average of at least 3 HbA1c measurements per year for up to 5 years before enrollment. All clinical records included the physician's evaluation of macrovascular complications, neuropathy, and retinopathy, for which we included in the analysis the status at the most recent available visit. Specifically, the presence of cardiovascular diseases (CVD) was assessed as

recent history or evidence of coronary heart disease, cerebrovascular disease, or peripheral arterial disease. Diabetic neuropathy evaluation was based on symptoms, quantitative sensory testing (temperature, vibration, and pressure perception) and quantitative motor testing (patellar and ankle reflexes) as assessed by the physician. Additionally, all patients underwent funduscopic examination to assess retinopathy. In order to assess the presence of reduced kidney function in our cohort, we considered the latest available serum creatinine and urine analyses. In particular, microalbuminuria and microalbuminuria, were diagnosed on the basis of albumin excretion rate between 30 and 300 mg/day or >300 mg/ day, respectively. Estimated glomerular filtration rate (eGFR) was calculated with the Cockcroft-Gault formula. Nephropathy was defined as the presence of both albumin-to-creatinine ratio (ACR) >30 mg/g and an eGFR <60 mL/min per 1.73 m^2. To evaluate the lipid profile, we analyzed the last cholesterol (total cholesterol, HDL, LDL) and triglycerides serum levels available.

4.2.1 Statistical analysis

Statistical analysis was performed using STATA Stata/IC 12.1 software (StataCorp, College Station, TX, USA). The distribution of variables was tested with the Kolmogorov-Smirnov test. The results are expressed as mean ± SD or median [interquartile ranges (IQRs)] as appropriate. Comparisons were done using Student's t-test, Kruskal–Wallis and Chi-square depending on distribution; Analysis of variance (ANOVA) was used for comparisons of continuous variables between more than two groups. Multinomial logistic regression was used to determine the contribution of the explanatory variables to the occurrence of a single and multiple (≥ 2) fractures. Each variable of interest was first assessed with minimal adjustment for age, sex and BMI. Variables were then tested to develop a final model using forward stepwise entry (p for retention <0.1). Age, sex and BMI were forced into the model. The effects of independent variables on the different categories of the dependent variable were tested for equality. Non-normally distributed continuous variables were ln-transformed before they were tested in the ANOVA and in the multinomial logistic regression model. Two-tailed p-value <0.05 was considered statistically significant.

4.3 Results

The average age of diabetic patients was 41.9±12.8 years old (range 18-79). Average BMI and disease duration were 24.4±3.7 kg/m^2 (15.6-44.8) and 19.9±12.0 years (1-63). The mean of the last 5 years HbA1c level was 7.6±1.0% (5.3-13.0). Insulin requirement (IR) was 20.7±10.3 IU/daily for fast-acting and 19.5±9.5 IU for long-acting. A total of 111 subjects (18.5%) reported at least one fragility fracture after diabetes diagnosis, and 29 of the fractured subjects reported ≥2 fractures. The most common fracture sites were: hand (18.6%), foot (17.1%), tibia/fibula (10.5%), wrist (9.3%) and ribs (8%).

Clinical factors associated with single and multiple fractures.

Clinical features of type 1 diabetes patients by previous fracture status (none, 1, 2+) are reported in Table 1.

	0 fractures (n=489)	1 fracture (n=82)	≥ 2 fractures (n=29)	p-value
Age (years)	40 (32-49)	43 (34-54)	47 (37-54)	0.018
BMI (Kg/m^2)	23.7 (21.6-26.5)	24.4 (21.6-27.1)	25.6 (23.1-28.1)	0.052
Sex (Men)	240	46	14	0.492
Family history of fracture (Y)	162	41	15	0.003
Age at onset	21 (11-31)	19 (13-31)	15(11-18)	0.061
Disease duration (years)	18 (10-27)	20 (12-31)	31 (23-39)	<0.001
Insulin Unit (IU/kg) (n=577)	0.54 (0.44-0.66)	0.55 (0.45-0.67)	0.53 (0.46-0.68)	0.774
Monthly Hypoglycemic episodes (n) (n=569)	6 (4-10)	8 (4-12)	8 (4-15)	0.707
eGFR (ml/min) (n=537)	105.8 (92.2-116.7)	101.6 (81.2-113.5)	96.9 (84.1-107.6)	0.002
Total Cholesterol (mg/dl) (n=542)	178 (155-197)	176 (156-193)	173 (160-195)	0.840
HDL (mg/dl) (n=531)	61 (51-75)	64 (50-74)	60.5 (52-73)	0.793
LDL (mg/dl) (n=516)	97 (80-115)	95 (74-115)	95 (86-104)	0.279
Triglycerides (mg/dl) (n=529)	66 (52-89)	72 (58-83)	72.5 (55-125)	0.252

A1C - **Tertile 1** - **Tertile 2** - **Tertile 3**	 164 152 155	 25 29 24	 4 11 13	0.181
CVD	31	8	6	0.012
Retinopathy	97	20	14	0.001
Neuropathy	47	15	8	0.002
Nephropathy	26	6	3	0.277
Celiac disease	19	1	0	0.440

Table 1 Characteristics of participants with type 1 diabetes; results are expressed as median [interquartile ranges (IQRs), ANOVA p<0.05

Variables that differed by fracture status included age (p=0.02), BMI (p=0.05), family history of fracture (p<0.01), disease duration (p<0.01) and eGFR (p<0.01). Diabetic retinopathy (p<0.01), neuropathy (p<0.01) and CVD (p=0.01) were more prevalent in subjects with higher number of fractures. In particular, subjects who experienced 2+ fractures showed higher prevalence of cardiovascular disease (p=0.004), retinopathy (p<0.001) and neuropathy (p=0.007) than subjects with negative history of fractures. On the contrary, cardiovascular disease and retinopathy did not differ between subjects with 1 fracture *vs* no fracture (Figure 1).

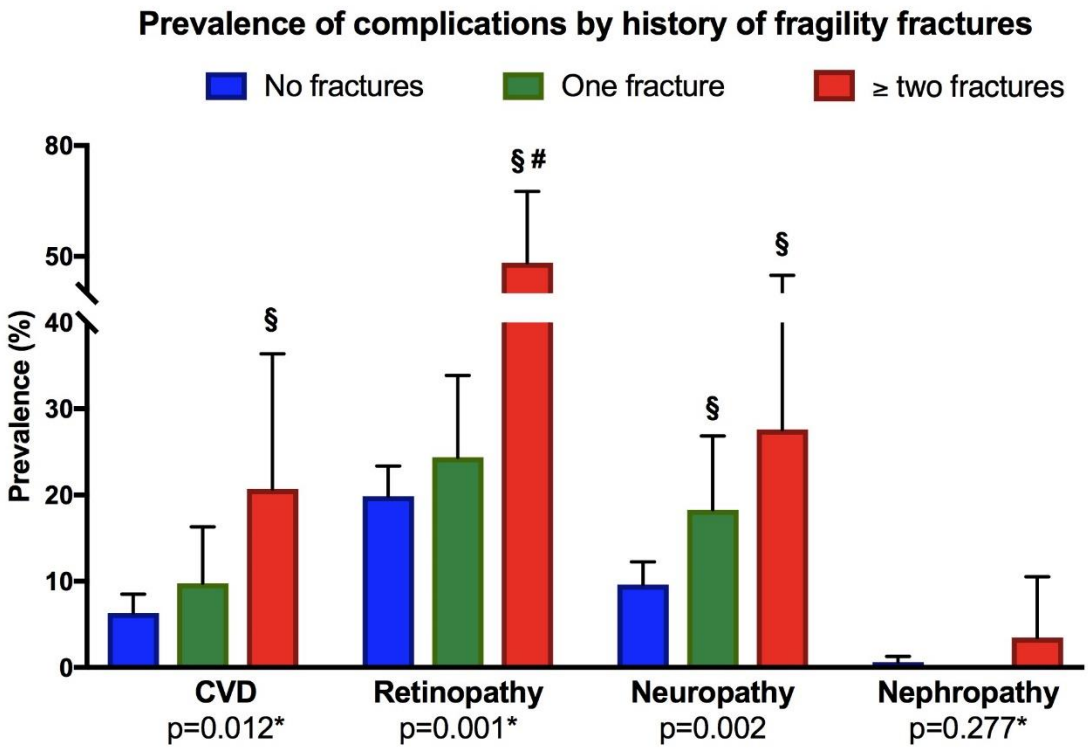

Figure 1 Prevalence of complications by history of fragility fractures; ANOVA, p<0.05

Only 4 patients had nephropathy, and only 20 had celiac disease. Because of this low prevalence, these two variables were not considered in adjusted analyses.

In models minimally adjusted for age, sex, BMI and family history of fracture, none of the variables were associated with history of a single fracture (Table 2).

VARIABLE	UNITS	Multinomial logistic model 1		Model 2	
		1 FX	2+ FX	1 FX	2+ FX
Ln eGFR	1 unit	0.31 (0.09-1.08)	0.27 (0.05-1.50)	0.22 (0.06-0.83)	0.23 (0.04-1.48)
Neuropathy	Yes/no	1.86 (0.96-3.62)	2.60 (1.03-6.60)	2.57 (1.21-5.46)	2.57 (0.92-7.15)
A1C					
Tertile 1	<=7.17	Ref	Ref	Ref	Ref
Tertile 2	7.18-7.9	1.19 (0.66-2.15)	2.54 (0.78-8.27)	1.44 (0.76-2.72)	3.42 (0.97-12.05)
Tertile 3	>7.9	1.02 (0.55-1.88)	3.00 (0.94-9.58)	0.98 (0.51-1.89)	3.50 (1-04-11.73)
Disease duration					
Tertile 1	<14 yrs	Ref	Ref	Ref	Ref
Tertile 2	14-25 yrs	1.66 (0.92-3.01)	4.28 (0.89-20.59)	1.68 (0.89-3.19)	3.43 (0.68-17.24)
Tertile 3	>=26 yrs	1.23 (0.63-2.39)	10.10 (2.20-46.4)	1.06 (0.52-2.18)	7.59 (1.60-35.98)
Family history of fragility fractures	Yes/no			2.08 (1.23-3.50)	2.83 (1.21-6.59)
Lipid profile					
Ln (Total chol)		0.69 (0.18-2.69)	1.25 (0.15-10.44)	---	---
Ln (HDL)		1.83 (0.65-5.13)	1.18 (0.24-5.87)	---	---
Ln (LDL)		0.49 (0.21-1.16)	0.97 (0.24-3.87)	---	---
Ln (Triglycerides)		1.13 (0.62-2.05)	1.46 (0.58-3.67)	---	---
Ln (Age at onset)		0.86 (0.60-1.23)	0.42 (0.26-0.68)	---	---
Ln (Hypo/month)		1.08 (0.82-1.43)	1.20 (0.78-1.85)	---	---
Ln (Insulin dose)		1.27 (0.66-2.47)	1.47 (0.51-4.24)	---	---
CVD	Yes/no	1.22 (0.50-2.95)	2.46 (0.81-7.49)	---	---
Nephropathy	Yes/no	0.00	4.76 (0.44-51.42)	---	---
Celiac disease	Yes/no	0.29 (0.04-2.19)	---	---	---
Retinopathy	Yes/no	1.13 (0.64-2.01)	2.94 (1.32-6.57)	---	---

Table 1 Variables from Table 1 adjusted for age, sex, BMI, and family history of fractures. Multinomial logistic regression analysis for 1 or 2+ more fractures

Neuropathy, retinopathy, disease duration and earlier age at onset were each associated with history of multiple fractures. After full adjustments, HbA1c, disease duration, eGFR and neuropathy were the independent variables retained in the final multivariate multinomial regression model (Table 2 and Figure 2).

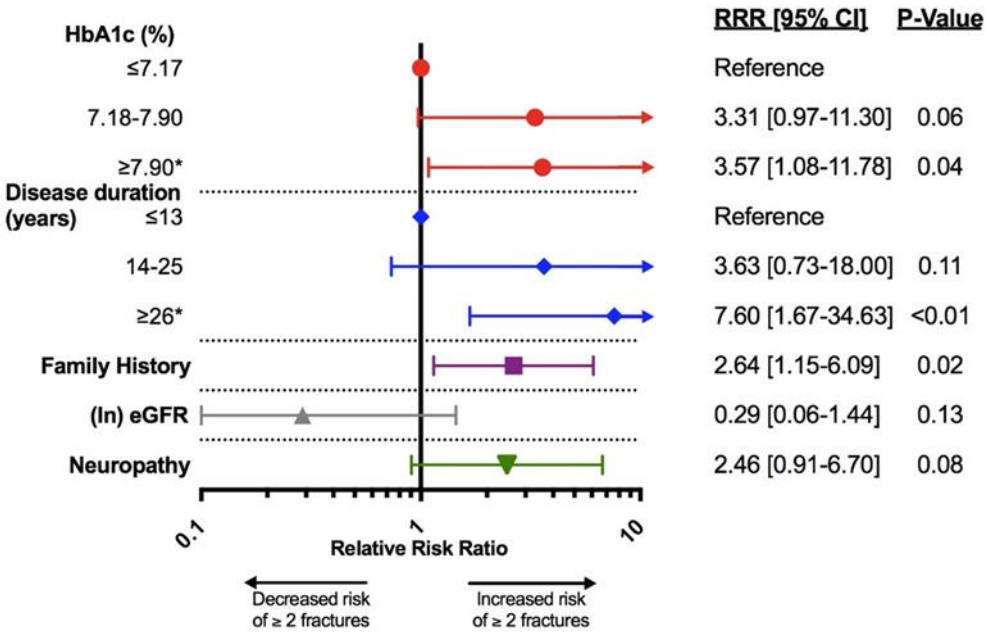

Figure 2 Relative risk ratio (RRR) in T1D patients with ≥2 fractures; Multinomial logistic regression model, p<0.05

Subjects in the highest tertile of HbA1c (HbA1c >7.9%) had an increased risk of ≥2 bone fractures (adjusted RRR: 3.50 [95%CI 1.04-11.73] compared to subjects in the lowest tertile (HbA1c <7.2%), but did not have increased

risk of a single fracture (adjusted OR: 0.98 [95%CI 0.51-1.89]). Similarly, subjects in the highest tertile of disease duration (disease duration >26 years) had an adjusted RRR for multiple fractures of 7.59 [CI 1.60 -36.0] when compared to subjects in the lowest tertile (disease duration <14 years), but no significant association was found with single fracture (adjusted RRR: 1.06 [95%CI: 0.52-2.18]). Testing for equality confirmed the differential association of HbA1c and disease duration with one and ≥2 bone fractures (p-values for the differences of the effects on outcome: p=0.043 for HbA1c; p=0.020 for disease duration).

Higher eGFR was protective for single fracture (adjusted RRR: 0.22 [0.06-0.83]) with a similar association for multiple fractures (adjusted OR: 0.23 [0.04-1.48]) (p-value for the difference of the effect on outcomes p=0.95) The presence of neuropathy increased the risk of single fracture (adjusted RRR: 2.57 [95%CI: 1.21-5.46]) and this association was similar (p-value for the difference of the effect on outcomes: 0.99) for multiple fractures (adjusted RRR 2.57 [95%CI: 0.92-7.15]).

3.4 Discussion

In this study we identify a population of well-characterized young adults with T1D experiencing a high prevalence (18.5%) of non-vertebral fragility fractures after disease onset, with family history of fragility fractures, impaired kidney function and diabetic neuropathy associated with an increased risk of these fractures. Furthermore, to our knowledge, this is the first study describing a sub-population of multi-fractured T1D patients. This allowed us to show that poor glycemic control over the past 5 years and longer disease duration also are associated with fractures, in particular if 2 or more fractures occur.

These results shed lights on a controversial topic. While in young adults T1D has been consistently associated with low BMD, in other long disease duration was not associated with a significant reduction in BMD [92-94]. This questioned whether a long-term exposition to exogenous insulin might benefit bone, as insulin is a major regulator of bone remodeling [94]. Here we show that subjects with longer disease duration have an increased prevalence of fragility fractures, which is independent from mean insulin dose [95].

Previous studies in T1D have not consistently identified an association between glycemic control and fracture. This may be due to lack of multiple HbA1c measurements, as reported in recent studies [96, 97]. Also, these studies did not consider those with multiple fractures as a separate group. Our study suggests that glycemic control and longer disease duration, associated with the presence multiple fractures but not a single fracture, identify a "severe bone fragility" phenotype in T1D. In type 1 diabetes, duration of disease is not strongly associated with glycemic control; instead, control remains relatively constant especially after adolescence. Thus, it is not surprising that both factors contribute independently to fracture risk as duration of disease provides a measure of how long the skeleton has been exposed to hyperglycemia while the average A1C provides a measure of the degree of hyperglycemia. This is in accordance with pre-clinical and clinical data showing that chronic hyperglycemia may impact on osteoblast function [84, 98] and bone quality specifically in subjects with T1D.

Vascular complications have been suggested as possible contributors to the increased bone fragility by impacting bone mineral density [92], bone quality [99] and final risk of fractures [87] and potentially as markers of reduced vascular function in bone. In this study we found a higher prevalence of

retinopathy, diabetic neuropathy and reduced renal function among T1D with positive history of fractures. With adjustment for glycemic control, disease duration and each other, neuropathy and renal function, but not retinopathy, were retained as independent risk factors for fracture.

Several studies have documented an increased risk of cardiac events in the presence of decreased skeletal health, but few studies have been done in those with T1D. In our study, CVD was more prevalent in subjects with higher number of fractures, but the association was not significant after adjustment for confounders (age and disease duration in particular). However, because of the known and strong association between CVD and these variables, we would suggest this association should be more appropriately tested in groups homogeneous for these confounders. In this regard, we have recently shown that an association between BMD at the femoral neck and history cardiovascular disease does exist in older people with long-standing type 1 diabetes who were homogeneous in terms of age, metabolic control and disease duration [92].

In studies of T2D, hypoglycemic episodes have been identified as risk factors for fracture. However, we did not find evidence of an association.

Lipid profile was associated with lower BMD in the Medalists but we did not find evidence of association with fracture risk.

Our study has several strengths. We were able to consider a full set of clinical risk factors, including glycemic control, insulin dose, hypoglycemic episodes, lipid profile and complications. Differently from previous studies conducted on population-based registries, we analyzed glycemic control through frequent HBA1c measurements in the past 5 years, which better describes the long-term glucose control than one single measurement. We also acknowledge this study has limitations. The design is cross-sectional so we cannot determine the temporal relationship between glycemic control and fractures. Although diabetic complications were established by the physician, fractures were self-reported. Any resulting misclassification of fractures is unlikely to differ with respect to the risk factors considered and any bias of associations would tend towards the null.

In conclusion, our study identified diabetes-specific factors that can be used to evaluate increased fracture risk in type 1 diabetic patients, namely disease duration, presence of neuropathy, HbA1c and eGFR values. Importantly, glycemic control and kidney function are modifiable risk factors that could be targeted for prevention of fractures in diabetes. Longitudinal studies of

fracture in T1D that include BMD as well as diabetes-specific risk factors are needed to confirm our findings.

5. Preclinical study: sclerostin resistance protects bone mass and improves glucose metabolism in a model of type 1 diabetes

5.1 Rationale

The incidence of T1D is increasing worldwide together with an increased life expectancy as a consequence of the improving quality of care and this is causing an exponential increase in the overall number of subjects with T1D in the age range at increased risk of fragility fractures. Despite the recognized increased incidence, ddiabetes-related mechanisms underlying a lower bone quality and increased risk of fractures in diabetics are, similarly to clinical factors, still not well established. As bone fractures are associated with increased morbidity and mortality, a better understanding of factors related to an increased bone fragility in T1D is crucial to identify risk factors to be tackled to decrease the incidence of fractures in this population.

A chronic hyperglycemia, oxidative stress and the accumulation of advanced glycation end products (AGEs) as well as an increase marrow adiposity and the alteration of osteocalcin and Wnt signaling are all possible contributors to a low bone formation and quality.

Specifically, the Wnt pathway is one of the best-known genetic determinants of bone strength, and it is believed to play an important role also in the context of diabetes-induced fragility [41].

The activation of canonical Wnt/β-catenin signalling promotes osteoblast differentiation and proliferation from mesenchymal stem cells (MSC), trough stimulation of osteogenic transcription factors, such as Runx2 and osterix, while repressing drivers of alternative differentiation programs such as PPRγ [41]. Attesting for its biological relevance, mutations and polymorphisms in the LRP-5 Wnt co-receptor gene are associated with changes in BMD [70, 100]. Interestingly, clinical studies have also shown that levels of sclerostin, which inhibits Lrp5 activation, are higher in patients with T2D compared with control subjects, inversely related with bone turnover markers and positively associated with spine and hip BMD [75, 82, 83, 101]. A more recent study documented also an increased in circulating sclerostin levels in T1D patients (24237244).

Animal models represent a powerful tool to address the mechanisms of bone disease in diabetes: Streptozotocin-induced diabetes in (STZ) mice and rats, non-obese diabetic mice (NOD, and the inflammation-independent insulin-deficient Ins2/Akita mice have all shown a similar degree of reduced trabecular and cortical bone mass, reduced bone formation rate and low bone turnover markers [66, 67, 102].

In order to determine the role in vivo of sclerostin and diabetes on bone fragility and bone anabolism, we set out to breed the *Ins2*^{Akita} mice (Akita), which become spontaneously diabetic at age 5 week, with the sost-resistant *Lrp5*^{A214V} (HBM) mutants. The inflammation-independent insulin-deficient *Ins2*^{Akita} mice are a genetically induced diabetic model with a mutation in one allele of the Ins2 gene. These mice become hyperglycaemic due to an intracellular accumulation of mutated pro-insulin that promotes endoplasmic reticulum (ER) stress in b-cells that leads to apoptosis [102]. In the male mutants there is a full penetrance and a more severe and progressive disease and for this reason we used for the breeding only male mice. Furthermore, Akita mice remain

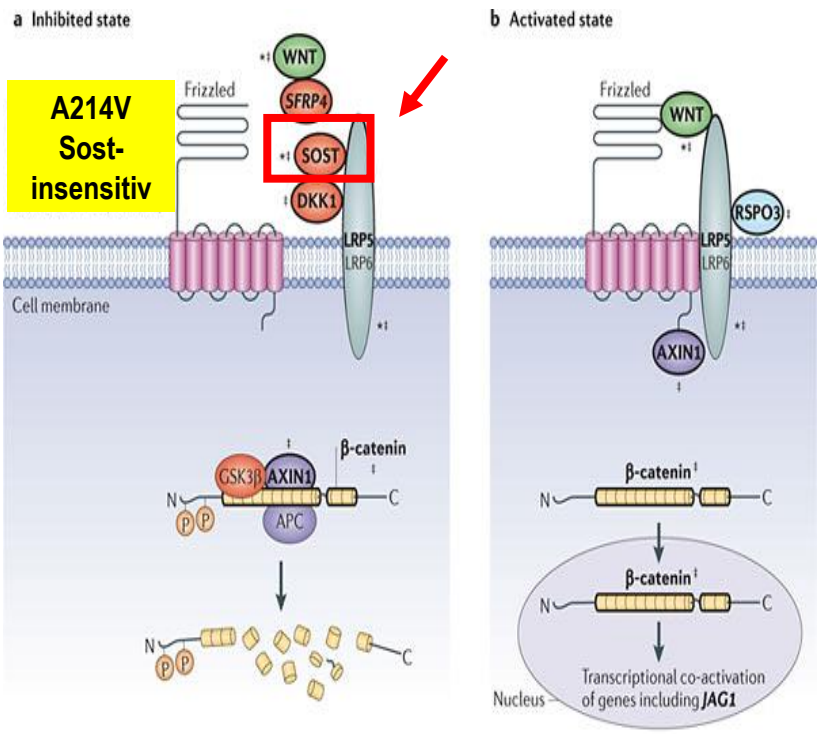

Figure 2 Schematic representation of the activated and inhibited state of the Wnt canonical pathway

relatively healthy under severe diabetic symptoms including hyperglycemia and hypoinsulinemia until 12 months old (www.jax.org) allowing to study bone phenotype later in the disease.

The *Lrp5*^{A214V} constitutive mutant is a knock-in mouse model (they knocked-in an alanine to valine substitution at amino acid 214) characterized by a high bone mass phenotype due to the Wnt pathway hyperactivation caused by the co-receptor insensibility to sclerostin and DKK1 [100].

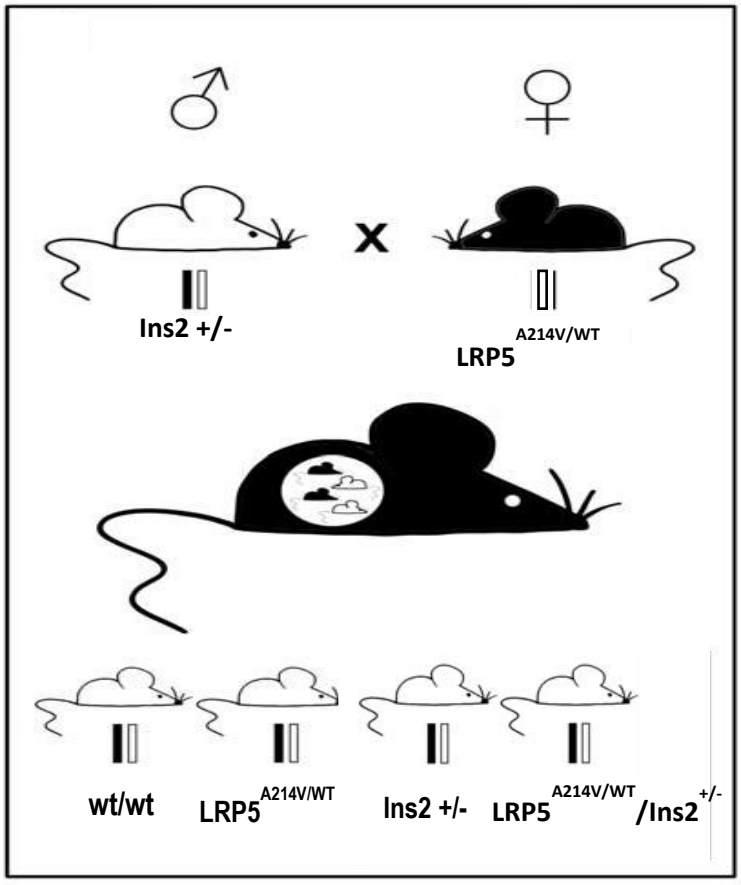

Figure 3 Breeding scheme

We hypothesize that compound mutant mice will target the effect of diabetes on sclerostin expression and they will show if the Wnt signalling is the main regulator of bone metabolism. Moreover, for a better understanding on the

effect of sclerostin and wnt signaling co-receptor Lrp5 on adipogenic differentiation of Mesenchymal Stem Cells we used heterozygous and homozygous $Lrp5^{A214V}$ mutant mice.

5.2 Research design and methods

5.2.1 Animals and in vivo experiments

All animal procedures were performed in accordance with a protocol approved by the Animal Studies Committee of Washington University in St. Louis (MO, USA).

Mice with the germline *Lrp5*^{A214V} mutant allele were a generous gift from Dr. Matthew L. Warman, Harvard University. The Ins2/Akita mice were purchased from the Jackson Laboratories (Bar Harbor, ME USA).

All the mouse lines used in this project were developed in a C57BL/6 background, and littermates were used as controls. Mice were waned at 28 days after birth and fed with regular chow ad libitum and housed in a room maintained at constant temperature (25°C) on a 12-h light and 12-h dark cycle.

Genotyping was performed by PCR on genomic DNA extracted from mouse tails using the HotSHOT method, as previously described [103]. Primers to detect *Lrp5*^{A214V} allele has also been described [104]. Genotyping for the *Ins2*^{Akita} allele was performed by TransnetYX INC. (Cordova, TN, USA).

4.2.2 Body composition and bone morphology

Weight (gr) and length (nose to tail mm) for monitoring the growth was registered on compound mutants (12-16/genotype) every week, starting from 4 weeks up to 18 weeks of age.

DXA procedure was performed by Faxitron UltraFocus100 scanner (Faxitron Bioptics, LLC Tucson, AZ, USA) at 6, 12, 18, and 26 weeks. The DXA machine was calibrated daily before use, one person performed all scans, and mice were always in the prostrate position on the imaging-positioning tray. For the imaging, mice were anesthetized with isoflurane vaporized at concentration of 2% (no impact on metabolic parameters) [105] and whole body measurements were made excluding head and tail. Analyses included BMD, bone mineral content (BMC), lean mass and fat percentage.

For in vivo longitudinal analysis of bone microarchitecture, mice (7-10/genotype) were subjected to in vivo proximal tibia scanning using μ-CT (VIVA CT40, Scanco Medical, AG, Switzerland) as previously described [106]. The leg to be scanned was fully extended and isolated from the body, while the mouse snout was kept in a mask to ensure the continuous flow of isoflurane (2%) during the entire scan. To minimize the variability due to leg repositioning for repeat in vivo μCT scanning, we developed a fixture

designed to orient the leg in a standard position. Microstructural analysis of cancellous and cortical bone was also performed as previously described [106, 107].

5.2.3 Glucose Metabolism

Glucose measurements were performed from the tail (On-call Express Blood Glucose Meter, ACON laboratories, San Diego, CA, USA). Blood sampling from the tail tip was made cutting 1–2 mm of tissue from the tail tip distal to the bone with sharp scissors and then blood is obtained by direct flow or by gently massaging the tail. The limit of detection was 600mg/dl, and glucose at/above this level is recorded as 600mg/dl but considered to be a lower limit of the true value, as previously described [108].

Random capillary blood glucose test was obtained weekly and at the same day time on compound mutants (12-16/genotype), starting from 4 weeks up to 18 weeks of age.

Intraperitoneal glucose tolerance test (IPGTT) was performed at 6 and 8 weeks of age after 6h morning or 14h overnight fasting for monitoring glucose metabolism on conscious mice. Glucose sampling was obtained at

baseline and 15, 30, 45, 60, 90, and 120 min after an intraperitoneal glucose (dextrose 50%) injection (1.5 mg/kg) [109].

Insulin tolerance test at 7 weeks of age (ITT) after 6h morning fasting was performed as follows. Insulin (0.50 U/kg body weight) was administered intraperitoneally [110]. Glucose measurements were performed at baseline and 15, 30, 45, 60, 90, and 120 min after insulin injection.

5.2.4 Cell line cultures

Bone-derived ST2 Mesenchymal cells were grown to confluence in Minimum Essential Medium-α (α-MEM) (GIBCO, Grand Island, NY) with no ascorbic acid, penicillin-streptomycin-amphotericin b, L- glutamine and 10% FBS (henceforth referred to as "basal media") at 37°C in a humidified atmosphere of 5% CO_2. Cell culture plates were from TPP Techno Plastic Products (AG, Switzerland).

Adipocytes differentiation, at 1 day post-confluence (day 0), was induced using 100nM dexamethasone, 5ug/ml insulin and 50mM indomethacin added to basal media (Adipogenic media) and cells were plated in a 96-well (10.000 cells/well) with rSOST at different concentration up to 2 um/ml (n=3

technical replicates). Recombinant mouse sclerostin was purchased from R&D Systems (Minneapolis, MN, USA). Adipogenic medium was replaced every 3 days for 10 days.

5.2.5 Primary Cell culture

Primary BMSC were obtained dissecting femur and tibia of 10-12 weeks old mice. The bones were cleaned, the epiphysis and bones were cut off end placed down in a 0.6ml tube in which the end has a hole bored with a 18g needle. The 0.6 ml tube were then put into a 1.7 ml tube and centrifuged at 15.000 rpm with a bench top centrifuge for 15 seconds in which the marrow pelleted down the 1.6 ml tube. The bone marrow (BM) was resuspended in culture media, filtered through 70µ filter, plated in a 15mm plate and grown for 2 weeks until 70-80% confluence. Cells were then plated in 96-wells tissue culture plates (TPP, see above) at the concentration of 150.000 cells/well, and adipogenic differentiation was induced, as well as increasing concentrations of rSOST up to 500 ng/ml [111] (n=2 biological replicates, 3 technical replicates). Adipogenic medium was replaced twice a week for 2 weeks, at end point cells were stained with Oil-Red-O and quantified.

5.2.6 Oil-Red-O staining

Cells were stained with Oil-Red-O method [112, 113]. For the Oil-Red-O staining cells were washed with phosphate buffered saline (PBS twice and fixed with neutral buffer formalin (NBF) 15 minutes at room temperature. Cells were then rinse 2x in distilled water and once in 60% isopropanol prior to a 45 minutes staining with Oil-Red-O solution. Oil-Red-O was rinsed off twice with 60% isopropanol and finally with distilled water [112, 113].

Representative images of differentiated cells were acquired at 20x and 40x by Axiovert S100 inverted microscope (Zeiss, Jena, Germany) and quantification was performed by counting lipid droplets (pre-adipocytes and adipocytes).

5.2.7 Statistical Analysis

Statistical differences between data sets were assessed using one-way and two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test (GraphPad Prism). A value for $P < 0.05$ was considered significant. Data were managed in Microsoft Excel and analyzed using the SigmaPlot 11.0 statistical package (Systat Software Inc., Chicago, Illinois). Group values are expressed as the mean ± SEM, unless otherwise noted.

5.3 In vivo results

5.3.1 Biometric parameters

In order to determine the potential role in vivo of sclerostin and Wnt signalling in T1D bone disease we introduce the sclerostin-resistant *Lrp5*^{A214V} mutation, associated with high bone mass (HBM), in mice carrying the *Ins2*^{Akita} mutation (Akita).

We first monitored growth of the different mutants by measuring weight and length (nose to tail) weekly starting from 4 up to 18 weeks.

We found no significant differences in body weight and length between HBM/Akita mutants and littermates at any age (Fig. 3-4).

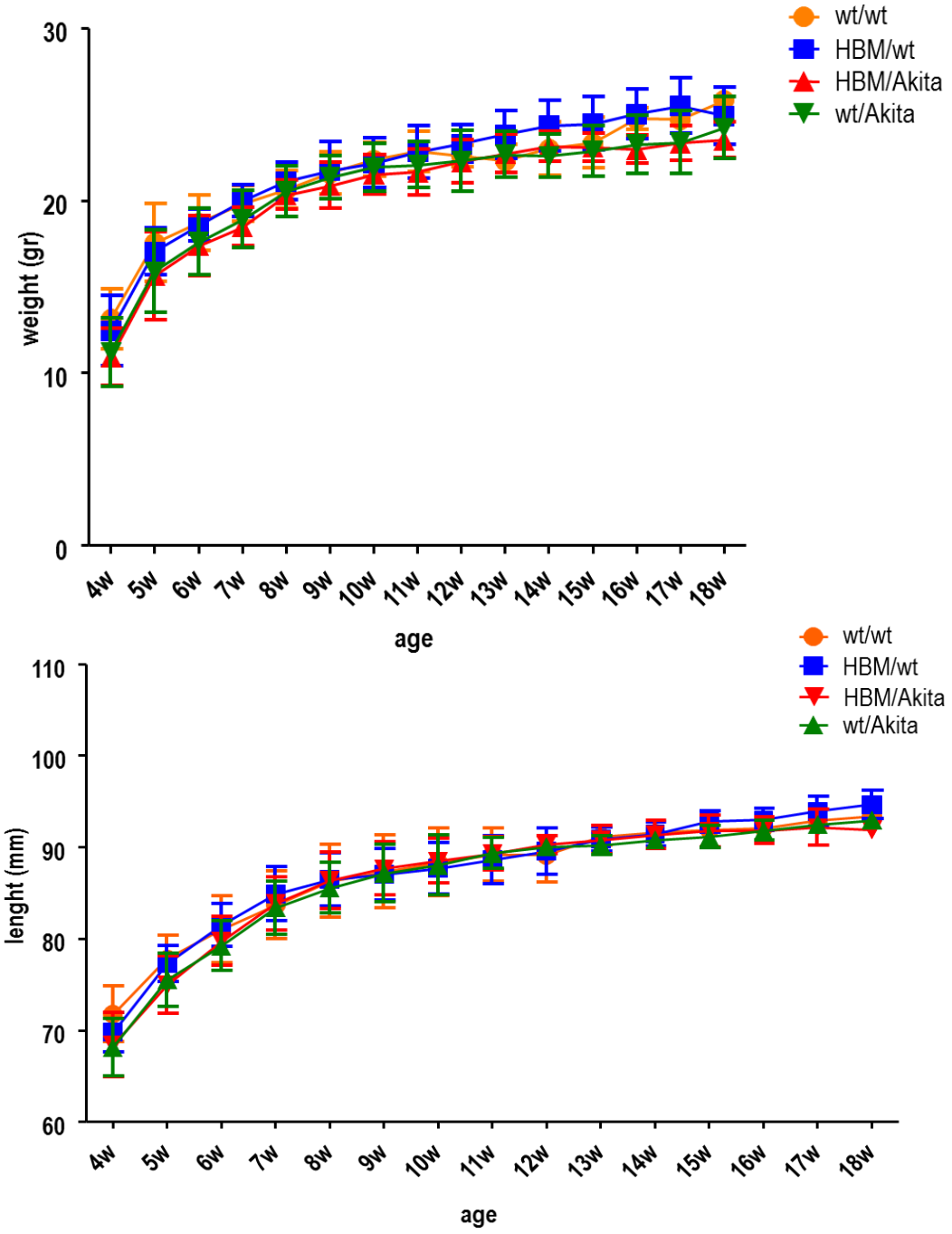

Figure 3-4 Body weight and length in male HBM/Akita mutants and control littermates (n=11-16/genotype); Two-way ANOVA and Bonferroni post-hoc analysis, p=ns

5.3.2 Bone microarchitecture

To determine whether the sclerostin-resistant *Lrp5*^{A214V} mutation, characterized by high bone mass rescues the bone loss previously documented in the *Ins2*^{Akita} diabetic mice, we longitudinally evaluated bone microarchitecture in vivo by µ-CT at 6 and 20 weeks of age in the same HBM/Akita mutants and control male mice. We found that at 6 weeks of age all cortical (Ct) and trabecular (Tb) morphometric parameters of the HBM/ Akita mutants were comparable to the HBM/wt littermates. We screened then all the mice at 20 weeks of age to evaluate the effect of the mutation on diabetes (Akita mice develop hyperglycemia at 5 weeks old). Trabecular bone volume/total volume (BV/TV) was lower in Akita compared to wild type (WT) mice (0.2±0.02 vs 0.3±0.06; $p<0.05$; n=7-10), consistently with the diabetic induced bone loss already documented (Fig.4).

Conversely, both Tb and Ct parameters were significantly higher in HBM/Akita mutants compared to Akita littermates, including total Ct area (1.5±0.06 vs 1.2 ±0.07mm^2), Ct bone area (0.9±0.1 vs 0.6±0.05mm^2), and Ct thickness (0.2±0.02 vs 0.1±0.01 mm, $p<0.001$, n=7-7). Tb BV/TV and Tb thickness were also higher in HBM/Akita mutants relative to Akita

littermates (0.4±0.05 vs 0.2±0.02; and 0.1±0.02 vs 0.09±0.03mm, respectively, $p<0.001$, n=3-5) (Fig. 4).

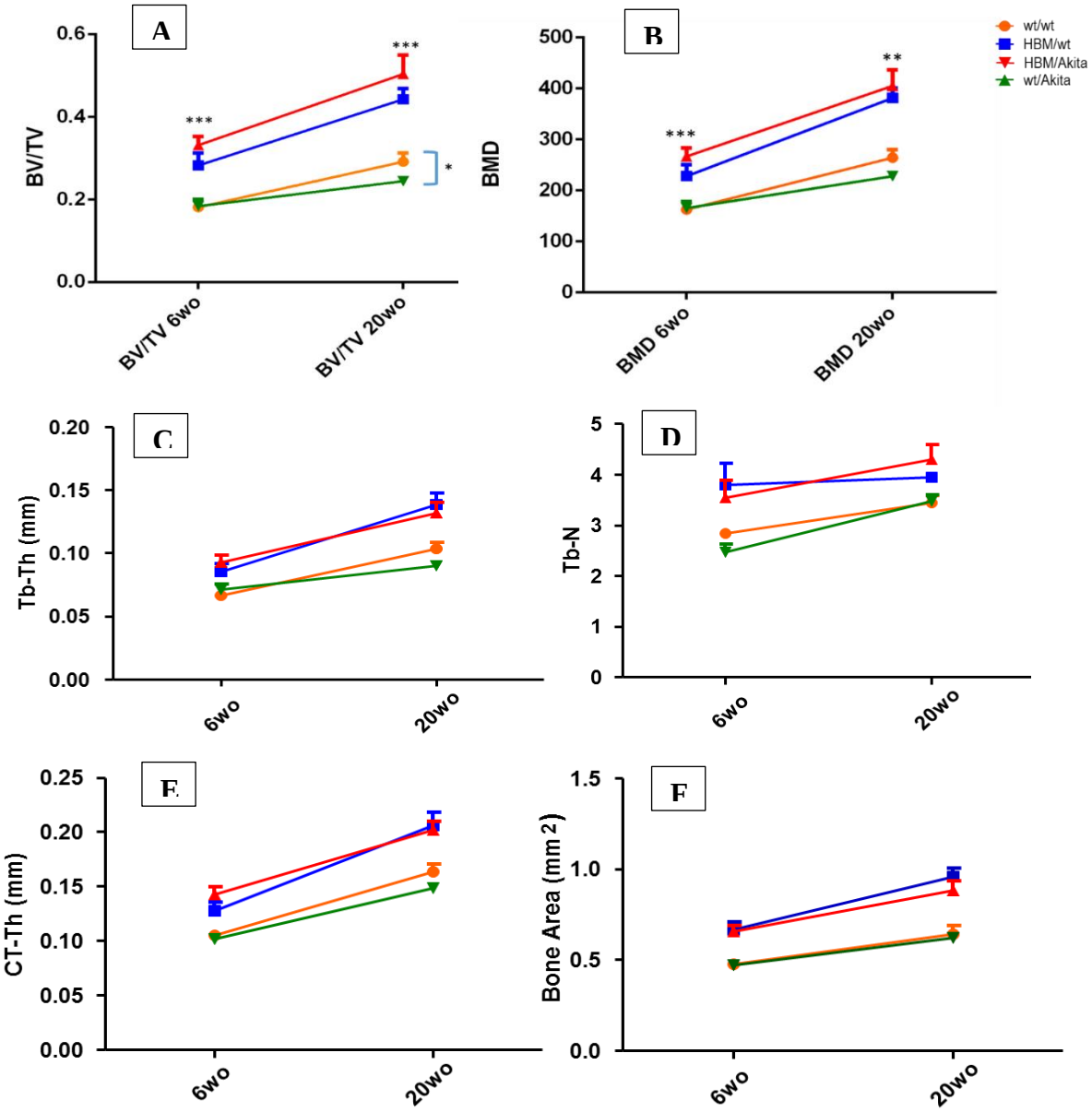

Figure 4 (A) Trabecular bone volule to total volume ratio (BV/TV), (B) trabecular volumetric bone mineral density (BMD), (C) and (D) Trabecular thickness (Tb-Th) and trabecular number (Tb-N), (E) Cortical thickness (CT-Th) and (F) cortical bone area at 6 and 20 weeks of age (n=6-10/genotype); Two-way ANOVA per repeated measures, $p<0.05$ and Bonferroni post-hoc analysis of HBM/Akita relative to littermates

There were no differences between HBM/wt and HBM/Akita either at 6 and 20 weeks of age demostrating that sclerostin insensitivity and Wnt hyperactivation develop high bone mass and protect from bone loss despite prolonged hyperglycemia.

We found no significant differences in total Ct area between Akita and WT mice, consistent with observations in humans with T1D.

5.3.3 Body composition

A phenotypic characterization of the compound mutants included monitoring of body composition by DXA. Total body bone mass by DXA was significantly higher in HBM/Akita relative to Akita littermates at 12 weeks (88.2±5.2 vs 67.9±4 mg/cm^2; $p<0.001$; n=7-11), persisting higher for up to

26 weeks (90.2±3.0 vs 70.4±1.7mg/cm^2; $p<0.001$ n=5-7) despite overt diabetes (Fig. 5).

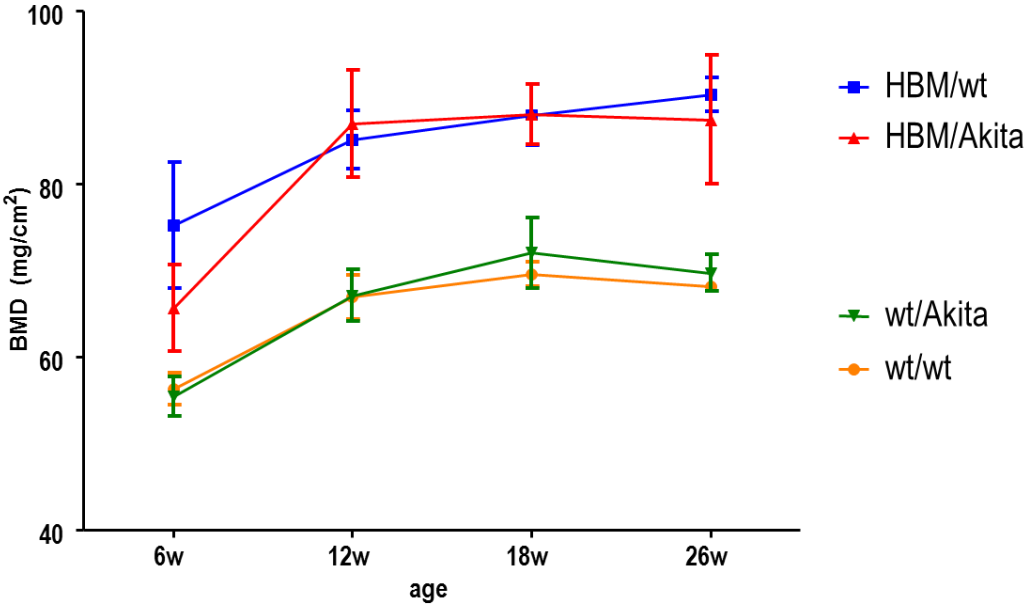

*Figure 5 Total-body bone mineral density (BMD) by DXA of HBM/Akita mutants and control littermates at 6 (3-7/genotype), 12 (6-11/genotype), 18 (2-6/genotype) and 26 (5-7/genotype). *p<0.5 by ANOVA (Bonferroni post-hoc test) relative to wt (for HBM) and Akita (for HBM/Akita)*

Conversely, there were no differences between HBM/Akita and HBM at all the age up to 26 weeks although the HBM/Akita mutants were all hyperglycemic since 12 weeks of age.

Regarding lean mass and fat mass screened by DXA, we found no significant differences in HBM/Akita mutants compared to compound mutants (Fig. 6).

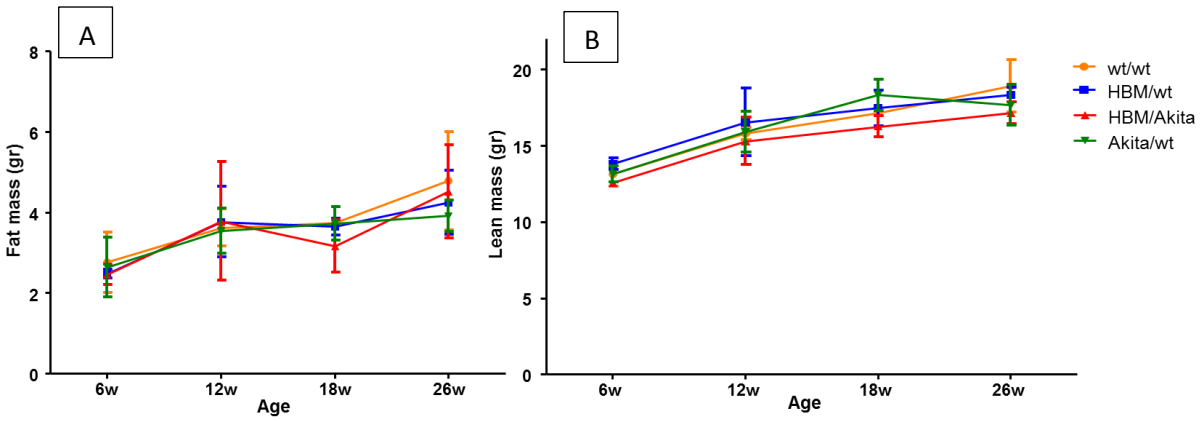

Figure 6 (A) Fat mass (gr) and (B) lean mass (gr) at 6,12,18 and 26 weeks of age by DXA (n=4-10/group); Two-way ANOVA, p=ns and Bonferroni post-hoc analysis of HBM/Akita and HBM/wt relative to littermates

5.3.4 Body fat analysis

To increase sensitivity for evaluating fat composition of sclerostin-insensitive Lrp5 mutants, we dissected the white adipose tissues (WAT), specifically gonadal and retroperitoneal fat pads, and the brown adipose

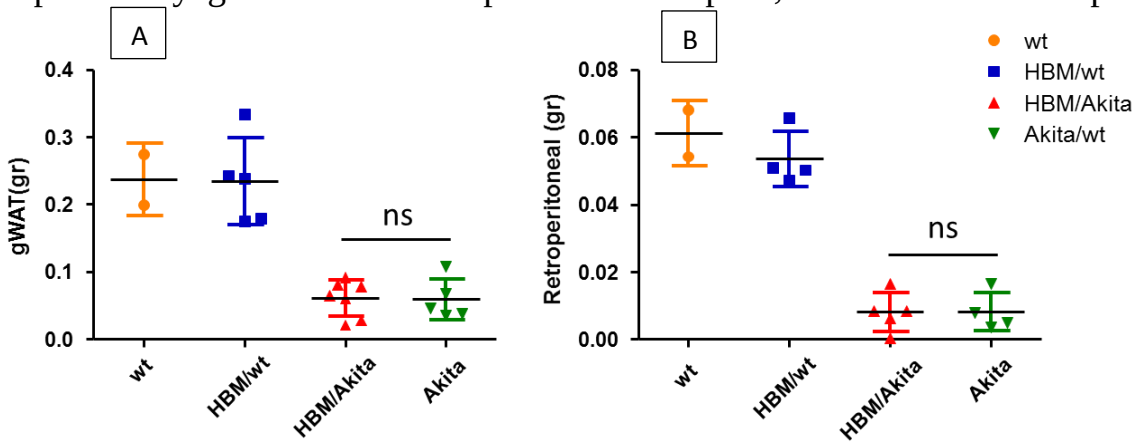

Figure 7 (A) Gonadal and (B) retroperitoneal white adipose tissues mass (gr) at 30 weeks of age (n=2-7/genotype); One-way ANOVA, p=ns and Bonferroni post-hoc analysis of HBM/Akita relative to Akita

tissue (BAT) of 30 weeks old mice. We found no significant differences in WAT between HBM/wt and wt. Consistent with the effect of hypoinsulinemia in T1D, both HBM/Akita and Akita mutants had less WAT compared to the non-diabetic mice (Fig. 7).

However, we found no differences in the WAT between HBM/Akita compared to Akita, which suggest the effect of the Ins2 mutation on the mutants that still have no insulin as the Akita mice.

Interestingly, the brown adipose tissue (BAT) mass was increased in the Lrp5 mutants (both HBM/wt and HBM/Akita) compared to littermates (Fig. 8).

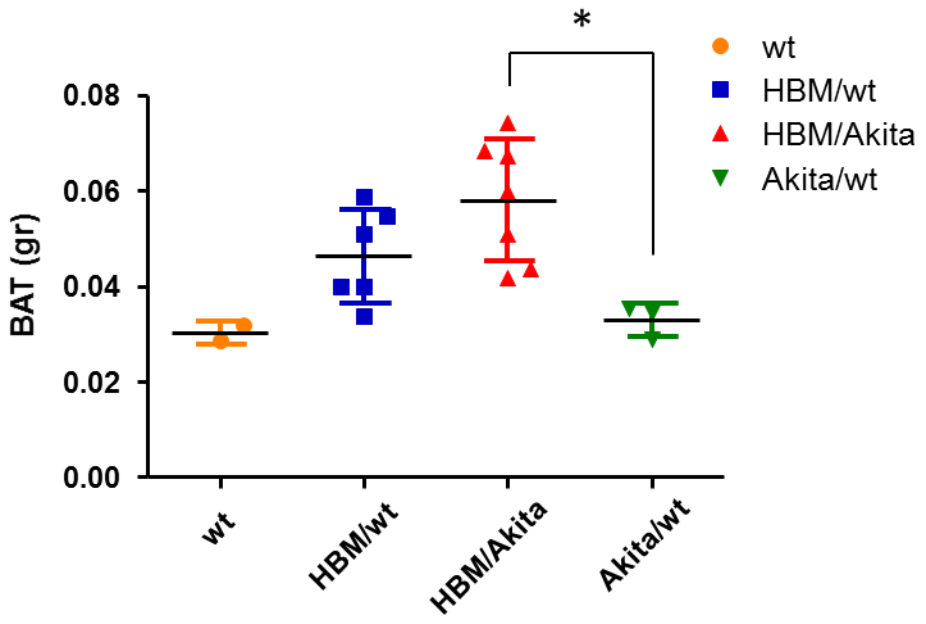

Figure 8 Brown adipose tissue (BAT) mass (gr) at 30 weeks of age (n=2-7/genotype); One-way ANOVA, $p<0.05$ and Bonferroni post-hoc analysis of HBM/Akita relative to Akita

5.3.5 Glucose metabolism

To verify the development of diabetes in compound mutants, we monitored blood glucose levels (random blood glucose weekly measurement, RBG) from 4 weeks up to 18 weeks of age (Fig. 9).

Wild type and HBM/wt mice had a stable glucose control at every time point showing no significant differences in glucose measurements between them. Akita mice, as expected, started to develop diabetes at 5 weeks of age and

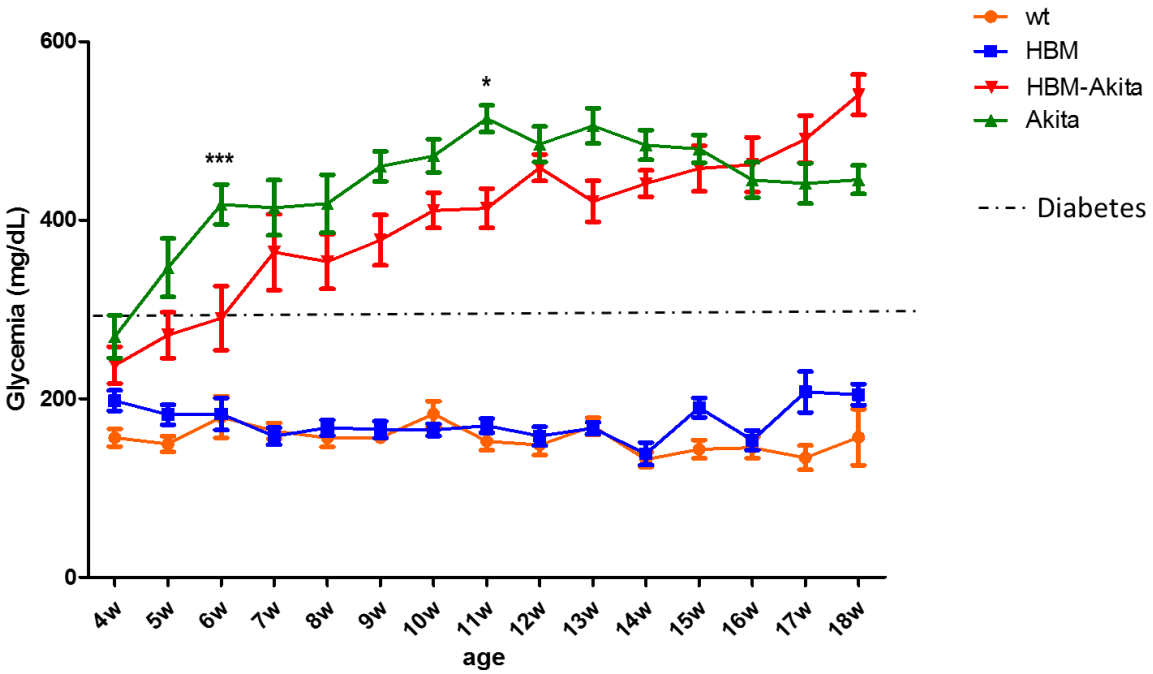

Figure 9 Random blood glucose measurements (n=10/genotype), Two-way ANOVA p<0.001 and Bonferroni post-hoc analysis relative to wt (for HBM) and Akita (for HBM/Akita)

showed very high glucose levels at every time point. Intriguingly, HBM/Akita mutants had milder RBG measurements and they developed

diabetes at a different age. At 6 weeks, HBM/Akita had significantly lower glucose levels relative to Akita littermates. Moreover, at 6 weeks only 30% of HBM/Akita mice developed hyperglycemia, compared to 90% of Akita

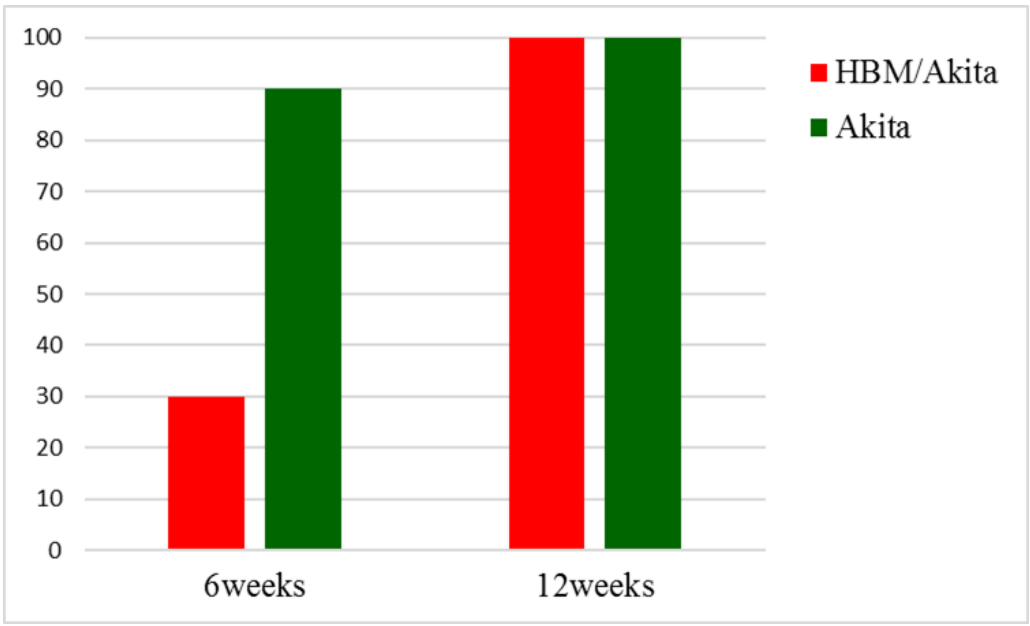

Figure 10 Percentage of HBM/Akita vs. Akita had developed diabetes at 6 and 12 weeks of age (n=10/genotype).

mice (n=10/genotype). At 12 weeks all HBM/Akita mutants were hyperglycemic (Fig. 10). In summary, both Akita and HBM/Akita mutants developed diabetes (non-fasting blood glucose >300 mg/dl), albeit with different onset timing.

Fasting blood glucose (14h overnight fast) at 6 weeks of age was even lower in HBM/Akita mutants relative to Akita (Fig. 11).

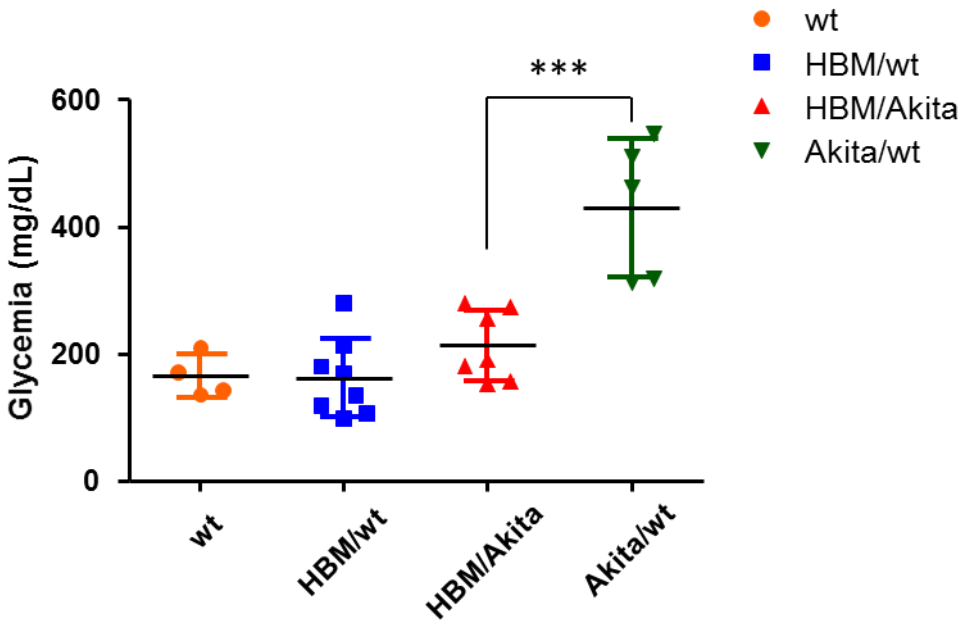

Figure 11 Fasting blood glucose after 14h overnight fast at 6 weeks of age (n=4-8/group); One-way ANOVA, p<0.001 and Bonferroni post-hoc analysis of HBM/Akita relative to Akita

Interestingly, circulating levels of sclerostin are increased in T2D relative to healthy controls and a recent study reported a negative correlation with fasting glucose and adipose insulin resistance.

To investigate the effect of sclerostin-resistance $Lrp5^{A214V}$ mutation on glucose metabolism we performed an ipGTT (at 6 and 8 weeks) and an ipITT (at 7 weeks) in all the mice.

We first examined the glucose tolerance in homozygous (HBM/HBM) and heterozygous Lrp5^{A214V} mutants compared to wild type (8-12 weeks) (Fig. 12).

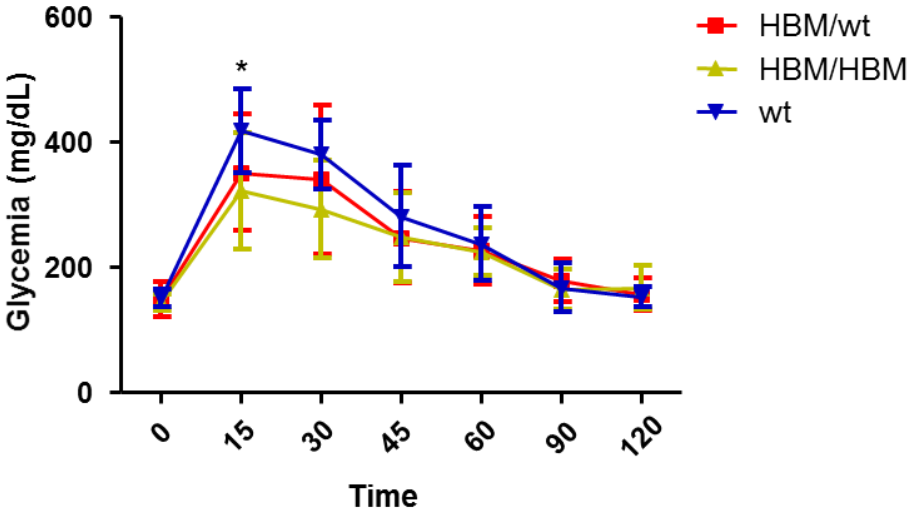

Figure 12 ipGTT after 6h morning fasting (n=7-10/genotype); Two-way ANOVA, p<0.05 and Bonferroni post-hoc analysis of HBM/HBM relative to wt

We found that glucose tolerance is significantly improved in the HBM/HBM compared to wt. There were no differences between the HBM/wt and wt mice.

To examine then if the Lrp5 mutation improve glucose tolerance also in a diabetic model, we performed an ipGTT at 4 (before the diabetes onset), 6 and 8 weeks of age in Akita/HBM mice and compound mutants.

At 4 weeks of age, glucose tolerance was no different in HBM/Akita compared to Akita littermates (Fig. 13).

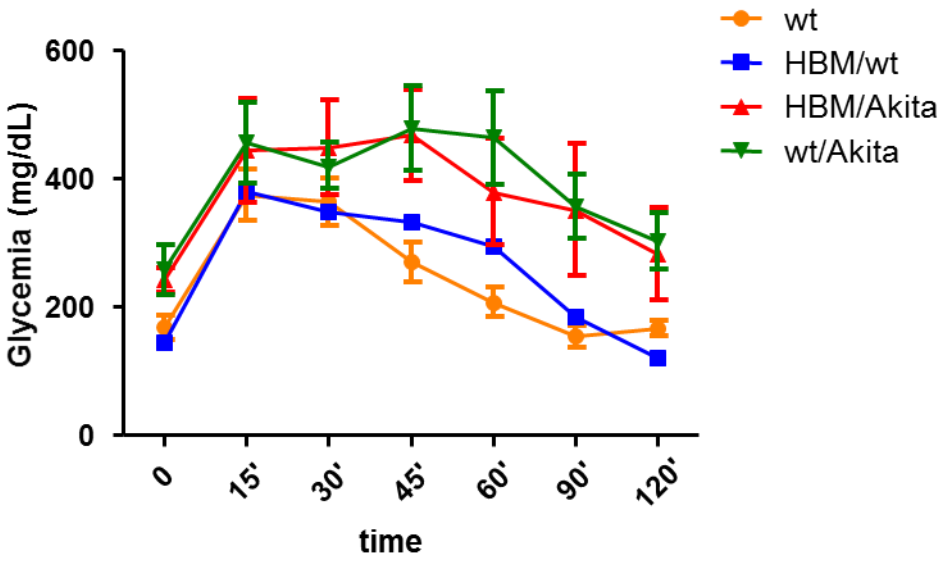

Figure 13 ipGTT after 6h morning fasting at 4 weeks of age (n=2-5/genotype); Two-way ANOVA, p=ns and Bonferroni post-hoc analysis of HBM/Akita relative to Akita

Conversely, at 6 weeks of age, when Akita mice were 90% diabetic, HBM/Akita had a significantly improved glucose tolerance compared to Akita (Fig. 14).

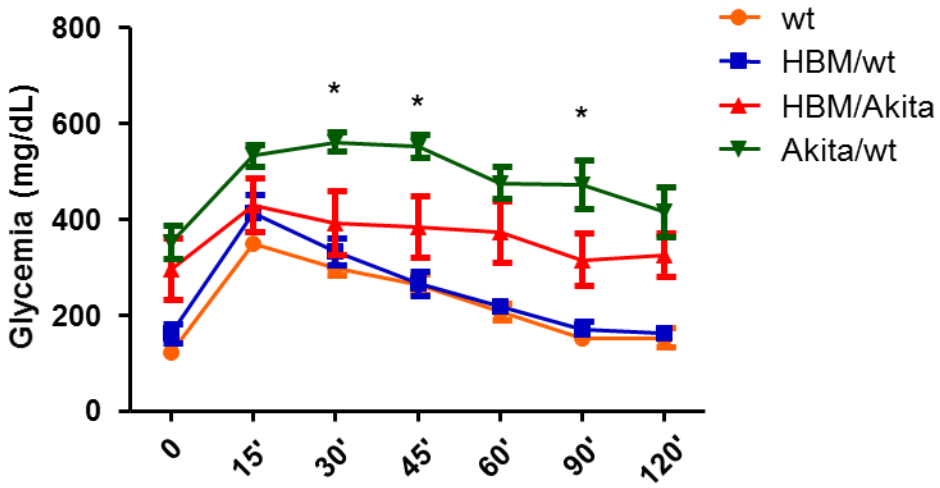

Figure 14 ipGTT after 6h morning fasting at 6 weeks of age (n=5-7/genotype); Two-way ANOVA, p<0.05 and Bonferroni post-hoc analysis of HBM/Akita relative to Akita

However, HBM/Akita at 8 weeks of age were glucose intolerants as the Akita mice (Fig. 15).

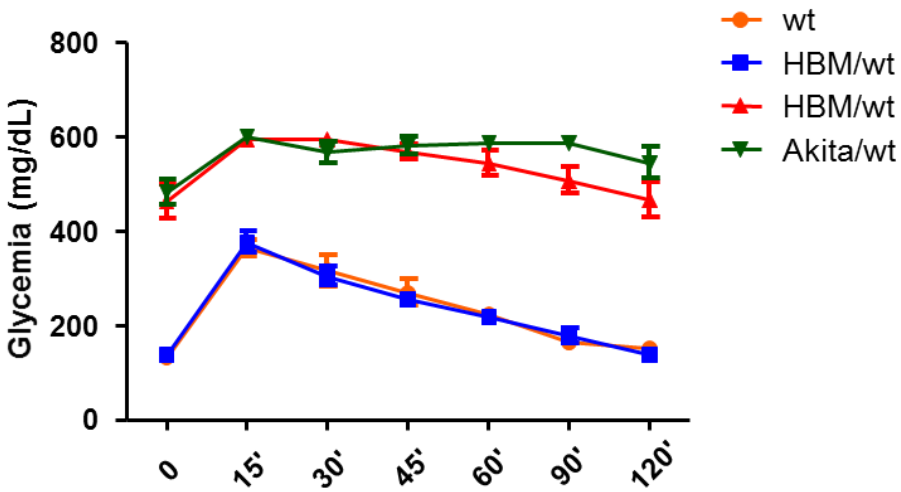

Figure 15 ipGTT after 6h morning fasting at 8 weeks of age (n=4-9/genotype); Two-way ANOVA, p<0.01 and Bonferroni post-hoc analysis of HBM/Akita relative to Akita/wt and HBM/wt

In all, analysis of area under the curve (AUC) of the ipGTT over the time (4 to 8 weeks), showed clearly the improved glucose tolerance of the HBM/Akita compared to Akita at 6 weeks but at 8 weeks they lost the glucose phenotype (Fig. 16).

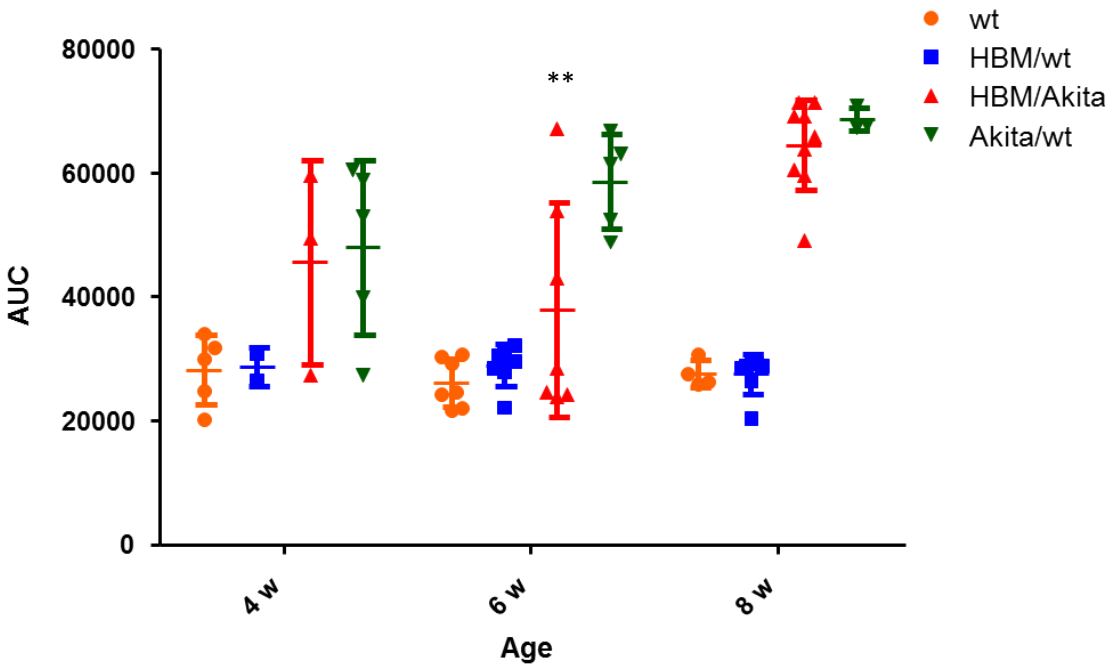

Figure 16 Area under the curve (AUC) analysis over the time for ipGTT (n=2-9/genotype); Two-way ANOVA, p<0.001 and Bonferroni post-hoc analysis of HBM/Akita relative to Akita/wt

Consistently with the improved glucose metabolism, insulin sensitivity (by intraperitoneal insulin tolerance test) was higher in the Akita/HBM compared to the Akita group at age 7 weeks (Fig. 17).

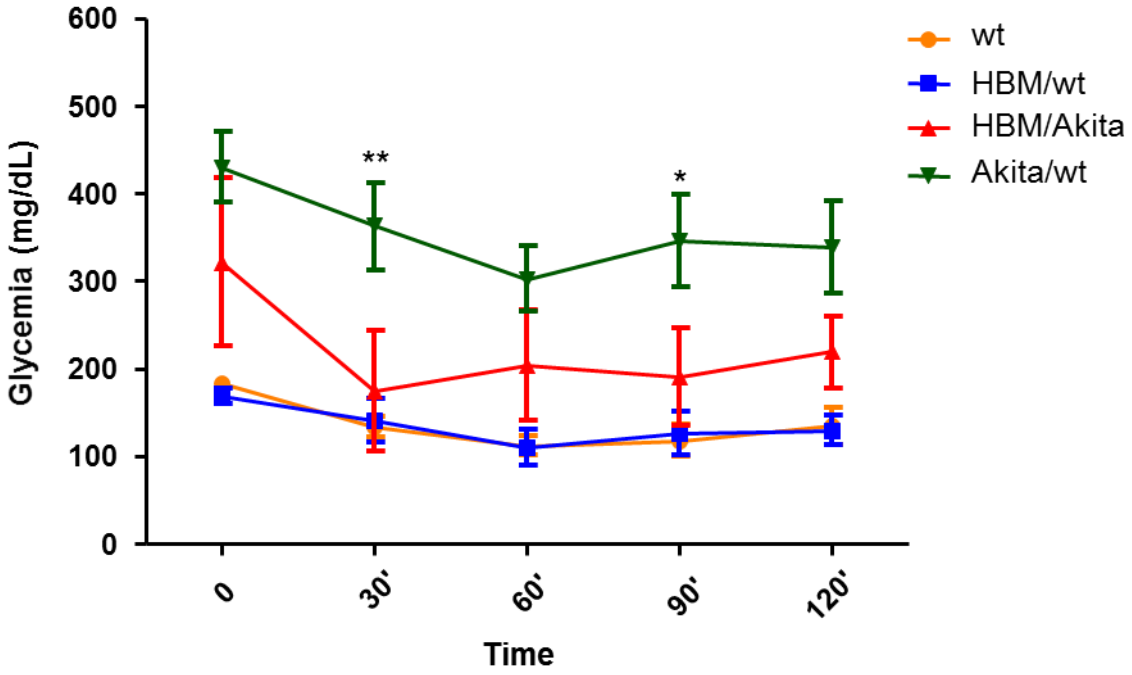

Figure 17 ipITT after 6h morning fasting at 7 weeks of age (n=4-8/genotype); Two-way ANOVA, p<0.01 and Bonferroni post-hoc analysis of HBM/Akita relative to Akita/wt

Taken together, these data imply that Wnt activation protects bone mass even in the presence of a chronic hyperglycemia and may also retard the onset of metabolic abnormalities.

5.4 In vitro Results

In order to test the effects of the Wnt signaling inhibitor sclerostin on bone marrow stromal cells (BMSC) adipogenic differentiation, we tested in vitro the capacity of multi-potent bone marrow-derived ST2 cells and primary BMSC to undergo adipogenic differentiation upon treatment with recombinant mouse sclerostin (rSOST).

First, ST2 bone-derived mesenchymal stem cells were used to assess the effect of rSOST on adipogenic differentiation. Regulation of adipogenesis was evaluated by lipid accumulation and change in cell morphology during

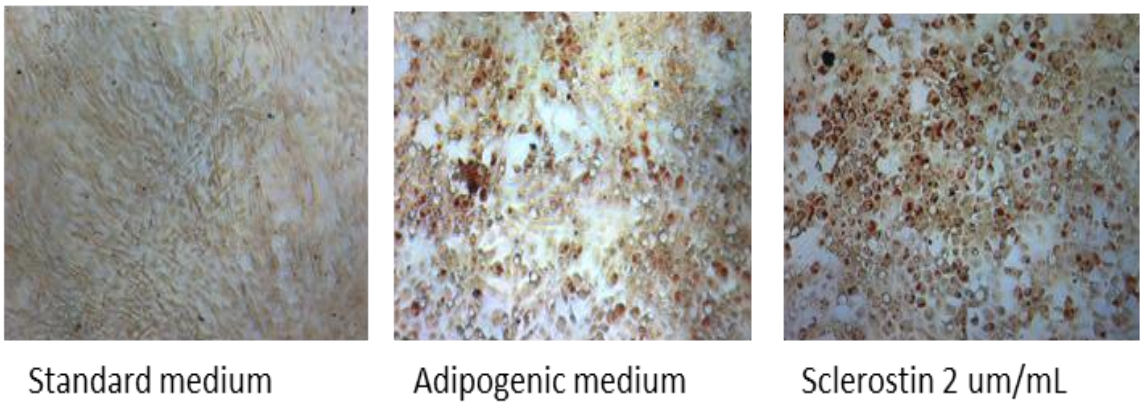

Figure 18 Representative images of the Oil-Red-O staining on ST2 bone-derived mesenchymal stem cells in alpha-MEM adipogenic media treated with mouse recombinant sclerostin (20x magnification).

differentiation in the presence of increasing concentration of rSOST (Fig. 18).

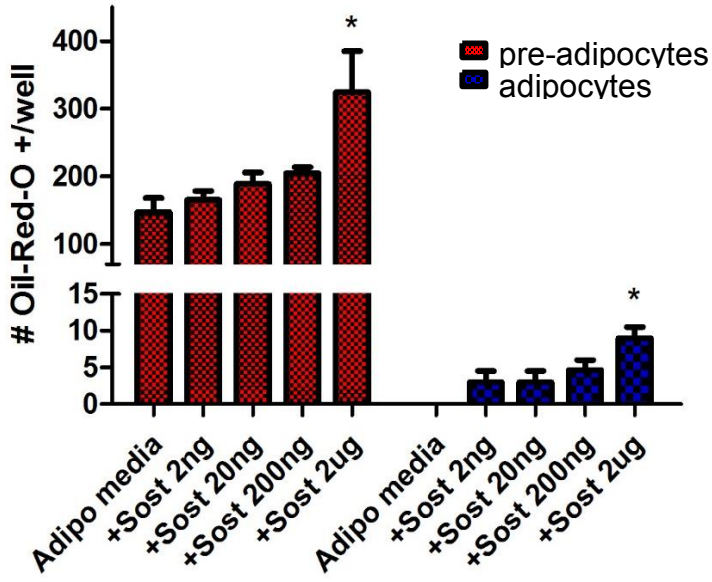

Figure 19 Adipogenic differentiation of ST2 cells in adipogenic media treated with increasing concentrations of rSOST. Number of Oil-Red-O positive cells/well (pre-adipocytes and adipocytes), (one-way ANOVA, $p<0.05$)

We observed a dose-dependent increase of the Oil-Red-O stained lipid droplets in both pre-adipocytes and mature adipocytes (Fig. 19).

To further investigate the effect of sclerostin on primary mesenchymal stem cells we used bone marrow stromal cells of heterozygous (HBM/wt) and homozygous (HBM/HBM) *Lrp5*^{A214V} mutants. We found that the number of

the Oil-Red-O positive cells was significantly reduced in cells isolated from heterozygous *Lrp5*^{A214V} and even lower in the homozygous mice (Fig. 20).

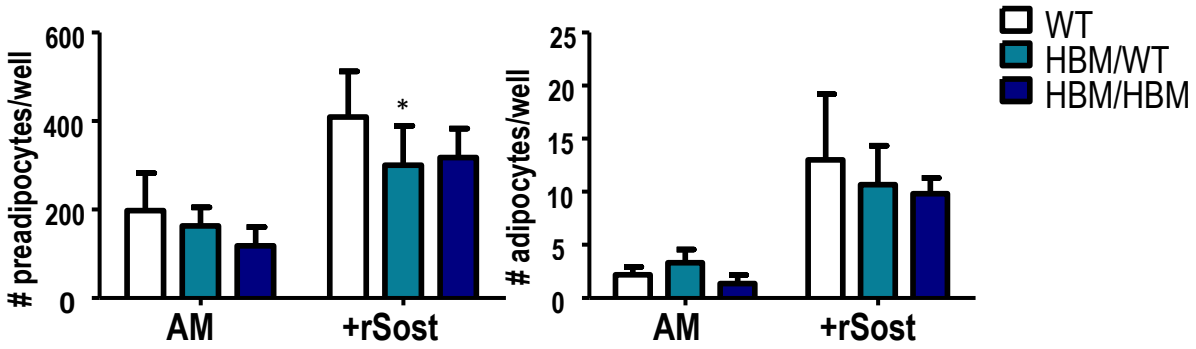

Figure 20 Adipogenic differentiation of mouse bone mesenchymal stem cells in adipogenic media vs. treated with 1um/ml of rSOST. Number of Oil-Red-O positive cells/well (pre-adipocytes and adipocytes), (Two-way ANOVA, $p<0.05$)

5.5 Discussion

Our study demonstrates that targeting Wnt signaling through sclerostin resistance in type 1 diabetes prevents bone loss despite chronic hyperglycemia. Here, we are the first to define in vivo the effect of the sclerostin resistance mutation in a T1D model and we suggest an additional role of Wnt signaling in regulating glucose metabolism increasing insulin sensitivity. Moreover, our study set the basis for further experiments to understand through which mechanism the sclerostin resistant Lrp5 mutation regulate this interaction between bone and glucose metabolism. A better understanding on these mechanisms may lay the basis for larger-scale studies to determine whether an anti-sclerostin or Wnt-targeting treatment may prevent bone fragility and improve glucose metabolism in diabetics.

We found that the double mutants HBM/Akita had high bone mass and no differences in BMD screened by DXA relative to HBM/wt starting from 6 weeks of age up to 26 weeks despite diabetes. All the 4 genotypes (wt, HBM/wt, HBM/Akita and Akita) reached a peak of bone density at 12 weeks of age and they had similar age-related progression. We found no differences measuring BMD by DXA in Akita male compared to wild type at all the time points. It has been reported in the literature that Akita mice had a lower BMD

measured by DXA at 12 months compared to sex-matched controls [114] but there were no other studies reporting body composition in male mice at a younger age. This suggests that bone loss in diabetic mice is detectable by DXA only at an older age after a prolonged exposition to hyperglycemia.

We did not find any differences between the genotypes at any time point regarding body weight and length, as has been reported [102].

To better investigate the effect of the Lrp5 mutation on bone microarchitecture in type 1 diabetes we then performed in vivo µ-CT at 6 and 20 weeks of age on the same group of mice. At 6 weeks we found that both trabecular and cortical parameters were significantly increased in HBM/Akita and HBM/wt compared to controls. We reported no differences at the same age between Akita and wt suggesting that the diabetes-induced bone loss developed after several weeks of exposition to hyperglycemia. At 20 weeks of age, we found that Akita mice had a significant lower BV/TV compared to littermates. No differences were found in cortical parameters, confirming that the bone defect in T1D is more related to the trabecular phenotype, as already reported in other studies [66, 102].

These data suggest that sclerostin resistance activate Wnt signaling protecting bone mass and preventing bone loss even in the presence of a

chronic hyperglycemia in a model of T1D where the osteopenia and bone fragility are more severe compared to T2D.

A very interesting and promising phenotype we described in this study was also the effect of sclerostin resistance on glucose metabolism. Our data showed a retard on the onset of diabetes. At 6 weeks of age 30% of the HBM/Akita mutants were diabetic compared to 90% in Akita mice and just at 12 weeks all the double mutants were frankly hyperglycemic. We also demonstrated that sclerostin insensitivity improved glucose metabolism acting on glucose tolerance and insulin sensitivity (respectively, at 6 and 7 weeks of age). HBM/Akita mutants become glucose intolerants as the Akita littermates at 8 weeks. We explain these short-term improvements on glucose metabolism with the effect of the Ins2 mutation on pancreatic β-cells. Mice become progressively insulin deficient and an improved peripheral insulin sensitivity is not sufficient to prevent the onset of an insulin dependent diabetes. Supporting our findings, in a recent paper Kim et al. also found an improved glucose tolerance and insulin sensitivity in SOST knock-out mice together with reduced white adipose depots, suggesting an endocrine function of sclerostin and the existence of a bone-adipose interaction [115]. Moreover, another study conducted on Lrp5-deficient mice showed a markedly impaired

glucose tolerance and decreased glucose-induce insulin secretion [116] strengthening our suggestions of an endocrine function of sclerostin in glucose metabolism.

Intriguingly, we also found an increased in brown adipose tissue (BAT) in HBM/wt and HBM/Akita that may explain the improved insulin sensitivity, but we didn't detect any differences in white adipose tissue (WAT) in HBM/wt relative to wt mice. More studies are needed to understand the mechanism behind this interaction sclerostin-fat.

In vitro, we confirmed the enhancing effect of sclerostin on adipogenic differentiation in both ST2 bone-derived mesenchymal stem cells and primary mouse BMSC as recently reported by Fairfield et al. [111].

We further tested in vitro the effect of sclerostin on BMSC differentiation of the sclerostin insensitive $Lrp5^{A214V}$ mutants. We reported a significant reduction on adipogenic differentiation of HBM/wt compared to controls that is even more dramatic in the HBM/HBM mutants compared to wt. As the differentiation was not completely abolished, this suggests that sclerostin could be interacting with another receptor to regulate adipogenic differentiation.

In summary, this study not only confirms the importance of Wnt signaling as the main regulator of bone metabolism, but also suggests the existence of a bone-glucose regulating interaction. Furthermore, we introduced the endocrine function of sclerostin in a model of type 1 diabetes disclosing a potential additional therapeutic advantage to sclerostin inhibition.

5.6 Limits of the study

Although this study investigates in vivo for the first time the role of the sclerostin resistant mutation giving a possible alternative therapeutic target in T1D bone fragility, there were some limitations.

For the limited available time, we did not perform the in vivo µ-CT at an older age (>26 weeks) to test the effect of the $Lrp5^{A214V}$ mutation on bone after a prolonged disease duration. For the same reason, we did not investigate the effect of the Lrp5 mutation on bone strength in T1D. Because of the limited time, we did not confirm whether Wnt signaling was hyperactivated in the bone and we didn't explore the molecular mechanism linking sclerostin resistance and Wnt activation to glucose metabolism.

6. Conclusions

Bone fragility has been reported as a concern in diabetic patients, but little data is available on mechanisms and there is not a final model and a strategy to be carried out in humans.

Risk factors for fractures as glycemic control and chronic diabetic complications have been poorly explored in association with T1D patients.

Our clinical study demonstrate that T1D patients should be carefully screened for fragility fractures and we proposed risk factors for any and multiple fragility fractures to use for a model of prediction for fractures. In summary, HbA1c, disease duration, presence of neuropathy and eGFR values could be targeted for prevention of fractures in diabetes.

As suggested by above mentioned studies the pathophysiological mechanism of bone fragility may be related to an overexpression in sclerostin levels. However, the involvement of Wnt signaling has been poorly explored and clinical data are lacking. With our preclinical study we explored for the first time in vivo the effect of type 1 diabetes and sclerostin resistance on bone. We also potentially target a role of sclerostin resistance to improve not only bone fragility but also glucose metabolism. Exploring the potential key role

of Wnt pathway and the effect on marrow fat, this study may have potential important clinical and scientific consequences.

We believe that a future characterization of bone strength and Wnt-pathway in diabetic patients will provide novel insights into the physiological effect of sclerostin on bone health and may lay the basis of future, larger-scale studies to determine whether an anti-sclerostin or Wnt-targeting treatment may prevent bone fragility in diabetic patients and improves glucose control. This study provides novel scientific inputs in the relationship between glucose homeostasis and bone health and may offer new avenues for assessment and treatment of bone alterations in diabetes.

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