GLICOMETABOLIC EFFECTS ON BONE METABOLISM: FROM NEW AND DIFFERENT PATHWAYS TO NEW DIAGNOSTIC AND THERAPEUTIC ASPECTS

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Submitted for the degree of
PhD in ENDOCRINOLOGICAL SCIENCES - *XXX Cycle*
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SAPIENZA UNIVERSITY OF ROME

In collaboration with
University Campus Bio-Medico, Rome, Italy
Barts and the London School of Medicine, London, UK
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STATEMENT OF ORIGINALITY

Unless otherwise stated, the work described in this thesis was carried out at the Sapienza University of Rome and at the University Campus Bio-Medico of Rome.

The author designed the studies that are reported in this thesis and/or analyse and described the results.

I hereby state that this thesis entitled "**Glicometabolic effects on bone metabolism: from new and different pathways to new diagnostic and therapeutic aspects**" has not been submitted for a degree or any other qualification at any other university.

Giuseppe Defeudis, July 2018

ABSTRACT

Background

Diabetes and bone fragility are rapidly growing diseases, as osteoporosis could be defined as a complication of type 2 diabetes (T2D) (Epstein et al. 2016). Aging of populations worldwide will be responsible for an increased risk in the incidence of these two diseases. There is a pathophysiological link between the high fracture incidence and diabetes if compared with the non-diabetic state, has recently been recognized but not totally clear and understood. In fact, several mechanisms are involved in bone homeostasis by impairing the function of bone cells as osteocytes, as osteoblasts and osteoclasts, and/or modifying the structural characteristics of the bone tissue (Jiang and Xia 2018; Epstein et al. 2016). Furthermore, as osteoblasts and adipocytes both derived from the mesenchymal stem cell (MSC), their physiological differentiation could be modulated by several interacting pathways on which diabetes may shows its influence and disruption. Finally, is well known the alteration of different organs and systems during diabetic disease, involved in bone metabolism, as kidney, gut or vitamin D pathway. In fact, In the last few years, studies are focusing not only to clarify old pathways but also to discover new pathways among diabetes, obesity and bone metabolism, as Wnt signaling and the role of sclerostin, as irisin, as different formulations of vitamin D like calcidiol. Moreover, some complications derived from diabetes, as cardiac neuropathy, were not yet fully evaluated on their link to the axis diabetes-bone, leading a gap of knowledge to fill (Epstein et al. 2016; Napoli, Strollo, et al. 2014).

So, many questions remain regarding the underlying mechanisms for greater bone fragility in diabetic patients and the best approach to risk assessment and treatment to prevent fractures.

Specific aims

1) to evaluate the role of sclerostin in patients with type 2 diabetes and patients with LADA.

2) to investigate the relationship between irisin and body composition in subjects with osteoporosis and the impact of irisin levels on fragility vertebral fractures.

3) to evaluate the role of calcidiol on metabolic parameters and ß-cell function in subjects with impaired glycaemic control and insufficient vitamin D levels.

4) to evaluate the relationship between cardiac autonomic neuropathy and BMD in patients with diabetes.

Materials and Methods

1) This cross-sectional study included 98 T2D and 89 LADA patients from the Action LADA and NIRAD cohorts. Patients were further divided according to MetS status. Non-diabetic subjects (n=53) were used as controls. Serum sclerostin, bone formation (P1NP) and bone resorption (CTX) were analyzed.

2) In this cross-sectional study, 36 overweight subjects affected by at least one vertebral osteoporotic fracture confirmed by a X-ray vertebral morphometry and 36 overweight non-osteoporotic subjects were enrolled. Serum irisin levels were measured using an irisin competitive ELISA. We evaluated lumbar spine and hip BMD and body composition using dual energy X-ray absorptiometry. To measure and monitor daily physical activity, each subject wore an armband for approximately 72 hours.

3) It is a double-blind placebo-controlled clinical trial enrolling subjects with IGT, IFG and T2D (20) and 25(OH)D <20 ng/ml. In this study were enrolled a total of 150 subjects and followed up for 6 months. Subjects were either assigned (50 per group) to 1) daily supplementation of 50 mcg of calcidiol (Arm A); 2) 25 mcg of calcidiol (arm B); 3) placebo (Arm C). Fasting blood glucose and Oral Glucose Tolerance Test (OGTT), HbA1c, 25 (OH) D, calcium, phosphorus, PTH, calciuria, phosphaturia, total

cholesterol, HDL cholesterol and triglycerides were measured with laboratory kits used in clinical settings. Measurements of Ox-LDL, Hs-CRP, TNF-α, IL-6, esRAGE, sRAGE were performed at the laboratory. To evaluate insulin resistance were used the ISOGTT index and the evaluation of the model of insulin-resistance homeostasis (HOMA-IR). Beta-cell function was evaluated using the insulin secretion sensitivity index-2 (ISSI-2)

4) Fourty-nine people with T2D were enrolled. Tests to determine heart rate response to deep-breathing (expiratory-to-inspiratory ratio), heart rate response to lying-to-stand test (30:15 ratio) and blood pressure response to standing were performed to detect cardiac autonomic neuropathy, and dual energy X-ray absorptiometry scan of both the lumbar spine and femoral neck were performed to evaluate bone mineral density.

Results

1) T2D subjects had higher sclerostin than LADA ($p=0.0008$, adjusted for sex and BMI), even when analysis was restricted to MetS subjects (adjusted $p=0.03$). Analyzing T2D and LADA separately, sclerostin was similar between subjects with and without MetS. However, a positive trend between sclerostin and number of MetS features was seen in T2D (p for trend=0.001) but not in LADA. Subjects with either T2D or LADA had lower CTX than controls ($p=0.0003$), and not significantly reduced P1NP. Sclerostin was unrelated to age or HbA1c but correlated with BMI ($\rho=0.29$; $p=0.0001$), HDL ($\rho=-0.23$; $p=0.003$), triglycerides ($\rho=0.19$; $p=0.002$) and time since diagnosis ($\rho=0.32$, $p<0.0001$).

2) No significant correlations were found between irisin and BMD at any site and between irisin with either lean or fat mass. Serum levels of irisin were not correlated with the daily physical activity. Serum irisin levels were lower in subjects with previous osteoporotic fractures than in controls ($p= 0.032$) and the difference in irisin

levels remained significant after adjustment for creatinine (p=0.037), vitamin D (p=0.046), lean mass (p=0.02), lumbar BMD (p=0.023) and femoral BMD (p=0.032).

3) At baseline, subjects were (mean±SD) 63.8± 2.1 y.o., BMI was 27.4±1.2 kg/m2; serum glucose 115.1±8.4 mg/dL, HbA1c 6.4±0.6%, 25OHD 16.3±2.5 ng/mL. There were significant associations of 25OHD with ISOGTT (β=0.35; 95% CI, 0.14, 0.46) and β-cell function (ISSI-2; β = 0.15; 95% CI, 0.02, 0.28). At six months, 25OHD increased up to 48±3 ng/mL in Arm A (P<0.01) and to 36±5 ng/mL in Arm B (P<0.01); no significant changes in the Arm C. Subjects in Arm A had a lower risk of dysglycemia (HR= 0.85, 95% CI, 0.75-0.97 per SD increase) while no significant effects were observed in the Arms B or C. Both ISOGTT and ISSI-2 were improved in Arm A (P<0.05) while no significant changes were observed in Arm B or placebo. Serum levels of sRAGE decreased in Arm A [median 1354 (1069-1680) pg/ml (P<0.01), as compared with levels at study entry, but not in Arms B or C. No significant differences were observed for hsCRP, IL6, TNFα or lipid panel.

4) We analyzed preliminary results among two evaluations of BMD and CAN, not finding any significative difference. In fact, subjects with no CAN showed a normal BMD in 35,7% while the remaining part had osteopenia or osteoporosis (64,3%: osteopenia: 60,7%; osteoporosis: 3,6%). Evaluating BMD in subjects with CAN, 48,1 had a normal BMD while 51,9 had osteopenia (37%) or osteoporosis (14,8%).

Conclusions

1) LADA patients present lower bone resorption compared to controls, similarly to T2D. Sclerostin is increased in T2D but not in LADA suggesting possible roles on bone metabolism in T2D only.

2) The data confirm an inverse correlation between irisin levels and vertebral fragility fractures, but no significant correlation was found with BMD or lean mass. Irisin may play a protective role on bone health independent of BMD.

3) Our findings indicate that high doses of calcidiol improved indices of glucose homeostasis in prediabetic subjects and decreased circulating sRAGE levels, suggesting a positive effect also on oxidative stress.

4) No significant correlations were found evaluating BMD in subjects with T2D and CAN.

TABLE OF CONTENTS

ABBREVIATIONS

ABPM: ambulatory blood pressure monitoring;

BMC: bone mineral content

BMD: bone mineral density

BMI: Body mass index;

BSI: Bending Strength Index

CAD: coronary artery disease

CAN: cardiovascular autonomic neuropathy

CART: cardiovascular autonomic reflex test

CRFs: clinical risk factors

CSI: Compression Strength Index

CTX: C-terminal crosslinking telopeptides of type I collagen

DFF: distal forearm fracture

GADA: Glutamic acid decarboxylase autoantibodies;

HRT: hormone replacement therapy

HRV: heart rate variability

IA-2A: Tyrosine phosphatase-related islet antigen 2 autoantibodies;

IQR: Interquartile range;

ISI: Impact Strength Index

LADA: Latent autoimmune diabetes of adults;

MetS: Metabolic syndrome;

P1NP: Pro-collagen type 1 N-terminal propeptide;

PKA: protein kinase A

PTH**:** Parathyroid hormone

RBMC: regional BMC

SCD: sudden cardiac death

SHBG: sex hormone binding globulin

SMI: silent myocardial ischaemia

TBMC: body compartments on total BMC

TRAP: Tartrate-resistant acid phosphatase

CHAPTER 1

GENERAL BACKGROUND

1.1 DIABETES

1.1.1 Definition, epidemiology and classification

Definition and epidemiology

Diabetes is a chronic condition that occurs when there are raised blood levels of glucose, hyperglycaemia, due to inability of body to produce any or enough of insulin or use insulin effectively ("IDF Diabetes Atlas 8th Edition" 2018; DeFronzo et al. 2015).

Hyperglycaemia, for long term, can damage various organs and leading to complications as cardiovascular disease, nephropathy, neuropathy, eye disease, erectile dysfunction, bone fragility etc.

Diabetes is one of the fasting growing diseases in the western world and is becoming a major problem in the emerging economic nations. The numbers of patients are staggering, and the rate of new diabetics continues to grow, especially as type 2 diabetes mellitus (T2D) is associated with obesity, the metabolic syndrome (MetS), cardiovascular and diseases and which itself is becoming a global health problem. The economic burden due the disease complications is enormous and will continue to increase unless public awareness of the disease, the curbing of obesity and cost-effective measures are instituted to prevent and treat the disease.

Diabetes is a multifactorial pathology, strongly influenced by lifestyle, food style and, among people aged 20 to 79 years, in 2017 about 425 million people present diabetes. In 2045 is estimated a substantial increase in incidence, involving 629 million people, an increase of 48% compared to 2017 (figure 1,2); while, undiagnosed cases are approximately 17.2 million, equivalent to 46.3%. In the specific case of the European countries, the diabetics are 58 million and will become 68 million in 2045, with a territorial prevalence that will rise from the current 6.4% to 7.1%.

In this scenario, is not less important the economic impact of diabetes on health care costs. In fact, worldwide the economic expenditure recorded in 2014 is 612 billion

dollars with a forecast in 2035 of about 627.3 billion dollars ("IDF Diabetes Atlas 6th Edition " 2013).

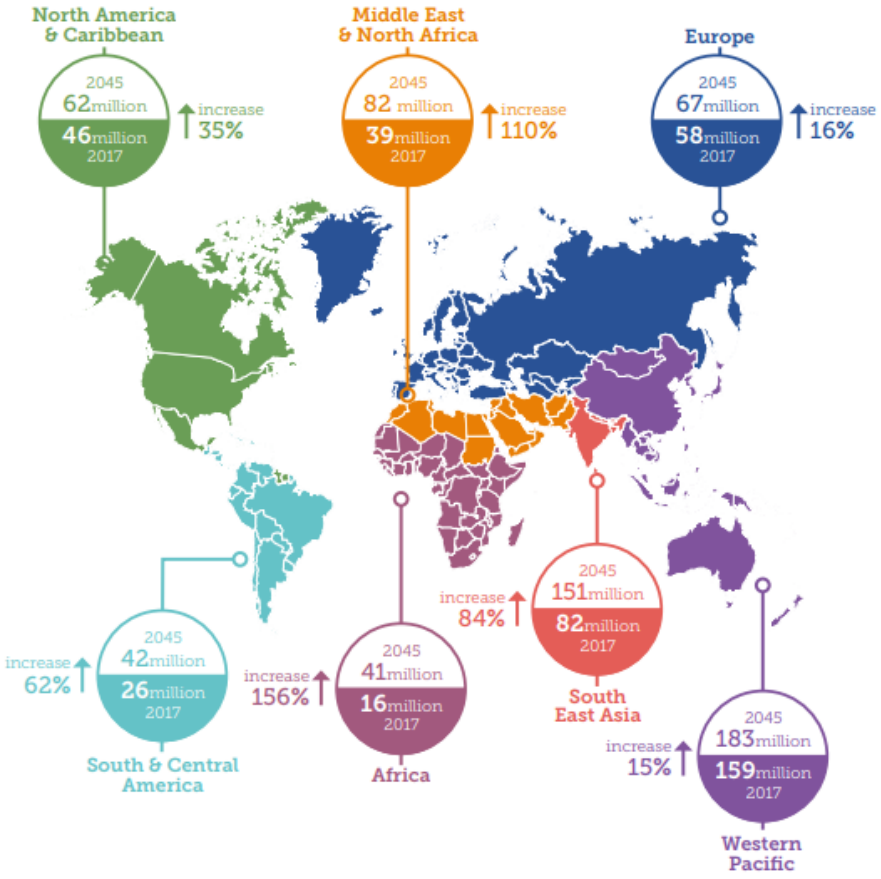

Figure. 1.1. Number of people with diabetes worldwide and per region in 2017 and 2045 (20-79 years) *(Atlas IDF 2018)*

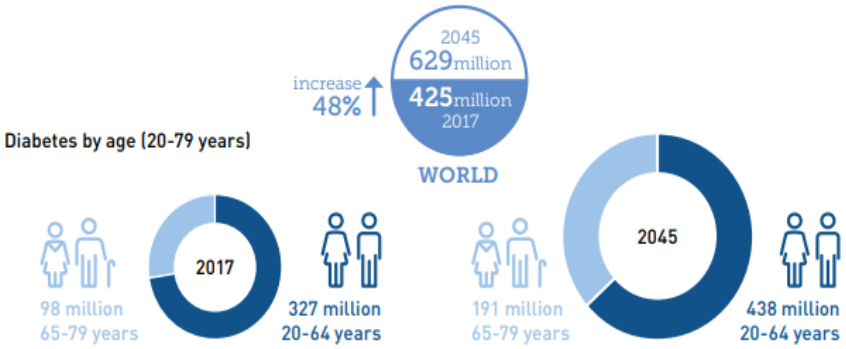

Figure 1.2. Number of people with diabetes in 2017 and 2045 (20-79 years) *(Atlas IDF 2018)*

Classification

Diabetes can be classified into four general categories:

1. **Type 1 diabetes (T1D)**: due to autoimmune b-cell destruction, usually leading to absolute insulin deficiency;
2. **Type 2 diabetes (T2D)**: due to a progressive loss of b-cell insulin secretion frequently on the background of insulin resistance;
3. **Gestational diabetes mellitus (GDM)**: diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation;
4. **Specific types of diabetes due to other causes**: e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY], diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation) ('Standards of Medical Care in Diabetes' 2018).

The majority of patients with diabetes fall into the first two classes, T1D for 5-10% of cases, and T2D for 90-95% of cases. In addition to the pathogenesis, autoimmune and metabolic, respectively, these two forms of diabetes also differ in the clinical presentation of patients and the typical course of the disease. In fact, T1D mainly affects young subjects, with a progressive increase in the incidence in the pediatric population less than 5 years, and often occurs at the onset with ketoacidosis. T2D affects subjects in advanced age, with an increase in incidence over 50 years, and shows a less rapid evolutionary trend compared to T1D. However, at the time of diagnosis, the distinction between T1D and T2D may not be easy, since T2D can start with ketoacidosis and T1D may have late onset and progression slowed over time, while presenting the immunological characteristics of an autoimmune activation towards of β-pancreatic cells. The latter type of diabetes, with the scientific support of numerous studies conducted in recent years, has acquired a significant clinical relevance, so as to merit an autonomous nosological classification in the Latent

Autoimmune Diabetes of the Adults (LADA), currently considered a variant of T1D, with late onset and will be discussed later ('Standards of Medical Care in Diabetes' 2018).

1.1.2 Pathogenesis

Insulin resistance and insulin secretory deficiency have a central role in T2D development. Visceral obesity is highly correlated with T2D and is one of its main risk factors. In the early stages of the disease, glucose tolerance remains normal, despite insulin resistance, due to compensatory activity of β pancreatic cells, increasing insulin secretion (DeFronzo, Cersosimo, and Mandarino 2000; Pozzilli, Strollo, and Bonora 2014) [1,2]. In subsequent stages, the pancreatic islets become unable to sustain the excess secretory load and glucose intolerance becomes manifest, as elevations of postprandial glycemia. Further and progressive reduction of insulin secretion, together with an increase in hepatic glucose synthesis, lead to onset of hyperglycaemia even on fasting, determining the stage of diabetes. Untreated or inadequately diabetes could lead to the natural evolution of pancreatic β-cellular insufficiency (AmericanDiabetesAssociation 2015).

Insulin resistance, a reduced ability of insulin to exert its action on target tissues, is the result of a complex interaction between genetic susceptibility and central obesity. The over physiological insulin levels normalize the blood sugar. In fact, the dose-response curves of insulin show reduced sensitivity to this hormone, by shifting the curve to the right, and also a diminished maximal insulin response by 30-60%, compared to healthy subjects. The insulin resistance activates the gluconeogenesis so the post-prandial glycemia undergoes elevation due to reduced glucose uptake by muscle tissue and adipose tissue, less insulin-sensitive tissues; moreover, fasting glucose increases, due to the induction of gluconeogenesis and glycogenolysis, with detriment of glycogenosynthesis and glycolysis. The exact molecular mechanism underlying insulin resistance has not yet been fully clarified (Khazrai, Defeudis, and Pozzilli 2014).

In terms of pathogenesis of T2D, the importance of the genetic component is demonstrated by the greater percentage of appearance of disease in monozygotic twins compared to heterozygotes. Familiarity also increases the risk of developing diabetes 2 to 4 times greater than the general population. In fact, approximately 15-25% of first-degree relatives of diabetic patients develop IGT or overt diabetes. In particular, with both parents affected by diabetes, the risk reaches 60% probability to be affected, after 60 years of age. The genes responsible for the inheritance of T2D, a complex polygenic pathology, have not all been identified. In the context of the large number of genes involved, a fundamental pathogenetic motive in mutations affecting the transcription factor PPAR-γ, involved in regulating the differentiation and maturation of adipocytes, has recently become the object of new therapeutic strategies through the drugs belonging to the class of Thiazolidinediones (Pioglitation and Rosiglitation).

T2D is considered a multifactorial pathology where genetic susceptibility is associated with environmental factors such as lifestyle, dominated by reduced physical activity, a relative increase in caloric intake and consequent obesity. This finding is confirmed by the increased incidence of diabetes in Western emigrated subjects, with a higher standard of living, or in populations with several contact with Western civilization. Glycotoxicity is a direct consequence of insulin resistance and insulin secretion deficiency and contributes to aggravating cellular β dysfunction, further compromising insulin production. The glucidic metabolism leads to the formation of oxygen free radicals, which are physiologically neutralized by cellular detoxification systems, such as catalase and superoxide dismutase. However, hyperglycemia leads to genesis of oxygen free radicals within the cytoplasm of β cells above the maximum neutralizable threshold, with subsequent functional impairment. The increase in the amount of triglycerides causes adipocyte hypertrophy, refractory to the suppression of insulin-mediated lipolysis. As consequence, there is a release of free fatty acids (FFA) into the bloodstream, contributing to the synthesis of small and dense triglycerides, VLDL and LDL, leading to increase atherogenic risk and steatosis and non-alcoholic steatohepatitis. Furthermore, the adipocytes release cytokines, as TNF-α and IL-6, compromise insulin-mediated intracellular signal transduction. Finally, thanks to

recent discoveries in the field of molecular biology, a true endocrine function has been identified in the adipose tissue: leptin, adiponectin and resistin, adipocyte secretion hormones, actively condition the glucose metabolism interfering with the sensitivity or resistance to insulin. Lipotoxicity derives from the excess of FFA released by the adipocytes, making up the central fat. So, FFA in the bloodstream would seem to be able to alter cellular β function and induce apoptosis. (figure 1.3).

Therefore, the resistance to insulin in muscles and in the liver and in β cells represent the fundamental pathophysiological defects in T2D. In addition to muscle, liver and β cell (the so-called "Triumvirate" of De Fronzo) (DeFronzo 1988), the fat cell (by acceleration of lipolysis), the gastrointestinal tract (for deficit / incretinic resistance), α cell (for hyperglucagonemia), kidney (for the increase of glucose reabsorption), and the brain (for insulin resistance), defining the "octet" of De Fronzo (Defronzo 2009). (figure 1.4).

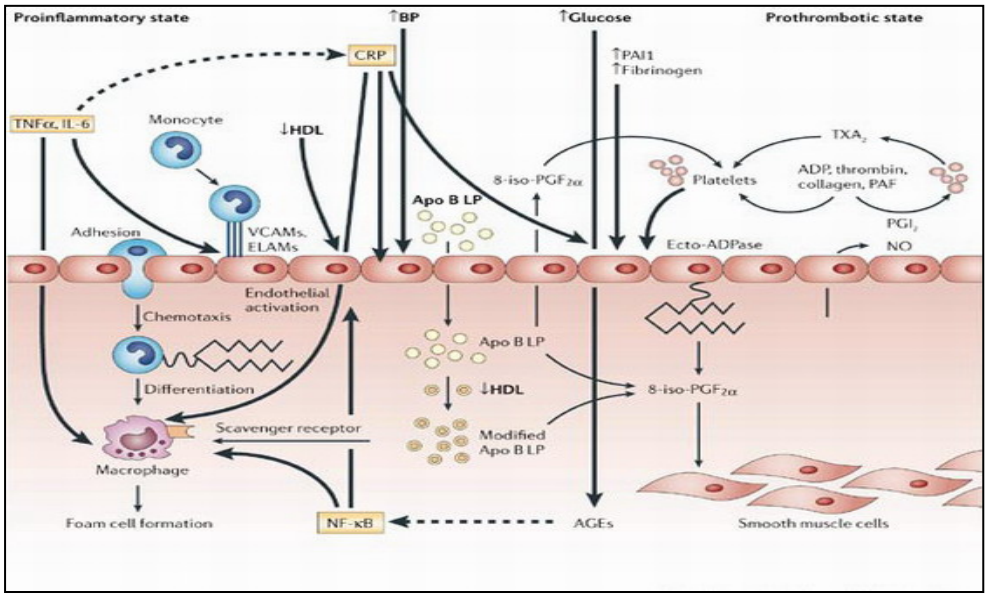

Figure 1.3 Pro-inflammatory and pro-thrombotic state in diabetes

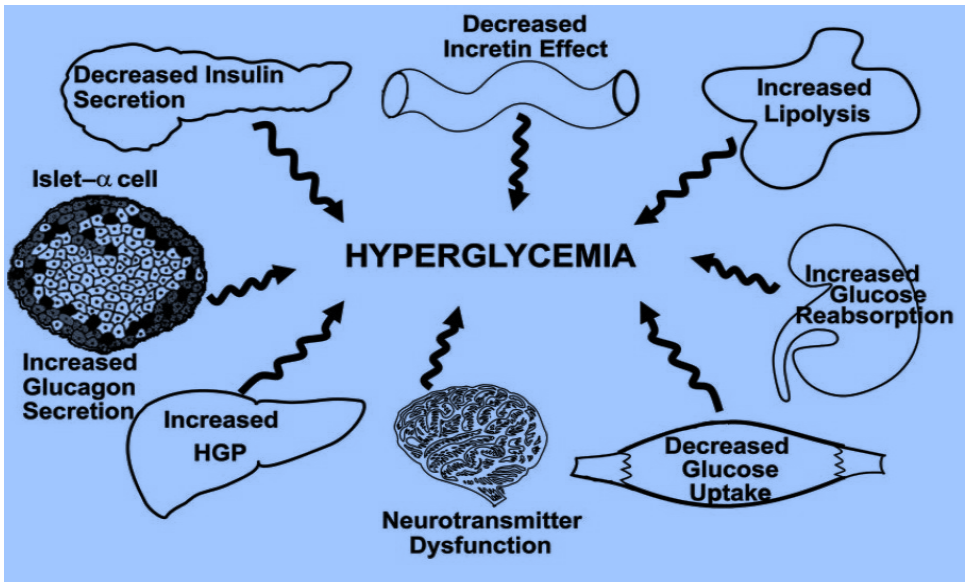

Figure 1.4 pathogenesis of T2D, the" ominous octet" by De Fronzo

1.1.3 LADA

Among different types of diabetes, there is the autoimmune type of adult-onset diabetes, characterized by insulin independence at the time of diagnosis and positivity to circulating islet-autoantibodies, most commonly anti-glutamic antibodies to decarboxylase (GAD-autoantibodies or GADA). This form of diabetes also occurs in a percentage of adult patients who initially do not require insulin therapy (Leslie, Williams, and Pozzilli 2006). For some, this form of diabetes is also called "diabetes with slow insulin dependence" (Kobayashi et al. 1993) or type 1.5 diabetes (Naik and Palmer 2003).

Therefore, LADA can and must be considered a form of diabetes clearly distinct from T2D, since it is associated with histocompatibility genes, Human Leucocyte Antigen (HLA), autoantibody positivity, reduced levels of insulin secretion, and necessary

insulin therapy after successful diagnosis and a lower correlation prevalence with MS (Alberti et al. 1998; Leslie et al. 2008; Hosszúfalusi et al. 2003). Furthermore, it was noted that subjects with LADA show lower genetic risk associated with HLA and fewer autoantibodies associated with diabetes than patients with childhood-onset T1D. From an epidemiological point of view, LADA is also influenced by geography, genetic susceptibility, environmental factors, gender and, obviously, the subject's age at the time of diagnosis. In North Europe and North America, approximately 5-10% of patients with a new diagnosis of noninsulin-dependent diabetes present LADA (Naik and Palmer 2003; Fourlanos et al. 2005; Leslie and Delli Castelli 2004).

A recent study, with subjects belonging to the ACTION LADA study group (Signore et al. 2015), showed the intercurrent relationship between insulitis and LADA, using scintigraphy (99m) Tc-IL-2 and magnetic resonance imaging at pancreas. Out of 25 patients with autoimmune diabetes (16 recently diagnosed with T1D and 9 with LADA) it was found that at the pancreatic level there was an accumulation of (99m) Tc-IL-2 in patients with autoimmune diabetes (61% positive) and in particular, in 6 of 9 patients with LADA, demonstrating that the presence of insulitis, especially in the first year of diagnosis, is similar in the two types of diabetes (Signore et al. 2015). Although LADA is associated with the same genetic and immunological characteristics of T1D, it also shares some genetic characteristics with T2D, thus raising the question of genetic heterogeneity predisposing to this form of disease.

The importance of screening for diabetes-associated autoantibodies in adult diabetes patients to identify those with LADA is highlighted by the lack of clinically distinct characteristics, different natural history from T2D and the potential need for a so-called "tailor made" therapeutic management. So, in some studies, patients with LADA show a worse glycemic control than patients with T2D, highlights the need for further therapeutic studies in this field in order to clarify classification, epidemiology, genetics, metabolism, immunology, clinical presentation and, therefore, an adequate treatment scheme (Laugesen et al. 2015). (Figure 1.5).

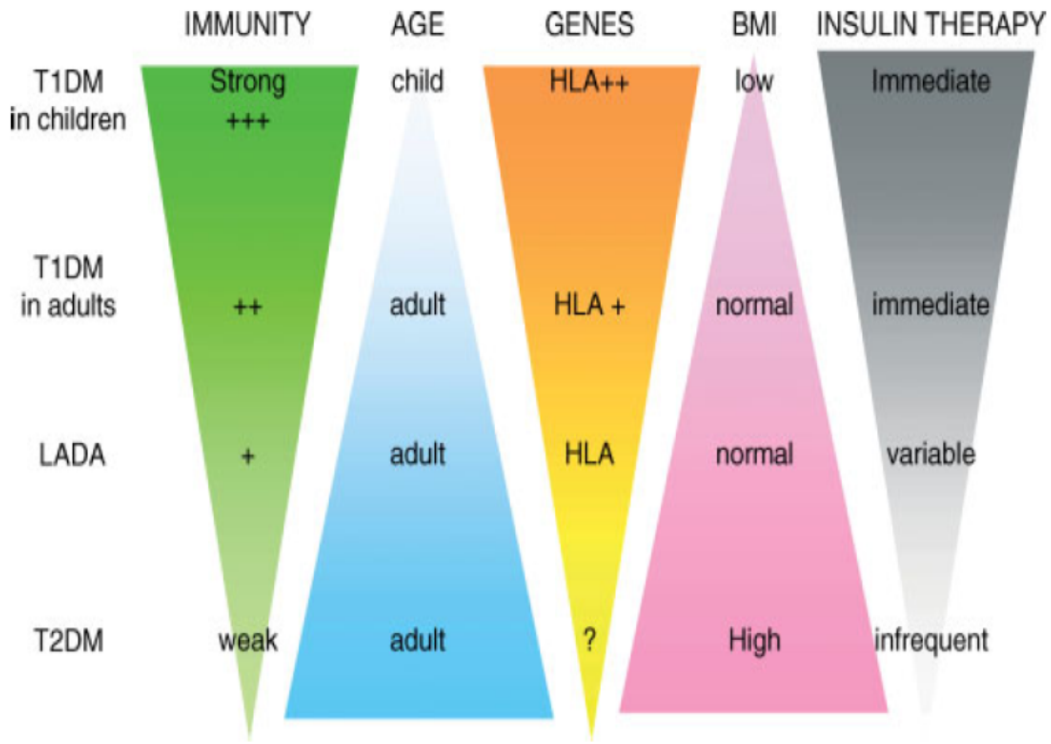

Figure 1.5 Spectrum of different form of diabetes related to immunity, age, genes, BMI, insulin therapy (from *Leslie RD & Pozzilli P, Diabetes Metab Res Rev 2008*)

1.2 METABOLIC SYNDROME

1.2.1 Definition, epidemiology and risk factors

Metabolic syndrome (MS) or insulin resistance syndrome consists of a set of metabolic abnormalities that can determine the onset of T2D and major cardiovascular events. The peculiar clinical features of this syndrome include: central obesity, hypertriglyceridaemia, reduced HDL cholesterol, hyperglycaemia and arterial hypertension. In epidemiological terms, the prevalence of MS increases with age and is greater in female sex (Hawa et al. 2009). The highest prevalence recorded worldwide, refers to an American population between 45 and 49 years of age with values equal to 60% among women and 45% among men. Compared to sex, the prevalence adjusted for age, was equal to 30% in men and 35% in women. (figure 1.6).

The global epidemic of the overweight / obesity is increasing the spread of MS. Central or visceral adiposity, in direct correlation with the value of the waist circumference, expressed in centimeters, is the dominant factor characterizing MS (Hawa et al. 2009; LeRoith and Cohen 2000).

Physical inactivity is a predisposing factor of considerable significance. The increase in deposition of central adipose tissue, hypertriglyceridaemia and hyperglycemia, the reduction of serum levels of HDL cholesterol, together with the increase in blood pressure beyond the physiological targets, are significantly related to a sedentary lifestyle, identifiable with less than 30 minutes a day of exercise.

Moreover, aging is a risk factor since about 44% of the American and world population over 50 years are affected, with a higher prevalence among women than men.

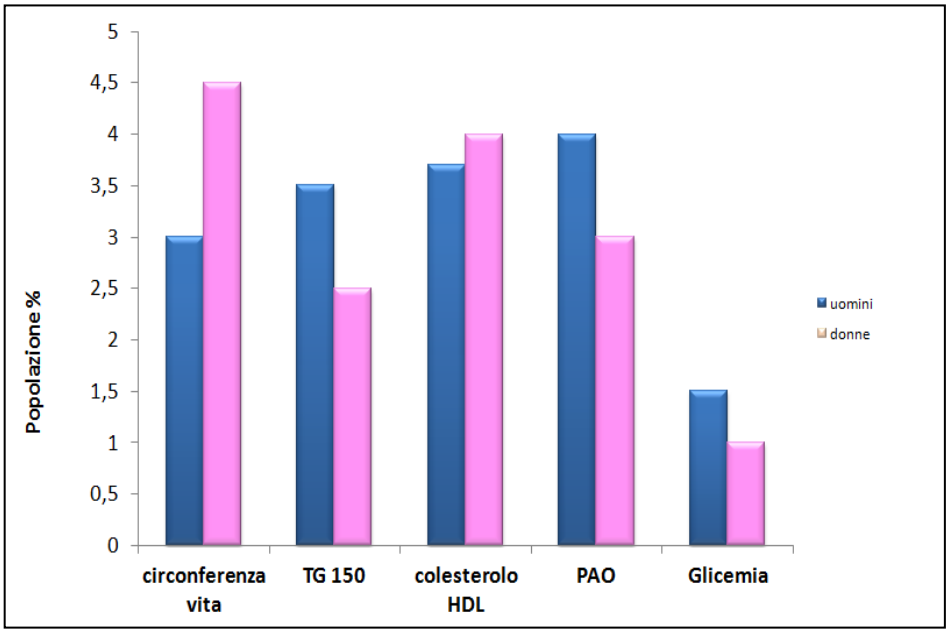

Figure 1.6 Prevalence of SM characters in population studied (*Jansen et al, Am J Clin Nutr, 2004). Circonferenza vita: waist circumference; TG: tryglicerides; Colesterolo HDL: HDL Cholesterol; PAO: blood pressure; glicemia: glycaemia*

1.2.2 Pathophysiology

Pathophysiology of MS appears to be extremely complex and not yet fully clarified, nevertheless the central element is represented by the insulin resistance, due to an anomaly of the mechanism of action of insulin on the target cells. The major initial contribution to the development of the MS is due to an excess of FFA released from the central adipose tissue in the blood. In fact, the exceeding visceral fat affects metabolism with different mechanisms linked to the lack of hormones with protective effects such as adiponectin, and its synthesis decreases in the presence of excessive quantities of visceral fat. The role of visceral fat in the pathogenesis of these phenomena can be illustrated by different ways, described below (LeRoith and Cohen 2000; Tangvarasittichai 2015).

The excess of FFA "compete" with glucose and are used in its place by the muscles, increasing glycaemia. The increase in blood glucose leads to the response of the pancreas, which increases the synthesis of insulin. Furthermore, even the elimination of exceeding insulin by the liver is not effective, so there is an increase in blood insulin in presence of hyperglycaemia, leading to insulin resistance.

Excess visceral fat liberates fatty acids that through portal circulation reach the liver, which produces triglycerides and lipoproteins rich in VLDL, special lipoproteins that can be subsequently transformed into LDL (Fig. 1.7). These represent the "bad cholesterol", which tends to accumulate in the wall of the vessels, favoring the onset of atherosclerosis. At the same time the "good cholesterol" linked to HDL lipoproteins decreases, which instead transport cholesterol from the peripheral tissues directly or indirectly to the liver for disposal. Finally, the excess of adipose cells in the abdomen can promote the onset of hypertension. In fact, in these conditions, the effect of adrenaline on the small vessels would increase, which are therefore more restricted (vasoconstriction), and the elimination of sodium by the kidneys would decrease. The mineral, remaining in the blood, tends to conserve the liquids inside the vessels. By associating these two mechanisms, is the narrowing of the conduits in which the blood runs and the increase in the contents inside the vessels, the pressure rises.

Excess of visceral fat favors the synthesis of inflammatory mediators such as interluechin-6 and Tumor Necrosis Factor (TNF) alpha and is associated with an increase in C-reactive protein, currently considered a fundamental marker of inflammation related to acute myocardial infarction. Moreover, this condition induces a decline in the synthesis of adiponectin, whose action instead contrasts the development of inflammation. For this reason, besides favoring endothelial dysfunction (is the innermost part of the arterial wall), it favors the instability of the atheromatous plaque whose rupture is at the origin of the thrombotic phenomena that reduce the flow of blood and oxygen to the heart and to the brain through the blood vessels, with the appearance of coronary syndromes and acute cerebral ischemia (Tangvarasittichai 2015) (figure 1.8).

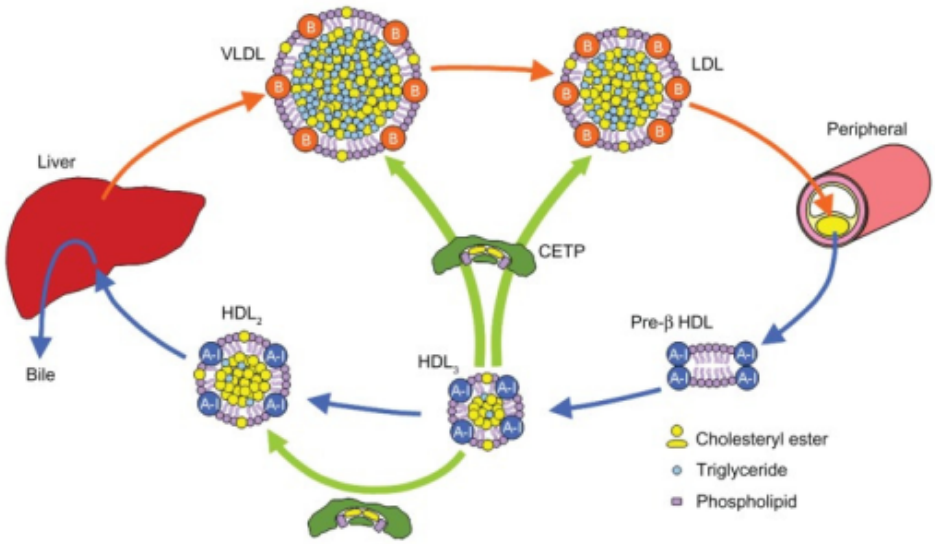

Figure 1.7 Physiology of lipid cascade *(from http://openi.nlm.nih.gov/)*

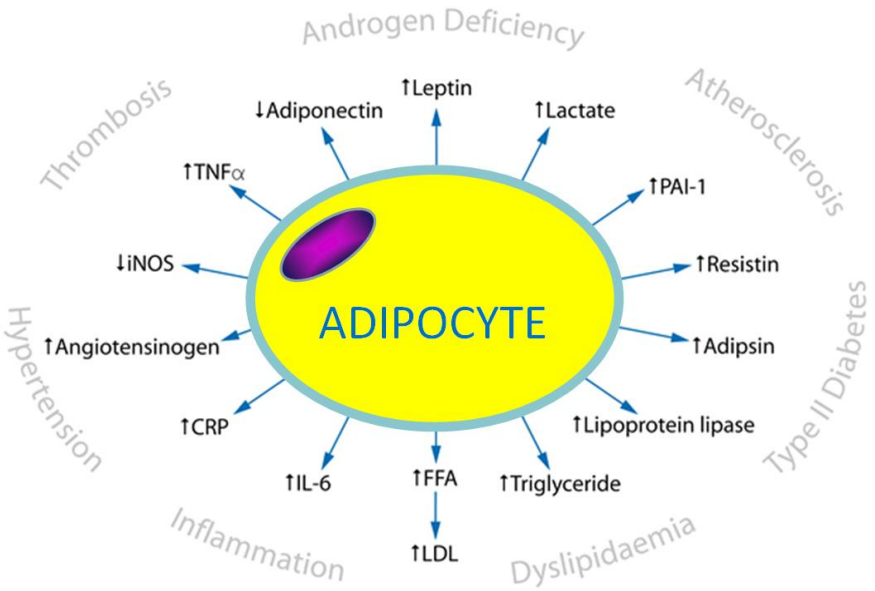

Figure 1.8 Adipocyte and physiopathological implications *(from http://openi.nlm.nih.gov/)*

1.2.3 Diagnosis

Diagnosis of MS is confirmed by the presence of specific clinical and laboratory criteria. The patient's medical history should always include the assessment of symptoms related to possible complications, such as chest pain, dyspnea, nocturnal apnea and joint pain, due to ischemic heart disease, heart failure, OSAS and gout. In women it is useful to investigate signs and symptoms linked to polycystic ovary syndrome: the prevalence of this syndrome in women with MS is 2 to 4 times higher than in general healthy population. Physical examination must always include weight and height measurement, BMI, waist circumference and blood pressure. Laboratory tests to be performed on all patients are represented by the lipid profile (total cholesterol, triglycerides and HDL cholesterol) and by glycemia, while selected patients can benefit from additional instrumental or laboratory tests such as polysomnographic examination, in case of suspected OSAS, or dosage of sex

hormones such as LH, FSH and testosterone, in case of suspected PCOS or hypogonadism.

Criteria used to diagnose MS have undergone numerous updates over the years. In 1998 the World Health Organization established that diagnostic criteria of the MS must necessarily be supported by the clinical-laboratory confirmation of insulin resistance by the detection of T2D, IGT or IFG or glucose consumption through the hyperinsulinemic euglycemic clamp technique below the 25th centile (in this test insulin was infused to maintain its plasma level constant and the amount of glucose needed to obtain euglycemia is considered a marker of insulin resistance). In addition, the required diagnostic criteria included at least two of the following: BMI> 30 Kg / m2, triglycerides> 150 mg / dL or HDL <35 mg / dL in humans and 40 mg / dL in women, values of arterial pressure> 140/90 mmHg and microalbuminuria (or albumin / creatinine ratio ≥30 mg / g).

In 1999 the European Insulin Resistance Study Group proposed alternative criteria and preferred to use the definition of insulin resistance syndrome. According to this group, the diagnosis was made in non-diabetic patients with plasma insulin levels higher than 75th centile compared to the healthy general population. In addition, for the diagnosis, at least two of the following criteria were required: waist circumference ≥ 94 cm in men and ≥ 80 cm in women, triglycerides ≥ 150 mg / dL, HDL <39 mg / dL or lipid lowering therapy, arterial pressure ≥ 140 / 90 mmHg or antihypertensive therapy and the presence of IFG or IGT.

Subsequently, the National Cholesterol Education Program - Third Adult Treatment Panel (ATP-III) [1] further modified the diagnostic criteria of the metabolic syndrome, since, unlike previous groups, it did not provide for the presence of a single patient as necessary for diagnosis, clinical-laboratory factor and in particular of insulin resistance. This choice was supported by the difficulty and inability to standardize the evaluation of insulin resistance, for which at least three of the following criteria were necessary for the diagnosis of MS according to ATP-III: waist circumference ≥ 102 cm in humans or ≥ 88 in women, triglycerides ≥ 150 mg / dL, HDL <40 mg / dL in humans and <50 mg / dL in women, blood pressure ≥ 130/85 mmHg and fasting

plasma glucose ≥ 110 mg / dL or latent diabetes. Lastly, the most recent diagnostic criteria for the metabolic syndrome were proposed in 2005 by the International Diabetes Federation and provide for the comparison of visceral obesity with waist circumference values ≥ 94 cm in men and ≥ 80 cm in women in association with two or more of the following criteria: triglycerides ≥ 150 mg / dL, HDL <40 mg / dL in humans and <50 mg / dL in women or lipid-lowering therapy, blood pressure ≥ 130/85 mmHg or antihypertensive therapy and fasting plasma glucose> 100 mg / dL or latent diabetes. The purpose of the IDF, which fundamentally differentiates the diagnostic system proposed by this group with respect to that of ATP-III and makes it in some respects more useful, is the possibility of identifying, through simple and effective tools, subjects at high risk for diseases cardiovascular and diabetes mellitus (Rodriguez et al. 2011).

Obesity

Obesity is a serious excess of body fat and, with cigarette smoking, currently represents the main risk factor for premature death in the world, with about 300,000 deaths each year. In the USA, the prevalence of obesity is increasing, because, adjusted for age, it has risen from the previous 22.9% of the years 1988-1994 to 30.5% from 2000 to 2010. At 50's, the prevalence of obesity is more than double compared to the age of 20's and, in relation to sex and socio-economic conditions, it is more frequent in women than in men. The prevalence of overweight, excess fat less severe than obesity, is also increasing and is around 64.5% of the years 2000-2010 compared to the previous 55.9% of the years 1988-1994 (Williams et al. 2015).

The etiology of obesity is complex and controversial since environmental, psychological, genetic and metabolic factors have effects on body weight. Almost all clinical cases of obesity derive from a wrong eating behavior and lifestyle of the patient: chronic hyperalimentation and inadequate exercise are the most frequent causes. The deposition of adipose tissue subcutaneously (about 50% of body fat) and visceral depends on the relationship between intake and caloric consumption. When

the caloric intake exceeds, and the adequate energy expenditure is not reached, the relative excess is deposited in the form of adipose tissue inside the adipocytes, with consequent weight increase. Therefore, overweight and obesity are caused, mostly, not by a slow metabolism of food, then by an imbalance in terms of caloric intake and elimination. Lastly, besides the absolute caloric intake, the type of diet followed by the patient is a crucial point: diets with high consumption of carbohydrates and saturated lipids strongly favor weight gain in spite of diets rich in fiber, vegetables and fruit, which reduce weight gain. Furthermore, some eating disorders may be a cause or contribute to the development of obesity (Gonzalez-Muniesa et al. 2017).

Finally, genetic predisposition is a defined risk factor for obesity, with an inheritance of BMI estimated at around 33%, but at the same time difficult to interpret. Different genetic mutations may alter the function of a discrete number of intercalated molecules in the regulation of bioenergetic metabolism, including adipokines and related hypothalamic gastrointestinal receptors involved in the regulation of hunger and satiety. Among these, there is ghrelin, produced by the stomach fasting, which evokes the sense of hunger, leptin, produced by adipose tissue, which induces the sense of satiety after the meal. The role of genetic factors seems to be involved in the regulation of basal caloric expenditure, such as thermogenesis or non-voluntary physical activity (peristalsis, respiration), while the genetic correlation inherent in the distribution of body fat is clearer.

Moreover, particular metabolic alterations, including some endocrinopathies, may be due to secondary obesity. Among these, Cushing's syndrome of adrenal and non-adrenal origin, hypothyroidism, acromegaly and hyperinsulinism, determined by insulin-secreting pancreatic and non-pancreatic neoplasia. Neoplastic or infectious brain disorders or the use of drugs, such as systemic corticosteroids, tricyclic antidepressants and monoamine oxidase inhibitors, benzodiazepines and antipsychotics, can interfere with the central ways of regulating caloric intake and consumption and determine significant weight gain (Gonzalez-Muniesa et al. 2017; Williams et al. 2015).

Diagnostic criteria

In adults, overweight and obesity are determined by calculating the body mass index or BMI (Body Mass Index), defined as the ratio between weight in kg and height in m2 of the subject. The BMI is considered normal between 19 and 24.9 mind is referred to as overweight when the value is included between 25 and 29.9. A BMI greater than or equal to 30 is indicative of obesity which is in turn distinguished in obesity of first degree (up to 34.9), obesity of the second degree (from 35 to 39.9) and obesity of III degree over 40. (Table 1)

BMI (kg / m ^{2})	DIAGNOSIS
19-24	**Normal**
25-29,9	**Overweight**
30-34,9	**Obesity-Class I**
35-39,9	**Obesity-Class II**
Over 40	**Obesity-Class III**

Table 1.1 BMI classification

Hypertension

Arterial hypertension is the most prevalent cardiovascular disease in the western industrialized population and is defined as the increase in blood pressure above the mean values in healthy subjects. Hypertension affects 50% of the population aged between 60 and 70 and more than 75% of the population over 70 years of age (Whelton et al. 2018).

Classification of blood pressure (Table 1.2):

BLOOD PRESSURE CATEGORY	SYSTOLIC mm Hg (upper number)		DIASTOLIC mm Hg (lower number)
NORMAL	LESS THAN 120	and	LESS THAN 80
ELEVATED	120 – 129	and	LESS THAN 80
HIGH BLOOD PRESSURE (HYPERTENSION) STAGE 1	130 – 139	or	80 – 89
HIGH BLOOD PRESSURE (HYPERTENSION) STAGE 2	140 OR HIGHER	or	90 OR HIGHER
HYPERTENSIVE CRISIS (consult your doctor immediately)	HIGHER THAN 180	and/or	HIGHER THAN 120

Table 1.2 classification of blood pressure (from *Whelton PK, et al. Hypertension, 2017)*

Pathogenesis

About 90% of patients have essential arterial hypertension, and not recognize a determining cause. In the remaining 10% of cases hypertension can be due to one or more of the following causes (Kaur 2014)(table 1.3):

• Renovascular: renal cystic diseases, acute and chronic glomerulonephritis, diabetic nephropathy, systemic connective tissue (Systemic lupus erythematosus, systemic sclerosis), renin-secreting tumors, renopriva hypertension, primary sodium retention, hydronephrosis, nephrovascular hypertension;

• Endocrine: pheochromocytoma, acromegaly, Cushing's syndrome, primary hyperaldosteronism, hyperthyroidism, hypothyroidism, hypercalcemia, carcinoid syndrome;

• Neurogenic: brain neoplasia, encephalitis, respiratory acidosis, acute stress;

• Cardiovascular: aortic stenosis, aortic insufficiency, coarctation of the aorta, patent arterial duct, arterio-venous fistula, Paget's disease, multiple myeloma, Waldestrom's macroglobuline, anemia, thyrotoxicosis;

• Others: alcohol, cyclosporine, oral contraceptives, tyramine, licorice, cocaine, steroids, antidepressants.

CAUSES	DISEASES
Renovascular	Urinary tract infections; urinary frequency and nocturia; obstruction, hematuria; analgesic abuse; abnormal urinalysis; family history of polycystic kidney disease; elevated serum creatinine
Endocrine	pheocromocytoma, acromegaly, Cushing's syndrome, primary hyperaldosteronism, hyperthyroidism, hypothyroidismo, hypercalcemia, carcinoid's syndrome
Neurogenic	cerebral neoplasms, encephalitis, respiratory acidosis, acute stress
Cardiovascular	aortic stenosis, aortic insufficiency, coarctation of the aorta, arterio-venous fistula, Paget's disease, multiple myeloma, Waldestrom's macroglobuline, anemia, thyrotoxicosis
Others	alcohol, cyclosporine, oral contraceptives, tyramine, cocaine, steroids, antidepressants

Table 1.3 causes of hypertension

Dyslipidemia

Dyslipidaemia is a heterogeneous group of alterations in lipid metabolism and occurs with changes in the blood concentration of circulating lipoproteins (LDL, VLDL, HDL triglycerides). Dyslipidemia could be classified in primitive, characterized by familiarity and more complex clinical management, and secondary, due to another causal pathology, for which the family history, evaluation of physiological and

pharmacological history, the objective examination, the assessment of global lipid assessment, glycemia, thyroid, renal and hepatic function is important in the diagnostic setting of the patient (Kaur 2014) (Table 1.4).

• Primitive dyslipidemias: caused by single (monogenic) genetic mutations or, more frequently, multiple (polygenic) mutations, with involvement of factors involved in weight regulation, the sense of hunger and satiety and the action of lipoproteins. The most common primitive dyslipidemia in the population, with a prevalence of 1/500 in heterozygosity and 1 / 1000,000 in homozygosis, is familial hypercholesterolemia, characterized by an increase in LDL in the blood secondary to a mutation affecting the LDL receptor (chromosome 19). The clinical effects are dominated by cutaneous, palpebral and tendinous xanthomatosis, especially yarrow, and by accelerated atherosclerosis involving the early onset of acute cardio and cerebrovascular events (heart attacks and strokes).

• Secondary dyslipidemias: caused by diseases not directly affecting the regulation of lipid metabolism. T2D, Cushing's syndrome, acromegaly, hypothyroidism, nephrotic syndrome, primary biliary cirrhosis, cholestasis and some pharmacological therapies (thiazide diuretics, beta-blockers, corticosteroids and oral contraceptives), especially when combined with sedentary lifestyle and irregular eating habits are the most frequent examples in the western population.

Phenotype	Lipoprotein(s) elevated	Type of disease
I	Chylomicrons	Familial Hyperchylomicronemia
IIa	LDL	Familial Hypercholesterolemia
IIb	LDL + VLDL	Familial combined Hypercholesterolemia
III	IDL	Familial dysbetalipoproteinemia
IV	VLDL	Familial Hyperlipemia
V	VLDL + chylomicrons	Endogenus Hypertriglyceridemia

Table 1.4 classification of dyslipidemia

Diagnostic classification

Diagnosis and typing of dyslipidemia requires the execution of blood tests with the measurement of total cholesterol, HDL and LDL cholesterol and triglycerides. The reference values of circulating lipids (Table 1.5), beyond which it is possible to identify a condition of dyslipidemia, can be summarized as follows:

type	Level	Values (mg/dl)
Total Cholesterol (mg/dL)	Optimal	<200
	Moderate	200-239
	High	>239
LDL cholesterol (mg/dL)	Optimal	<130
	Moderate	130-159
	High	>159
HDL cholesterol (mg/dL)	Optima	>50 (>60 in women)
	Low	<50 (<60 in women)
Triglycerides (mg/dL)	Optimal	<150
	Moderate	150-199
	High	200-399
	Very high	> 400

Table 1.5 normal and pathological ranges of total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides

Impaired glycaemia

Glycemia is the level of glucose circulating in the blood and normal values are typically between 80 and 100 mg / dL. The finding of increased levels of fasting glucose can be interpreted as a signal of the development of carbohydrate metabolism modifications that can be defined as "pre-diabetes" status. It is of fundamental importance, since the patients affected are able to benefit from targeted, pharmacological and non-pharmacological treatments to prevent the development of free diabetes mellitus. The impaired fasting glucose is the most early indicator of the metabolic changes that underlie diabetes and evolves first in a reduced tolerance to carbohydrates, is increase in blood sugar two hours after the meal, and then in the confirmed diabetic pathology.

1.3 DIABETES, FAT AND BONE METABOLISM

1.3.1 Introduction

Bone fragility has recently proved to be a new complication of T2D. The pathophysiological link between bone fragility and diabetes has not been fully clarified. Some mechanisms may influence bone homeostasis by altering the function of osteoblasts, osteoclasts and osteocytes, others may also exhibit changes in the structural properties of bone tissue. It is known that adipocytes and osteoblasts are derived from a common precursor, mesenchymal stem cells (MSC) and their differentiation is modulated by several interacting factors that may be disturbed in diabetes. Furthermore, other organs and endocrine systems such as the intestine, the kidney, the cardiovascular system and the regulatory systems of vitamin D are also involved in the alteration of the bone metabolism, since they are also affected by the coexistence of diabetes. Therefore, all these pathophysiological assumptions are at the base of the diabetes-related osteometabolic complications. It should also be noted that while bone mineral density (BMD) is decreased in patients with T1D, T2D is normal or even increased in T2D patients (Napoli, Strollo, et al. 2014). So, what has been recently recognized is the link between diabetes and osteoporosis with fractures being more common in diabetics than non-diabetics. Diabetes is now considered to be an extremely important risk factor for fractures, and it is advocated that diabetes be incorporated as one of the risk factors for fractures in osteoporotic and similarly fractures in diabetic patients. At the same time, many questions remain regarding the underlying mechanisms for greater bone fragility in diabetic patients and the best approach to risk assessment and treatment to prevent fractures (Epstein et al. 2016).

Osteoporosis

Osteoporosis is another disease which is increasing due to the aging populations, and the fracture burden globally is alarming. Currently, it is estimated that about 200 million people in the world suffer from osteoporosis. About 30 % of all

postmenopausal women have osteoporosis in the USA and in Europe. At least 40%of these women and 15–30%of men will present one or more fragility fractures in their rest of life. Aging of populations worldwide will be responsible for an increased risk in the incidence of osteoporosis in postmenopausal women ('Epidemiology | International Osteoporosis Foundation' 2015). The mortality after a hip fracture is around 20–50 % in the first year, besides the morbidity which also includes loss of independence. Despite effective drugs reducing the fracture burden by about 50–60 %, the disease is still poorly recognized, diagnosed, or treated.

1.3.2 Epidemiology

The prevalence among patients with diabetes treated for osteoporosis varies widely, from less than 10 % in Europe to more than 25 % in some US Medicare populations (Lochner and Cox 2013). Most, but not all, cohort studies have found increased not decreased BMD in T2D (Dede et al. 2014; Valderrabano and Linares 2018). Many studies have defined the linkage between diabetes and risk fractures in women, but only a few in men. In 2014, Napoli et al. in the MrOs study evaluated the relationship between diabetes and prospective non-vertebral fractures in elderly men. This showed an increased risk of non-vertebral fracture for a given age and BMD (Napoli, Strotmeyer, et al. 2014). In 2015, the same author about the same study presented an abstract at ASBMR and showed that T2D was not associated with increased risk of prevalent or incident vertebral fractures (VFs) in elderly men and lower BMD was associated with higher risk of prevalent VFs in diabetics as well as non- diabetics men (Nicola Napoli 2015). Also interesting were the conclusions of Abrahamsen at the annual meeting of ASBMR in 2015, who demonstrated that the increase of risk in fractures in diabetic patients does not manifest itself in a clinically significant earlier age a first major osteoporotic fracture (Bo Abrahamsen 2015). The gradient of risk for fracture for a 1 SD decrease in BMD may be steeper in diabetes, perhaps due to altered bone material quality properties, though the higher gradient of risk has also been disputed recently. The etiologies for fracture are multifactorial, and the risk of falling

is higher in diabetes, which also contributes to a greater risk in fracture than what would be expected for a given BMD (Vinik et al. 2014; Abrahamsen, Osmond, and Cooper 2015).

1.3.3 Pathophysiology

Diabetic bone is more fragile than would be expected for a given bone density by dual energy X-ray absorptiometry (DXA). The reasons for this greater fragility are not clearly understood, but there are a number of recent provocative findings demonstrating differences in bone in the setting of T2D. Cortical porosity appears to be increased with T2D (Schaffler and Burr 1988; Patsch, Burghardt, et al. 2013). With the development of high-resolution peripheral QCT (HR-pQCT), it is possible to non-invasively assess bone micro- architecture at the radius and tibia. Using HR-pQCT, Patsch et al. found increased cortical porosity in diabetic postmenopausal women with prevalent fracture, compared with diabetic women without fracture history (Patsch, Burghardt, et al. 2013). However, this study as well as another small study by Farr et al. did not find differences in cortical micro-architecture between women with and without diabetes (Farr et al. 2014). These somewhat puzzling findings appear to have been resolved with findings from the Framingham cohort, presented as an abstract at ASBMR (Samelson E and Carroll D 2014). In this cohort of older men and women, cortical porosity was higher at the tibia in those with diabetes (11.2 vs. 10.0 %; $p < 0.01$). A new HR-pQCT analysis technique, cortical laminar analysis, provides a measure of the distribution of pores across the layers of cortical bone. Heilmeier et al. (Heilmeier et al. 2015) in a recent work, using this technique of cortical laminar analysis, reported greater cortical porosity in the mid-cortical layer in diabetic women with prevalent fracture compared with diabetic women without fracture, while porosity in the endosteal and periosteal layers did not differ between these groups. These results suggest that the mechanisms underlying development of cortical porosity may differ and lead to speculation that compromised vasculature in the mid-cortical layer may contribute to cortical porosity in diabetic women at high fracture risk. Trabecular bone

score (TBS) derived from DXA radiographs is used exclusively for lumbar spines, showing a prediction of osteoporotic fractures in postmenopausal white woman with diabetes and capturing a larger part of the fracture risk associated with diabetes than does BMD (Kim et al. 2015; Leslie et al. 2013) (see below under skeletal imaging for more details). Material properties of diabetic bone may also be compromised, as indicated by greater susceptibility using micro-indentation. In a small study of postmenopausal women, Farr et al. reported greater indentation depth associated with diabetes (Farr et al. 2014). Use of microindentation in vivo is still a relatively untested technique; but an outstanding question is the degree to which indentation depth is influenced by strictly material properties vs. microstructure of the cortical bone (Napoli, Strollo, et al. 2014). Animal and clinical studies provide evidence that accumulation of advanced glycation end products (AGEs) in diabetic bone collagen contributes to reduced material properties and greater susceptibility to fracture. Higher levels of circulating AGEs are reported to increase fracture risk (Schwartz and Sellmeyer 2007). This process of non-enzymatic glycation is due to a different number of stages (Napoli, Strollo, et al. 2014). The presence of glucose induces the formation of a Schiff's base from which intermediating products containing highly reactive dicarbonyls are formed. Carbonyls and the NH2 side chain lead to the production of irreversible AGE compounds such as Nε-carboxymethyllysine and pentosidine (Singh et al. 2001). Accumulation of AGEs leads to flawed collagens by forming irreversible cross-links between the fibers in triple helix (Viguet-Carrin et al. 2006), as well as a source of reactive oxygen species (ROS), inducing structural changes via posttranslational modifications (Strollo et al. 2013). Results of this reaction include reduced strength and impaired biomechanical properties of both cancellous and cortical bone become evident (Leslie et al. 2012). In fact, in patients with diabetes and increased levels of serum pentosidine, the latter is independently associated with spine fractures (Yamamoto et al. 2008). Moreover, impaired bone formation occurs due to the action of AGE, by disturbing osteoblast function (Sanguineti et al. 2008), attachment to collagen matrix (McCarthy et al. 2004), and interfering with their normal development (Kume et al. 2005). Additionally, increased circulating AGE link

receptors, receptor for advanced glycation endproducts (RAGE), located on osteoblasts and immune cells (Manigrasso et al. 2014; Hein 2006) enhances production of inflammatory cytokines and ROS with a related increase of bone resorption due to chronic inflammation. Other important factors playing an important role are acute and chronic hyperglycemia downregulating osteocalcin (OCN) expression and activity (Botolin and McCabe 2006), decreasing calcium uptake in osteoblast cultures (Balint et al. 2001), while increasing, PPAR-γ2 expression (McCabe 2007). The picture of DO is further complicated by the effect of low vitamin D levels (see below), impaired vascular supply, and neuropathy. Well described etiological factors involve the contribution of lifestyle and anti-diabetic drugs to impaired bone quality and material properties (Napoli, Strotmeyer, et al. 2014; Palermo et al. 2015). Weight loss although strongly advocated as the first-line therapy in diabetics itself may be associated with bone loss and increase in bone resorption. A 10 % weight loss may increase levels of sclerostin (Villareal et al. 2011). This bone loss maybe attenuated or prevented by exercise (Shah et al. 2011). The list of contributing factors to fracture risk should include medications commonly used for treating diabetes such as thiazolidinediones (TZDs), sulfonylureas, and insulin (Napoli, Strotmeyer, et al. 2014; Palermo et al. 2015). Overzealous blood glucose control with consequent hypoglycemia may lead to impaired cognition, and dizziness which when combined with poor vision and peripheral neuropathy adds to the risk for falls. Recently, a new class of medication to treat diabetes, SGLT1 and SGLT2 inhibitors which act by blocking intestinal glucose uptake and renal glucose reabsorption via the sodium-glucose transporters have been linked to an increased risk for fractures. Mechanisms may include alteration of renal tubular transportation of minerals [18]. Thrailkill et al. showed that in control mice, long term exposure to SGLT2 inhibition was associated with adverse effects on the trabecular compartment of bone (Thrailkill et al. 2015). By evaluating eight clinical trials comprising 6177 patients using canagliflozin, it was found that those subjects treated with canagliflozin showed more frequent extremity fractures than the comparator (Napoli, Strollo, et al. 2014; "INVOKANA (canagliflozin) " 2013). Furthermore, in 2015, Taylor et al. underlined that SGLT2 inhibitors increase the

frequency of treatment-emergent bone fractures, and the risk of fractures seems to show an increase over time (Taylor, Blau, and Rother 2015). If the diabetic patient has impaired renal function, is on dialysis, or has benefited from an organ transplant, then renal osteodystrophy and immunosuppressant therapies (see below) will be exerting an influence on bone quality, leading to a fragility fracture.

1.3.4 Bone histomorphometry and bone markers

Bone histomorphometry which can provide information both at a static and dynamic level of bone biology is an extremely useful tool. In fact, this is considered to be the gold standard for assessing these parameters. However, patient reluctance to undergo this procedure and lack of skilled histomorphometrists limit this procedure clinically. There is now an abundance of information from pre-clinical studies showing that in diabetes, a low turnover state exists which is confirmed by low bone markers such as osteocalcin (EPSTEIN 1988). The bones also reveal a qualitative defect with the bones not being able to withstand the load and strain compared with non-diabetic bones. One of the issues with animal models (of which there are now many) is that it is hard to mimic in animal models, the clinical picture with all the complications that diabetic patients experience as these may take decades to develop (see below). Thus, the translation to fracture risk from pre-clinical models is implied but not definite.

There are several studies in which bone histomorphometry has been employed to gain a better understanding of the deleterious effects of diabetes on the skeleton. A study showed reduced bone formation in rib cortical bone from ten diabetics (Wu et al. 1970). Krakauer et al. studied cancellous bone from eight subjects, six with T2D, and two with T1D (Jesse C 1995). The bone formation rate was reduced by approximately 70 % due to reductions in both mineralizing surface and mineral apposition rate. Reduced bone formation was confirmed in a larger study of T2D (Leite and Da Silva 1995), which demonstrated marked reductions in osteoblast surface and osteoid parameters. Cancellous bone volume and cortical thickness were also decreased. However, the largest histomorphometric study of T1D (Armas et al. 2012) did not

reveal any significant differences between diabetics and controls in histomorphometric or micro-architectural parameters. In 5 of 18 patients who had a history of fracture, these subjects showed a trend towards poorer microarchitecture and lower turnover. In general, biochemical markers of bone turnover support the histomorphometric evidence of low turnover in diabetes. A recent meta-analysis of 22 studies (Starup-Linde and Vestergaard 2015) revealed that serum osteocalcin and C-terminal cross-linked telopeptide were significantly lower in diabetics (both types 1 and 2) than in non-diabetic controls. In summary, bone histomorphometry of patients with T2D shows a low turnover state with disordered micro-architecture which leads to impaired bone quality and susceptibility to fracture.

1.3.5 Vitamin D and Diabetes

Vitamin D has been promoted as an immune modulator which influences beta cell function as well as other organ systems (Hypponen et al. 2001). These immunomodulatory effects of vitamin D may require higher vitamin D levels than the classical effects on bone metabolism and calcium homeostasis. Effects of vitamin D on other health-related outcomes, e.g., neurological (multiple sclerosis) cancer, cardiovascular disease, immune modulation, and the prevention of diabetes mellitus remain controversial.

Vitamin D deficiency has been linked to beta cell dysfunction, insulin resistance, and abnormal glucose homeostasis (Sheth et al. 2015). Vitamin D receptors and vitamin D activating enzymes are present, both in insulin producing tissues and target organs. In animal models, vitamin D deficiency pre-disposes to diabetes whereas vitamin D supplementation prevents disease (Mathieu 2015; Girgis, Baldock, and Downes 2015). Furthermore, in obese patients, higher amounts of lean mass are directly linked to a lower inflammatory profile and to better insulin sensitivity, but also to the presence of higher level of vitamin D, suggesting that higher levels of lean mass, in subjects affected by obesity, correlate with a better metabolic profile (Fornari et al. 2015).

The vitamin D-calcium axis is also impacted adversely by hyperglycemia per se (Chaiban and Nicolas 2015) firstly by impairing renal calcium absorption, secondly by reducing 1,25-dihydroxyvitamin D (1,25(OH)2D3) receptor number on the osteoblast and limiting the ability of the osteoblast to synthesize osteocalcin in response to 1,25(OH)2D3, and thirdly by inactivating vitamin D receptors and 1a OHlase synthesis (Somjen et al. 2015). Despite these negative effects, vitamin D supplementation aimed at raising 25OHD levels above 30 ng/mL had no effect on insulin secretion, insulin sensitivity, or the development of diabetes compared with placebo (Davidson et al. 2003). RCTs conducted to date although suggestive do not convincingly show positive effects of vitamin D supplementation on the prevention of either T1D or T2D (Issa, Zantout, and Azar 2015). These trials are often limited by small size, short duration of follow-up, or lack of a control group. Also, dosage variation and relatively high baseline vitamin D levels may influence results. Thus, the role of vitamin D at present in affecting T2D and fracture is unresolved.

1.3.6 The relationship among bone cells and glucose regulation

Bone and bone cells are no longer considered merely as foundations for muscle, tendon, and ligament attachments as well as regulating calcium phosphate metabolism. There has been an evolution in rethinking the role of a whole host of functions, including cell-mediated immunology, energy homeostasis, central nervous system (CNS) functions, obesity, etc. Earlier data demonstrated that the bone forming cells are controlled by leptin in the central nervous system via a β-adrenergic axis that inhibits bone formation. However, the effects of leptin on energy and skeletal function are not without controversy and the route of administration appears to be very important in central or peripheral as the results may be opposite on the skeleton. The bone Gla protein osteocalcin has recently received prominence as a factor heavily involved in insulin–glucose homeostasis. Observations that mice genetically deficient in Bglap (encoding for osteocalcin (OC) are hyperglycemic, glucose intolerant, and insulin resistant pointed to OC as a possible link to glucose and insulin homeostasis

(Wei and Karsenty 2015). OCdeficient mice also have decreased β cell mass, leading to glucose intolerance and increased fat deposition. There appears to be a difference in metabolic/skeletal function between carboxylated and uncarboxylated osteocalcin with uncarboxylated being primarily a bone "hormone." Infusion of uncarboxylated osteocalcin (uc-OC) improves glucose metabolism and decreases peripheral fat accumulation and serum
triglycerides in normal mice fed a high fat diet, supporting the notion that uc-OC can function as a hormone that positively regulates energy homeostasis. The action of OC is dependent on its binding to a receptor in β cells, GPCR6A which stimulates insulin secretion and that insulin in turn, via insulin receptor (InsR) stimulates OC production while suppressing OPG in osteoblasts. This results in stimulation of bone resorption, which is sufficient to decarboxylate OC that in turn acts on β cells and peripheral tissues. The osteoblast appears to be integral to this regulatory loop, as ablation or activation of insulin signaling selectively in osteoblasts results in worsening or improvement of insulin resistance induced by a high fat diet, associated with decreased or increased circulating OC, respectively. The direct translation of these pre-clinical studies to humans poses a lot of questions. In fracture trials using powerful anti-resorptive agents where osteocalcin values are low or are suppressed, an outbreak or emergence of diabetes in the trial patients which one would have anticipated did not appear. Another study failed to show any changes in bone turnover in healthy and diabetic subjects by changing insulin levels. Studies have looked at levels of uncarboxylated and carboxylated osteocalcin and glucose homeostasis, and the results appear to be mixed. Many questions remain to be answered, as we are just beginning to understand the relationship between bone and energy metabolism.

1.3.7 Osteocyte and diabetes

The WNT signaling and the peroxisome proliferator-activated receptors-γ (PPAR-γ) pathways (Sadie-Van Gijsen et al. 2013; Kim et al. 2013) respectively act to maintain the balance existing between osteoblastogenesis (Gong et al. 2001; Little et al. 2002;

Gimble et al. 2006) and adipogenesis (Bennett et al. 2002; Ross et al. 2002). The reciprocal activity of these pathways could under various influences lead to the prevalence of one lineage dominating the other. The WNT signaling pathway consists of the canonical (or Wnt/β-catenin) and the non-canonical pathways (Baron and Kneissel 2013). WNT canonical pathway regulates MSC differentiation to three specific lineages, adipocytes, osteoblasts, and chondrocytes. The Wnt/β-catenin, when activated, induces osteoblast differentiation and proliferation from MSC through stimulation of osteogenic transcription factors as Runx2 and osterix (Hu et al. 2005). This cascade of events leads to an activation of a negative feedback control with Dkk-1 and sclerostin production by osteocytes. The glycoprotein sclerostin, an antagonist for the WNT/β-catenin canonical signaling pathway, produced by osteocytes and binding to LRP-5 and/or 6 on osteoblasts, exerts an inhibitory effect. In mice with streptozotocin-induced T1D, WNT signaling is suppressed but also sclerostin is downregulated (Portal-Nunez et al. 2010). In these types of mice, increased osteocyte apoptosis and lower total and nuclear β-catenin was shown (Portal-Nunez et al. 2010). Sclerostin is secreted almost exclusively by mature osteocytes to maintain an inhibition of bone formation (Canalis, Giustina, and Bilezikian 2007), until mechanical forces applied to the bone inhibit the secretion from osteocytes leading to bone formation (Fig. 1.8). Serum sclerostin levels in diabetic patients are significantly higher than in non-diabetic patients, positively correlated with HbA1c, negatively associated with bone turnover markers, and positively correlated with BMD of the lumbar spine, femoral neck, and total hip (Zhou et al. 2013). A role for sclerostin in vascular pathology has been identified. In patients with diabetes and atherosclerosis, serum sclerostin concentrations were significantly higher compared with a control group without atherosclerosis. Furthermore, it is possible to use sclerostin as a marker of increased osteocyte activity in postmenopausal patients with T2D (Zhou et al. 2013). Patients with T1D and microangiopathy showed a significant correlation with serum sclerostin levels (Catalano et al. 2014) (Fig. 1.9). In addition, some data shows that sclerostin may protect against progression of vascular complications in diabetic patients (Gaudio et al. 2014).

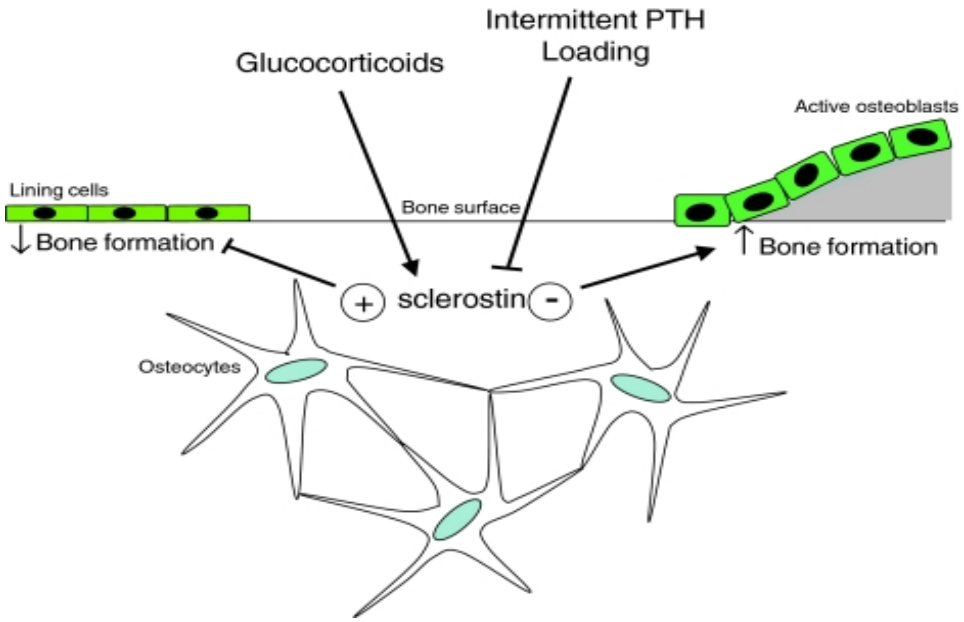

Figure 1.8 Functional mechanism of Sclerostin *(from www.naturalheightgrowth.com)*

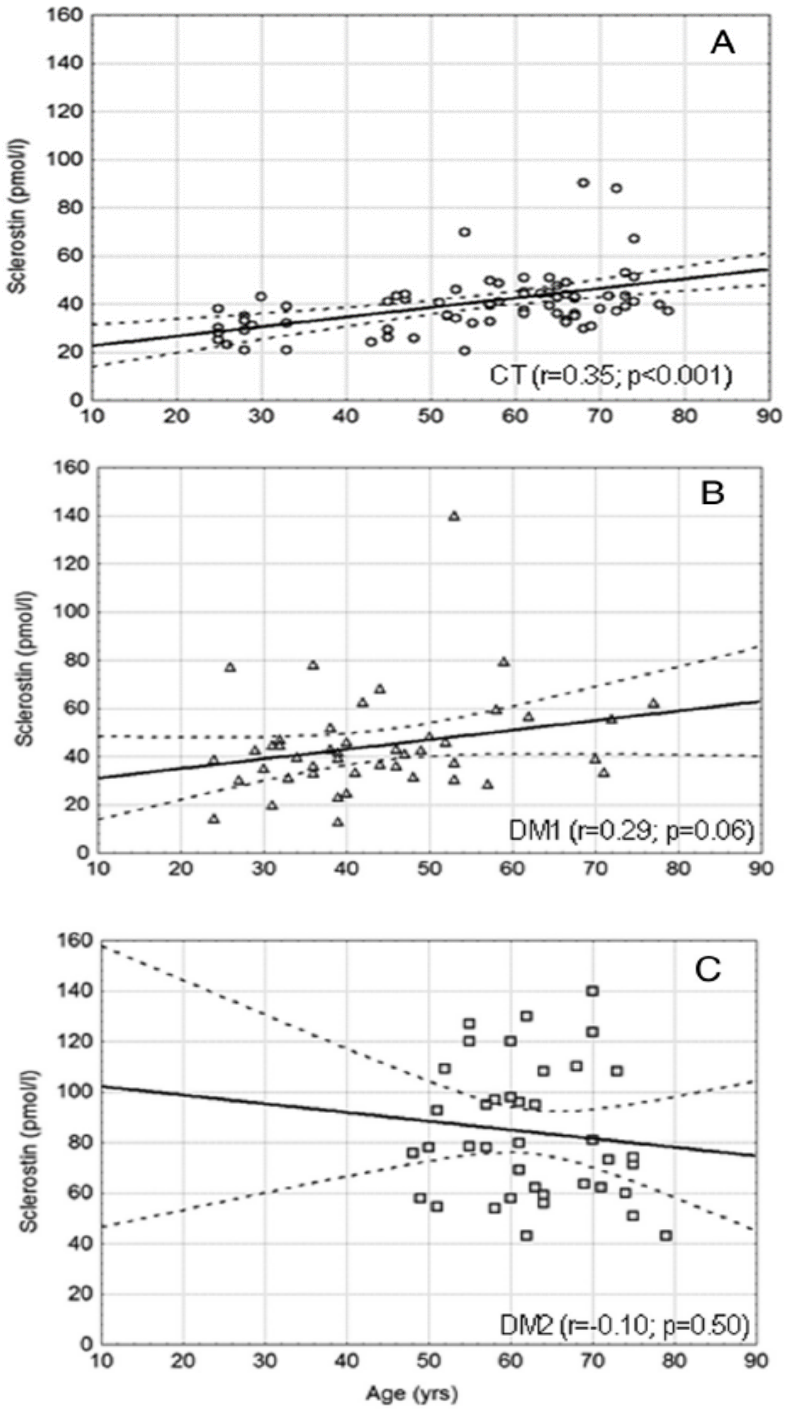

Figure 1.9 Correlation of sclerostin with age of control group, (A), in subjects with T1D (B) and T2D (C). Dotted lines are the confidence range *(Gennari et al. JCEM 2012)*

1.3.8 Fat and bone

In the USA, no state has a prevalence of obesity less than 20 %, and in Europe it is >13 %, according to the latest surveys. Asia is not immune to this with Japanese school children having an incidence of 30 % of being overweight. Furthermore, worldwide prevalence of both overweight and obesity have been steadily rising, going from 28 % in 1980 to 36 % in 2013. There is no doubt that the prime factors include eating much more of the less nutritious more convenient high calorie food, exercising less, i.e., life style changes with more TV watching, internet "surfing," etc. and genetic predisposition. Recently, was confirmed the role of physical activity and hypocaloric diet have on bone metabolism in women, recovering osteoblast activity (Bimonte et al. 2017; Migliaccio et al. 2013). Anyway, the belief that obesity has a protective role against osteoporosis has recently been revised. In fact, latest studies showed that a high level of fat mass, but also reduced muscle mass, might be a risk factor for osteoporosis and fragility fractures (Migliaccio et al. 2014).

For sure, is now clear that there are both potential positive and negative influences between adipose and bone tissue (Greco, Lenzi, and Migliaccio 2015). It will also focus on the hypothesis that osteoporosis might be considered the obesity of bone. Bone marrow fat is regulated differently from visceral fat and subcutaneous fat, and it seems that marrow fat could be a depot for energy stores in the setting of starvation or relative starvation (Devlin 2011; Scheller and Rosen 2014; Schafer et al. 2015). Furthermore, some studies suggested a role of bone marrow in the decline in bone mass after weight loss in humans (Chao et al. 2000; Ensrud et al. 2003; Villareal et al. 2008; Ensrud et al. 2005). Patients without diabetes-maintained marrow fat content after RYGB despite declines in overall fat mass. While, among patients with diabetes, RYGB lead to reduce marrow fat content maybe due to an improvement of glycemic control.

A link between adipose tissue and metabolic disorders could be related to inflammation and adipose tissue pathophysiology in the development of obesity, metabolic syndrome, and diabetes. Paracrine function, macrophage infiltration, cytokine, and adipokine secretion (Aguirre et al. 2014) (see below) have consequences

on aberrant ectopic adipose deposition, insulin resistance, and endothelial dysfunction, all promoting dyslipidemia and hyperglycemia. In fact, the skeleton is also compromised as an inverse relationship exists between bone marrow fat and trabecular bone mineral density (Greco, Lenzi, and Migliaccio 2016). Cytokines secreted by inflamed adipose tissue impair bone formation, and increased FFA and hyperglycaemia stimulate resorption. Results from more recent epidemiological studies show that obesity is not protective against fracture in postmenopausal women, at variance with previous data, confirming the hypothesis of a role for adipose tissue inflammation and dysfunction in bone formation.

The association between obesity, BMI, and bone mass becomes negative when the mechanical loading effect of total body weight is statistically removed (Zhao et al. 2007; Napoli, Strollo, et al. 2014) (see below). So, risk of osteoporosis and hip fractures is independent of BMD (Compston 2013; Compston et al. 2014), suggesting an independent effect of obesity. Therefore, there are different factors that contribute to the homeostasis between adipogenesis and osteogenesis. Fat is rich in biologically active molecules that regulate metabolic homeostasis and also interact with bone metabolism (Sadie-Van Gijsen et al. 2013).

Fractures are frequent in T1D and are often associated with marrow adiposity, emphasizing the different links between adipocyte dysfunction and mesenchymal progenitors and osteoblastic differentiation. Marrow adipose tissue (MAT) is reported to have high metabolic activity (Rahman et al. 2013; Fazeli et al. 2013; Krings et al. 2012; Sheu and Cauley 2011; Qin, Bauman, and Cardozo 2010) and responds to physiological stimuli such as calorie restriction, in turn effecting skeletal homeostasis. Endosteal adipocytes are more numerous in the marrow cavity of long bones, and their quantity and quality are dependent upon aging and disease conditions such as osteoporosis, diabetes, and many other metabolic diseases. In fact, an inverse relationship exists between bone marrow fat and trabecular bone mineral density. A number of studies are now focusing on the precise effects between MAT and white fat, which maybe a source of inflammatory cytokines (Bredella et al. 2009; Ecklund et al. 2010; Cohen et al. 2012). Some studies revealed a direct correlation between bone

mineral density and bone marrow adiposity; other studies are in opposite direction showing an inverse correlation (Di Iorgi et al. 2010; Di Iorgi et al. 2008; Wren et al. 2011; Shen et al. 2012). T1D and T2D present different amounts of MAT. In T1D patients, there is a high quantity of MAT (Moyer-Mileur et al. 2008; McCabe, Zhang, and Raehtz 2011; McCabe 2007), in contrast to the amount in T2D patients (Baum et al. 2012; Schwartz et al. 2011). Furthermore, in this last group of T2D particularly in patients with previous fracture, more saturated lipid and less unsaturated lipid is found compared with those who had not had previous fractures (Greco, Lenzi, and Migliaccio 2015) (Patsch, Li, et al. 2013). Marrow saturation or unsaturation seems to be related to the overall amount of fat, and consequently to fracture risk, but the reason remains unexplained.

Finally, overweight/obesity pre-disposes to diabetes and many obese people may have also had diabetes in the studies cited which may confound the findings.

Adiponectin

Adiponectin produced by fat tissue, is negatively correlated with visceral fat mass and BMI in humans, and low levels are shown in patients with diabetes (Weyer et al. 2001; Pischon et al. 2004; Nakashima et al. 2006; Shinoda et al. 2006).

Studies have shown that adiponectin has a pre-anabolic effect on osteoblasts and inhibits osteoclastogenesis (Williams et al. 2009; Oshima et al. 2005). Clinically, results of adiponectin and BMD are conflicting.

Richards showed that in a population of 1735 non-diabetic women, a negative correlation with BMD in postmenopausal was observed, but not in pre-menopausal women confirming that the status of menopause plays an important role (Richards et al. 2007). The use of peripheral quantitative computed tomography (pQCT), revealed that serum levels of adiponectin was inversely associated with bone mass in women but not in men (Napoli et al. 2010).

Resistin

Resistin, an adipocyte-secreted factor which acts as an inflammatory (Steppan et al. 2001) cytokine, is produced by fat tissue (Fain et al. 2003) and peripheral mononuclear cells (Patel et al. 2003) and is expressed by bone marrow. High levels of resistin are involved in the atherogenic process particularly in diabetic and obese patients (Steppan et al. 2001; Vendrell et al. 2004; Yannakoulia et al. 2003). Another crucial role is its involvement in bone remodeling, promoting both osteoblast and osteoclast differentiation (Thommesen et al. 2006). An inverse relationship between serum resistin and lumbar spine BMD (Oh et al. 2005) has been found.

Cytokines

Adipose tissue produces different cytokines which are associated with bone loss. In obesity and in T2D, there is an increase of cytokine levels directly related to marrow fat and insulin resistance. In T1D, this relation is due to the autoimmune activation. IL-6 is increased in overweight and obese individuals (Das 2001; Fernandez-Real and Ricart 2003), affecting glucose homeostasis and energy expenditure either directly or indirectly by acting on adipocytes, hepatocytes, skeletal muscle, and pancreatic β cells (Kristiansen and Mandrup-Poulsen 2005). Interesting is also the association between T2D and obesity due to a genetic polymorphism of IL-6 (−174 G/C) (Berthier et al. 2003). IL-6 (Richards et al. 2000) stimulates bone resorption via osteclastogenesis but indirect stimulation on osteoblast proliferation or differentiation may also occur (Taguchi et al. 1998; Franchimont, Wertz, and Malaise 2005). Tumor necrosis factor (TNF) stimulates osteoclastogenesis enhancing expression of RANKL in different target cells, e.g., osteoblasts, and increasing the lifespan of osteoclasts in a pro-inflammatory environment (Weitzmann 2013). Furthermore, high TNF levels can block differentiation and proliferation of osteoblasts and their progenitors, thus inhibiting bone formation.

Oxidative stress

Oxidative stress is common in diabetes and obesity (West 2000; Stadler 2012). ROS are increased in adipose tissues, controlling pro-inflammatory genes expression

(Zmijewski et al. 2007). Furthermore, ROS play direct effects on the differentiation of osteoclasts, osteoblasts, and osteocytes (Manolagas 2010; Frassetto and Sebastian 2012). An important action on the immune system indirectly promotes osteoclastogenesis by altering the immunoskeletal interface.

Sex hormones

Estrogens, via aromatase expression, increase as a function of body weight and advancing age (Nelson and Bulun 2001), leading to a positive effect on bone. Estrogen deficiency has, also, been associated with a decrease in SIRT1 (Elbaz, Rivas, and Duque 2009) (see below), a longevity factor previously associated with increased expression of the osteogenic factor Runx2 in MSC (Tseng et al. 2011) and leading also to an increased oxidative stress within bone tissue. MS is associated with higher estradiol levels, with a protective effect to bone. High visceral adipose tissue (VAT) is linked to higher risk of MS. In obese men with MS, low estradiol levels promote a negative effect on bone (Ornstrup et al. 2015).

Testosterone is another sex hormone where studies are positive, negative, or inconclusive, regarding diabetes and bone. Wang et al. demonstrated that in obese patients with MS and T2D and low levels of testosterone, they present with an increased risk of cardiovascular disease and loss of sexual function (Wang et al. 2011). Furthermore, testosterone prevents osteoclastogenesis, in an osteoblasts dependent way thus low levels would favor bone resorbtion (Michael et al. 2005). However, lack of consensus of normal age-related values, assay variability with different technologies has not helped define its role in obesity MS and osteoporosis.

Physiopathology

Osteoblasts and adipocytes derive from a common mesenchymal stem cell. While osteoblastogenesis is induced by the Wnt/-catenin signaling pathway, peroxisome proliferator-activated receptor gamma (PPAR- ɣ) is responsible for the differentiation of adipose tissue. In fact, bone marrow-derived mesenchymal stem cells treated in vitro with PPAR- and interleukin-1 (which suppresses its function) showed an inhibition of

the adipogenesis pathway and a switch to the osteoblastogenesis one, confirming PPAR-as an essential component of adipose tissue differentiation (Takada et al. 2005). PPAR- activity could thus be involved in the age-related bone marrow fat accumulation associated with suppressed production of osteoblasts and decreases in bone mass (Moerman et al. 2004). Moreover, PPAR-mRNA expression in adipose tissue is increased in obese subjects, suggesting that its more intense activity may be involved in reduced bone formation (Pei et al. 2004; Vidal-Puig et al. 1997). The activity of PPAR- also appears to be implicated in body fat distribution according to evidence from animal studies (Kirkland et al.2002). In fact, not all fat depots are the same: location (Sepe et al. 2011, Goodpaster t al. 2000) and type (Yim et al 2007) of excessive adipose tissue, rather than simply total body adiposity, may be crucial in the systemic increase of circulating cytokines and the upsurge of metabolic diseases such as diabetes (Yim et al 2007, Wronska et al.2012).

Subcutaneously stored adipose tissue depots, particularly those in the gluteal-femoral region, are negative predictors of metabolic syndrome and appear to be cardioprotective (Ryan et al. 1999, Snijder et al 2005). However, those stored in ectopic locations such as muscle, liver and abdominal cavity are linked with chronic inflammation (Yim et al. 2008, Cartier et al. 2009), impaired glucose tolerance (Addison et al 2012, Prior et al 2007), increased total cholesterol (Yim et al. 2008, Cartier et al. 2009, Dubè et al. 2011) and decreased strength and mobility in older adults (Durheim et al. 2008, Goodpaster et al. 2001). Advancing age results in a redistribution of fat depots, despite stable or decreasing overall fat, with adipose storage sites switching from subcutaneous locations to more harmful ectopic ones (Sepe et al. 2011, Yoshida et al. 2012, Hughes et al. 2004). This process is also known as “the overflow hypothesis” (Raguso et al. 2006). Moreover, fat tissue location and distribution relate to several bone health parameters in healthy premenopausal women independently of obesity per se (Bredella et al. 2011, Ng et al. 2013). Recent evidence suggests that abdominal fat, VAT and bone marrow adipose tissue are associated with lower BMD, greater cortical porosity, lower bone formation rate and lower bone trabecular volume and stiffness. In contrast, subcutaneous adipose tissue (SAT)

appears to be protective or neutral regarding bone health. A shift in allocation of resources from bone to other compartments and vice versa is mediated by a cross communication between all fat compartments, several organs and bone tissue. The endocrine system, inflammation, and adipokines may be some of the components of such coordination.

It is known that during perimenopause a gradual decrease in estrogen levels occurs. The link between estrogen deficiency and accelerated bone loss has been well documented. Obese women show lower serum levels of SHBG thus leading to higher levels of free hormones compared with normal-weight women (Haffner et al. 1989). Higher adrenal production of androstenedione with a subsequent increased pool of precursors ready for peripheral conversion is observed in these subjects as well (Mac Donald et al. 1978).

As aromatase expression also increases with age in adipocytes (Cleland et al. 1985), fat tissue activity in terms of estrogens production is one of the potential mechanisms that can explain the protective effect of obesity on bone health.

Although the relationship between estrogen metabolism and bone tissue is well established, less is known about estrogens and body composition. Napoli et al. (Napoli et al. 2012) showed that an increase in the metabolism of estrogen towards the inactive metabolites is associated with lower body fat and higher lean mass. These results suggest that a subset of women with a specific pattern of estrogen metabolism may be somewhat protected from obesity, leading to both advantages and disadvantages of this condition.

Research in the last decade has revealed that bone tissue has connections with several other circulating hormones (Hannemann et al. 2013). Osteocalcin (Ocn), an osteoblast-derived hormone considered a marker of bone formation but also released from the bone matrix during the resorption phase (Ferron et al. 2010), stimulates testosterone production in mice, acting on Leydig cells (Oury et al. 2011). In fact, Ocn-deficient male mice show reduced levels of testosterone, testis size and fertility (Oury et al. 2011).

Men demonstrate a correlation between age and bone loss which is apparent even though it is less marked compared to the one occurring in women (Cawthon et al. 2016). In fact, aging men present bone loss in both trabecular and cortical compartments with increased cortical porosity (Seeman et al. 2002, Sundh et al. 2015), thus increasing the risk of fracture after the age of 70 (Donaldson et al. 1990). As for women, male age-related bone loss is due to decreased circulating sex steroid hormones, necessary for bone growth and maintenance (Karsenty et al. 2012, Khosla et al. 2001, Riggs et al. 2002, Nakamura et al. 2007). Furthermore, the possible correlation between androgen deficiency and metabolic syndrome (MetS) deserves further attention (Bobjer et al. 2016, Haring et al. 2009) as it is not yet fully elucidated. Several studies have shown the beneficial effects of testosterone replacement on bone and fat mass in hypogonadic men (Yassin et al. 2016, Nieschlag 2015) confirming the necessity of filling this lack of knowledge.

Systemic inflammation due to several conditions such as aging, insulin resistance/metabolic syndrome/diabetes and sexual hormone deficiency appears to impair the balance of body metabolism leading to bone loss. The pathological process characterized by the up-regulation of the inflammatory response that occurs with advancing age due to the elevation of the main inflammatory cytokines like interleukin IL-1, IL-6 and Tumor Necrosis Factor-alpha (TNF-α) has been recently named "inflammaging" (Prats-Puig et al. 2010). This process is mainly due to reduced gonadal hormone levels and aging, conditions leading to the characteristic increase of catabolic cytokines shown in the elderly (Franceschi et al. 2007). The molecular action of TNF in bone resorption is in large measure a consequence of its ability to stimulate activation of the Nuclear Factor kappa-B (NF-κß) transcription factor. This pathway is also a great mediator of Receptor activator of nuclear factor kappa-B ligand (RANKL)-induced signal transduction, and not surprisingly TNF potently augments RANKL-induced osteoclast formation. In fact, RANKL, a member of the TNF cytokine family, has a crucial role in the differentiation of osteoclast precursors into activated osteoclasts, and it is up-regulated during the inflammatory response (Targownik et al. 2013).

Confirming that inflammation is itself capable of jeopardizing bone health, it has been demonstrated that inflammatory systemic conditions such as Crohn's disease and rheumatoid arthritis are associated with reduced BMD, osteoporosis and fragility fractures (Gautier et al. 2013). It is well established that obese subjects have lower serum levels of adiponectin compared to normal-weight individuals, and its levels increase after weight loss (Weyer et al 2001, Lenchik et al 2003). Adiponectin serum levels are inversely correlated with insulin resistance (Yamauchi et al. 2001). However, the effects of adiponectin on bone health remain controversial. Adiponectin activity favors osteoblastogenesis and inhibits osteoclast formation in vitro, potentially contributing to an increase in bone mass (Berner et al. 2004). In contrast, adiponectin knock-out mice show increased bone density, suggesting an indirect effect of adiponectin on bone tissue, possibly through modulation of circulating growth factor activity or insulin sensitivity (Wang et al. 2005). For example, this adipokine decreases circulating insulin levels, reducing its anabolic effect, which in turn might inhibit bone growth (Williams et al. 2009). Several authors have shown an inverse correlation between serum adiponectin and BMD in both women and men (Richards et al. 2007, Jürimäe et al. 2007, Peng et al. 2008). Other authors, in an Italian population of 600 elder men and postmenopausal women, have failed to confirm such a finding in men while confirming it in women (Napoli et al. 2010). Tamura et al. showed instead a positive correlation with BMD (evaluated in distal radius) in patients with T2D (Tamura et al. 2007). Given the controversial evidence currently available, further studies are warranted to understand whether the characteristically low adiponectin levels in obese subjects are protective or detrimental with regards to bone health.

Leptin is an adipokine that decreases appetite and increases energy expenditure in malnutrition and circulates at higher levels in obese subjects compared with normal-weight ones. Both negative and positive correlations between leptin and BMD have been described in humans (Kontogianni et al. 2004, Pasco et al. 2001); in fact, while leptin seems to promote the differentiation of osteoblasts (Elefteriou et al. 2004), it also seems to inhibit bone formation acting through the sympathetic nervous system and cocaine-amphetamine regulated transcript (Ducy et al. 2000). In peri-and

postmenopausal women a positive correlation between leptin and BMD and a negative correlation with markers of bone resorption have been observed (dependent on BMI and fat content) (Blain et al. 2002). The above correlations are weaker in postmenopausal women with osteoporosis, in comparison with healthy women in the same age group (Shaarawy et al. 2003). In obese postmenopausal women the correlations between leptin and BMD and bone turnover markers are stronger (mainly for bone resorption markers) than in lean women in the same age group (Holecki et al. 2010). Leptin resistance in the central nervous system may explain the previous assumption, in fact an imbalance between leptin levels in serum and cerebrospinal fluid is present in obese subjects (leptin cerebrospinal fluid levels are much lower than serum leptin levels in obese subjects compared with normal weight ones) (Shaarawy et al. 2003, Couce et al. 2001). High leptin levels in obese individuals can have a protective effect on bone tissue due to the interaction between leptin and the RANKL/ RANK/Osteoprotegerin system. It was proposed that the beneficial effect of leptin on bone metabolism was a result of the inhibition of the receptor activator of NF-κß ligand and the improved expression of osteoprotegerin (Lamghari et al. 2006).

Ghrelin is a gut-derived hormone, which increases food intake in both rodents (Shintani et al. 2001, Wren et al. 2001a) and humans (Wren et al. 2001b) and decreases metabolic rate (Asakawa et al. 2001) and fat catabolism (Tschöp et al. 2000). Ghrelin also appears to be involved in bone metabolism via modulation of osteoblast differentiation and function (Fukushima et al. 2005). Although some in vitro findings suggest that ghrelin has protective effects on bone health, the available data are controversial. Napoli et al. have recently shown that ghrelin is associated with trabecular BMD but not with total or cortical BMD in post-menopausal women (Napoli et al. 2010).

Traditionally, bone marrow fat function has always been conceived as a physical support (Tavassoli 1984). However, it has been recently reported that its role is far more complex and active, appearing to be directly implicated in bone metabolism (Rosen et al 2006, Rosen et al. 2009, Takeda et al. 2003). As mentioned above, both osteoblasts and adipocyte progenitors have roots in a common mesenchimal

progenitor, whose ability in differentiating into both lineages is impaired in some conditions, such as obesity, where adipogenesis becomes the preferential pathway (Rosen et al 2006, Rosen et al. 2009). Moreover, it has been reported that bone marrow fat inversely relates to bone strength (Schellinger et al. 2001). A study in obese young men and women conducted by Miriam et al. has recently shown a strong correlation between several lipid parameters such as serum triglyceride, intrahepatic and intra-myocellular lipids and bone marrow fat, maintaining statistical significance even when controlled for potential confounders like BMI, age, level of physical activity and serum insulin levels. Moreover, HDL levels were found to be inversely related to marrow fat content. As bone marrow adiposity is known to be inversely correlated to BMD, the authors suggested that ectopic and serum lipid levels are modulated by the same factors as bone marrow fat and may be potentially detrimental to bone health (Bredella et al. 2013).

The role of lipid and lipoprotein oxidation in the pathophysiology of osteoporosis has been suggested by several studies (Parhami et al. 2003, Rajamannan et al. 2008). In a recent study on mice fed an atherogenic high fat diet, it was reported that T-lymphocytes may have a role in the hyperlipidemia-induced bone loss. In fact, in this study, it was demonstrated that T-lymphocytes isolated from the spleen and bone marrow from the high-fat group showed increased expression of RANKL and not only became hyperlipidemic but also showed significantly reduced mineral content. T-lymphocytes from the high fat group tested ex vivo showed an increased expression of IL-6, TNF-alpha, IL-1beta and INF-gamma, cytokines that have a well-documented association with inflammation and bone loss. Several potential mechanisms have been suggested to elucidate the complex relationship between bone and adipose tissue. The endocrine system, adipokines and inflammation have been proposed as some of the components of such interplay. Fat tissue is one of the major sources of aromatase that has a crucial role in the maintenance of skeletal health. Several adipokines, such as leptin and adiponectin, have shown a direct effect on bone metabolism. An inflammation markeras TNF potently augments RANKL-induced osteoclast formation. However, the effects of these factors on bone health remain controversial,

especially because some of them presented both potential positive and negative impacts. Anyway, more studies are needed to elucidate this complex relationship.

Irisin in the glucose and bone metabolism

In recent years, numerous studies focused their attention on the role of irisin, which is a cytokine secreted from fibronectin type III domain containing 5 (FNDC5) of skeletal muscle by an unknown protease. Irisin has a hormone-like effect, influencing energy consumption, in response to physical activity (Pedersen 2012). Its action could influence weight loss, reducing fat-induced insulin resistance, and so significant reductions in insulin levels and blood glucose levels (Bostrom 2012). Furthermore, irisin has a role on bone metabolism (Briganti et al. 2017), as showed in recent *in vivo* studies. In fact, irisin exerts a role of connection between physical activity and bone metabolism (Yan 2017) as induces differentiation of bone marrow stromal cells into mature osteoblasts (Colaianni 2015, Qiao et al. 2016). So, as showed by Quiao et al, the levels of one of the main protein in bone matrix and related to mechanical stimulation , the osteopontin, were higher in mice treated with irisin (Qiao et al. 2016). About irisin, this performs a loading-mimetic function and could mediate loading-induced increases in expression of osteopontin. Similar results were obtained by Colaianni who also found a significant reduction in the number of osteoclasts.

Anastasilakis et al 2014 and Palermo et al 2015 investigated the effect of irisin on vertebral fractures in postmenopausal women with severe osteoporosis (Anastasilakis et al. 2014, Palermo et al. 2015), but no significant correlation was observed between irisin and BMD at any site, or between irisin and lean mass. In subjects with previous fractures due to osteoporosis, the irisin levels were lower than in control subjects.

Furthermore, Yan et al. 2017 confirmed those findings: women showed higher irisin levels had fewer fractures (Yan et al. 2017).

1.3.9 Skeletal imaging

To determine bone strength, evaluation of both bone mineral density (BMD) and skeletal micro-architecture are necessary the former using DXA (Silva and Bilezikian 2014). In fact, BMD measurement at the femoral neck with DXA is clearly recognized to be a strong predictor of hip fractures both in men and women with a similar predictive ability (Johnell et al. 2005). In 2011, Schwartz et al. evaluating T2D patients demonstrated their higher fracture risk for a given femoral neck BMD T-score and age or for a given FRAX probability (Schwartz et al. 2011). Bone micro-architecture is demonstrated variously by histomorphometric analysis, using transiliac crest bone biopsies, micro-computed tomography (μCT) (Kulak and Dempster 2010; Hildebrand et al. 1999), HRpQCT (Boutroy et al. 2005), and magnetic resonance imaging (MRI) (Krug et al. 2008). To add to these diagnostic tools, there is TBS, a gray-level texture measurement based on the use of experimental variograms of two-dimensional (2D) projection images acquired during a DXA lumbar spine scan. It is possible to use this system in addition to BMD to subcategorize patients according to their fracture risk (Eller-Vainicher et al. 2012). TBS is used exclusively for the lumbar spines, showing a prediction of osteoporotic fractures in postmenopausal white woman with diabetes and capturing a larger part of the fracture risk associated with diabetes than does BMD (Kim et al. 2015; Leslie et al. 2013). Furthermore, patients with low BMD and low TBS show more likely fractures than patients with low BMD, but high TBS (Eller-Vainicher et al. 2012; Pothuaud, Carceller, and Hans 2008; Pothuaud et al. 2009; Bousson et al. 2012). A low TBS value could indicate few, less well-connected, and more widely distributed trabeculae, while high TBS values are correlated with better trabecular structure (Hans et al. 2011). To use the new diagnostic tool, a specific software to be applied to DXA image is required (Silva et al. 2014). Iliac crest biopsies

are expensive, invasive, and time consuming, and so non-invasive imaging techniques are needed for assessment of bone microstructure in vivo. High-resolution peripheral quantitative CT HR-pQCT assesses trabecular and cortical bone micro-architecture of the radius and tibia in vivo. HR-pQCT provides measurements analogous to those obtained by 2D histomorphometry and 3D μCT of biopsy specimens. In addition to these measurements, trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp) (Cohen et al. 2012) are included. In a study by Cohen et al., a relatively weak and a largely non-predictive relationships between transiliac biopsies and HR-pQCT scans was seen while the relationship between HR-pQCT scans and central DXA of the spine and hip was considerably stronger. The issue is that different technologies measure different aspects of bone density, quality, and properties which may also depend on the site examined. Amstrup et al. in 2015, analyzing 125 postmenopausal women, underlined that various techniques measure different aspects of the bone, suggesting a common use in clinical practice (Amstrup et al. 2015).

Finally, in recent years, the second-generation HR-pQCTscanner became more accurate rather than the first generation. The first can assess human bone micro-architecture of peripheral limbs with a 61-μm nominal isotropic voxel size (vs. 82 μm of first generation) and is at the limit to accurately determine the thickness of individual human trabeculae (Manske et al. 2015). A relatively recent innovation is the in vivo micro-indentation test of the tibia to directly measure bone mass strength (BMS). In contrast to traditional approaches to fracture risk assessment, this technique permits direct assessment of bone quality. Jenkins et al. observed that micro-indentation at the femoral neck discriminated fracture cases from controls independent of BMD and traditional risk factors but was dependent on location (Jenkins et al. 2015). In support of this diagnostic tool, Coutts et al. confirmed that this technique will be useful in the mid-shaft region where cortical thickness is greatest (Coutts et al. 2015).

1.3.10 Diabetes, bone metabolism and cardiac autonomic neuropathy

Definition and epidemiology

Cardiac autonomic neuropathy (CAN) is a critical diabetes-related complication caused by damage of small nerve fibers (classes Aδ, B and C) (Gandhi et al. 2010). Currently, tests available for studying status of their function use quantitative sensory tests of thermal and pain perception and invasive biopsy methods, such as sural nerve biopsy with electron microscopy and the ex vivo confocal microscopy of skin biopsy (Cruz-Almeida et al. 2014, King et al. 2013, Lauria et al. 2005).

The prevalence of CAN is different among studies on this topic; this due to adopted tests, the diagnostic criteria used, and the population studied. In both subjects with T1D and T2D, the prevalence of confirmed CAN varied from 16.6 to 20% (Spallone et al. 2011, Ziegler et al. 1993, Valensi et al. 2003). Prevalence rates increased both with age (up to 38% in subjects with T1D and 44% in subjects with T2D aged 40–70 years) and diabetes duration (up to 35% and 65% respectively) (Low PA et al. 2004, Pop-Bosui et al. 2009).

Screening for CAN is recommended 5 years after diagnosis of T1D and at the time of T2D diagnosis (Boulton et al. 2005) but is not so simple to perform these tests in the daily clinical practice because these are time-consuming, difficult to perform or not sufficiently accurate in non-collaborative patients (Ziegler et al. 1992) so, this form of neuropathy remains under-diagnosed (Vinik et al. 2007). So, CAN is usually documented using several cardiovascular autonomic reflex tests (CARTs) (Spallone et al. 2011, n.a. 1996)

Pathogenesis

The pathogenesis of CAN in subjects with diabetes is multi-factorial (Edward et al. 2008): hyperglycaemia could lead to neuronal ischaemia or direct neuronal death or its dysfunction (Fig. 1.10), by increasing oxidative and nitrosative stress (Vinik et al. 2003); in fact, mitochondria in the neuronal axons are susceptible to the direct and indirect effects from these type of stress (Leinninger et al. 2006). So, the toxic effect of hyperglycaemia leads to downstream pathways including advanced glycation end

products production, the polyol pathway, protein kinase C and the hexosamine pathway (Soriano FG et al. 2001, Obrosova et al. 2005, Yamagishi et al. 2003, Giacco et al. 2010). Moreover, could succeed changes in gene expression, transcription factors, disrupting several cellular functions, leading to neuronal dysfunction and death (Williams et al. 1997, Russell et al. 1999, Ramasamy et al. 2005). Finally, impaired microvascular regulation and endothelial dysfunction (Kolm-Litty et al. 1998, Wada et al. 2005), may lead to decrease of neurovascular perfusion and to dysfunction and cellular apoptosis (Sayeski et al. 1996).

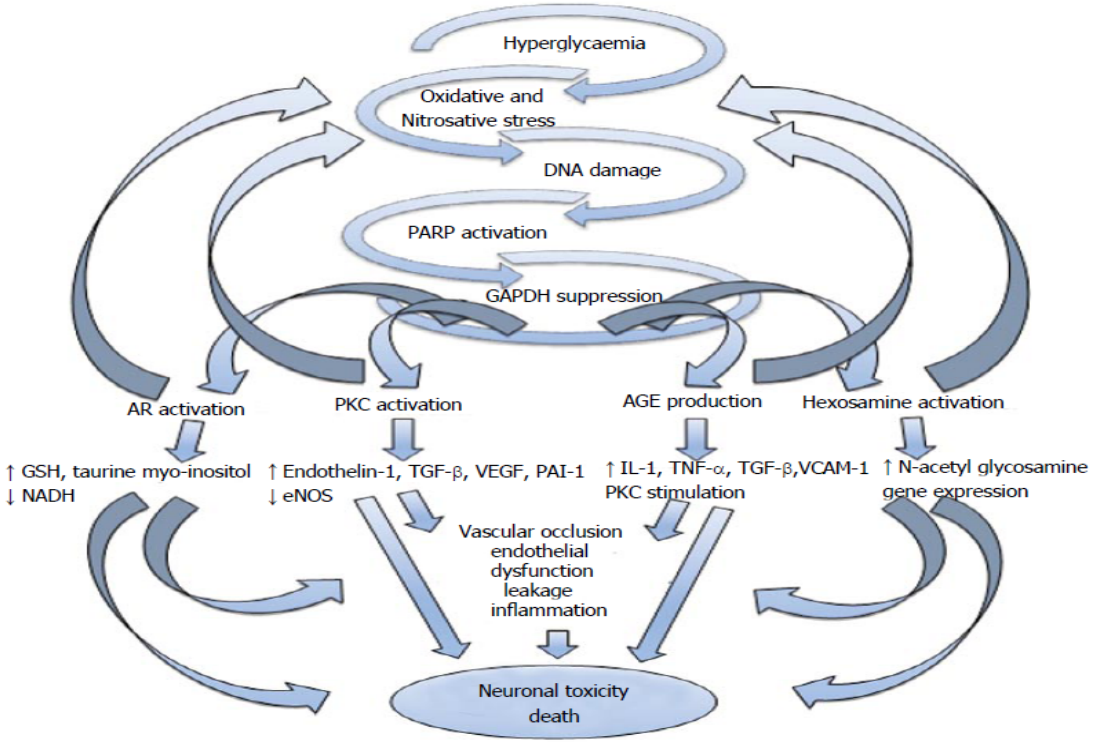

Figure 1.10 Mechanisms that relate hyperglycaemia to microvascular complications in patients with diabetes *from Dimitropoulos G et al. World J Diabetes 2014*
PKC: Protein kinase C; AGE: Advanced glycation end-products; PARP: Poly ADP-ribose polymerase; GAPDH: Glyceraldehyde-3 phosphate dehydrogenase; GSH: Glutathione; NADH: Nicotinamide adenine dinucleotide; TGF-β: Transforming growth factor; VEGF: Vascularendothelial growth factor; PAI-1: Plasminogen activator inhibitor-1; eNOS: Endothelial nitric oxide synthase; IL-1: Interleukin 1; TNF-α: Tumournecrosis factor-α; VCAM-1: Vascular cell adhesion molecule 1.

Clinical manifestations

There are different types of symptoms associated to CAN. Among these: sinus tachycardia, exercise intolerance, and orthostatic hypotension. The last one was in 6–32% of patients with diabetes (Vinik et al. 2003, Valensi et al. 2003, Low et al. 2004, Ewing et al. 1985, Kempler et al. 2001, Low et al. 2008).

These symptoms may play an important dangerous role, leading to excess in mortality and in morbidity (CAN based on at least two abnormal CART results determined a relative risk of mortality of 3.65 (95% confidence interval 2.66–4.47) (Maser et al 2003).

CARDIOVASCULAR SYSTEM
Perioperative instability - Resting tachycardia - Hypertension - Orthostatic hypotension - Exercise intolerance - Loss of reflex heart rate variations - Postprandial hypotension - Left ventricular dysfunction and hypertrophy - QT interval prolongation - Silent myocardial ischaemia - Impaired baroreflex sensitivity - Non-dipping, reverse dipping - Sympathovagal imbalance - Dysregulation of cerebral circulation - ⇓ Sympathetically mediated vasodilation of coronary vessels - ↑ Arterial stiffness
PERIPHERAL VASCULAR FUNCTION
↑ Peripheral blood flow and warm skin - ↑ Arteriovenous shunting and swollen veins - ↑ Venous pressure - Loss of protective cutaneous vasomotor reflexes - Leg and foot oedema - ↑ Transcapillary leakage of macromolecules - Loss of venoarteriolar reflex with microvascular damage - Medial arterial calcification

Table 1.6 Abnormalities associated with CAN at the level of cardiovascular system and peripheral vascular function.

Cardiac autonomic neuropathy assessment

i. Questionnaires

The use of questionnaires allows to investigate orthostatic symptoms and their severity in dysautonomic conditions, although they have not been specifically validated for CAN (Spallone et al. 2011).

ii. Cardiac autonomic cardiovascular autonomic reflex tests (CARTs)

CARTs are considered the gold standard to evaluate CAN; these tests assess cardiovascular autonomic function through provocative physiological manoeuvres and by evaluation of the end-organ response, i.e. blood pressure and heart rate changes. Parasympathetic function can be evaluated by heart rate variations during deep breathing, Valsalva maneuver, and lying-to-standing (heart rate tests), whereas the orthostatic hypotension, the blood pressure response to a Valsalva maneuver and sustained isometric muscular strain provide indices of sympathetic function (American Diabetes Association and American Academy of Neurology 1988; Anonymous et al. 1996, Ewing et al. 1985).

These tests are performed to detect CAN using a standard electrocardiograph. Prior to the tests, at least 24 hours, subjects need to discontinue any interfering drugs (i.e. anti-hypertensive or antidepressant drugs) (Spallone et al. 2011).

1) *Heart rate response to deep breathing* (expirium: inspirium ratio). Electrocardiographic QRS complexes were recorded while patients were consecutively repeating 10-sec cycles 8 times, including 5-sec periods of inspirium and expirium. The proportion of the longest R-R interval in expirium to the shortest in inspirium yields the expirium to inspirium (E : I) ratio.

2*) Heart rate response to lying to stand test*. Basic heart rates were recorded after patients had rested for 10 min. Subjects were asked to stand-up in 2 or 3 secs without help and heartbeats were recorded in the orthostatic position. The R-R intervals between the 10th and the 15th heartbeat and between the 20th the 30th heartbeat were measured to find the shortest and the longest R-R intervals. The proportion of the longest interval to the shortest interval yields the 30/15 ratio.

4) *Valsalva manoeuvre*. Evaluates the HR response during and after a provoked increase in the intra-thoracic and intra-abdominal pressures (the patient normally exhales for a period of 15 seconds against a resistance);

3) *Blood pressure response to standing*. Baseline systolic and diastolic blood pressures were measured after patients had rested for 10 min. Patients were asked to quickly stand up within 2 or 3 secs without help. Four consecutive blood pressure recordings were obtained in 1-min intervals in the orthostatic position. Results were defined as abnormal when the difference between the systolic blood pressures was above or equal to 30 mmHg, or the difference between diastolic blood pressures was above or equal to 15 mmHg, or the difference between the mean blood pressures was above or equal to 20 mmHg in the supine and standing positions.

Subjects could be considered as affected by CAN in the presence of one abnormal result on heart rate test (early), or of two or more abnormal results on heart rate tests (moderate) or in the presence of orthostatic hypotension (severe).

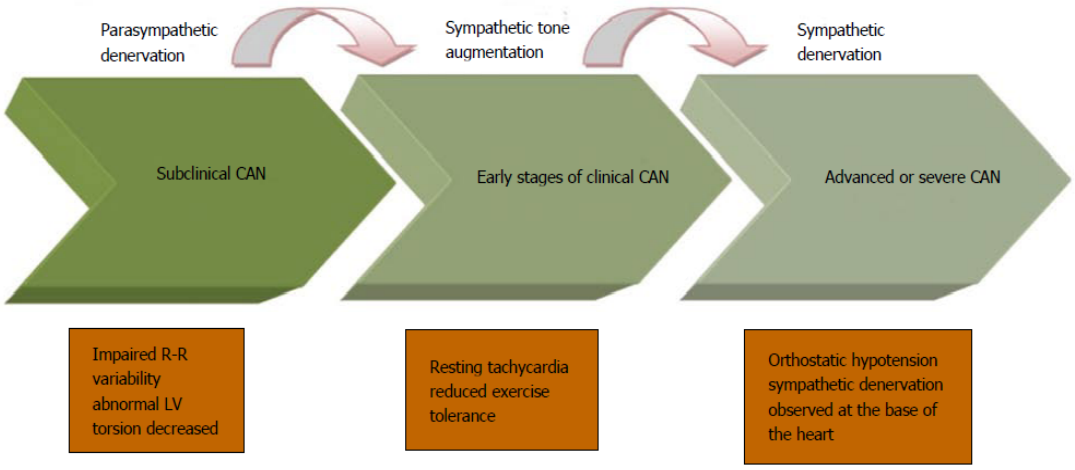

Figure 1.11 Natural progression of CAN and correlation with clinical signs and symptoms
CAN: Cardiac autonomic neuropathy; LV: Left ventricle.
From Dimitropoulos G et al . World J Diabetes 2014

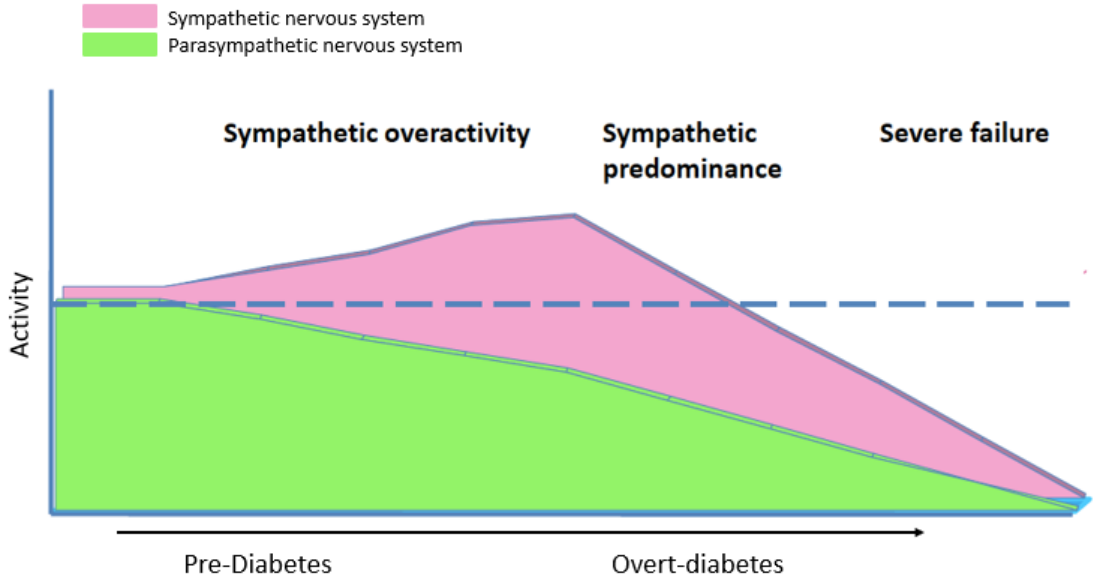

Figure 1.12 Progression of CAN and Diabetes (from Spallone V., Downloaded from https://www.excemed.org/manage-diabetes online/resources)

CAN and bone metabolism in diabetes

The physiological role of bone metabolism is remodeling and consists of an alternation between a bone resorption and formation phase (Shy et al. 2010; Rodan and Martin 2000). Recent studies analyzed the not yet clear mechanism of regulation of bone remodeling. These studies identificated that neuronal control has an important role, whereby the adipocyte-derived hormone leptin regulates bone mass (Ducy et al. 2000). In fact, after intracerebroventricular infusion of leptin in wild-type (WT) or leptin-deficient (ob/ob) mice before or after chemical lesioning of hypothalamic neuronal populations, the authors revealed that leptin inhibits bone mass accrual by acting solely through neuronal means (Shi et al. 2008). It was showed that leptin inhibits synthesis and release of serotonin, after binding to its receptor on brainstem neurons (Yadav et al., 2009), so, brain-derived serotonin signals through the Htr2c receptor present on ventromedial hypothalamic (VMH) neurons, decreasing activity of the sympathetic nervous system (SNS). In fact, SNS is a negative regulator of bone mass accrual acting

through the b2 adrenergic receptor (Adrb2) present on osteoblasts (Elefteriou et al. 2005; Fu et al. 2005; Takeda et al. 2002) (Fig. 1.13). About the role of parasympathetic nervous system (PNS), it is known that its main neurotransmitter is acetylcholine, which binds to the muscarinic acetylcholine receptors, a family of G protein-coupled receptors, (Caulfield et al.1993; Wess et al. 1996). Shi et al. studied the bone phenotype of mice lacking muscarinic receptors and showed that M3 receptor (M3R), five different muscarinic receptors (MRs), is the only receptor subtype influencing bone remodeling (Shy et al. 2010), decreasing sympathetic activity. So, PNS could be defined as a positive regulator of bone mass accrual, dampening the negative influence of the sympathetic tone on bone mass.

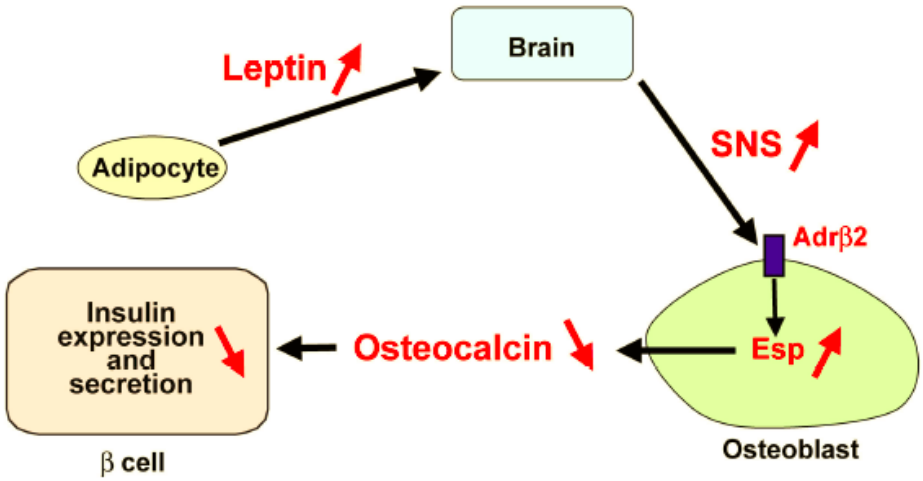

Figure 1.13 In the presence of leptin, sympathetic tone is high; thus, Esp expression in the osteoblast is high, and osteocalcin bioactivity is low. This contributes to the inhibition of insulin secretion by leptin.

CHAPTER 2

RESEARCH PROJECT N°1

SERUM SCLEROSTIN AND BONE TURNOVER IN TYPE 2 DIABETES AND IN LATENT AUTOIMMUNE DIABETES IN ADULTS*

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2.1 Introduction

The aim of this study was to evaluate serum sclerostin and bone turnover markers in LADA in comparison with T2D. LADA is clearly different from T2D, in that LADA is associated with HLA genes, islet-autoantibodies, reduced insulin secretion, and less prevalence of metabolic syndrome. Based on this and following the evidence that Wnt is differently regulated in T1D compared to T2D, we hypothesized that LADA would have a compromised skeletal phenotype, different from T2D, and closer to that showed for T1D. Given the relatively high prevalence of MetS in both LADA and T2D and the potential impact of MetS features on sclerostin and bone turnover, we also studied whether sclerostin or bone turnover are affected by MetS associated with these two types of diabetes, and our hypothesis was that they would be. We studied LADA and T2D cases with similar age, disease duration and prevalence of MetS. Compared to T1D, LADA is the ideal model for studying bone turnover in the context of autoimmune diabetes, because it is not affected by the juvenile age of onset, impaired peak bone mass and insulin therapy proper of T1D.

2.2 Methods

2.2.1 Subjects

This was a cross-sectional study involving 89 subjects with LADA and 98 subjects with T2D. Serum of patients with LADA and type 2 diabetes was obtained from the European Union Action LADA project (Hawa et al. 2009) and the NIRAD study in Italy (Buzzetti et al. 2007). Samples were selected where sufficient serum and data was available from four Action LADA centres in Spain, France, Belfast and London, and from the NIRAD group in Italy. The Action LADA multicentre study was performed to identify immune and clinical risk factors for adult-onset autoimmune diabetes, including its epidemiology, genetic susceptibility, metabolic characteristics and clinical progression (Leslie et al 2008). Diabetes was designated according to standard criteria, and LADA was defined as patients aged 30–70 years with GADAs who did not require insulin treatment for at least 6 months after diagnosis (Leslie et al 2008, Report 1997). Patients came from Europe and almost all of them were of Caucasian ethnicity (96% Caucasian, 3% Asian, 1% African, and 1% mixed race). The NIRAD Study is a nationwide survey sponsored by the Società Italiana di Diabetologia with the aim of assessing the prevalence and characteristics of autoimmune diabetes within adult patients attending diabetes clinics in Italy with a clinical diagnosis of non–insulin-requiring diabetes. Adult-onset autoimmune diabetic subjects were selected using the following inclusion criteria: *1*) an initial diagnosis of T2D according to the American Diabetes Association, *2*) documented antibody positivity for GADA and/or IA-2° (Petrone et al. 2008), *3*) no insulin requirement and no evidence of ketosis from diagnosis to screening time, and *4*) time since diagnosis between 6 months and 5 years. All subjects from the NIRAD study were unrelated and of exclusively Italian origin (with parents and grandparents of Italian origin). Exclusion criteria included prior insulin therapy, pregnancy, renal disease with a raised creatinine level or proteinuria, and the presence of any other severe disease. MetS was assessed according to the NCEP criteria, as described below. T2D and LADA samples were selected where enough sample volume was available, aiming at ensuring a numeric balance between the following four groups, namely LADA with MetS; LADA without MetS; T2D with MetS; T2D without MetS. There was no other selection at all. We selected from the Action LADA cohort, LADA cases with MetS (n=31) and LADA cases without MetS

(n=29); from the NIRAD group we selected LADA cases with MetS (n=11) and LADA cases without MetS (n=17). Overall, selected T2D cases were limited by availability of sufficient sera (n= 60 from the Action LADA, and n=38 form the NIRAD group), being of similar age, sex and disease duration to the LADA cases. Waist circumference and blood pressure, at least twice in the sitting position, were measured in each subject. Lipids and lipoproteins (total and HDL cholesterol, triglycerides) were determined by standardized assays at each center. Patients with T2D or LADA were divided into four groups according to the presence or absence of MetS; 1) type 2 diabetes with MetS (n=57); 2) type 2 diabetes without MetS (n=41); 3) LADA with MetS (n=42); 4) LADA without MetS (n=47). Sera from 53 individuals without diabetes (fasting blood glucose lower than 126 mg/dl) were used as control. Subjects treated with thiazolidinediones or sodium glucose transporter 2 inhibitors were not included in this study. Control subjects were recruited through the Endocrinology outpatient clinics of Universitá Campus Bio-Medico di Roma. Subjects with diseases (e.g. osteoporosis, hyperparathyroidism, hyper or hypothyroidism, chronic kidney disease, etc.) or drugs (glucocorticoids, bisphosphonates, etc.) known to affect bone metabolism were excluded.

2.2.2 Diagnostic criteria for the metabolic syndrome (MetS)

MetS was assessed according to the NCEP criteria (Expert panel 2001), with modifications by the AHA/NHLBI (Grundy et al. 2005), as follows: waist circumference >102 cm in men and >88 cm in females, triglycerides ≥150 mg/dl, HDL cholesterol <40 mg/dl in men and <50 mg/dl in women, blood pressure ≥130/85 mmHg or taking antihypertensive medication, and fasting glucose ≥100 mg/dl. All diabetic patients in this study were identified as fulfilling the criteria for hyperglycemia. MetS was defined by the presence of three of five criteria, including blood glucose.

2.2.3 Sclerostin and bone turnover markers

Sclerostin serum levels were assessed by quantitative sandwich ELISA (Biomedica, Vienna, Austria). Bone turnover was evaluated analyzing serum levels of a bone formation marker, the total pro-collagen type 1 N-terminal propeptide (P1NP) by ELISA (Biomedica, Vienna, Austria) and a bone resorption marker, the CTX by ELISA (IDS, Boldon, UK). P1NP is a specific marker of bone formation, while CTX is an accurate marker of bone resorption. CTX and P1NP have been suggested by the International Osteoporosis Foundation as the appropriate bone markers when exploring bone resorption and formation in clinical and research settings (Vasikaran et al. 2011).

2.2.4 Sample size and power calculation

Sample size was calculated for the primary aim on the hypothesis that circulating sclerostin would be different between T2D and LADA. The calculation was based on previous published data of increased circulating sclerostin in T2D compared to non-diabetic control subjects (Gaudio et al. 2012). Using a significance level of 0.05 and 80% power, the minimum sample size required was of 46 subjects per group (T2D, LADA and controls). To investigate the relationship between sclerostin and MetS status, the samples size was further increased on the base of available serum and clinical/biochemical information on the MetS status.

2.2.5 Statistical analysis

Continuous variables are presented as mean±SD. Normality was tested with the Shapiro-Wilk test. When data were not normally distributed, logarithmic transformation was performed. Categorical variables are expressed as absolute frequencies. For continuous variables, mean differences across groups were compared by Generalized Linear Models/ANOVA for normally distributed variables. Homoscedasticity was tested with Levene and Brown-Forsythe test'. For post-hoc analyses, Tukey and Games-Howell tests were applied. Pearson (normal distribution) and Spearman (non-normal distribution) correlations were used to assess the

correlations between serum sclerostin levels and other continuous variables. Multiple backward model linear regression analysis was performed to identify independent predictors of serum sclerostin levels (dependent variable, sqrt transformed) in the overall population and in LADA/ T2D groups. The models included time since diagnosis, BMI, HDL-cholesterol levels and triglycerides levels, while age and sex were not included in the multiple linear regression model as they were not correlated with serum sclerostin levels. Furthermore, after adjusting for age and sex, the results did not change. A two tailed p-value <0.05 was considered significant. A multiplicity adjusted p value was reported when multiple comparisons were performed. Data were analyzed with SAS version 9.4 statistical software (SAS Institute Inc., Cary, NC).

2.3 Results

2.3.1 Features of the studied population

Clinical and biochemical features of subjects with T2D, LADA and non-diabetic control subjects are shown in Table 2.1. Overall, patients with LADA were younger and leaner than those with T2D [median body mass index (BMI) (range) of 24.7 (17.6-41.8) vs. 28.3 (18.0- 44.5) Kg/m2, respectively; p<0.0001], while time since diagnosis was similar in patients with LADA and T2D [median years of disease with range 2.9 (0-7) vs. 2.0 (0-11) years; p=0.25]. However, when divided according to presence or absence of MetS, the four groups were comparable in terms of age and time since diagnosis (Table 2.1). The reference non-diabetic group consisted of 53 subjects (36 females) without diabetes (age 48.2±21.1 years; median BMI 26.2, range 18.5-41.0, kg/m2). Non-diabetic subjects were of similar age compared to LADA but younger than T2D (p<0.001); the non-diabetic control group consisted of more female than the LADA and T2D groups (p<0.01). BMI of controls was not significantly different compared to LADA or T2D. Among control subjects, 5/53 (9.4%) had impaired fasting glucose, 9/53 (17%) had a diagnosis of primary hypertension, 5/53 (9.4%) had dyslipidemia and 9/53 (17%) were obese (BMI>30 kg/m2).

	1	2	3	4	5		
	T2D		LADA		Non-diabetic controls	p between T2D and LADA	p vs. group 5
Variables	With MetS	Without MetS	With MetS	Without MetS			
N	57	41	42	47	53		
Sex (Male/Female)[a]	35/22	24/17	22/20	27/20	17/36	ns	<0.05
Age (years)[b]	52.7±9.6	52.4±9.2	51.5±11.1	47.5±11.2	48.2±21.1	ns	ns
Time since diagnosis (years)[b]	2.9±1.7	2.5±1.8	2.7±1.9	2.4±2.3	NA	ns	NA
Waist Circumference (cm)[b]	104.9±9.1	90.2±12.8	98.7±12.4	82.3±11.4	90.0 (24.0)	<0.0001	≤0.01
BMI (Kg/m^2)[c]	30.4 (6.3)	25.6 (5.9)	27.6 (7.7)	22.6 (4.6)	26.2 (6.7)	<0.0001	<0.0001
HbA1c (%)[b]	6.7±1.4	6.5±1.2	7.9±1.5	7.2±1.5	NA	ns	NA
Creatinine (mg/dl)[b]	0.79±0.13	0.80±0.08	0.95±0.26	0.85±0.20	0.81±0.17	ns	ns
Triglycerides (mg/dl)[c]	157.0 (139.0)	101.9 (35.4)	165.0 (124.0)	70.0 (31.3)	97.0 (53.0)	<0.0001	<0.0001
HDL-cholesterol (mg/dl)[c]	37.1 (13.0)	52.6 (19.4)	46.4 (20.9)	65.0 (22.6)	54.5 (22.5)	<0.0001	<0.0001
Systolic Blood Pressure (mmHg)[b]	134.9±12.0	123.1±13.4	134.3.1±16.9	120.0±11.1	123.7±10.9	<0.0001	<0.0001
Diastolic Blood Pressure (mmHg)[c]	80.0 (10.0)	80.0 (11.0)	77.0 (20.0)	75.5 (10.0)	75.0 (10.0)	0.005	ns

Table 2.1 Clinical characteristics of LADA (Latent Autoimmune Diabetes in Adults) and T2D (Type 2 Diabetes) patients categorized according to the Metabolic Syndrome (MetS) status, and non-diabetic control subjects.

[a]Data are expressed as absolute frequencies; [b]Data are expressed as mean±SD; [c]Data log trasformed and are expressed as median (IQR); NA: not available/not applicable
Waist Circumference: 1 vs. 2, 1 vs 4., 2 vs. 3, 2 vs. 4, 3 vs. 4, p≤0.03
BMI: 1 vs. 2, 1 vs. 3, 1 vs. 4, 2 vs. 4 and 3 vs. 4, p≤0.019;
Triglycerides: 1 vs. 2, 1 vs. 3, 2 vs. 3, 2 vs. 4 and 3 vs. 4, p≤0.01;
HDL-cholesterol: 1 vs. 2, 1 vs. 4, 2 vs. 4 and 3 vs. 4, p≤0.007;
Systolic Pressure: 1 vs. 2, 1 vs. 4, 2 vs. 3 and 3 vs. 4, p≤0.007;
Diastolic Pressure: 1 vs. 2 and 1 vs. 4, p≤0.048.

2.3.2 Circulating sclerostin in LADA and T2D

Patients with T2D had higher serum sclerostin than those with LADA or controls (29.8±11.9 vs. 23.0±11.8 vs. 24.3±5.7 pmol/l, p=0.0001; p≤0.002 adjusted for sex and BMI) (Figure 2.1). In the combined group of diabetic subjects, sclerostin tended to be higher in the group with MetS but this was not significant (25.0±12.7 vs. 28.3±12.8 pmol/l; p=0.08). Within MetS patients, serum sclerostin was higher in T2D than LADA (p=0.01; p=0.03 adjusted for sex and BMI). When analyzing separately T2D

and LADA, in both groups serum sclerostin was similar between subjects with and those without MetS ($p \geq 0.15$). However, when all diabetic individuals were included in the analysis and divided in four groups according to diabetes type and MetS (namely LADA without MetS, LADA with MetS, T2D without MetS, T2D with MetS), we found a trend for increased sclerostin from LADA without MetS towards T2D with MetS ($p<0.0001$ for trend) (Figure 2.2). In the whole group of diabetic subjects, sclerostin progressively increased with the number of MetS features ($p=0.002$ for trend); when analysis was performed according to diabetes type, sclerostin increased with the number of MetS features in T2D ($p=0.001$ for trend) but not in LADA.

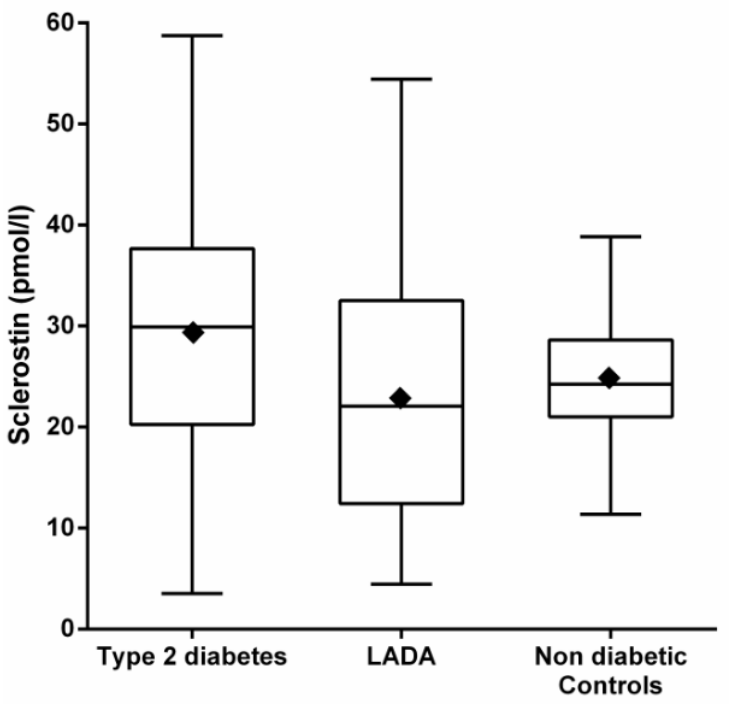


Figure 2.1 Sclerostin in patients with type 2 diabetes, LADA and non-diabetic control subjects. Patients with type 2 diabetes (T2D) had higher sclerostin than LADA ($p=0.0007$) or control subjects ($p=0.002$). Box plots show the 25th and 75th percentile, and the horizontal line shows the median (50th percentile). Bars outside the box indicate the minimum and maximum value. Diamond symbol represents mean.

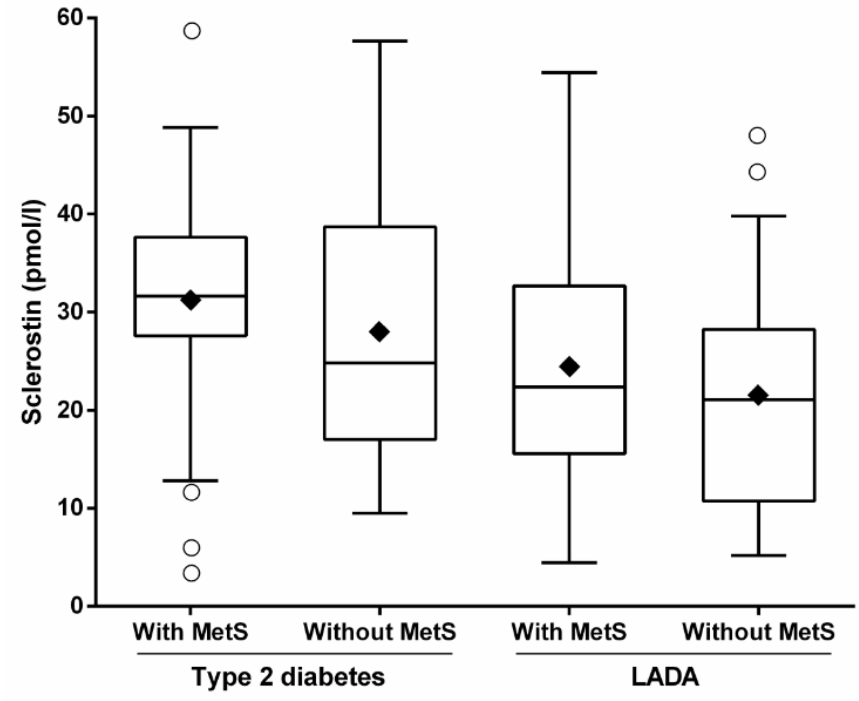


Figure 2.2 Sclerostin in T2D and LADA according to metabolic syndrome (MetS) status. When analyzing separately T2D and LADA, in both groups the presence of MetS did not influence significantly serum sclerostin ($p \geq 0.15$); however, within MetS patients, serum sclerostin was higher in T2D than LADA ($p=0.01$). T2D with MetS had higher sclerostin than LADA without MetS ($p=0.001$). A trend for increased sclerostin across the four groups shown in the figure, from LADA without MetS to T2D with MetS, was found ($p<0.0001$ for trend). Box plots show the 25th and 75th percentile, and the horizontal line shows the median (50th percentile). Bars outside the box indicate the minimum and maximum value. Circle and diamond symbols represent outliers and mean, respectively.

2.3.3 Bone turnover markers in LADA and T2D

Subjects with T2D had 11% and 13% lower P1NP compared with LADA or non-diabetic subjects, respectively, although the differences were not significant (57.3±16.6 vs. 64.2±19.5 vs. 66.5±25.2 pg/ml, respectively). The bone resorption marker CTX was 43% lower in subjects with diabetes, either T2D or LADA, compared with non-diabetic subjects (0.16±0.06 vs. 0.16±0.10 vs. 0.28±0.16 ng/ml, respectively; $p=0.0003$) (Figure 2.3). When LADA and T2D were divided according to the presence of MetS we found no significant differences in either P1NP or CTX levels across the

four groups. Levels of bone turnover markers were unrelated to age, time since diagnosis, BMI or other clinical and biochemical parameters in all study groups. Bone turnover markers were not correlated with serum sclerostin ($0.006<rho<0.04$; $p\geq0.67$) in all study groups.

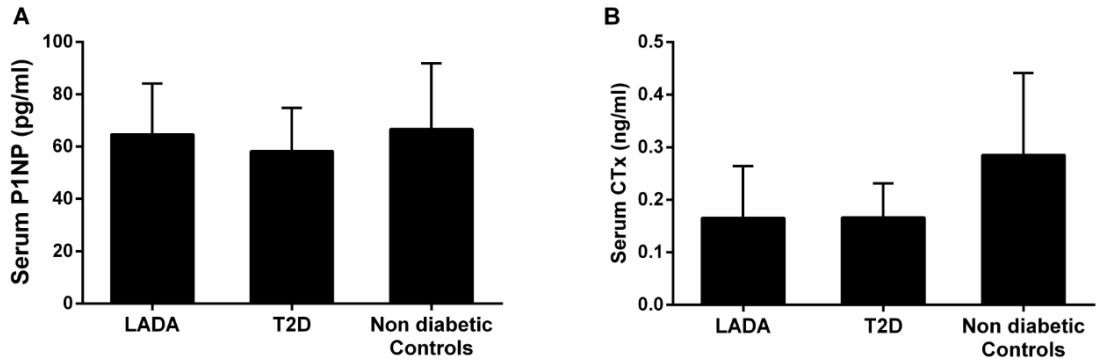


Figure 2.3 Bone turnover markers in T2D, LADA and non-diabetic control subjects. (a) Subjects with T2D had 11% and 13% lower P1NP compared with LADA or non-diabetic subjects, respectively, although the differences were not significant; (b) the bone reabsorption marker CTX was significantly reduced in both types of diabetes compared with control subjects ($p\leq0.006$). Data are presented as mean ± standard deviation.

2.3.4 Relationship of sclerostin with clinical and biochemical features

In the control group, serum sclerostin were unrelated with age (rho=0.08; p=0.63), BMI (rho=-0.06; p=0.73) or blood glucose (rho=0.21; p=0.22). In the overall cohort of subjects with diabetes, serum sclerostin was similar between males and females (p=0.92) and unrelated with age (rho=0.05; p=0.49), HbA1c (rho=0.27; p=0.053) or creatinine (rho=-0.18; p=0.16), but increased significantly with BMI (rho=0.29; p=0.0001), time since diagnosis (rho=0.32, p<0.0001), triglycerides (rho=0.19; p=0.02), and was inversely correlated with HDL cholesterol (rho=-0.23; p=0.003). In multiple regression analysis of the overall population with diabetes, we found that time since diagnosis (β =0.19; p=0.002) and triglycerides (β=0.003; p=0.03), but not BMI and HDL-cholesterol, were independent predictors of sclerostin levels (Supplementary

Table 1). Altogether, time since diagnosis and triglycerides explained 11% of sclerostin variance.

2.4 Discussion

To our knowledge, this is the first study reporting sclerostin and bone turnover in patients affected with LADA. We found that sclerostin is increased in T2D but not in LADA, while the bone resorption marker CTX was equally reduced compared to control subjects in both types of diabetes. These data indicate that low bone resorption is a feature of both LADA and T2D. MetS did not affect bone turnover markers in either LADA or T2D. In contrast, sclerostin was positively associated with the number of MetS features in patients with T2D, suggesting a relationship between MetS severity and T2D. The finding that bone resorption is reduced in LADA compared to controls is novel. Previous studies have shown consistently lower levels of CTX and the bone formation marker osteocalcin in T1D and T2D compared with controls, regardless of diabetes type, suggesting that both bone resorption and formation are reduced in these types of diabetes (Hygum et al. 2017, Starup-Linde et al. 2014). Our data follow a similar trend showing that also patients with LADA, as well as patients with T2D, have lower CTX than controls. As highlighted by a recent metanalysis, most of the studies have reported reduced P1NP levels in T2D compared to non-diabetic controls (Hygum et al. 2017). In our study, we found a non-significant reduction of P1NP in T2D compared to non-diabetic and LADA subjects. However, the magnitude of P1NP reduction (over 10%) was similar to that reported by the metanalysis of Hygum et al. when comparing T2D to non-diabetic controls (Hygum et al. 2017). This may suggest that the P1NP difference found in our study did not reach statistical significance, probably due to increased variance among the groups or the small sample size. Despite the similar reduction in bone turnover, we found that serum sclerostin was increased in T2D but not in LADA. The role of sclerostin in diabetic bone turnover is controversial and there are no data in LADA so far. According to the literature, our data clearly mirror those reported by other groups in T1D. T1D patients have sclerostin levels similar to (Gennari et al. 2012, Tsentidis et al. 2016, Catalano et al. 2014) or

only slightly higher than controls (Neumann et al. 2014). Conversely, increased sclerostin has been consistently found in individuals with T2D (Gaudio et al. 2012, Ardawi et al. 2013, van Lierop et al. 2012). In a recent metanalysis, the magnitude of sclerostin increase in comparison to controls was four times higher than that reported for T1D (24). In the study by Gennari et al. sclerostin was increased in patients with T2D but not in those with T1D despite the similar reduction of bone turnover in both groups (Gennari et al. 2012). This is consistent with the experimental evidence that sclerostin expression is down-regulated in a mouse model of T1D (Portal-Nunez et al. 2010), while the SOST/sclerostin gene is up-regulated in T2D rats (Nuche-Berenguer et al. 2010). Taken together, these findings may support a different role for sclerostin in the impairment of bone metabolism associated to T2D or autoimmune diabetes (including LADA and T1D). Drake et al. have shown that peripheral serum sclerostin correlates with bone marrow plasma levels (Drake et al. 2010). Although sclerostin is a locally active molecule, we may speculate that circulating levels could reflect activity in the bone microenvironment. In T2D, the increased sclerostin release by osteocyte may attenuate osteoblastogenesis via inhibition of the canonical Wnt pathway. On the other hand, this pathway may not be significantly affected by the autoimmune types of diabetes (LADA and T1D), where other T1D/LADA-specific elements, such as insulin deficiency (Campos Pastor et al. 2000), autoimmunity (Sugai et al. 2002, Strollo et al. 2013) or an intrinsic osteoblast defect, may be the primary cause. This is plausible considering that T1D, T2D and LADA present a number of differences in genetic, metabolic and immunologic profile. It follows that bone metabolism/turnover is an additional element that can differentiate these forms of diabetes. MetS is more common in T2D than autoimmune diabetes, and its features are clearly linked to insulin resistance (Eckel et al 2010). In this study, sclerostin and bone turnover markers were similar between subjects with MetS and those without MetS, regardless of diabetes type. However, when all diabetic individuals were included in the analysis and divided in four groups according to diabetes type and MetS (namely LADA without MetS, LADA with MetS, T2D without MetS, T2D with MetS), we found a trend for increased sclerostin from LADA without MetS towards

T2D with MetS. Such increase may reflect an association with the progressive increase in insulin resistance across the four groups analyzed, where LADA without MetS is the group with the lowest degree of insulin resistance, while T2D with MetS is the group characterized by the highest degree of insulin resistance, respectively. Of note, in patients with T2D we found a correlation between sclerostin and the number of MetS features. These observations may partially resemble those provided by Daniele et al., who showed a correlation between sclerostin and insulin resistance in skeletal muscle, liver, and adipose tissue (12). Sclerostin has been studied in association with other features of MetS, such as body weight, providing mixed results. A positive correlation with body weight or BMI has been shown in a report (Gennari et al. 2012), while others have reported no association (Yamamoto et al. 2013, Garcia-Martin et al. 2012) such as in the present study, or even increased levels in response to weight loss, as also recently shown by our group (Strollo et al. 2017). According to multivariate analysis, time since diagnosis (which is an estimate of disease duration) was the strongest predictor of sclerostin levels. This may imply that when diabetes progresses, inhibition of Wnt/β-catenin pathway by sclerostin becomes more significant leading to impaired bone turnover. Although bone turnover markers did not correlate with disease duration in our study, others have shown a negative correlation between diabetes duration and markers of bone formation (Abd El Dayem et al. 2011) and resorption (Reyes-Garcia et al. 2013). Our study has several strengths and limitations. To our knowledge, this is the first analysis of bone turnover and sclerostin in a large and well characterized population of patients with LADA. The study population consisted of subjects with relatively short time since diagnosis without chronic complications. The significance of our findings may be limited by the lack of bone mineral density measurements and by the cross-sectional nature of the study. An additional limitation is the lack of data on post-menopausal status, vitamin D status or treatment with bisphosphonates or other bone-active drugs in subjects with T2D and LADA, all factors that may potential alter bone turnover and/or sclerostin levels. Additional studies with larger cohorts (cross-sectional and longitudinal and at-risk

individuals) are required to assess the role of sclerostin and the Wnt pathway on bone turnover and fragility in diabetes.

In conclusion, our findings indicate that bone resorption is reduced in both T2D and LADA compared to non-diabetic subjects, while circulating sclerostin is increased in T2D only. These data suggest that pathways involved in bone metabolism are different between the two types of diabetes. Furthermore, MetS does not seem to affect bone turnover in both types of diabetes while its features may additively influence sclerostin in T2D. Larger longitudinal studies are needed to confirm these findings and to explore the potential role of sclerostin and Wnt pathway on bone fragility associated with diabetes.

CHAPTER 3

RESEARCH PROJECT N°2

IRISIN IS ASSOCIATED WITH OSTEOPOROTIC FRACTURES INDEPENDENTLY OF BONE MINERAL DENSITY, BODY COMPOSITION OR DAILY PHYSICAL ACTIVITY*

**This article has been published in Clinical Endocrinology (Oxf) in 2015*

3.1 Introduction

A metaphor "The hazardous duet" has been used to describe the concurrent onset of sarcopenia and osteoporosis (Cepaldi et al. 2005), underlining the deep relationship between bone health and muscle mass in the aging population. As extensively shown by several studies in different clinical conditions and ages, skeletal muscle is crucial for bone health and a

complex cross-talk through several mechanisms has been described (LeBlanc et al. 2007, Karasik et al. 2010). It is commonly recognized that gains in bone mass resulting from exercise are basically related to an increase in mechanical strain, but recently endocrine factors secreted by skeletal muscle, especially during exercise, have been identified as possible anabolic messengers from muscle (myokines) to bone and fat tissue (Pedersen and Febbraio 2012, Huh et al. 2014). Irisin is a recently discovered myokine induced in exercise, that acts by stimulating adipose tissue browning (Boström et al. 2012). Brown Adipose Tissue (BAT) volume is a positive predictor of femoral

bone structure and correlates positively with thigh muscle (Bredella et al. 2014). Furthermore, in a recent in vitro study, it has been shown that irisin enhanced osteoblast differentiation (Colaianni et al. 2014) and that irisin levels were lower in women with previous osteoporotic fractures (Anastasilakis et al. 2014). Moreover, it has also been shown that irisin is not only secreted by skeletal muscle, but also by adipose tissue, in particular by subcutaneous adipose tissue in rat, underlining its role

in the complex muscle fat- bone axis (Roca-Rivada et al. 2013). However, although there is evidence of correlation between irisin levels and osteoporotic fractures, previous studies have not elucidated the relationship between irisin and either lean or fat mass. Indeed the main aim of this study is to investigate the relationship between irisin and body composition and the impact of irisin levels on fractures. To our knowledge, this is the first study to evaluate the effect of daily physical activity on irisin levels, using a novel wearable metabolic Holter in postmenopausal women affected by fragility fractures.

3.2 Methods

3.2.1 Study Design

This cross-sectional study was carried out from December 2013 to April 2014 in the out-patient clinic of the Department of Endocrinology at the University Campus Bio-Medico of Rome.

3.2.2 Study Population

During the study period, 65 post-menopausal women affected by osteoporosis (lumbar spine and/or non-dominant total hip and/or femoral neck T score < 2.5) (group A) and 60 post-menopausal women with either osteopenia (lumbar spine or non-dominant total hip or femoral neck T score < -1 but > -2.5) or normal bone mineral density (BMD) (lumbar spine or non-dominant total hip or femoral neck T score > -1) (group B) were screened. All subjects were assessed by X-ray vertebral morphometry and were enrolled according to the following inclusion/exclusion criteria. Subjects were considered eligible for the group A if they were affected by at least one vertebral osteoporotic fracture. Subjects were considered eligible for the group B if they were free from vertebral osteoporotic fracture. All subjects were excluded from the study if: age<50 years or >80 years; severe renal failure (EPI <30 mL/min); severe liver failure; moderate and severe heart failure (NYHA III and IV); ischemic heart disease history; musculoskeletal disorders other than osteoporosis; neoplasia; BMI>30 kg/m2; diagnosis of diabetes; medications such as corticosteroids and aromatase inhibitors

that could interfere with bone metabolism. Finally, we enrolled 36 subjects affected by at least one vertebral osteoporotic fracture (Group A) and 36 nonosteoporotic patients (Group B). All the postmenopausal women with severe osteoporosis were treated with either Denosumab (77.8%) or teriparatide (22.2%).

At the time of the study visit an accurate medical history was collected, a complete physical examination was performed and data about therapy, BMI, wrist, waist and hips circumferences were acquired. Thereafter blood samples (8-9 am, fasting) were drawn to measure creatinine, calcium and phosphate (using standard methods) and 25(OH) vitamin D (RIA, diasorin INC, MN, USA). Serum irisin concentrations were measured by a competitive ELISA (AG-45A-0046EK-KI01; Adipogen AG, Liestal, Switzerland) according to the manufacturer's instruction. In this assay the native irisin is recognized by a polyclonal antibody reacting with serum samples under competition in the irisin coated plate.

We estimated patients' BMD using dual energy X-ray absorptiometry (DXA Discovery Wii, Hologic), by scanning the hip (total hip and femoral neck) and spine (L1-L4) and also the whole body to evaluate the body composition. To measure daily physical activity, patients were invited to wear an armband (Sense Wear armband SensorMedics, Milan, Italy) on the back of the triceps of the left arm, for about 72 hours. This was to enable tracking burned calories during daily physical activity, calculating the Metabolic Equivalent of Task (MET defined by "the ratio of the work metabolic rate to the resting metabolic rate", in particular 1 MET is equal to the oxygen cost of sitting quietly, equivalent to 3.5 ml/kg/min) and monitoring daily physical activity intensity. It was also employed to record quantity and quality of sleep.

3.2.3 Statistical analysis

Student T-test was used to compare quantitative variables between groups and Chi-square test used to compare categorical variables. The non-parametric Kruskal-Wallis test and the Fisher exact test were used when appropriate. One-Way Analysis of Covariance (ANCOVA) was used for adjusted analysis. A two-tail Pearson test was used to evaluate correlations between continuous variables. A p-value of less than 0.05

was considered statistically significant at 80% power. SPSS 21.0 for Windows was used to compute the analysis.

3.2.4 Ethics

This study was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization (ICH) principles of Good Clinical Practice (GCP). The research protocol was approved by the Ethical Committee of the University Campus Bio-Medico of Rome and all subjects gave their informed consent.

3.3 Results

Study groups were homogeneous for age, sex and BMI. In the overall study population (72 post-menopausal women, mean age 64.3 ± 6.1 years) BMD at all sites and lean mass were higher in the control group than in the fractured group (Table 3.1). As expected, femoral and total BMD were strongly correlated with age while lean mass correlated with BMD at the lumbar spine, total femur and femoral neck (Table 3.2). No significant correlations were found between irisin and BMD at any sites or between irisin with either lean or fat mass (Table 3.2). Serum levels of irisin were not correlated with METs.

Serum levels of creatinine were significantly lower in the fractured group and 25-OH Vitamin D was higher in the fractured group. No significant differences were found between the two groups in terms of calcium and phosphorus. Serum irisin levels were lower in subjects with previous osteoporotic fractures than in controls (Table 3.1 and fig. 3.1). However, the difference in irisin levels remained significant after adjustment for creatinine ($p=0.037$), 25-OH Vitamin D ($p=0.046$), lean mass ($p=0.02$), for lumbar BMD ($p=0.023$) and for total femur BMD ($p=0.032$).

	Fractured group (n=36)	Control group (n=36)	P value
Age, mean ± SD (years)	65.6 ± 6.7	62.9 ± 5.1	ns
Body Mass Index, mean ± SD (Kg/m^2)	25.7 ± 2.8	26.6 ± 3.0	ns
Irisin, (µg/mL)	0.688 ± 0.224	0.799 ± 0.204	**0.032***
Creatinine, (mg/dL)	0.76± 0.18	0.84 ± 0.12	**0.037**
Calcium (mg/dl)	9.26 ± 1.19	9.37 ± 0.55	0.606
Phosphate (mg/dl)	3.44 ± 0.64	3.59 ± 0.54	0.276
Vitamin D (ng/ml)	28.69 ± 14.31	20.48 ± 8.31	**0.005**
Vertebral BMD (g/cm^3)	0.725 ± 0.166	0.948 ± 0.110	**<0.001**
Total femur BMD (g/cm^3)	0.692 ± 0.138	0.858 ± 0.086	**<0.001**

Lean subtotal Body Mass (g)	29753.29 ± 2865.99	33328.09 ± 3898.15	**<0.001**
Total Fat Mass (g)	24848 ± 5564.25	26847.58 ± 5807.99	ns
METs**	1.314 ± 0.193	1.361 ± 0.222	ns

Table 3.1. Baseline comparative data of fractured group and control group. Values are mean ± SD BMD (Bone Mineral Density)

* The difference in irisin levels remained significant also after adjustment for creatinine (p=0.037), 25 (OH) vitamin D (p=0.046), lean mass (p=0.02), for lumbar BMD (p=0.023), for total femur BMD (p=0.032).

** METs (Metabolic Equivalent of Task. One MET is equal to the oxygen cost of sitting quietly, equivalent to 3.5 ml/kg/min)

	Correlation Coefficient (r)	p
Total femur BMD		
vs Age	**-0.403**	**<0.001**
vs Lean mass	**0.438**	**<0.001**
vs Irisin	0.083	0.489
vs METs	0.077	0.519
Neck BMD		
vs Age	**-0.275**	**0.019**
vs Lean mass	**0.489**	**<0.001**
vs Irisin	0.135	0.256
vs METs	-0.013	0.916
Lumbar BMD		
vs Age	-0.189	0.112
vs Lean mass	**0.376**	**0.001**
vs Irisin	0.070	0.560
vs METs	0.078	0.516
Irisin		
vs Age	-0.165	0.167
vs Lean mass	0.033	0.782
vs METs	0.120	0.314
Lean mass		
vs Age	-0.210	0.077
vs METs	0.032	0.791
METs*		
Vs Age	-0.080	0.504

Table 3.2. Correlations between BMD, irisin, lean mass and daily physical activity level (measured by METs). In bold significant correlations.

BMD: Bone Mineral Density;
* METs (Metabolic Equivalent of Task. One MET is equal to the oxygen cost of sitting quietly, equivalent to 3.5 ml/kg/min)

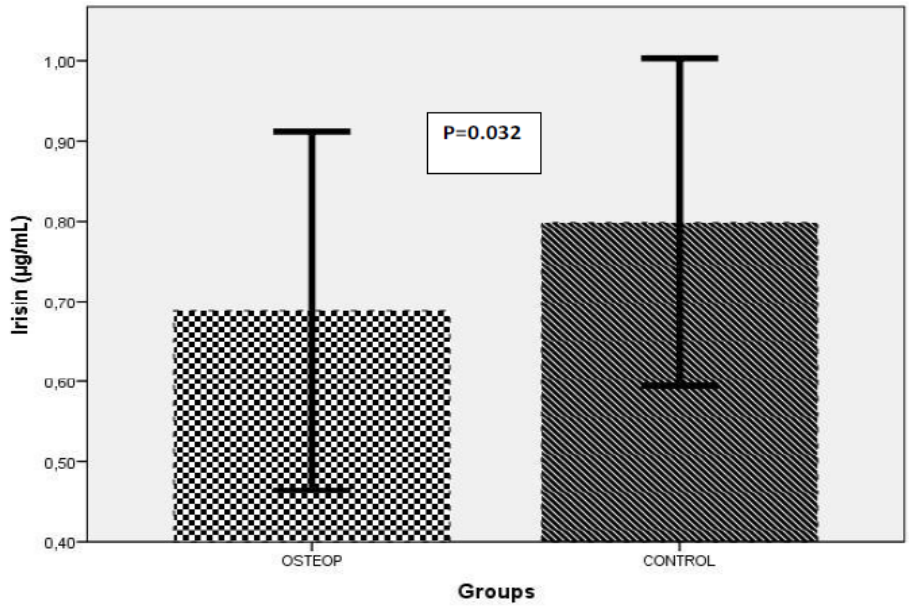


Figure 3.1. Irisin levels in postmenopausal women with severe osteoporosis (OSTEP) and control subjects (CONTROL)

3.4 Discussion

In the present study, we show that irisin is inversely related with vertebral fractures in postmenopausal women, independently of bone mineral density and body composition. Serum irisin was not associated with MET, a physiologic measure of the energy cost of daily physical activity. Since its discovery in 2012 (Boström et al. 2012), irisin has raised interest as potential therapeutic and diagnostic target for metabolic diseases such as obesity (Novelle et al. 2013), type 2 diabetes (Eckardt et al. 2014), gestational diabetes (Kuzmicki et al 2014), non-alcoholic fatty liver disease (Polyzos et al. 2014) and myocardial infarction (Emanuele et al. 2014). Based on the known interplay between bone and muscle, an involvement of this molecule in bone metabolism has been suggested. However, this has not been consistently substantiated either in vitro or in vivo. To our knowledge, the main preclinical evidence linking irisin to bone metabolism was provided by Colaianni et al. showing osteoblast differentiation enhancement in conditioned media collected from mature exercised myoblasts expressing high levels of irisin (Colaianni et al. 2014). Indeed, osteoblasts treated with

the exercised media had increased expression of ALP and Collagen I mRNA that was fully reversed in presence of an irisin neutralizing antibody, suggesting a direct effect of irisin on bone formation. Vertebral fractures are a relevant clinical problem in post-menopausal women and elderly men. Vertebral fractures are also strong predictors of either future vertebral or femur fractures. According to Melton, the presence of a vertebral fracture is associated with a 12.6-fold increase (95% CI, 11-14) in additional vertebral fractures and a 2.3- fold increase (95% CI, 1.8-2.9) in hip fractures (Melton et al. 1999).

Our current data confirm those by Anastasilakis et al. who have shown lower serum irisin levels in postmenopausal women with previous osteoporotic fractures independent of BMD (Anastasilakis et al. 2014a). Although lean mass was not analysed, authors independent of BMD and lean mass. Studies of the relationship between irisin and daily physical activity are so far inconsistent as well. In their original report, Bostrom et al found increased irisin levels in both mice and humans during exercise training (Boström et al 2012). Similarly, acute vibration exercise rather than chronic training has been shown to increase irisin levels in humans (Huh et al. 2014). Other recent studies have shown a positive correlation between irisin and physical activity (Anastasilakis et al. 2014b, Daskalopoulou et al. 2014, Tsuchiya et al. 2014). On the contrary other studies show no significant changes in either irisin levels or FNDC5 expression after physical training (Pekkala et al. 2013). Five more exercise intervention trials have independently showed no correlation between irisin levels and daily physical activity (Huh et al. 2012, Kurdiova et al. 2014, Norheim et al. 2014, Hecksteden et al. 2013, Ellefsen et al. 2014). In our study there was no correlation between irisin levels and daily physical activity measured with Armband. However, it has been recently reported that irisin secretion after acute running exercise is affected by exercise intensity. Indeed, high intensity exercise caused greater exercise-induced irisin response than low intensity exercise, regardless of similar energy consumption (Tsuchiya et al. 2014). In our study the daily physical activity intensity recorded trough Armband (mean METs 1.3) was not comparable to an

exercise training intensity, therefore this result could explain the non-correlation between irisin level and daily physical activity in our study.

We further confirm previous findings of Pardo M et al who have recently shown that irisin levels did not correlate with daily physical activity, measured trough a wearable watch (Actiwatch AW7), in a heterogeneous population of females with a wide range of BMI and age. (Pardo et al. 2014). Compared to Pardo, we used a homogeneous population and daily physical activity trough was measured with a "metabolic holter". Our findings are also in agreement with recent evidence showing no relationship between irisin and BMI, sarcopenia or waist circumference (Choi et al. 2014). Indeed, the authors demonstrated that in a large cohort of subjects (401 subjects) with and without sarcopenia defined by skeletal muscle mass index and appendicular skeletal muscle/height2 using DXA, serum irisin levels were not different. The lack of significant association was confirmed by the use of two different ELISA kits to measure irisin making reliable the findings obtained with our assay (Choi et al. 2014) Moreover even if our ELISA kit is different from that used by Anastasilakis et al (Anastasilakis et al. 2014a), we obtain the similar inverse correlation between irisin levels and fragility fractures (Pardo et al. 2014). Although data from Bostrom supported a directed correlation between irisin and BMI, later studies have provided mixed evidence with some supporting a positive association (Huh et al. 2012, Park et al. 2013, Stengel et al. 2013), while other reported null (Timmons et al. 2012) or even a negative relationship (Moreno-Navarrete et al. 2013). Adipose tissue is another abundant source of irisin implying a possible role of irisin on energy metabolism (Moreno-Navarrete et al. 2013). However, using DEXA total body, we did not find significant correlations between irisin levels and either total fat mass or trunk fat. Although experimental studies may suggest a relationship between irisin and lean or fat mass, reproducibility of these data in humans has been challenging so far, due to the heterogeneity of subjects selected by different studies and the lack of adjustment for possible factors influencing irisin, such as BMI, kidney function and age (Anastasilakis et al. 2014a, Moreno-Navarrete et al. 2013, Ebert et al. 2014). As obesity could be a confounding factor to correctly determine the irisin levels, we have

recruited overweight women excluding subjects with BMI higher than 30 kg/m2. Our study was limited by the small sample size, by the cross-sectional design and by the lack of availability of bone turnover markers. Strengths of this study include the analysis of daily physical activity through METs measured by armband and body composition by DEXA. speculated that low irisin levels may result from sarcopenic obesity and decreased muscle strength, factors associated with osteoporotic fractures (Aubertin-Leheudre et al. 2008). Our findings confirm an inverse relationship between irisin and fractures. In conclusion, our data confirm an inverse correlation between irisin levels and vertebral fragility fractures, but no significant correlation was found with BMD or lean mass. However, confirming the lack of correlation between irisin and BMD, our data suggest that this myokine might positively influence the bone strength probably by affecting the bone quality more than the bone mass. The absence of other studies that have investigated the role of irisin on bone metabolism doesn't allow us to formulate more detailed theories. Even if the study population is completely different from ours, our belief might help to explain why Singhal V et al found a significant positive correlation between irisin levels and some bone quality parameters (volumetric bone mineral density, stiffness and failure load) measured by high resolution peripheral quantitative CT and finite element analysis (Singhal et al. 2014). Our data might suggest also that level of daily physical activity doesn't widely affect the irisin levels in humans. Anyway, further preclinical and prospective clinical studies are needed to shed more light on the real effect of irisin on bone quality.

CHAPTER 4

RESEARCH PROJECT N°3

EFFECT OF CALCIDIOL ON INSULIN-RESISTANCE AND β-CELL FUNCTION IN SUBJECTS WITH IMPAIRED GLYCAEMIC CONTROL (IGT, IFG AND TYPE 2 DIABETES) *

*manuscript in preparation

4.1 Introduction

As diabetes, vitamin D deficiency could be recognized as a global pandemic (Zhang et al. 2017). Several studies have evaluated the effects played by vitamin D on metabolic outcomes, as insulin secretion, insulin sensitivity or glucose tolerance (Alvarez et al. 2010). In fact, at the end of the last century, Baynes et al. showed that hypovitaminosis D may be a significant risk factor for glucose intolerance (Baynes et al. 1997), suggesting being a protective compound for diabetes (Maddaloni et al. 2018, Epstein et al. 2016).

Among different types of vitamin D, calcidiol (25-hydroxyvitamin D3 (25(OH)D3)) is the main circulating form; it is the best indicator of vitamin D status, due to its hydrophilic properties (Ng et al. 2008, Yang et al. 2016). Studies showed that the 25(OH)D3 concentration is lower in individuals with IGT and T2D than in those with normal glucose tolerance (Targher et al. 2006), and low levels might play a significant role in the pathogenesis of T2D (Ozfirat et al. 2010). However, this role on insulin resistance and β-cell function has not been well understood in subjects with diabetes. So, recent studies analyzed the role of inflammation, of which it is well known that contributes to the pathophysiology of T2D (Zhang et. al. 2017, Badawi et al. 2010, Donath et al. 2011, Luotola et al. 2000, Pickup et al. 2004, Grossmann et al. 2015, Pittas et al. 2010, Thorand et al. 2011). In fact, 1,25(OH)2 D3, could be able to down-

regulate the production of pro-inflammatory cytokines (Flores et al. 2005). So, the effect of inflammatory pathways on interfering balanced metabolism, disrupting proper insulin signaling, may influence glucose homeostasis by modulation of typical response of inflammatory cytokines (Chagas et al. 2012). In particular, some inflammatory markers, as high-sensitivity C-reactive protein (hs-CRP), tumor necrosis factor (TNF-α), and interleukin-6 (IL-6), were inversely correlated to vitamin D status in subjects with IGT, IGF and T2D (Zhang et al. 2017, Thorand et al. 2011, Chagas et al. 2012, Kabaldi et al. 2013).

To the best of our knowledge, there are no clinical studies evaluating the effectiveness of systemic treatment with calcidiol on insulin-resistance and beta-cell function parameters, in subjects with impaired glycaemic control as in IGT, IFG or T2D. Anyway, those clinical trials that focused their attention to the efficacy of vitamin D supplementation on the glycaemic control in subjects with pre-diabetes or T2D have yielded inconsistent results (Maddaloni et al. 2018).

So, the aim of this study was to evaluate the effect of calcidiol supplementation on insulin resistance, β-cell function and markers of inflammation and oxidative stress in subjects with IGT, IFG and T2D and insufficient vitamin D levels.

4.2 Materials and Methods

It is a double-blind placebo-controlled clinical trial enrolling subjects with IGT, IFG and T2D (American Diabetes Association 2018) and 25(OH)D <20 ng/ml.

4.2.1 Study design and population

In this study were enrolled a total of 150 subjects and followed up for 6 months. Subjects were either assigned (50 per group) to 1) daily supplementation of 50 mcg of calcidiol (Arm A); 2) 25 mcg of calcidiol (arm B); 3) placebo (Arm C).

Inclusion criteria included: 1) males and females over 40 years old; 2) Fasting plasma glucose > 100 mg / dl or 2-hour glycaemia after oral glucose tolerance test of 75 g of glucose > 140 mg / dL or Hb A1c> 5.8%; 3) 25 (OH) D Serum levels <20 ng / ml. Exclusion criteria included: 1) Vitamin D supplementation in progress; 2) Pregnant or lactating women; 3) eGFR <40ml / min / 1.72m2; 4) Diabetes; 5) Hyperparathyroidism; 6) Hypercalcemia; 7) Nephrolithiasis; 8) Conditions that may have affected the metabolism of vitamin D or calcium (eg Sarcoidosis). Finally, each subject enrolled signed an informed consent.

Subjects were visited at the time of enrollment (Visit 1) and for regular checks after 1 month (Visit 2), after 3 months (Visit 3) and after 6 months (Visit 4) since the first visit. In Visit 1 the subjects were assessed on the basis of inclusion and exclusion criteria. The study protocol was approved by the Ethics Committee of Campus Bio-Medico University of Rome University. Written informed consent was obtained from all participants.

4.2.2 Biochemical measurements

Fasting blood glucose and Oral Glucose Tolerance Test (OGTT), HbA1c, 25 (OH) D, calcium, phosphorus, PTH, calciuria, phosphaturia, total cholesterol, HDL cholesterol and triglycerides were measured with laboratory kits used in clinical settings. Measurements of Ox-LDL, Hs-CRP, TNF-α, IL-6, esRAGE, sRAGE were performed at the laboratory of the Unit of Endocrinology and Diabetes of the Campus Bio-Medico University of Rome.

4.2.3 Evaluation of insulin resistance and β-cell function

To evaluate insulin resistance were used the ISOGTT index of Matsuda and DeFronzo (Matsuda et al. 1999) and the evaluation of the model of insulin-resistance homeostasis (HOMA-IR) by Matthews et al. (Matthews et al. 1985).
Beta-cell function was evaluated using the insulin secretion sensitivity index-2 (ISSI-2) (Retnakaran et al. 2010). This index is defined on the basis of the relationship

between the area under the insulin curve (AUC insulin) and the area under the blood glucose curve (AUCglucose), multiplied by ISOGTT.

4.2.4 Statistical analysis

A sample size of 50 patients per group was calculated to obtain a significant difference in variation in insulin resistance and beta-cell function markers, among the three groups. We used T-test, assuming a significant level of $p = 0.05$, a power of 80% and a standard deviation of 1.3%.

4.3 Results

At baseline, subjects were (mean±SD) 63.8± 2.1 years, BMI was 27.4±1.2 kg/m2; serum glucose 115.1±8.4 mg/dL, HbA1c 6.4±0.6%, 25(OH)D 16.3±2.5 ng/mL. There were significant associations of 25(OH)D with ISOGTT (β=0.35; 95% CI, 0.14, 0.46) and β-cell function (ISSI-2; β = 0.15; 95% CI, 0.02, 0.28). At baseline, the ISOGTT index values were 2.72 ± 0.61 in group A, 2.62 ± 0.63 in group B and 2.64 ± 0.67 in the group C. The ISSI-2 index at the beginning of the study was 220.3 ± 26.4 in group A, 229.1 ± 28.6 in group B and 225.1 ± 26.3 in group C. Starting levels of sRAGE were 155 ± 42.3 ng / ml in group A, 135.6 ± 36.9 ng / ml in group B and 126.4 ± 47.3 ng / ml in group C (fig. 4.1). At six months, 25(OH)D increased up to 48±3 ng/mL in Arm A (P<0.01) and to 36±5 ng/mL in Arm B (P<0.01); no significant changes in the Arm C (fig. 4.1).

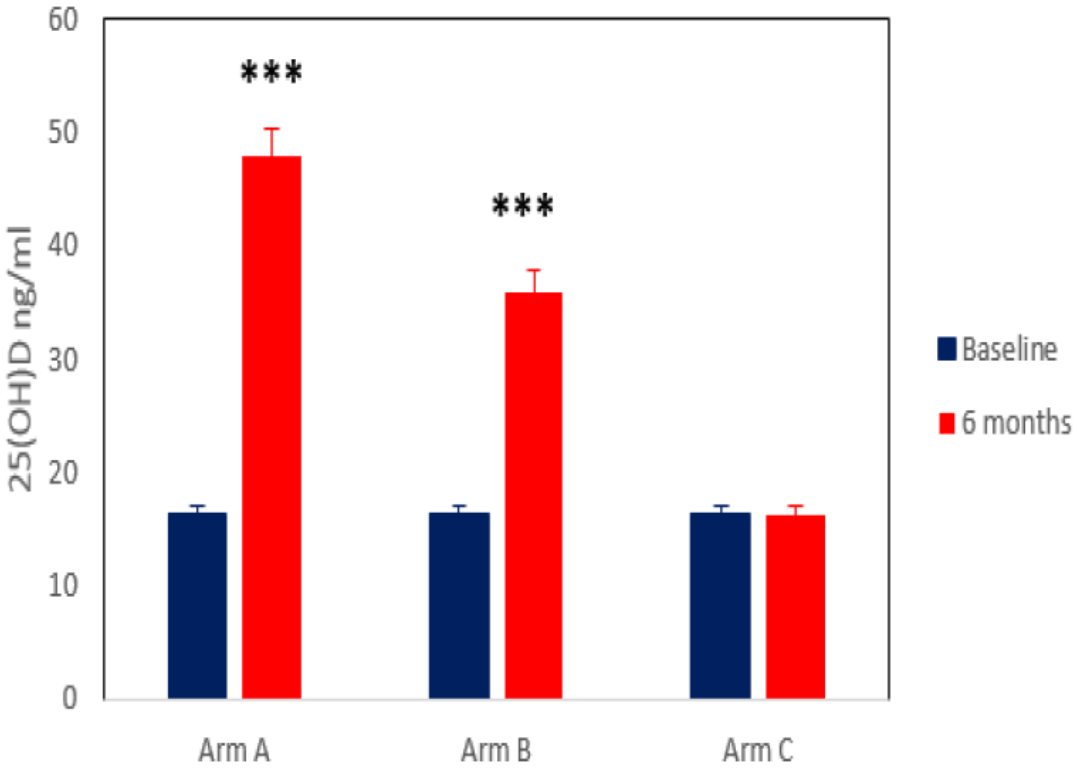


Figure 4.1 Variations in levels of 25 (OH) D: at baseline and 6 months

Subjects with daily supplementation of 50 mcg of calcidiol (Arm A) had a lower risk of dysglycemia (HR= 0.85, 95% CI, 0.75-0.97 per SD increase) while no significant effects were observed in the Arms B or C. Both ISOGTT and ISSI-2 were improved in Arm A ($P<0.05$) while no significant changes were observed in Arm B or placebo (fig. 4.2, fig 4.3, tab. 4.1).

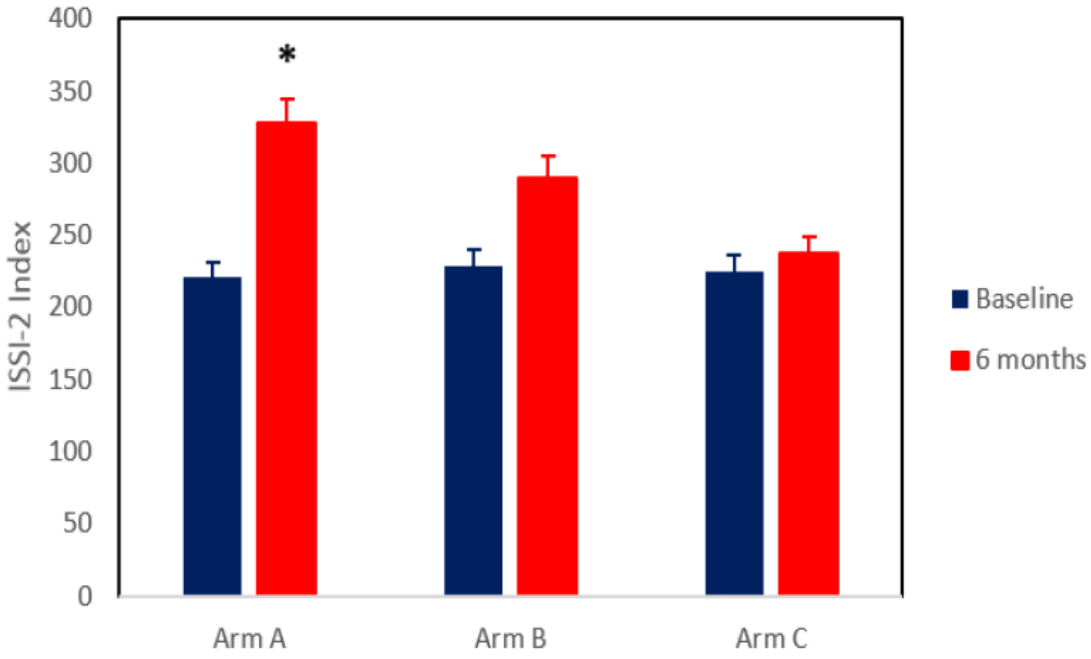


Figure 4.2 Variations of ISSI-2 Index: at baseline and 6 months

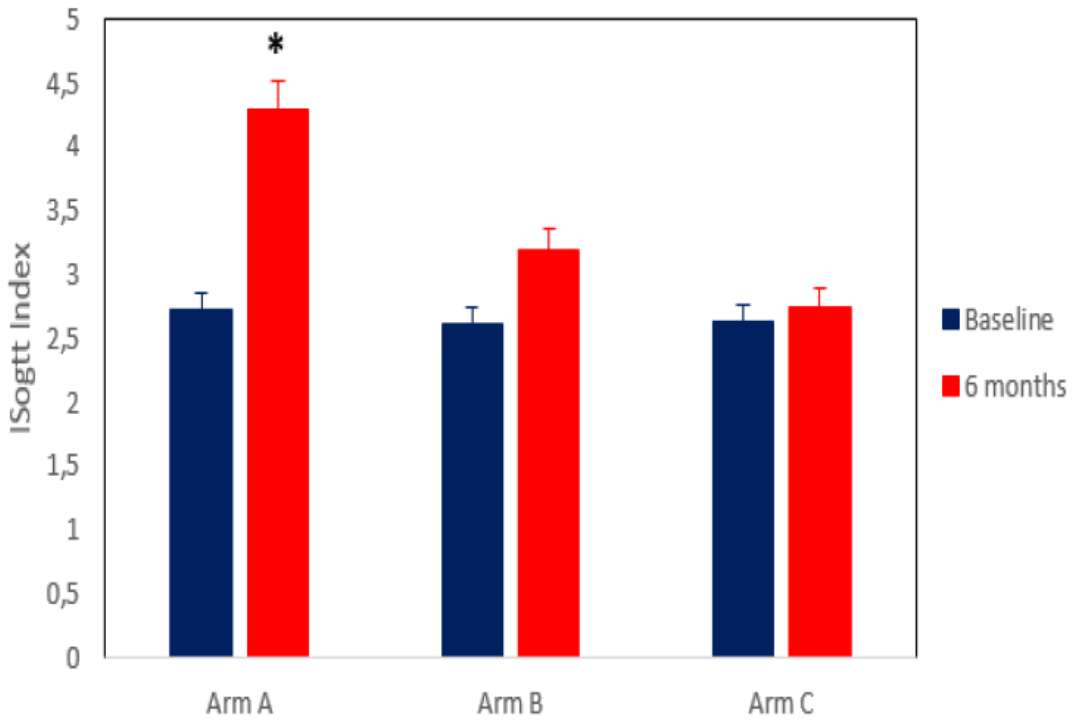


Figure 4.3 Variations in levels of ISOGTT: at baseline and 6 months

	ISOGTT Index		***ISSI-2 Index***	
Values	*basal*	*6 months*	*basal*	*6 months*
GROUP A	2,72 ± 0,61	4,3±1,1	220.3 ± 26.4	327.8 ± 25.8
GROUP B	2,62 ± 0,63	3,2±0,8	229.1 ± 28.6	289.8 ± 22.6
GROUP C	2,64 ± 0,67	2,75±-1	225.1 ± 26.3	236.8 ± 24.1

Table 4.1 Variations among ISOGTT Index and ISSI-2 Index at baseline and 6 months in group A, B, C

Serum levels of sRAGE decreased in Arm A [median 1354 (1069-1680) pg/ml ($P<0.01$), as compared with levels at study entry, but not in Arms B or C (fig. 4.4). No significant differences were observed for hsCRP, IL6, TNFα or lipid panel.

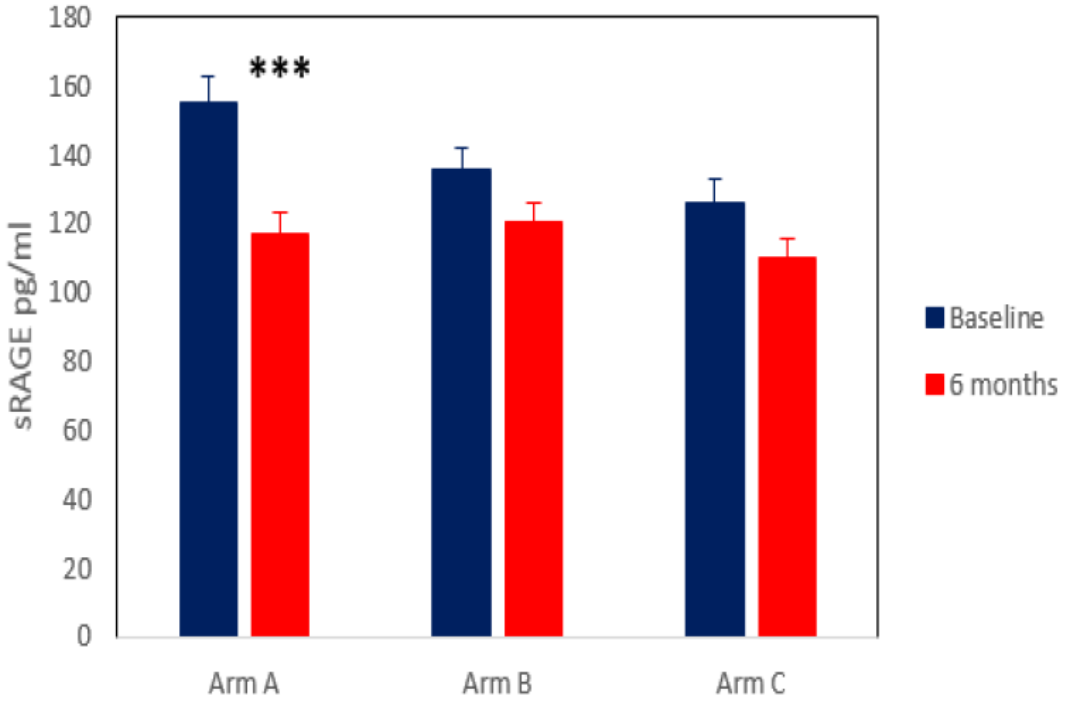


Figure 4.4 Variations in levels of sRAGE: at baseline and 6 months

4.4 Discussion

Hypovitaminosis D is a very common condition in the general population, and it represents a public health problem, primarily for the risk of developing pathological fractures. The attention of scientific community to integrate this hormone to achieve normal ranges is becoming increasingly, but these targets are standardized only in relationship to bone metabolism. Recent scientific evidences suggest that bone metabolism influences the regulation of glucose homeostasis and bone-related

metabolites are associated to pancreatic function. Therefore, the relationship between bone and glucose metabolism could be closely linked to the development of major metabolic alterations, such as impaired fasting glucose (IFG) and reduced glucose tolerance. In fact, some studies showed that vitamin D play an important role on etiopathogenesis of T2D, as higher basal levels of vitamin D are an independent predictor for the detection of improved beta-cellular function and lower blood sugar levels during follow-up (Kayaniyil et al. 2010). Furthermore, vitamin D receptor (VDR) is widely expressed, also on cells involved in the processes of chronic inflammation. This concept is quite interesting from the metabolic point of view, given the well-known role played by chronic inflammation in the development of alterations in glucose metabolism.

Anyway, the literature has not yet revealed certain data on the possible effects of vitamin D supplementation on the development of carbohydrate metabolism alterations, nor on the dosage necessary to prevent the development of T2D (El-Hajj Fuleihan et al. 2016). So, in this gap of knowledge, this study evaluated the influence that calcidiol treatment exerts on glucose metabolism.

In fact, these results indicate that daily supplementation with 50 mcg of calcidiol is associated with an improvement of the ISOGTT insulin resistance index and an improvement of the beta-cellular function, identified by the ISSI-2 index, as well as a reduction in the circulating levels of soluble receptor of the final products of advanced glycosylation (sRAGE).

These data suggested the importance of vitamin D supplementation in patients at high risk of diabetes, in which the alterations of glucose metabolism are present but not yet evident. In fact, 25 (OH) D supplementation can reduce insulin resistance and improve beta-cell function, to a greater extent if taken at higher doses, expressing a protective effect against micro and macro-vascular complications, as showed by sRAGE results.

Anyway, no changes were found in the levels of circulating inflammatory markers (CRP, IL-6, TNFα) or in the lipid panel. Muscogiuri et al. showed that low levels of 25 (OH) D are associated to a reduction in insulin sensitivity, in particular in obese subjects (Muscogiuri et al, 2010). Furthermore, there is a strong evidence that 25 (OH)

D modulates the signaling of intracellular ionized calcium in adipocytes, which in turn promotes the increase of lipogenesis and decreased lipolysis, probably by inhibiting the UCP-2 decoupling protein. So, vitamin D could have an active role in the physiology of adipocytes, but the clinical data for which obesity is strongly associated with low levels of 25 (OH) D are in contrast with the evidence derived from animal models, in which the absence of vitamin D in null mice for VDR (VDR - / -) determines atrophy of adipose tissue, a general decrease in fat mass, a reduction in serum leptin and an increase in resting energy expenditure. This data could be justified in consideration of the fact that adipose tissue is the main deposition site of 25 (OH) D and that obesity is the most common cause of insulin resistance. The association between levels of 25 (OH) D and insulin resistance could simply be the result of an increase in BMI, independently of the action of vitamin D on adipocytes. Hence, we used calcidiol to bring the levels of 25 (OH) D back to adequate values in prediabetic patients, who tend to be overweight or obese and therefore have a high volume of distribution, in which vitamin D is easily dispersed.

A 2016 study (Yang et al., 2016) showed that serum 25 (OH) D levels are significantly lower in patients with type 2 diabetes mellitus neodiagnosticated, compared to the group of healthy controls, and the prevalence of hypovitaminosis D exceeds 60% in diabetic subjects, supporting the results of previous studies in which it was shown that the concentration of 25 (OH) D at the basal level was inversely associated with the incidence of type 2 diabetes mellitus. Studies have reported a risk of developing doubled diabetes due to vitamin D <20 ng / l.

Kayaniyil et al. in 2011 conducted a study in which the baseline vitamin D value was an independent predictor of beta-cell function and glucose AUC after 3 years of follow-up within the cohort of the PROMISE (The PROspective Metabolism and ISlet cell Evaluation Cohort Study). This was the first study to examine the prospective association of 25 (OH) D with beta-cell function, with a significant association of 25 (OH) D with the ISSI-2 index and the IGI / IR ratio emerged.

Trying to explain the mechanism of suboptimal concentrations of 25 (OH) D in obese subjects, Wortsman et al. have evaluated whether obesity can alter the production of

vitamin D or its absorption (Wortsman et al. 2000). Both processes were similar in lean subjects, confirming that low levels of 25 (OH) D were caused by the increased sequestration of vitamin D in the large subcutaneous adipose tissue reserve. Some studies claim that vitamin D does not play a fundamental role in the pathogenesis of insulin resistance due to the fact that the supplementation of Calcitriol or its analogs did not reduce insulin resistance either in diabetic subjects or in healthy controls.

Supplementation with single dose vitamin D3 400 IU + 200,000 IU in subjects with diabetes did not have any effect on insulin-sensitivity between the placebo group and the vitamin D group, nor any change due to treatment in the of the single group. These data confirmed the results of other clinical trials, which had previously suggested the absence of effect of vitamin D supplementation on the metabolic compensation of diabetic patients. It must be taken into account that the measurement of insulin-sensitivity and insulin secretion has been detected through gold standard procedures rather than with poorly reproducible surrogate measures. However, it is possible the role of concomitant therapies (insulin sensitizers and / or secretagogues) that influenced the effect of vitamin D supplementation on insulin sensitivity and insulin secretion (Gulseth et al., 2017).

CRP and IL-6 are strongly correlated with the development of T2D and usually in long-term diabetic patients' TNF-alpha is high, contributing to insulin resistance. In addition, IL-10, one of the most important anti-inflammatory cytokines, has been inversely correlated with obesity, metabolic syndrome, hyperglycemia and T2D. Despite this, in this and some other studies it was not possible to demonstrate an effect of vitamin D supplementation on IL-6, IL-10, TNF-alpha, or CRP. Probably the follow-up period should be extended in order to observe the effects of vitamin D on inflammation. Furthermore, the inflammatory status of patients not yet frankly diabetic, such as those included in our study, is not yet at very high levels, unlike long-standing diabetic patients, where insulin resistance is presumed to be less reversible and less easily influenced by the effects of vitamin D.

Among the strengths of this study, is clear that the sample examined consists exclusively of patients with pre-diabetes, whose insulin resistance has not yet led to the degeneration of beta-cell function. In addition, the patients did not take any therapy for diabetes and, finally, the choice to use calcidiol, safe and effective, which allows a rapid and constant increase in vitamin D levels, avoiding that peaks are reached.
Furthermore, the stratification of the dosage in two treatment groups Vs placebo allowed to evaluate the most suitable dosage of calcidiol necessary to produce effects on the glucose metabolism and on the presence of sRAGE.

Anyway, this study presents some limitations: 1) no direct methods have been used for the measurement of insulin resistance and beta-cellular function, such as the hyperinsulinemic euglycemic clamp and the intravenous glucose tolerance test (IVGTT), preferring surrogate measures that have made it possible to carry out less demanding evaluations for patients, without the need to carry out a hospitalization for the evaluation of metabolic indices; 2) the duration of the follow-up, six months.
For sure, future clinical trials are certainly needed that examine a larger population to verify the effects of the high dosage of calcidiol in prediabetes.

CHAPTER 5

RESEARCH PROJECT N°4

CARDIAC AUTONOMIC NEUROPATHY AND BONE MINERAL DENSITY IN SUBJECTS WITH DIABETES*

*preliminary results from
ongoing STUDY

5.1 Introduction

Cardiac autonomic neuropathy (CAN) is a critical diabetes-related complication caused by damage of small nerve fibers (classes Aδ, B and C) (Gandhi et al. 2010). The prevalence of CAN is different among studies on this topic; this due to adopted tests, the diagnostic criteria used, and the population studied. In both subjects with T1D and T2D, the prevalence of confirmed CAN varied from 16.6 to 20% (Spallone et al. 2011, Ziegler et al. 1993, Valensi et al. 2003). Prevalence rates increased both with age (up to 38% in subjects with T1D and 44% in subjects with T2D aged 40–70 years) and diabetes duration (up to 35% and 65% respectively) (Low et al. 2004, Pop-Busui et al. 2009). Screening for CAN is recommended at the time of T2D diagnosis (Boulton et al. 2005).

Bone fragility has recently proved to be a new complication of T2D. The pathophysiological link between bone fragility and diabetes has not been fully clarified. Some mechanisms may influence bone homeostasis by altering the function of osteoblasts, osteoclasts and osteocytes, others may also exhibit changes in the structural properties of bone tissue. It is known that adipocytes and osteoblasts are derived from a common precursor, mesenchymal stem cells (MSC) and their differentiation is modulated by several interacting factors that may be disturbed in diabetes. Furthermore, other organs and endocrine systems such as the intestine, the

kidney, the cardiovascular system and the regulatory systems of vitamin D are also involved in the alteration of the bone metabolism, since they are also affected by the coexistence of diabetes. Therefore, all these pathophysiological assumptions are at the base of the diabetes-related osteometabolic complications. It should also be noted that while bone mineral density (BMD) is decreased in patients with T1D, BMD is normal or even increased in T2D patients (Napoli et al. 2014). So, what has been recently recognized is the link between diabetes and osteoporosis with fractures being more common in diabetics than non-diabetics.

Furthermore, bone remodeling is regulated by various neuronal inputs, including sympathetic tone, which is known to inhibit bone mass accrual. This aspect of sympathetic nervous system (SNS) function raises the prospect that the other arm of the autonomic nervous system, the parasympathetic nervous system (PNS) may also affect bone remodeling. So, PNS could be defined as a positive regulator of bone mass accrual, dampening the negative influence of the sympathetic tone on bone mass (Shi et al. 2010).

So, as CAN has an impact on PNS, directly linked to bone remodeling, the aim of this study evaluates, for the first time, the relationships of CAN and BMD in subjects with T2D.

At the moment, the enrollment of subjects is ongoing, so only preliminary results have been established and showed.

5.2 Materials and Methods

5.2.1 Study design and population

At the moment, a total of 62 adults with T2D diabetes were recruited in the outpatient clinic of the Department of Experimental Medicine of Sapienza University of Rome and Unit of Endocrinology and Diabetes of the University Campus Bio-Medico of Rome. Six of 62 subjects did not perform DXA and 1 of 62 subjects were not able to

perform CAN test. So, totally 55 subjects (age: 64,8 ±6,9years) with T2D (mean ± SD disease duration ± 12,8±7,7 years) were evaluated with CAN test and DXA (Tab. 5.1).

Participants were excluded from the study if they had a previous diagnosis of neuropathy/arrhythmia from any cause other than diabetic neuropathy.

5.2.2 Evaluation of cardiac autonomic neuropathy

Participants were asked about symptoms of impaired cardiac autonomic function. In addition, participants were asked about the presence of resting tachycardia. The following cardiovascular provocative tests (Bernardi et al. 2011) were performed to detect cardiac autonomic neuropathy (CAN): heart rate response to deep breathing (expiratory-to-inspiratory ratio), heart rate response to lying-to-stand test (30:15 ratio), blood pressure response to standing. Participants were asked to discontinue any interfering drug (i.e. antidepressant drugs and/or anti-hypertensive) at least 24 h before the tests. Normal values were adapted for age (Ziegler et al. 1992). Subjects were considered to have CAN if they had one abnormal result on the heart rate test (early CAN) or two or more abnormal results on heart rate tests (moderate CAN) or if they had orthostatic hypotension (severe CAN) (Spallone et al. 2011).

5.2.3 Evaluation of bone mineral density

BMD gr/cm2 was assessed in the femoral neck (FN) and lumbar spine (LS) regions by dual x-ray absorptiometry (DXA). The results of bone densitometry were compared with the reference data supplied by the manufacturer and expressed as BMD g /cm2, BMD T-score, for each measurement site.
Diagnosis of osteopenia and osteoporosis were confirmed by the WHO criteria (WHO Study Group. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis Report World Health Organization. Geneva: 1994) (respectively: BMD-T -score less than -1.0 and more than -2.5 for osteopenia, while less than - 2.5 for osteoporosis).

5.2.4 Ethics

The study was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Principles of Good Clinical Practice. The research protocol was approved by the Ethical Committee at the Sapienza University of Rome and all participants gave informed consent.

5.2.5 Statistical Analysis

Continuous data were described as absolute values, mean ± Standard Deviation (SD), and range.

Student's t-test was used to compare quantitative variables between groups and the chi-squared test as used to compare categorical variables. Fisher's exact test was used when appropriate. A $p < 0.05$ was considered statistically significant.

5.3 Results

A total of 55 subjects (age: 64,8 ±6,9years) with T2D (mean ± SD disease duration: 12,8±7,7 years; males:11,2±7,2 years; females: 7,3±1,3 were evaluated with CAN test and DXA. This group showed a mean of A1c 7,2%±1,4 (males:6,9%±1,5; females: 7,3%±1,3). All these parameters not showed any significant difference among males and females group (Table.5.1).

	Males (n=20)	Females (n=35)	Total (n=55)	p value
Age (years, mean±SD; min-max)	62,6±6,9 (50-75)	66,1±6,7 (45-78)	64,8±6,9 (45-78)	NS
A1c (%, mean±SD; min-max)	6,9±1,5 (4,7-10)	7,3±1,3 (5,6-10)	7,2±1,4 (4,7-10)	NS
Duration of diabetes (years, mean±SD;min-max)	11,2±7,2 (2,5-25)	13,6±8,0 (3-39)	12,8±7,7 (2,5-39)	NS

Table. 5.1 Characteristics of population study

The evaluation of BMD showed that about 41,8 % of DXA evaluated subjects had a normal BMD (males: 18,2%, females: 23,6% of entire population), while 49,1% had osteopenia (males: 30,9%, females: 18,2% of entire population) and 9,1% osteoporosis (all females) (Figure 5.1-A).

About CAN evaluation, subjects with no evidence of CAN were 28 (50,9%: males 21,8% and females 29,2%); while, patient with CAN were 27 (49,1%: males 14,5% and females 34,5%) (Figure 5.1-B).

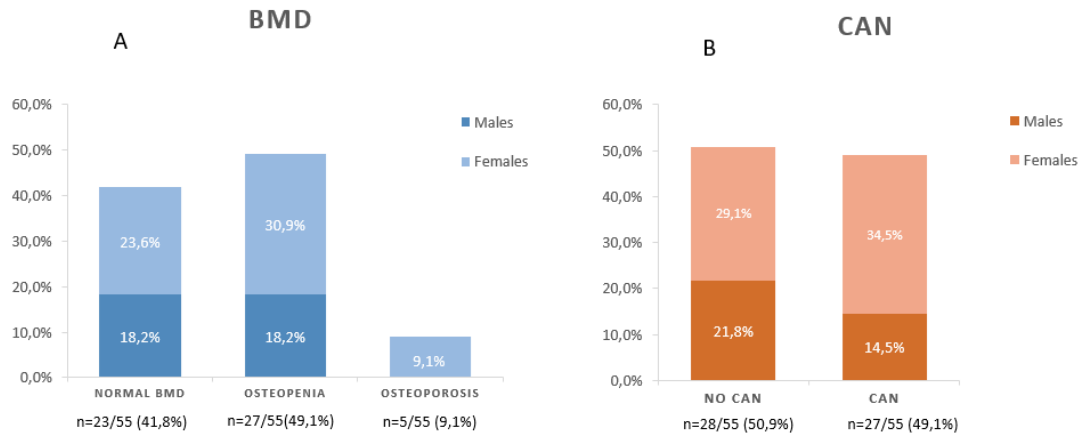


Figure 5.1 A, Prevalence of subjects with normal BMD, osteopenia, osteoporosis (males and females); B, Prevalence of subjects with and without CAN (males and females).

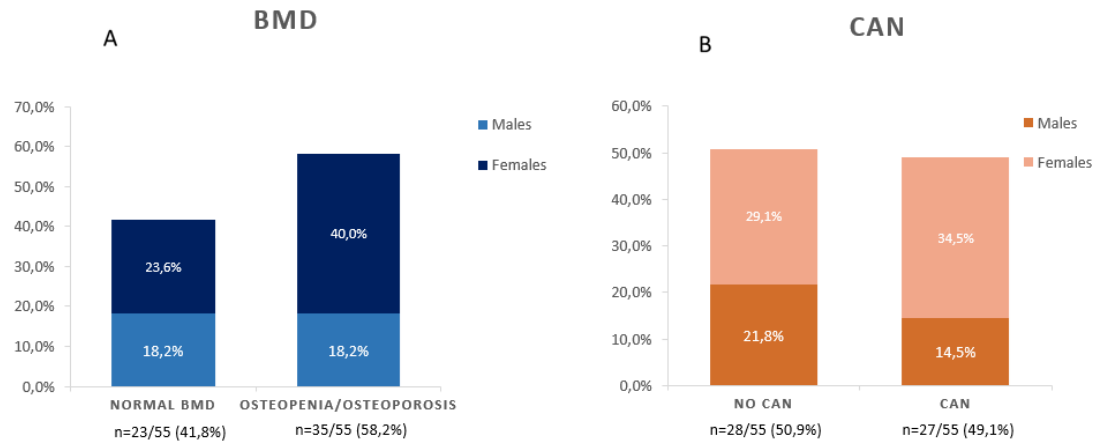


Figure 5.2 A, Prevalence of subjects with and without normal BMD, (males and females); B, Prevalence of subjects with and without CAN (males and females).

Evaluating in detail the different level of CAN in those subjects positive to tests (49%), 59,3% of subjects showed a mild form of CAN, 33,3% showed a moderate form of CAN and 7,4% showed a severe CAN (fig. 5.3).

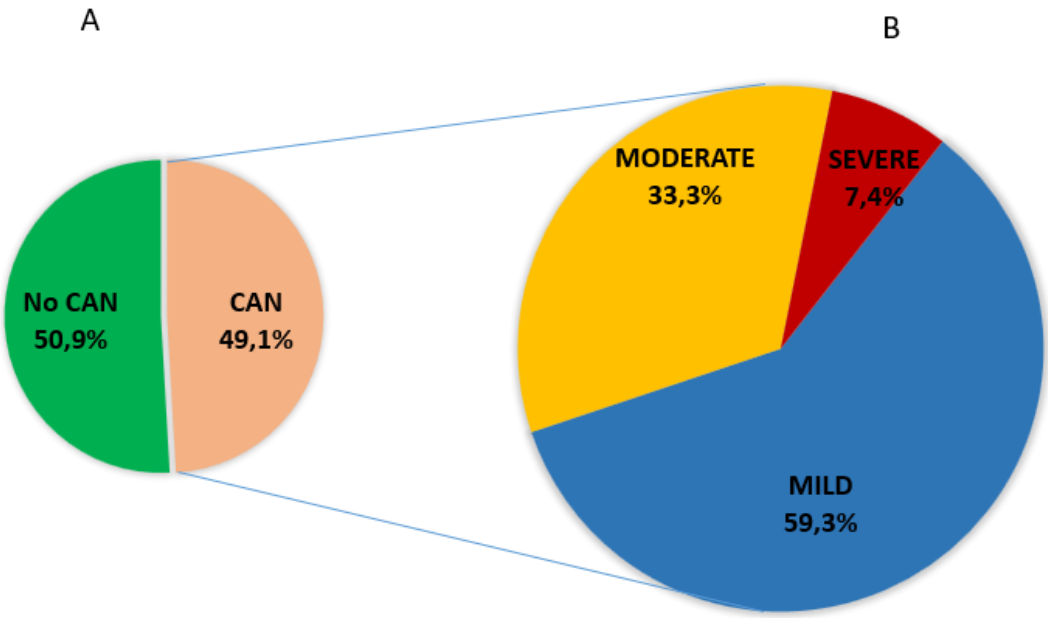
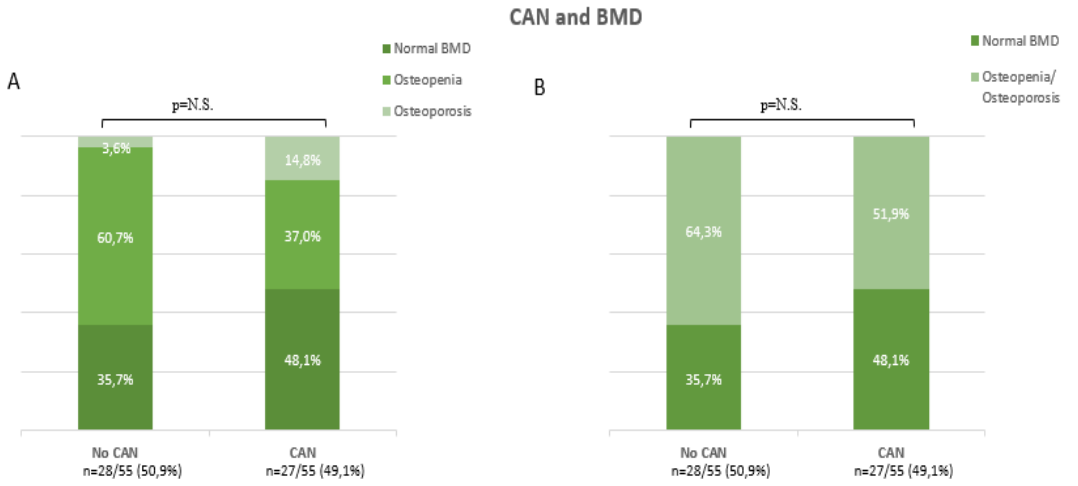


Figure 5.3 A, Percentage of subjects with and without CAN; B, Distribution of severity of CAN in study population.

We analyzed preliminary results among two evaluations of BMD and CAN, not finding any significative difference. In fact, subjects with no CAN showed a normal BMD in 35,7% while the remaining part had osteopenia or osteoporosis (64,3%: osteopenia: 60,7%; osteoporosis: 3,6%). Evaluating BMD in subjects with CAN, 48,1 had a normal BMD while 51,9 had osteopenia (37%) or osteoporosis (14,8%).



C

	No CAN (n=25)	CAN (n=27)	Total (n=55)	p value
Age (years, mean±SD; min-max)	65,1±8,0 (45-78)	64,5±5,6 (50-73)	64,8±6,9 (45-78)	N.S.
A1c (%, mean±SD; min-max)	7,1,±1,2 (5,3-10)	7,3±1,6 (4,7-10)	7,2±1,4 (4,7-10)	N.S.
Duration of diabetes (years, mean±SD; min-max)	11,3±6,3 (2,5-22)	14,1±8,8 (3-39)	12,8±7,7 (2,5-39)	N.S.

Figure 5.4, Prevalence of: A, Normal BMD, Osteopenia, Osteoporosis; B, Normal BMD and Osteopenia/Osteoporosis, in subjects with and without CAN; C, Mean of Age, A1c and Duration of diabetes in study population with and without CAN.

5.4 Discussion

CAN is one of the most overlooked but life-threatening complications of diabetes mellitus (Vinik and Ziegler 2007). Furthermore, bone remodeling is regulated by various neuronal inputs, including sympathetic tone, which is known to inhibit bone mass accrual. This aspect of SNS function raises the prospect that the other arm of the autonomic nervous system, the PNS may also affect bone remodeling. So, PNS could be defined as a positive regulator of bone mass accrual, dampening the negative influence of the sympathetic tone on bone mass. To the best of our knowledge, the present study shows, for the first time, an evaluation of BMD in a population of patients with T2D and CAN, to evaluate potentially effects of neuropathy on BMD too.

Due to a not complete enrollment, these are preliminary data on 55 subjects. About half of this population had CAN (49,1%). The main stage showed by these subjects was the lower, the mild with 59,3% while moderate stage was in 33,3% of subjects and the highest stage of gravity of CAN was 7,4%. Evaluating BMD with DXA, a little bit more than 50% of subjects with CAN showed impaired BMD (osteopenia or osteoporosis while subjects with CAN and normal BMD were 49,1%. There was non-significative difference among patients with CAN or without CAN and those with impaired BMD. These data are not conclusive, and it is not possible to reach find a real effect of CAN on BMD analyzed by DXA. It is plausible speculate that there are some limitations to this study at this stage of progression of the trial: firstly, the number of subject need to be increased to allow a more large population to analyze; secondly, it is possible that BMD evaluated with DXA show false negative results (Napoli et al. 2017); thirdly, the duration of T2D, and so the duration of CAN maybe was not so long to have effects on BMD; finally, a moderate glycaemic control of the subjects evaluated do not allow a worsening of BMD in a larger number of patients at the time of evaluation.

In conclusion, to our knowledge the present study is the first to evaluate BMD with DXA in a population of patients with T2D and CAN. For sure, it opens the way for larger studies to further define the role of CAN on BMD.

PUBLICATIONS AND PAPERS DERIVED FROM THIS WORK

6.1 Diabetes and disordered bone metabolism (diabetic osteodystrophy): time for recognition.

Epstein S*, **Defeudis G***, Manfrini S, Napoli N, Pozzilli P; Scientific Committee of the First International Symposium on Diabetes and Bone.

*Equal contribution

Osteoporosis International 2016 Jun;27(6):1931-51.

Impact factor: 3.591

6.2 The Importance of Evaluating Body Composition with Dual-Energy X-Ray Absorptiometry in Men: The Structure of the Aging Men's Bones (STRAMBO) Study.

Defeudis G.

Journal of Clinical Densitometry. 2017 Oct - Dec;20(4):462-463.

Impact factor: 3.240

6.3 BMI and BMD: The Potential Interplay between Obesity and Bone Fragility.

Palermo A, Tuccinardi D, **Defeudis G**, Watanabe M, D'Onofrio L, Lauria Pantano A, Napoli N, Pozzilli P, Manfrini S.

International Journal of Environmental Research and Public Health. 2016 May 28;13(6).

Impact factor: 2.101

6.4 Irisin is associated with osteoporotic fractures independently of bone mineral density, body composition or daily physical activity.

Palermo A, Strollo R, Maddaloni E, Tuccinardi D, D'Onofrio L, Briganti SI, **Defeudis G**, De Pascalis M, Lazzaro MC, Colleluori G, Manfrini S, Pozzilli P, Napoli N.

Clinical Endocrinology (Oxf). 2015 Apr;82(4):615-9.

Impact factor: 3.487

6.5 Serum sclerostin and bone turnover in latent autoimmune diabetes in adults.

Napoli N, Strollo R, **Defeudis G**, Leto G, Moretti C, Zampetti S, D'Onofrio L, Campagna G, Palermo A, Greto V, Manfrini S, Hawa MI, Leslie RD, Pozzilli P, Buzzetti R; NIRAD (NIRAD 10) and the ACTION LADA study groups.

Journal of Clinical Endocrinology and Metabolism 2018 Mar 1.

Impact factor: 5.455

OTHER PEER-REVIEWED PAPERS PUBLISHED DURING MY PhD

A pilot study of D-chiro-inositol plus folic acid in overweight patients with type 1 diabetes.

Maurizi AR, Menduni M, Del Toro R, Kyanvash S, Maggi D, Guglielmi C, Pantano AL, **Defeudis G**, Fioriti E, Manfrini S, Pozzilli P.

Acta Diabetologica 2017 Apr;54(4):361-365

Impact factor: 3.340

Erectile dysfunction and its management in patients with diabetes mellitus.

Defeudis G*, *Gianfrilli D*, Di Emidio C, Pofi R, Tuccinardi D, Palermo A, Lenzi A, Pozzilli P.*

**Equal contribution*

Reviews in Endocrine & Metabolic Disorders 2015 Oct 26.

Impact factor: 4.817

Conversation Maps™, an effective tool for the management of males and females with type 2 diabetes and mildly impaired glycemic control

Defeudis G, KhazraiYM, Di Rosa C, Secchi C, Montedoro A, Maurizi AR, Palermo A, Pozzilli P, Manfrini S

Hormones, 2018. doi.org/10.1007/s42000-018-0005-9

Impact factor: 1.712

The CATCH Checklist to Investigate Adult-Onset Hypogonadism

Defeudis G, Mazzilli R, Gianfrilli D, Lenzi A, Isidori AM

Andrology, 2018 doi: 10.1111/andr.12506

Impact factor: 2.427

A Case of Pheochromocytoma With Negative MIBG (VHL Included) And A Rare Case of Post-Operative Erectile Dysfunction

Defeudis G, Fioriti E, Palermo A, Tacchinardi D, Minucci A, Capoluongo E, Pozzilli P and Manfrini S.

Hormones, 2018 in press

Impact factor: 1.712

H-index 2017: 5

ABSTRACTS AND ORAL COMMUNICATIONS DURING PhD Course

1. Erectile Dysfunction and Cardiac Autonomic Neuropathy: A Study of Prevalence in Patients with Diabetes
   G. Defeudis, E. Maddaloni, R. Strollo, R. Del Toro, A. Palermo, S. Manfrini, P. Pozzilli. Congresso Nazionale Società Italiana di Endocrinologia. Taormina 2015. *Abstract*
2. The Efficacy of Continuous Glucose Monitoring to Improve BMI and Glycaemic Control in Prediabetes: the OBIG Trial
   Giuseppe **Defeudis**, Chiara Di Emidio, Rocky Strollo, Maria Verona Rinati, Dario Tuccinardi, Andrea Palermo, Silvia Angeletti, Silvia Manfrini, Paolo Pozzilli American Diabetes Association Congress, Boston, June 5-9th 2015
3. Serum Sclerostin and Bone Turnover in Relation to Metabolic Syndrome in Patients with Type 2 Diabetes or LADA N. Napoli, R. Strollo, **G. Defeudis**, G. Leto, M.I. Hawa, R.D.G. Leslie, P. Pozzilli, R. Buzzetti, ACTION LADA GROUP, NIRAD GROUP American Diabetes Association Congress, Boston, June 5-9th 2015
4. Erectile dysfunction and cardiac autonomic neuropathy: a study of prevalence in patients with diabetes. **G. Defeudis**, E. Maddaloni, R. Strollo, R. Del Toro, A. Palermo, S. Manfrini, P. Pozzilli. Congresso Nazionale della Società Italiana di Endocrinologia, Taormina, 27-30.5.2015
5. Serum sclerostin and bone turnover in relation to metabolic syndrome in patients with type 2 diabetes or LADA: ACTION LADA AND NIRAD GROUPS, oral presentation. N. Napoli, R. Strollo, **G. Defeudis**, G. Leto, M.I. Hawa, R.D.G. Leslie, P. Pozzilli, R. Buzzetti, ACTION LADA GROUP, NIRAD GROUP Congresso Nazionale della Società Italiana di Endocrinologia, Taormina, 27-30 Maggio 2015
6. Disfunzione erettile e neuropatia autonomica cardiaca: studio di prevalenza in una popolazione con diabete tipo 1 e tipo 2. **Giuseppe Defeudis**, Ernesto Maddaloni, Rocky Strollo, Rossella Del Toro, Andrea Palermo, Silvia Manfrini, Paolo Pozzilli. Incontri Italiani Di Endocrinologia e Metabolismo. Pisa, 19-20.5.2016
7. Sexual, Erectile Function and Cardiac Autonomic Neuropathy in Patients with Type 1 And Type 2 Diabetes: A Population Study **Giuseppe Defeudis**, Ernesto Maddaloni, Rocky Strollo, Claudia Caggiano, Rossella Del Toro, Andrea Palermo, Silvia Manfrini, Paolo Pozzilli. Congress of Italian Society of Andrology and Sexual Health – 1,2,3 Sex- Rome 1-3rd/12/16. *Poster*
8. Cardiac autonomic neuropathy is efficiently diagnosed by Valsalva manoeuvre alone. Maddaloni E, Del Toro R, Fioriti E, Tabacco G, **Defeudis G**, Caggiano C, Manfrini S, Pozzilli P. American Diabetes Association Congress, San Diego, California June 9-13 2017 *Poster*

9. Effect of calcidiol on insulin resistance an β-cell function in subjects with pre-Diabetes. Maddaloni E., Strollo R., **Defeudis G**., Pozzilli P., Napoli N. Italian Congress of Italian Society of Endocrinology (SIE) 21-24.6.17; *oral communication.*
10. I livelli di HDL sono associati ai livelli di testosterone totale in soggetti affetti da patologie andrologiche. Briganti S. **Defeudis G**, Maddaloni E, Piralide S, Manfrini S, Napoli N, Pozzilli P.III update Endocrino-Metabolico in ambito andrologico. Cassino, 6-7 aprile 2018. *Oral Communication*

CONGRESSES AND CONFERENCES AS SPEAKER and/or CHAIRMAN

- Serum sclerostin and bone turnover in relation to metabolic syndrome in patients with type 2 diabetes or LADA: ACTION LADA AND NIRAD GROUPS, oral presentation. Congresso Nazionale della Società Italiana di Endocrinologia, Taormina, 27-30.5.2015
- Serum sclerostin and bone turnover in relation to metabolic syndrome in patients with type 2 diabetes or LADA: ACTION LADA AND NIRAD GROUPS, oral presentation, EYES European meeting, Modena, 24-26.9.2015
- Erectile dysfunction and cardiac autonomic neuropathy in diabetes, oral presentation, EYES European meeting, Modena, 24-26.9.2015
- La disfunzione erettile ed il suo trattamento nel paziente con diabete. XXXI Congresso Medicina della Riproduzione. Abano, febbraio 2016
- Endocrinology and Metabolic diseases: Residency and training in Italy. 43rd Annual Meeting Hellenic Endocrine Society Athens 20-23.4.2016
- Diabete e Disfunzione Erettile. Update endocrino-metabolico in ambito andrologico. Frosinone, 14.5.16
- V Clinical Update in Endocrinology and Metabolism (CUEM) Chairman - Brescia, 7-8. 7. 2016-
- Vitamina D e andrologia: è vera sinergia? – oral presentation- VII giornate Andrologiche e Ginecologiche del Sant'Andrea. 29-30.9.2016
- Diagnosi di ipotiroidismo- oral presentation – University Campus Bio-Medico di Roma – 15.10.2016
- Funzionalità Erettile E Sessualità Nei Pazienti Con Diabete Tipo 1 E Tipo 2: Uno Studio Di Popolazione – Oral Presentation – Abano Terme – 25.2.17
- Diabete, disfunzione erettile e nuove tecnologie di somministrazione IPDE5 as speaker, Nuove evidenze per la terapia delle patologie endocrino-metaboliche, Cassino 17.6.17

- Erectile dysfunction and cardiac autonomic neuropathy in patients with type 1 and type 2 diabetes: the Deca study. European Young Endocrine Scientists (EYES) Congress. Porto (Portugal) 8-10.9.2017
- Adult onset hypogonadism: dalla diagnosi alla terapia. VIII giornate Andrologiche e Ginecologiche del Sant'Andrea Roma, Osp. Sant'Andrea – 29.9.17
- Diabete e fertilità maschile. XI Giornate di Andrologia e Medicina della Riproduzione. Sabaudia, 19.10.17
- Terapia androgenica sostitutiva: comorbilità e correlati sistemici. Ipogonadismo, diabete e sindrome metabolica- I Congresso Nazionale Androyoung. Rimini, 17-18.11.17
- Percorso integrato andro-ginecologico: terapie potenzialmente risolutive. As Discussant. XXXIII Convegno di Medicina della Riproduzione. Abano 22-24 February 2018.

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