.

Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in
Scienze Biomediche Integrate e Bioetica
XXXI ciclo a.a. 2015-2016

ADULTS WITH AUTOIMMUNE DIABETES: VASCULAR RISK, EMERGING COMPLICATIONS AND NOVEL DISEASE PATHWAYS

Ernesto Maddaloni

Coordinatore
Prof. Paolo Pozzilli

Tutore
Prof. Raffaella Buzzetti

20 Marzo 2019

Truth needs calm and simplicity
[Immanuel Kant]

Table of contents

Abstract

Background. The population of adults with autoimmune diabetes has grown worldwide. This is largely the result of better care and increased longevity of people with young-onset type 1 diabetes (T1D), but also of the increased number of latent autoimmune diabetes of the adult (LADA) diagnoses. Adults with autoimmune diabetes differ from those with type 2 diabetes (T2D), with challenges and uncertainties about the impact of aging in people with autoimmune diabetes. In the immediate future, we will have to face complications of autoimmune diabetes in the context of aging and long exposure to the disease, but a paucity of data is available in this regard. Therefore, studies elucidating the pathophysiology, epidemiology and clinical features of autoimmune diabetes in adults and elderly people, both those with T1D and LADA, are needed.

Hypothesis: We hypothesized that clinical features and risk of diabetic chronic complications may differ in adults with different forms of diabetes. Therefore, we also hypothesize that the study of vascular and bone disorders in adults with autoimmune diabetes may unveil novel risk factors and pathways of chronic complications.

Aims and methods. The overall aim of this project was to investigate the pathophysiology and clinical features of autoimmune diabetes and its complications during the adulthood and the senescence. More specifically we aimed to:

1. Evaluate whether rates and risk factors for vascular complications differ between LADA and T2D.

To this aim data from the UK Prospective Diabetes Study (UKPDS) have been retrieved and analyzed. Diabetes autoantibodies (AAb) were measured in 5,062 UKPDS participants. The incidence of major adverse CV events (MACE), defined as CV death, nonfatal myocardial infarction or nonfatal stroke, was compared in those with LADA (≥1 AAb test positive) with those without LADA (AAb negative).

2. Evaluate bone health and its relationship with vascular complications in adults and aging people with autoimmune diabetes.

To this aim adults with T1D followed in the centers of the IMDIAB group and elderly people enrolled in the 50-Years Joslin Medalist Study were fully characterized in terms of history of metabolic control, chronic complications and bone fractures. Bone mineral density was measured by DEXA in a subgroup of these subjects. Risk factors for impaired bone health and the relationship between bone fragility and vascular complications were investigated in both young adults and elderly with long standing type 1 diabetes.

3. To investigate circulating osteoprogenitors as a new mechanism of vascular complication in type 1 diabetes

Osteocalcin (OCN) + monocytes were studied in a unique population with ≥ 50 years of T1D, the 50-Year Joslin Medalists. CD45 bright/CD14+/OCN+ cells in the circulating mononuclear blood cell fraction were quantified by flow cytometry in and reported as percentage of CD45 bright cells. Mechanisms were studied by inducing OCN expression in human monocytes in vitro.

Results

Specific aim 1. There were 567 participants with LADA (11.2%). Compared with T2D, they were younger, with higher mean HbA_{1c} and HDL-cholesterol values but lower body mass index, total cholesterol and systolic blood pressure values (all $p<0.01$). After median (25^{th} – 75^{th} percentile) 17.3 (12.6-20.7) years follow-up, MACE occurred in 157 (17.4 *per* 1000 person-years) LADA and 1544 (23.5 *per* 1000 person-years) T2D participants respectively (HR 0.73, 95% Confidence Interval [CI] 0.62–0.86, $p<0.001$). However, after adjustment for confounders, this difference was no longer significant (HR_{adj} 0.90, 95% CI 0.76–1.07, $p=0.22$).

Specific aim 2. Among 600 adult subjects with T1D (age: 41.9±12.8 years, disease duration: 19.9±12.0 years; BMI: 24.4±3.7 kg/m^2; 5-year average HbA1c: 7.6±1.0%), 18.5% experienced at least one fragility fracture (73.8% had only one and 26.2% had more than one fracture). In this population, increased risk for ≥2 fractures was found in subjects in the highest tertile of HbA1c

(≥7.9%) compared with the lowest tertile (≤7.17%) (RRR 3.50 [1.04-11.7], p=0.04) and of disease duration (≥ 26 years versus < 14 years) (RRR 7.59 [1.60-35.98], p=0.01). The presence of neuropathy increased the risk of single fracture (RRR_{adj}: 2.57 [95%CI: 1.21-5.46]), and multiple fractures (p-value for the difference of the effect on outcomes: 0.99).

Differently, in a selected population of elderly T1D subjects (age: 66.0±7.6 years) with an extreme disease duration (>50 years) we found a lower prevalence of fragility fractures (1.12%). Because of the low prevalence of chronic complications in this population (cardiovascular disease: 39.9%; retinopathy: 46.4%; nephropathy: 12.5%), we hypothesized an association between vascular complications and bone health. This was confirmed by a significant association found between history of cardiovascular disease and low bone mass at the femoral neck (RR: 4.6 [1.2–18.1], p=0.03).

Specific aim 3. Subjects without history of CVD (n=16) showed lower levels of OCN+ monocytes than subjects with CVD (n=14) (13.1±8.4% vs 19.9±6.4%, p = 0.02). OCN+ monocytes level was inversely related to total high-density lipoprotein (HDL) cholesterol levels ($r = -0.424$, $p = 0.02$), large ($r = -0.413$, $p = 0.02$) and intermediate ($r = -0.445$, p=0.01) HDL sub-fractions, but not to small HDL. In vitro, incubation with oxidized low-density lipoprotein (OxLDL) significantly increased the number of OCN+ monocytes ($p < 0.01$). This action of OxLDL was significantly reduced by the addition of HDL in a concentration dependent manner ($p < 0.001$). Inhibition of the scavenger receptor B1 (SR-B1) reduced the effects of both OxLDL and HDL ($p < 0.05$).

Conclusions

This project evaluated in depth the risk of cardiovascular disease, bone fragility and their intimate relationship in adult and elderly subjects with autoimmune diabetes. Our data show that the healthier cardiometabolic profile of subjects with LADA compared with T2D translates in a lower incidence of major cardiovascular events, which is mostly explained by traditional cardiovascular risk factors, including age, lipids and blood pressure. This highlights the importance of aggressively tackling these cardiovascular risk factors in autoimmune diabetes to keep the lower risk of CVD. On the other hand, we are showing an alarming increased risk of bone fractures in adults with T1D. As bone fragility

fractures are among the most important causes of reduced life expectancy in elderly and because of the ageing of T1D population, our data claim for immediate action to tackle this emerging complication. Of note, we are showing a close relationship between bone fragility and chronic complications of diabetes, which has been confirmed also in a special population of elderly subjects with T1D protected from vascular complications. This might suggest that strategies to prevent vascular complications may also aid in preventing fragility fractures in T1D. Furthermore, this led to the hypothesis that common mechanisms of disease are shared between bone and vascular complications. This was explored by looking at the role of circulating osteoprogenitors in CVD, which were found lower in T1D subjects protected from CVD. Results regarding the regulation of OCN expression on monocytes by OxLDL and HDL through SR-B1 and its relationship with CVD provide new information on vascular pathophysiology specifically in T1D. Indeed, these findings may provide new insights on the mechanism of HDL-mediated cardiovascular protection in autoimmune diabetes and promote advances in therapeutic strategies in this population.

1. State of the art

1.1. Autoimmune diabetes in adults, an overlooked issue

Autoimmune diabetes is a multifactorial and polygenic disorder characterized by the destruction of pancreatic beta cells, on an autoimmune basis, resulting in absolute insulin deficiency [1]. Type 1 diabetes (T1D) is the most aggressive form of autoimmune diabetes and is mostly diagnosed during childhood. Therefore, historically, autoimmune diabetes has been largely considered a disorder of children and adolescents and most of the clinical studies on autoimmune diabetes focused on this population or on young adults. However, this opinion has changed over the past decade due to the increased life-expectancy of people with T1D which is drastically changing the epidemiology of the disease. The number of elderly subjects with long standing T1D is projected to quickly rise in the immediate future because of the increasing T1D incidence and the improved quality of care allowing people to survive longer despite the disease. Thus, in the immediate future we will have to face age-related disorders in the unusual setting of T1D, with the uncertainties related to the effect of long history of diabetes and insulin therapy on these disorders and their relationship with the chronic vascular complications of diabetes.

Furthermore, it has been recognized that an increasing number of new autoimmune diabetes cases occur during adulthood. In particular, there is a substantial number of people with an initial clinical diagnosis of type 2 diabetes (T2D), but having detectable serum markers of beta-cell autoimmunity. These subjects are affected by a form of autoimmune diabetes called "Latent Autoimmune Diabeets of Adults" (LADA). While these subjects do not require insulin at the time of diabetes diagnosis, their clinical features may significantly differ from people with T2D, reflecting a different pathophysiology.

Overall, few is known about the implications of autoimmune diabetes during adulthood and the heterogeneity of the disease complicates clinical research in this field. The increased number of adults

with autoimmune diabetes claims for studies specifically investigating the complex relationship between aging, diabetes and chronic complications, eventually describing new complications of autoimmune diabetes emerging during senescence.

1.1.1. Long-standing type 1 diabetes

Despite the prediction of an exponential increase in the number of elderly subjects with T1D, there is a paucity of data describing the clinical features of this people. Therefore, in the last decade there has been an effort in recruiting elderly people with extreme duration T1D in order to provide a picture of what is to come as the number of individuals with T1D grows and their longevity increases. The study of this population also provides a unique opportunity to characterize the effects of autoimmune diabetes on age-related disorders. Moreover, since a substantial percentage of this people has survived the disease for decades without developing the deadly complication of diabetes, it also provides an extraordinary chance to study protective factors versus the deleterious effects of autoimmune diabetes. The Joslin 50-Years Medalist Study has been the first study of this kind, enrolling from all-over the United States subjects with at least 50-years of insulin requiring diabetes. The Study has been conducted by researchers at the Joslin Diabetes Center (Harvard Medical School) in Boston and has identified a cohort of individuals (n=1000) who have survived with over 50 years of T1D, with the main objective of characterizing individuals with extreme duration T1D. The Study aimed to understand how individuals live to extreme duration with T1D and to identify genetic, environmental, psychological and physiological factors, which may contribute to survival with extreme duration of diabetes. Furthermore, the mean age of the subjects enrolled (>65 years) offers the possibility to investigate the relationship between autoimmune diabetes and the aging process. In the Joslin 50-Year Medalist cohort a large number have survived without any complications after an average of 55 years of insulin dependence. The 50-Year Medalists (54.3% female) have had T1D for a median [q1, q3] of 53 [51, 57] years, age of 65 [60, 70] years and HbA1c 7.1% [6.6, 7.7]. Medalists are lean with a body mass index (BMI) of 25.6 kg/m^2 [23.0, 28.6], have a favorable lipid profile and little renal

disease with a median estimated glomerular filtration rate of 71.1 (ml/min/1.73 m^2) [55.3, 85.7]. Despite the common dogma complications are inevitable, 20.2% of Medalists are without microvascular complications.[2] Only 46.6% diagnoses with PDR, 65.5% with diabetic neuropathy and 40.2% with cardiovascular disease. This population does have the hallmarks of type 1 autoimmune diabetes with a 94.8% frequency of the DR3 and/ or DR4 risk alleles and over 40% retaining autoantibody positivity (IA2 or GAD). The study of the Medalists has already led to significant advances in knowledge in the field of T1D and its complications, such as the successful identification of target molecules for the prevention and intervention of diabetic retinopathy and nephropathy.

Other groups from Canada and Europe are also enrolling subjects with a long history (>50 years) of T1D, highlighting the relevance of describing the clinical features of this people.

1.1.2. Latent Autoimmune Diabetes of Adults

Even though autoimmune diabetes is generally regarded as a condition that presents in childhood or adolescence, a substantial proportion of patients experience onset in adulthood. Epidemiological studies have highlighted that the majority of patients with onset in adulthood does not require treatment with insulin at the time of diagnosis, and these patients are defined as LADA. This term identifies a form of autoimmune diabetes with later mean age at onset, slower rate of beta-cell loss and longer period of insulin independence after onset if compared to T1D. In 2005, the Immunology of Diabetes Society proposed three main criteria for the diagnosis of LADA: adult age of onset (>30 years); the presence of any islet autoantibody; and the absence of insulin requirement for at least 6 months after the diagnosis. Based on these criteria, which are still debated, LADA can be identified in 4-14% of patients with a clinical diagnosis of T2D. Epidemiological studies have shown that the frequency of glutamic acid decarboxylase autoantibody (GADA), tyrosine phosphatase IA2

autoantibody (IA-2A), islet cell autoantibody (ICA) and or autoantibody against the Zinc transporter 8 (ZnT8), varies considerably depending on ethnicity. We have shown a prevalence of 2.6% among Arab people,[3] in contrast to a prevalence of 4.5% described in Italy[4] and of 12% described in the United Kingdom. The highest rates of LADA were reported in people of northern European origins.

LADA differs both genetically and clinically from T1D and T2D. A recent genome-wide association study showed that the genetic background of patients with LADA is more similar to T1D than to T2D.[5] However, LADA has a lower 'genetic load' than T1D. This is consistent with a less severe functional deterioration of the beta-cells at disease onset. Compared with young-onset T1D, LADA represents the other extreme of the autoimmune diabetes spectrum, whereby genetic susceptibility, an autoimmune response and non-insulin-requiring presentation converge in a mild form of diabetes mellitus. This translates in a wide clinical heterogeneity, which is reflected into the broad definition of LADA. Indeed, LADA may be diagnosed in any adult with diabetes who does not require insulin and who is positive for any islet autoantibody, regardless of titre, number or epitope specificity. Nevertheless, evidence exists showing that autoantibody titre and number are both related to different metabolic and clinical phenotypes of the disease. Indeed, the heterogeneity of LADA manifests in different clinical phenotypes, ranging from prevalent insulin resistance to prevalent insulin deficiency, each of which might be associated with different autoimmune and metabolic markers. In this regard, the NIRAD Study highlighted the presence of a 'bimodal distribution' of the GADA titre in patients with LADA that identified two subpopulations, with high and low GADA titres.[4] Compared with patients with LADA who had a low GADA titre, those with a high titre had more severe autoimmunity, which resulted in higher levels of HbA1c, a lower BMI and a lower prevalence of the metabolic syndrome.

Despite the genetic and humoral similarities with T1D, at the individual level, patients with LADA and patients with T2D share clinical and metabolic characteristics, making it very difficult to diagnose LADA solely on the basis of the clinical phenotype. Therefore, individuals with LADA are often misdiagnosed as having T2DM. On the other hand, studies conducted in large groups of diabetic

subjects highlighted some clinical differences which might aid in the correct identification of LADA. relative to T2DM, LADA is characterized by higher fasting levels of glucose and HbA1c, a lower prevalence of the metabolic syndrome, a higher frequency of thyroid peroxidase autoantibodies, a higher frequency of *HLA* risk haplotypes and a consistently greater likelihood of insulin requirement [**Table 1.1**].

To identify patients with diabetes who have the highest odds of testing positive for autoantibodies, a screening tool was developed to identify LADA in the clinical setting.[6] In the retrospective phase of a two-stage study, five clinical parameters were found to occur more frequently in LADA than in T2DM: age of onset <50 years; acute symptoms before diagnosis (polydipsia, polyuria and unintentional weight loss); BMI <25 kg⁄m^2; personal history of other autoimmune diseases; and a family history of autoimmune disease. In the second prospective phase, adults with newly diagnosed diabetes were enrolled to determine if a 'LADA clinical risk score' based on the five aforementioned parameters could identify LADA. The presence of at least two of these clinical features had 90% sensitivity and 71% specificity for identifying LADA, and the negative predictive value for a LADA clinical risk score ≤1 was 99%.

Table 1.1. Genetic, metabolic and clinical features of LADA compared with T1D and T2D. Modified from Buzzetti R, Zampetti S, Maddaloni E., Nat Rev Endocrinol 2017.[7]

	Type 1 Diabetes	LADA	Type 2 Diabetes
Age at diagnosis	Childhood-young adults	30-70 years	Adulthood
Onset	Acute onset	Slow	Slow
Autoimmunity	↑↑↑	↑	←→
Ketosis	Frequent	Rare	Rare
Insulin-resistance	←→	↑ ←→	↑↑↑
Beta-cell function	↓↓↓	↓	↑ ←→
Insulin-dependence	At the onset	> 6 months (or years)	Late
Body Mass Index	Normal-underweight	Normal-overweight	Overweight-obese
Metabolic syndrome	←→	↑ ←→	↑↑↑
HLA susceptibility	↑↑↑	↑	←→

1.2. The open research field of cardiovascular disease and emerging complications in autoimmune diabetes

The changing epidemiology of autoimmune diabetes, with the increasing number of people surviving with T1D for decades and the increasing number of diagnoses in the adulthood, has been accompanied by a change in the diabetes complications' panorama. The longer the duration of T1D, the higher is the prevalence of cardiovascular disease (CVD). Diabetes also accelerates age-related disorders, such as osteoporosis and cognitive decline, which are also emerging as new complications of diabetes mellitus. All CVD and the newly emerging complications of diabetes pose relevant challenges in adults and elderly with autoimmune diabetes. The peculiar features of this population (such as the long exposure to hyper- and hypoglycaemias, decades of exogenous insulin use, autoimmunity, oxidative stress, etc.) make it difficult to generalize to autoimmune diabetes the knowledge about pathophysiology and treatment goals acquired from the general population or from people with T2D.[8] Therefore, there is a need for studies investigating CVD and the age-related complications of diabetes specifically focusing on the population of adults with autoimmune diabetes.

1.2.1. Autoimmune diabetes and Cardiovascular Disease

Atherosclerotic CVD is a major burden for patients with autoimmune diabetes and healthcare providers. The Pittsburgh Epidemiology of Diabetes Complications (EDC) study demonstrated that the incidence of major coronary artery disease events in young adults (aged 28–38 years) with T1D was 0.98% per year and surpassed 3% per year after age 55 years, which makes it the leading cause of death in that population.[9] Cardiovascular events are more common and occur earlier in patients with T1D than in nondiabetic populations, with an age-adjusted relative risk (RR) for CVD in T1DM being about 10 times that of the general population.[10] The large UK General Practice Research

Database (GPRD), comprising data from >7400 patients with T1D showed that cardiovascular events occur 10 to 15 years earlier in subjects with T1D than in matched nondiabetic control subjects.[11] However, CVD prevalence rates in T1D varies substantially based on diabetes duration, age of cohort, and sex, as well as possibly by race/ethnicity. In particular, studies have suggested significant sex differences in cardiovascular events rates associated with T1D. A meta-analysis collecting data from 26 studies including 214114 individuals and 15273 events showed pooled women-to-men ratio of the standardized mortality rates (SMR) for all-cause mortality of 1.37 (95% CI 1.21–1.56), for incident stroke of 1.37 (1.03–1.81), for fatal cardiovascular diseases of 1.86 (1.62–2.15) and for incident coronary artery disease of 2.54 (95% CI 1.80–3.60).[12] These data clearly show that women with T1D have twice the excess risk of fatal and nonfatal vascular events, compared with men with T1D. The huge impact of sex on CVD in T1D is only the first of several differences in CVD between T1D and T2D. Indeed, risk factors appear to affect the risk for CVD differently in T1D versus T2D (**Figure 1.1**), which may have a significant impact in the stratification of cardiovascular risk in T1D. Hyperglycaemia is the most important risk factor for CVD in autoimmune diabetes, as definitely supported by the findings of the Diabetes Control and Complication Trial (DCCT). However, a recent registry-based observational study on 33915 T1D subjects and 169249 controls showed patients with T1D and optimal glycaemic control still had a risk of death from any cause or from cardiovascular causes that was twice as high as the risk for matched controls.[13] Interestingly, a study conducted in T1D patients >18 years of age included in the Swedish National Diabetes Registry showed risks for

	T1DM	T2DM
Hypertension	+++	++
Cigarette smoke	++	++
Inflammation	++	++
High LDL-C	+	+++
Low HDL-C	0, +	++
Triglycerides	No data	++
Microalbuminuria	+++	+++
Insulin resistance	+	+++
Poor glycemic control	+++	+++

Figure 1.1. Different association between cardiovascular risk factors and cardiovascular events in type 1 diabetes (T1DM) and type 2 diabetes (T2DM). *From: de Ferranti SD et al., Diabetes Care 2014*

all major cardiovascular events were numerically higher for patients with T1DM compared with controls, even when all risk factors were at target, with risk for acute myocardial infarction and heart

failure hospitalization statistically significantly higher.[14] This suggests additional specific cardiovascular risk factors in autoimmune diabetes should exist. In this regard, post-hoc analyses of the DCCT showed that beta-cell activity is beneficial to vascular complication in T1D, with preserved C-peptide being associated to lower risk of both microvascular and macrovascular complications of diabetes.

A different impact of cardiovascular risk factors on cardiovascular outcomes in autoimmune diabetes is also supported by epidemiological evidence in subjects affected by LADA. people affected by LADA show a healthier lipid profile, lower blood pressure values, are leaner and have less central adiposity than T2D subjects. All these factors should suggest a lower risk of coronary heart disease, stroke and peripheral artery disease in patients with LADA. On the contrary, the evidence published so far failed to demonstrate this hypothesis. In the Botnia Study 56% of the LADA subjects enrolled had positive history for coronary artery disease and 5% for stroke after 13 years of disease.[15] These rates were similar to those registered in people affected by T2D: 58% and 7% respectively. In the same study the overall mortality after a follow-up of 5.7 years was 18% in LADA and 20% in T2D (p=ns), while the cardiovascular mortality was 7.4% in LADA and 12.4% in T2D (p=ns). The Fremantle study also showed no differences in cardiovascular disease prevalence and mortality between LADA and T2D.[16] As well no differences in terms of cardiovascular outcomes were found in a post-hoc analysis of the Collaborative Atorvastatin Diabetes Study [**Figure 1.2**].[17] The similar rates of CVD, despite the healthier vascular risk profile of LADA patients, together with the evidence that the excess mortality in autoimmune diabetes is not completely explained by hyperglycaemia,[13] suggests a different pathophysiology underling the development of cardiovascular disease in LADA, claiming for studies investigating potential pathways of atherogenesis in autoimmune diabetes

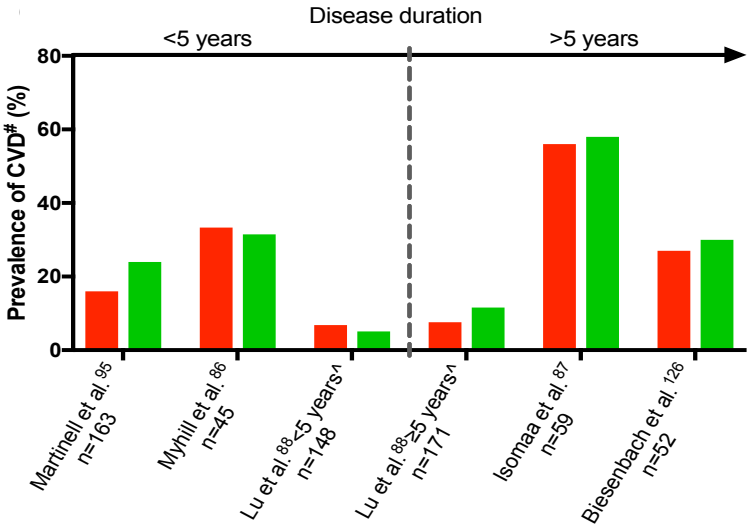


Figure 1.2 CVD in LADA and T2D. Prevalence of coronary artery disease (y axis) in people with LADA (red bars) compared to T2D (green bars) according to disease duration. The x axes show the studies in which the data were reported. The studies reported on the x axes are stratified by a disease duration threshold of 5 years (depicted with a dashed line.) n= number of subjects with LADA included in each study. ^Data from this study were split according to years of disease duration and are depicted separately in the graph. *Adapted from Buzzetti R, Zampetti S, Maddaloni E., Nat Rev Endocrinol 2017.*[7]

Despite the highlighted differences, there are no CVD risk-prediction algorithms specifically designed for patients with T1D in widespread use and cardiovascular risk stratification and management still relies on scores and experience from T2D or the general population. This is a urgent issue to tackle to allow a tailored management of macrovascular complications in autoimmune diabetes.

The clinical differences described above could also result in a different pathology of the arterial wall between T1D and T2D. However, pathology data on atherosclerosis in T1D are limited. Coronary findings may differ between T1D and T2D and from those in the general population, with some studies suggesting atherosclerosis in T1D is more diffuse and more concentric. This consideration mostly comes from an autopsy study performed in eight patients with T1D died in young age (mean death age: 29 years) for CVD.[18] This study suggested plaques in T1DM were soft and fibrous and had a more concentric (less eccentric) location of lesions. A small computed tomography study comparing patients with T1D to those with T2D demonstrated similar coronary artery calcium scores

but more obstructive lesions, more non-calcified lesions, and more lesions overall in patients with T2D than in those with T1D.[19]

In conclusion, despite the known higher risk of CVD in individuals with T1D, the pathophysiology underlying the relationship between cardiovascular events, CVD risk factors, and T1D is not well understood. Management approaches to CVD reduction have been extrapolated in large part from experience in T2D despite the longer duration of disease in T1D than in T2D and the important differences in the underlying pathophysiology. Overall, there is a growing interest in better understanding the adverse effects of glycaemia and the prevalence and impact of the traditional cardiovascular risk factors (dyslipidemia, hypertension, obesity, smoking, etc.) in people with autoimmune diabetes compared to T2D and to the general population. Open research questions include, but are not limited to: do the standard cardiovascular risk factors operate as in T2D and in the nondiabetic population? Are there additional specific cardiovascular risk factors in autoimmune diabetes? Do we need specific risk scores for people with autoimmune diabetes?

1.2.2. Bone fragility as a new complication of autoimmune diabetes

Osteoporosis is an epidemic condition strongly associated with age and characterized by increased risk for low-trauma (fragility) fractures. 50% of Caucasian women will experience an osteoporotic fracture, as will 20% of Caucasian men. Several studies have documented increased rates of mortality after fractures, particularly after hip fractures.[20,21] However, osteoporosis is not only a disease of the elderly, but many other conditions are associated with secondary osteoporosis, especially endocrine diseases. Diabetes is the most prevalent endocrine disease shown to affect bone strength. Diabetic osteopathy has recently been acknowledged as a rising complication of diabetes. This is particularly relevant for people with T1D who are up to 12 times more likely to experience major fragility fractures.[22,23] Additionally the number of elderly subjects with long standing T1DM is projected to quickly rise in the immediate future because of the increasing T1D incidence and the improved quality

of care allowing people to survive longer despite the disease. This indicates that there will be a fast and large increase in the number of individuals at risk for aging-related diseases on a background of long term T1D. Beside hyperglycemia, published reports highlight hyperinsulinemia as a factor related to structural bone defects in T2D as insulin plays a pivotal role in the maintenance of balanced bone turnover. Yet, hyperinsulinemia is not a long term feature of T1D. Therefore, much less is understood regarding the structural and biochemical profile of bone pathology in those with T1D, a disease of insulin deficiency. Decreased rate of bone apposition compared to resorption is hypothesized to be the center of imbalance in the process of bone remodeling, causing the phenotype of lower bone mineral density (BMD) in T1D. Exogenous insulin therapy by-passing the portal system cannot reproduce the physiological liver exposure to insulin, resulting in a lower expression of growth hormone receptors on hepatocytes, likely reducing production of IGF1 which contribute to decreased osteoblast stimulation. This would reduce what would be the expected level of bone formation at normal levels of portal insulin.[24] Oxidative stress and the formation of advanced glycation end products (AGEs) are also likely to have common effects making it important to compare factors responsible for diabetic osteopathy. This is particularly important as current therapies including bisphosphonates, denosumab, anabolic agents like teriparatide and PTH1-84 and hormone therapies, like calcitonin and selective estrogen receptor modulators, have not been evaluated for T1D clearly indicating a practical need to understand the different mechanisms to effectively treat patients. Of note, Dual-energy X-ray Absorptiometry (DXA) studies assessing BMD indicate an increase in risk for fracture in this group, but do not explain the excess risk in hip fracture experienced by those with T1D. In a further effort to understand the paradox of a relatively small shift in BMD curve and an almost fivefold increase in risk over those with T2D for fracture, it has been hypothesized that there are differences in the geometry and/or microarchitecture of those with T1D.[24] Studies evaluating bone geometry and microarchitecture in T1D are summarized in **table 1.2** and in **table 1.3** respectively. High-resolution peripheral quantitative computed tomography (HR-pQCT) is the most advanced technique for studying bone quality *in vivo* in human subjects enabling a sensitive and

specific noninvasive assessment of bone geometry and microarchitecture. Studies using HR-pQCT reported trends of higher cortical porosity among those with T1DM than in those without. However, the largest difference was seen in trabecular features: trabeculae thickness, trabeculae spacing, and trabecular bone volume/total volume ratio.[25,26] The most striking finding was by Shanbhogue et al., who showed differences in bone microarchitecture not between those with and without diabetes, but rather those with T1DM with and without microvascular complication, especially proliferative diabetic retinopathy. This finding suggests common pathways of disease between vascular complications and bone fragility in autoimmune diabetes. The association of vascular diabetes complications and decline of skeletal health in those with T1DM has been also hypothesized based on a study by Armas et al. examining the histomorphometry of the iliac crest of subjects with and without T1D. While no differences were found between parameters of formation and microarchitecture in the absence of complications, complications in the presence of T1D were the differentiating factor for at risk bone microarchitecture.[27] To the aims of this project, it is particular relevant to highlight the intimate relationship which is emerging between bone health and the macrovascolar complications of autoimmune diabetes.

Several studies have documented an increased risk of cardiac events and mortality in the presence of decreased skeletal health. This link has been documented in the Prospective Epidemiological Risk Factors Study which found an increased risk for hip fracture with aortic calcification (adjusted RR 2.3 95 % [1.1–4.8], p = 0.03) and in a Swedish population-based case-control in which women without diabetes but with CVD were twice as likely as those to have fractured than those who did not (adjusted odds ratio for hip fracture 2.38 [95CI 1.92–2.94]).[28,29] Additionally, yearly rate of vertebral trabecular bone loss evaluated by CT was associated with the rate of change in aortic calcification in postmenopausal women independently from confounding factors such as age, BMI, blood pressure, and physical activity [28]. In vitro data from Arnett et al. supports the epidemiologic studies by demonstrating hypoxia as a major stimulator of bone resorption and osteoclast formation as it causes a significant VEGF response in healthy tissue and shows a diminished response in tissues with

vascular complications.[30] Hypoxia causes acidosis due to increased anaerobic metabolism, reducing pH in bone inhibiting osteoblasts, and enhancing bone resorption by osteoclasts. [31] Also alterations in bone hemodynamics such as vascular calcification and arteriosclerosis, which occur frequently in people with T1DM, have been shown to influence osteoblast activity and bone remodeling.[32–34] Factors including inflammatory cytokines, oxidative stress, vitamin K and vitamin D deficiency, BMPs, osteocalcin, sclerostin, and other osteokines have also been implicated in the bone-vascular axis.[35] However, to date scares evidence exists about the relationship between vascular complications and bone health in adults and elderly with autoimmune diabetes.[24]

Table 1.2 Summary of human studies of bone volumetric BMD and geometry in type 1 diabetes. Means are presented. T1D: Type 1 Diabetes; T2D: Type 2 Diabetes; NDM: non-diabetic controls; pQCT: peripheral quantitative computed tomography *calculated. *Adapted from Keenan HA, Maddaloni E, Curr Osteopor Rep 2016*[24]

Study	Year	Design	Method	Age	Duration	n T1D	Conclusion	Sites studied
Roe et al.[36]	1991	Case-control	QCT	F:12.9 T1D; 12.9 NDM; M:12.7 T1D; 12.7 NDM	5.2	48	Ct BMD decreased, no differences in Tb BMD	Lumbar vertebrae
Lettgen et al.[37]	1994	Case-control	pQCT	12.6 T1D; 12.8 NDM	5.3	42	Lower trabecular Tb vBMD inversely related to HbA1c	Ultra-distal Radius
Moyer-Mileur et al. [38]	2004	Longitudinal 12 months	pQCT/ DXA	14.9 T1D; 15.0 T2D	4.2	42	Adolescents with T1D have small bones despite normal growth patterns	Spine DXA/ tibia pQCT
Bechtold et al. [39]	2007	Longitudinal 2-4 years	pQCT	9.87 T1D; 15.4 ND	4.3	41	Early stage impairment ameliorated over time	Distal metaphysis and proximal of the diaphysis radius
Heap et al.[40]	2004	Case-control	pQCT/ DXA	F:14.7 T1D; 14.8 ND M:14.6 T1D; 14.5 ND	5.5*	55	Tb vBMD was inversely related to glycemic control	Spine and FN DXA/ Radius and tibia
Saha et al. [41]	2009	Case-control	pQCT	F: 15.1T1D; 15.5 ND M: 15.2 T1D; 15.9 ND	6.8	48	Bone mineral content and cross-sectional area is affected with dominant effect in boys	Radius and tibia
Roggen et al. [42]	2013	Case-control	pQCT	17.9 T1D; 18.1 T2D	4.7	56	Smaller bone size in those with T1; Tb was similar.	Distal radius

Table 1.3 Summary of human studies of bone microarchitecture in those with type 1 diabetes. T1D: Type 1 Diabetes; T2D: Type 2 Diabetes; NDM: non-diabetic controls; TBS: Trabecular bone score; HR-pQCT: High resolution-peripheral quantitative computed tomography; uCT: micro-computed tomography. *Adapted from Keenan HA, Maddaloni E, Curr Osteopor Rep 2016*[24]

Study	Year	Design	Method	Age	Duration	N T1DM	Conclusion	Sites studied
Armas et al. [27]	2012	Case-control	uCT biopsy	31T1DM; 34.1 NDM	15	18	Tb differs between non-fracture T1 and fracturing. Non fracturing T1 not different than controls	Iliac crest
Shanbohugue et al. [25]	2015	Case-control	HR-pQCT/ DXA	45 T1DM; 45.8 T2DM	17	55	Deficiencies of Ct and Tb appear driven by MVD	Ultradistal radius and tibia
Starup-Linde et al. [26]	2016	Case-control	HR-pQCT/ DXA	24.4 T1DM; 14.6 T2DM	24.4	51	Increased Ct area, bone stiffness lower in tibia T1, radius Ct pore volume lower in T1	Tibia and radius
Abdalrahaman et al. [43]	2016	Case-control	Micro-MRI	22.0 T1DM; 21.8 NDM	12.8	30	Deficits in trabecular bone. Among T1D, lower BV/TV with retinopathy	Tibia
Neumann et al. [35]	2016	Case-control	TBS	43.4 T1DM; 42.8 NDM	21.5	119	TBS are lower in inT1D with existing fractures	Lumbar spine

2. Hypothesis and Aims

2.1. Hypothesis

We hypothesized that clinical features and risk of diabetic chronic complications may differ in adults with different forms of diabetes. Therefore, we also hypothesize that the study of vascular and bone disorders in adults with autoimmune diabetes may unveil novel risk factors for and pathways of chronic complications.

2.2. Overall aim

The overall aim of this Ph. D. project was to investigate the pathophysiology and clinical features of autoimmune diabetes and its complications during the adulthood and the senescence. In particular, the below mentioned specific aims have been pursued.

2.3. Specific aims

1. **To evaluate whether rates and risk factors for vascular complications differ between autoantibody positive and autoantibody negative subjects with adult-onset diabetes**

To this aim data from the UK Prospective Diabetes Study (UKPDS) will be retrieved and analyzed in collaboration with the Oxford Centre for Diabetes Endocrinology and Metabolism. Autoantibodies to islet-cell cytoplasm (ICA) and to glutamic acid decarboxylase (GADA) were measured in 5062 patients with type 2 diabetes enrolled in the UKPDS and resulted positive in 11.2%. Incidence rates of vascular complications of diabetes will be compared between autoantibody positive and autoantibody negative subjects. Moreover, we will evaluate whether the measurement of diabetes-related autoantibodies improves cardiovascular risk stratification in subjects with adult-onset diabetes

and whether the relationship between traditional cardiovascular risk factors and cardiovascular events differ between autoantibody positive vs negative subjects.

2. **To evaluate bone health and its relationship with vascular complications in adults and aging people with autoimmune diabetes**

To this aim adults with type 1 diabetes followed in the centers of the IMDIAB group and elderly people enrolled in the 50-Years Joslin Medalist Study have been fully characterized in terms of history of metabolic control, vascular complications and bone fractures. Bone mineral density has been measured by DEXA in a subgroup of these subjects. Risk factors for impaired bone health and the relationship between bone fragility and vascular complication have been investigated in both young adults and elderly with long standing type 1 diabetes.

3. **To investigate circulating osteoprogenitors as a new mechanism of vascular complication in type 1 diabetes**

To this aim, CD45+CD14+Osteocalcin+ cells will be evaluated in people enrolled in the 50-year Joslin Medalist Study and mechanisms of physiological and pharmacological procalciphic drift will be investigated both *in vitro* and *in vivo*

3. Specific aim 1: Vascular complications in latent autoimmune diabetes of adults

Maddaloni E, Coleman RL, Pozzilli P, Holman RR. Long-term Risk of Cardiovascular Disease in Individuals with Latent Autoimmune Diabetes of Adults. Presented at the 54th Annual Meeting of the EASD, Berlin, 1-5 October 2018. Full manuscript currently under review.

3.1. Background

Cardiovascular (CV) events represent the main cause of morbidity and mortality in subjects with type 2 diabetes (T2D).[44] Subjects with type 1 diabetes (T1D) are also at increased risk of CV disease,[11] although precise CV risk estimates remain an open research question deserving of long-term prospective studies.[8] It is also uncertain whether CV risk factors for macrovascular complications contribute equally in T1D and T2D.[8] Autoantibodies (AAb) to islet-cell cytoplasm (ICA), to glutamic acid decarboxylase (GADA) or to islet antigen-2/2ß (IA-2A), are detectable in up to 12% of adults with a clinical diagnosis of T2D [45,46]. The presence of these AAb identify a subset of patients who have Latent Autoimmune Diabetes of Adults (LADA). LADA patients tend to be leaner, younger and to have a healthier CV risk profile than non LADA subjects.[3,47] However, available data from studies with a relatively short follow-up show no difference in CV outcomes for those with LADA compared with T2D,[7] suggesting that the impact of CV risk factors may differ between these two types of diabetes.

We have examined the long-term risk of CV events in patients with LADA, compared with T2D, in the large population with newly-diagnosed T2D enrolled into the United Kingdom Prospective Diabetes Study (UKPDS), and have evaluated whether the relationship between traditional CV risk factors and CV events might differ between these two groups. Furthermore, we have evaluated whether the effect of intensive glucose-lowering treatment with insulin/sulfonylureas or metformin impacts CV outcomes differently in those with LADA or T2D.

3.2. Methods

Study design

The UKPDS protocol, design and methods have been reported previously.[48] Briefly, after a 3-month dietary run-in period, those patients with a fasting plasma glucose (FPG) concentration >6 mmol/l and <15 mmol/l were assigned randomly to a conventional glucose control strategy (primarily diet) or to an intensive glucose control strategy (sulfonylurea or insulin, or [if >120% of ideal body weight] metformin), and followed quarterly in UKPDS clinics. Following closeout of the interventional trial on September 30, 1997, all surviving patients entered a 10-year post-trial monitoring program as has been reported previously.[49] During this post-trial monitoring period no attempt was made to maintain previously randomized therapies. Participants were seen annually for 5 years in UKPDS clinics, with continued standardized collection of outcome data, measurements of blood pressure, fasting plasma glucose, glycated hemoglobin, plasma creatinine, and the ratio of urine albumin to creatinine. For the final 5 years of follow-up participants were followed remotely via questionnaires.

Study population

A total of 5102 patients aged 25–65 years with T2D newly diagnosed by their general practitioner, and with a FPG >6 mmol/l on two subsequent occasions, were recruited into the UKPDS. A further 2514 subjects were excluded according to the following criteria: severe vascular disease (myocardial infarction in the past year, current angina or heart failure); accelerated hypertension; proliferative or pre-proliferative retinopathy; renal failure with plasma creatinine >175 µmol/l; other life-threatening disease such as cancer; an illness requiring systemic steroids; an occupation precluding insulin treatment; unfamiliarity with English; and ketonuria greater than 3 mmol/l suggestive of type 1 diabetes.[48]

ICA, GADA and/or IA-2A were measured as previously described[45,47,50] in 5096 (99.9%) of UKPDS participants. Only 1.2% of AAb samples were taken >2 years after diagnosis.[47] Participants were identified as having LADA if they tested positive for ≥1 AAb (ICA, GADA or IA-2A). Participants

were confirmed as T2D if they tested negative for all measured AAb. A total of 5062 subjects are included in this study, as participants with one negative Ab measurement and no data for any of the other two antibodies (n=34) were excluded as one negative Ab test does not exclude LADA [**Figure 3.1**].

Statistical analysis

The primary CV outcome for this study was the first occurrence of a major adverse CV event (MACE), defined as CV death, nonfatal myocardial infarction or nonfatal stroke, with deaths of unknown cause considered to be CV deaths. Secondary analyses examined the first occurrence of the three MACE components separately. Descriptive statistics are presented for categorical variables as numbers and percentages, and for continuous variables as appropriate measures of central tendency and dispersion. The distribution of variables was tested with the Shapiro-Wilk normality test. Groups were compared by Student's t-test, Kruskal–Wallis, Chi-square, or Fisher's exact tests as appropriate. Time-to-event analyses comparing LADA with T2D participants were performed for the primary (MACE) and secondary outcomes using Cox proportional-hazards models. The proportional-hazard assumption was shown not to be violated when tested using Schoenfeld residuals and graphically. Models were assessed first with adjustment for age, and then for assigned glycemic control strategy as well as conventional CV risk factors including, sex, ethnicity, smoking (yes/no), as well as baseline HbA_{1c}, total cholesterol, LDL-cholesterol, HDL-cholesterol, systolic blood pressure (SBP) and body mass index (BMI). Variables were retained in final models if their effect remained significant at $p<0.1$. Heterogeneity of LADA status effect across components of the composite end-point was tested. Subgroup analyses were performed to investigate whether the effect of the randomized glycemic control strategy on MACE or CV death differed between LADA and T2D. Missing risk factor data (2 values for smoking, 18 values for BMI, 734 values for HbA1c, 665 values for total cholesterol, 757 values for HDL-cholesterol, 791 values for LDL-cholesterol, 672 values for SBP) were imputed using multiple imputation.[51]

Two-sided tests at the 0.05 level of significance were used for all statistical comparisons, with Stata/IC 12.1 software (StataCorp) and Prism 7.0d Software (GraphPad Software) used for data analysis and graphical representations.

Ethics

The study was performed according to the Helsinki guidelines and all patients gave written informed consent to participate. The study protocol was approved by the ethics committee of all 23 UKPDS clinical centers

3.3. Results

Population

Overall 567 (11.2%) of 5062 UKPDS participants were categorized as having LADA. At baseline, compared with T2D participants, they were younger ($p<0.001$), more frequently Caucasian ($p<0.001$), with a lower BMI ($p<0.001$), higher HDL-cholesterol ($p=0.011$), lower total cholesterol ($p=0.006$), lower systolic blood pressure ($p<0.001$), and higher HbA_{1c} ($p<0.001$) [**Table 3.1**].

Of the 567 LADA participants, 381 (67.2%) were positive for only one AAb: 291 (51.3%) had GADA alone, 75 (13.2%) had ICA alone, 15 (2.7%) had IA-2A alone. The remaining 186 (32.8%) subjects were positive to two or more antibodies: 101 (17.8%) had GADA and ICA, 12 (2.1%) had GADA and IA-2A, 3 (0.5%) ICA and IA-2A, and 70 (12.4%) had all three AAb. Participants positive to two or more AAb, compared with those positive to only one AAb, were younger ($p<0.001$), more frequently Caucasian ($p=0.006$), had lower median BMI ($p<0.001$), higher HbA_{1c} (<0.001), higher HDL-cholesterol ($p=0.034$) and lower systolic blood pressure ($p<0.001$) [**Table 3.1**].

Cardiovascular outcomes

Over a median (IQR) follow-up period of 17.3 (12.6-20.7) years the primary MACE outcome occurred in 1701 participants, of which 157 had LADA (27.3%; incidence rate 17.1 *per* 1000 person-

years) and 1544 had T2D (34.4%; incidence rate 23.5 *per* 1000 person-years), with a Hazard Ratio (HR) of 0.73 (95% CI 0.62–0.86, p<0.001). The age-adjusted HR was 0.86 (95% CI 0.73-1.02, p=0.078). The HR was no longer statistically significant when the other conventional CV risk factors were introduced in the model (HR_{adj} 0.90, 95% CI 0.76–1.07, p=0.22) [**Figure 3.2**].

Incidence rates for each of the MACE components were also lower in LADA, compared with T2D participants. Following adjustment for age, the differences in the incidence rates for non-fatal myocardial infarction between the two groups remained statistically significant, whilst the differences for CV death and for non-fatal stroke became statistically non-significant [**Table 3.2**].

The 186 LADA participants with ≥2 positive AAb tests had a lower MACE risk than those with only one positive AAb test (interaction p<0.001), with an unadjusted HR of 0.46 [95% CI 0.33–0.65] when compared with T2D participants. The age-adjusted HR was 0.65 (95% CI 0.46–0.90) and remained statistically significant (interaction p=0.035), but became non-significant following adjustment for the other conventional CV risk factors (HR_{adj} 0.76, 95% CI 0.54–1.07, interaction p=0.28) [**Table 3.3**]. There was no significant interaction between groups and the degree of AAb positivity (zero, 1 or ≥2) and CV risk factors with respect to the primary CV outcome.

Effect of assigned glycemic strategy on CV outcomes by LADA status.

Overall during the interventional trial participants randomized to the intensive glycemic control strategy with insulin or sulfonylureas, compared with those randomized to the conventional glycemic control strategy therapy, had similar risks for MACE (HR 0.90, 95% CI 0.77-1.05, p=0.19) and for CV death (HR 0.94, 95% CI 0.76-1.16, p=0.57). However, by the end of the post-trial monitoring period those in the intensive glycemic control strategy group had reduced risks of MACE (HR 0.88, 95% CI 0.78-0.98, p=0.026) and CV death (HR 0.68, 95% CI 0.51-0.92, p=0.011).

For participants with LADA, there was a reduced risk CV death during the interventional trial (HR 0.46, 95% CI 0.23-0.90, p-value for interaction: 0.029), driven primarily by those randomized to insulin (HR 0.29, 95% CI 0.08-0.74, p-value for interaction: 0.013) than by those randomized to

sulfonylureas (HR 0.77, 95% CI 0.54-1.11, p-value for interaction: 0.18). However, at the end of the post-trial monitoring period the effect of insulin/sulfonylureas on CV outcomes did not differ between LADA and T2D [**Figure 2**]. These results did not change after adjusting for HbA1c and BMI, which differed significantly between LADA patients randomized to intensive glycemic control compared with those randomized to conventional glycemic control. No other imbalances in baseline characteristics were found in these subgroups.

Overall during the interventional trial participants randomized to metformin had reduced risks of MACE (HR 0.61, 95% CI 0.44-0.86, p=0.005) and CV death (HR 0.59, 95% CI 0.37-0.94, p=0.025), compared with those randomized to the conventional glycemic control strategy therapy. Statistically significant risk reductions were also seen at the end of the post-trial monitoring period (MACE HR 0.73, 95% CI 0.57-0.93, p=0.010; CV death HR 0.68, 95% CI 0.51-0.92, p=0.011). The impact of intensive treatment with metformin on the risks for MACE and CV death did not differ between LADA and T2D (p-values for interaction >0.05 in all analyses) [**Figure 2**].

3.4. Conclusions

Among patients enrolled into the UKPDS with a clinical diagnosis of new-onset T2D, those found to have LADA, compared with those without LADA, had a lower long-term risk of CV events. This lower risk of MACE in LADA patients is explained predominately by their lower age and more favorable conventional CV risk factor values, including sex, race, smoking, metabolic control, lipid profile and BMI. As the difference in MACE risk is no longer statistically significant after risk factor adjustment there would appear that measuring AAb in people with T2D will not assist with CV risk stratification.

Our study provides further insights into the somewhat controversial topic of macrovascular complications in people with LADA, and the hypothesis that their healthier metabolic profile should result in lower CV event rates. To date a few studies, mostly cross-sectional, have described a similar

prevalence of coronary artery disease in LADA and T2D patients matched for age and disease duration.[7] In the BOTNIA study LADA patients had lower markers of central adiposity, lower rates of hypertension and higher HDL, but this did not result in a lower prevalence of CV disease.[15] An analysis conducted on 45 LADA subjects from the cohort of the Fremantle Diabetes Study found similar crude incidence rates of myocardial infarction, stroke or cardiac mortality than T2D, again despite the significant differences in CV risk factors.[16] On the contrary, in a recent cohort study LADA seemed to be associated with lower short-term risk of CV events and mortality,[52] even though patients with insulin-dependent diabetes were not considered as possibly affected by LADA. These results were in contrast with findings from the prospective population-based HUNT2 study, showing equal all-cause and CV mortality in LADA and in T2D.[53] Unlike the UKPDS, however, HUNT2 did not evaluate non-fatal events, had a shorter mean follow-up (9.1 years among patients with diabetes) and the diagnosis of LADA was based on the measurement of only one AAb. Here we showed that LADA patients have a similar age-adjusted incidence of fatal CV events with T2D, confirming over a long follow-up that CV mortality does not differ with T2D after correction for age. On the other hand, an age-independent lower risk of non-fatal CV events compared with T2D was found, which is explained by the lower prevalence of CV risk factors. In our study, patients with two or more AAb tests positive differed from those with only one AAb test positive in having an age-independent lower risk of CV events, which was explained fully by their more favorable traditional CV risk factor values. Comparing rates and features of CV complications between LADA and T2D patients could contribute to clarifying the relationship between vascular complications, risk factors and the presence of autoimmunity, which is poorly understood. Hyperglycemia, obesity, dyslipidemia and hypertension often coexist in T2D, contributing to the increasing burden of macrovascular complications. However, this is less often the case in LADA patients. Prior suggestions that LADA and T2D patients might be at similar CV risk implied that there might have been a different impact of the conventional CV risk factors in LADA. Our study confutes this hypothesis as we found no significant interactions between AAb status and conventional CV risk factors. This suggests that conventional CV risk factors

contribute equally to major CV events in LADA and T2D and should be tackled similarly in both these forms of diabetes.

Although different recommendations exist for the treatment of adults with T1D and T2D, there is a wide uncertainty regarding the best treatment options in LADA. While CV safety and efficacy of glucose-lowering drugs has become crucial in the therapeutic algorithm for patients with diabetes,[54,55] no data are available about the CV safety and efficacy of glucose-lowering drugs in LADA. We have previously reported that insulin therapy might be associated with a better preservation of beta-cell function.[47] This post-hoc analysis of the UKPDS by AAb subgroups suggests that early intensive insulin therapy might be associated with protection from CV death in LADA within the first 10 years from diagnosis. However, in the post-trial longer-term follow-up of the UKPDS cohort, the CV protection associated with insulin therapy, sulfonylureas and metformin did not differ between LADA and T2D patients.

This study has several strengths. To our knowledge this is the largest LADA population with the longest follow-up that has been evaluated for CV complications. The enrollment of UKPDS participants with newly-diagnosed T2D, measurement of all three of main diabetes-related AAbs, and the extended follow-up has permitted a complete description of the natural history of vascular disease in LADA and T2D. This also allowed us to study vascular complications in two LADA AAb subgroups which differed in clinical features, and probably in pathophysiology, contributing to a better definition of the clinical heterogeneity of the disease and helping to promote a personalized approach to LADA management.

Some limitations are acknowledged. In our study the 920 patients missing one Ab measurement, and with the other two Ab tests being negative, were considered as having T2D. In theory, we may have misdiagnosed some of these patients who could have had LADA. However, the vast majority (93.2%) of these patients were GADA and ICA negative, missing only a measurement of IA2A. Given the low prevalence of LADA positive only to IA2A (2.7%), we estimate that the risk of misdiagnosis

was low and unlikely therefore to have influenced our overall findings. We have no data concerning the post-trial use of concomitant medications that might have impacted on CV disease, such as statins. Theoretically, statins might have been used more frequently in T2D than in LADA patients because of their less favorable lipid profile. Finally, post-trial questionnaires used to identify potential UKPDS endpoints may not have captured all nonfatal outcomes.

In conclusion, in adults with newly-diagnosed T2D the long-term risk of MACE was lower in those with LADA, but did not differ after adjustment for conventional CV risk factors. This suggests that measurement of AAb in T2D will not aid stratification of their CV risk

3.5. Tables

Table 3.1. Baseline characteristics. Values are median (IQR) for continuous variables and number (%) for categorical variables. Abbreviations: WC, White-Caucasian; B, Black; HbA1c, glycated hemoglobin; LDL, Low-Density Lipoprotein; HDL, High-Density Lipoprotein.

	Whole population			LADA population		
	LADA (n=567)	**T2D (n=4495)**	**P-value**	**One Ab+ (n=381)**	**≥2 Ab+ (n=186)**	**P-value**
Age (years)	50.4 (40.4-58.1)	54.0 (47.4-59.5)	<0.001	52.4 (45.0-59.0)	45.5 (35.6-54.7)	<0.001
Sex (male)	315 (55.6)	2663 (59.2)	0.093	218 (57.2)	97 (52.2)	0.254
Race			<0.001			0.006
- WC	531 (93.7)	3638 (80.9)		349 (91.6)	182 (97.9)	
- B	11 (1.9)	365 (8.1)		11 (2.9)	0	
- Other	25 (4.4)	492 (11.0)		21 (5.5)	4 (2.2)	
Smoker			0.066			0.079
- Current	198 (34.9)	1370 (30.5)		122 (32.0)	76 (40.9)	
- Ex	171 (30.2)	1527 (34.0)		124 (32.6)	47 (25.3)	
- Never	198 (34.9)	1596 (35.5)		135 (35.4)	63 (33.9)	
Body Mass Index (kg/m^2)	24.2 (21.8-27.5)	26.8 (24.1-30.4)	<0.001	25.1 (22.4 29.4)	22.7 (21.1-25.0)	<0.001
HbA1c (%)	7.3 (6.1-9.2)	6.8 (5.9-8.0)	<0.001	7.0 (6.0-8.6)	8.3 (6.9-10.2)	<0.001
Total cholesterol (mmol/L)	5.19 (4.44-5.84)	5.31 (4.59-6.03)	0.006	5.20 (4.49-5.83)	5.10 (4.37-6.02)	0.564
LDL-cholesterol (mmol/L)	3.30 (2.73-4.12)	3.43 (2.80-4.14)	0.098	3.32 (2.78-4.13)	3.23 (2.54-4.09)	0.353
HDL-cholesterol (mmol/L)	1.07 (0.93-1.24)	1.04 (0.91-1.20)	0.011	1.06 (0.91-1.23)	1.14 (0.95-1.27)	0.034
Systolic Blood Pressure (mmHg)	128 (116-140)	135 (122-148)	<0.001	130 (120-143)	120 (113-130)	<0.001

Table 3.2. Incidence rate and Hazard Ratios (HR) of macrovascular outcome. Abbreviations: MACE, Major Adverse Cardiovascular Events; MI, Myocardial Infarction

	Incidence rate per 1000 person-years [95% CI]		HR [95% CI]	Age-Adjusted HR [95% CI]	Fully adjusted HR
	LADA	T2D			
MACE	17.4 [14.9-20.3]	23.5 [22.4-24.7]	0.73 [0.62-0.86]	0.86 [0.73-1.02]	0.92 [0.77-1.09]
Cardiovascular death	11.8 [9.8-14.2]	15.0 [14.2-16.0]	0.77 [0.64-0.94]	0.93 [0.77-1.13]	0.97 [0.79-1.18]
Fatal MI	9.0 [7.3-11.1]	10.9 [10.1-11.7]	0.81 [0.65-1.01]	0.98 [0.79-1.23]	1.01 [0.81-1.28]
Fatal stroke	1.5 [0.9-2.5]	2.7 [2.3-3.1]	0.54 [0.31-0.92]	0.68 [0.39-1.16]	0.77 [0.44-1.32]
Sudden death	1.0 [0.5-1.8]	0.9 [0.7-1.2]	1.02 [0.51-2.05]	1.12 [0.56-2.26]	1.09 [0.53-2.25]
Unknown death	0.3 [0.1-1.0]	0.4 [0.3-0.6]	0.72 [0.22-2.35]	0.79 [0.24-2.60]	0.75 [0.22-2.54]
Non-fatal MI	4.7 [3.5-6.3]	7.5 [6.8-8.1]	0.63 [0.46-0.86]	0.70 [0.51-0.96]	0.73 [0.53-1.00]
Non-fatal stroke	2.7 [1.8-4.0]	4.7 [4.2-5.2]	0.56 [0.38-0.85]	0.68 [0.45-1.02]	0.73 [0.48-1.11]

Table 3.3. Cox regression models for MACE in LADA by number of positive autoantibodies

	HR [95% CI] (vs T2D)	P	P for interaction
Unadjusted			
LADA	0.73 [0.62-0.86]	<0.001	
• Single AAb+	0.87 [0.73-1.05]		<0.001
• Two or more AAb+	0.46 [0.33-0.65]		
Age-adjusted			
LADA	0.86 [0.73-1.02]	0.078	
• Single Ab+	0.96 [0.79-1.15]		0.035
• Two or more Ab+	0.65 [0.47-0.90]		
Fully adjusted model*			
LADA	0.92 [0.77-1.09]	0.217	
• Single Ab+	0.98 [0.81-1.18]		0.322
• Two or more Ab+	0.77 [0.55-1.08]		

* Final fully adjusted model for MACE included age, sex, BMI, HbA1c, HDL-c, Total cholesterol, SBP, Smoke, Race, Main randomization (LDL non significant at p<0.1)

3.6. Figures

Figure 3.1 CONSORT Diagram

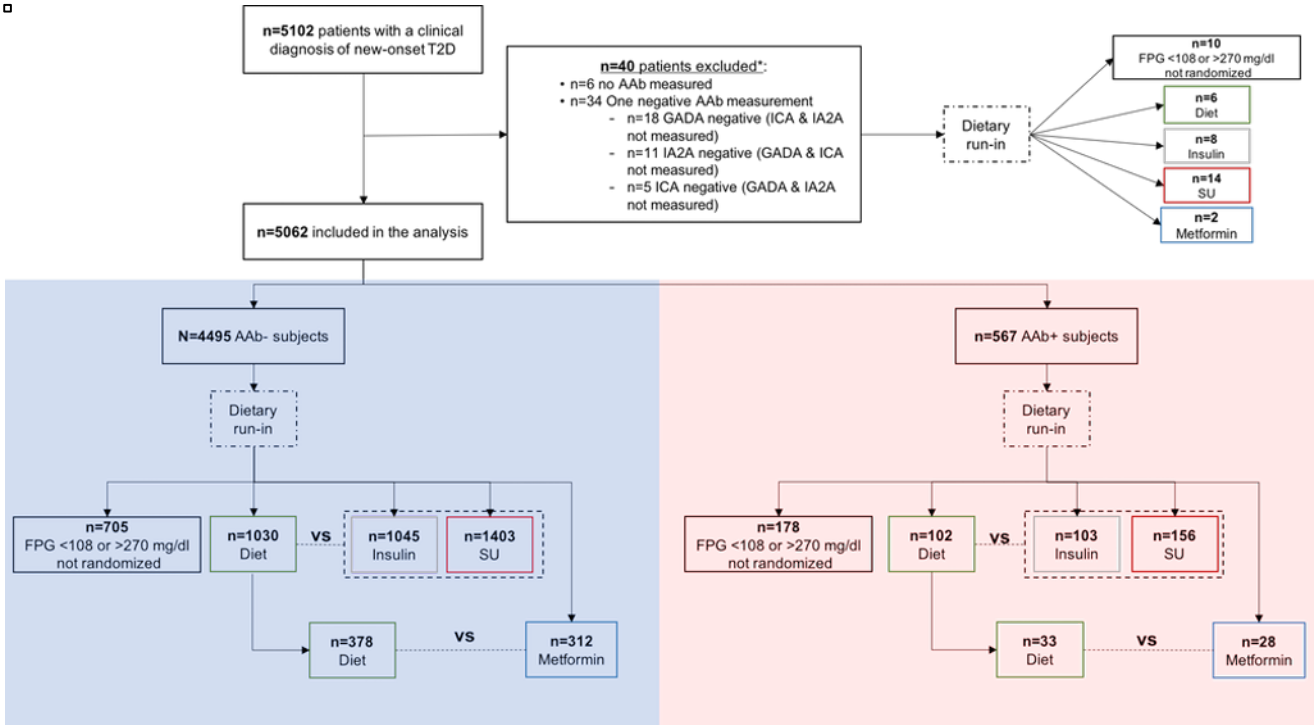


Figure 3.2. Cox-proportional hazard regression survival curves for the primary composite outcome (MACE) in T2D (blue line) and LADA (red line). A) unadjusted model; B) age-adjusted model; C) Adjusted for age, sex, race, smoking, BMI, HbA1c, HDL-c, Total cholesterol, SBP.

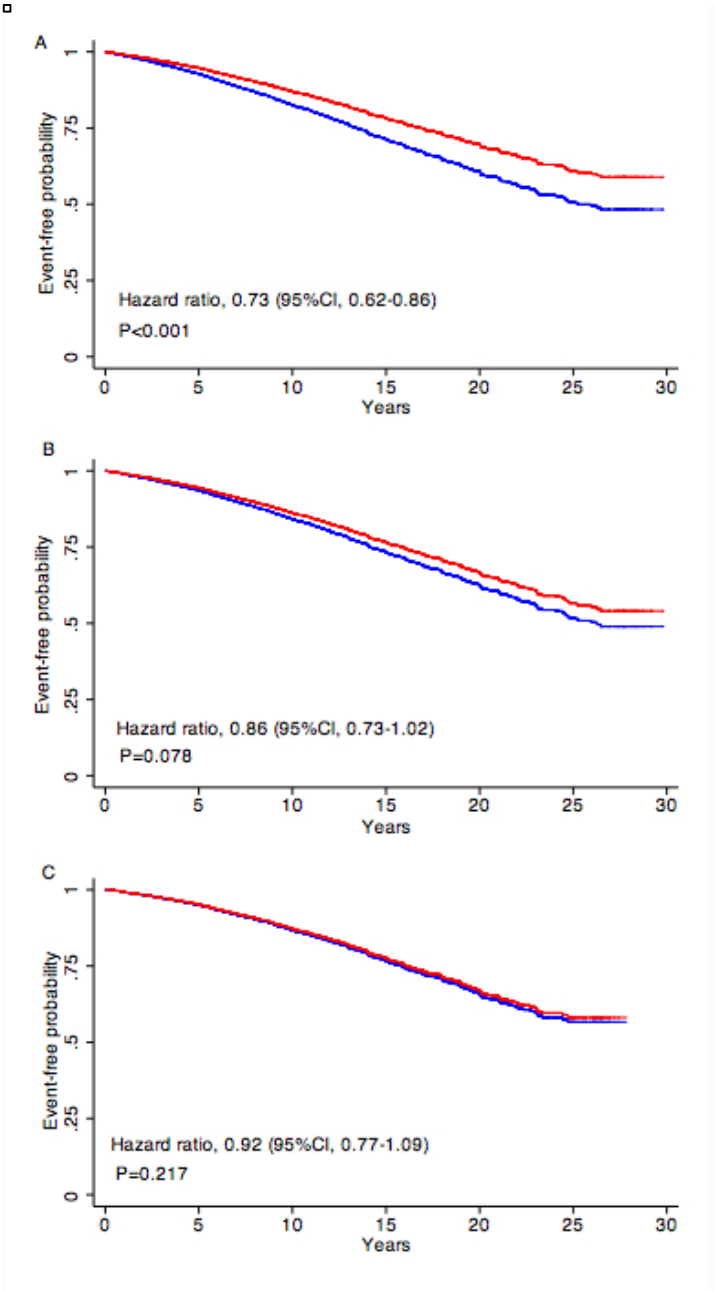
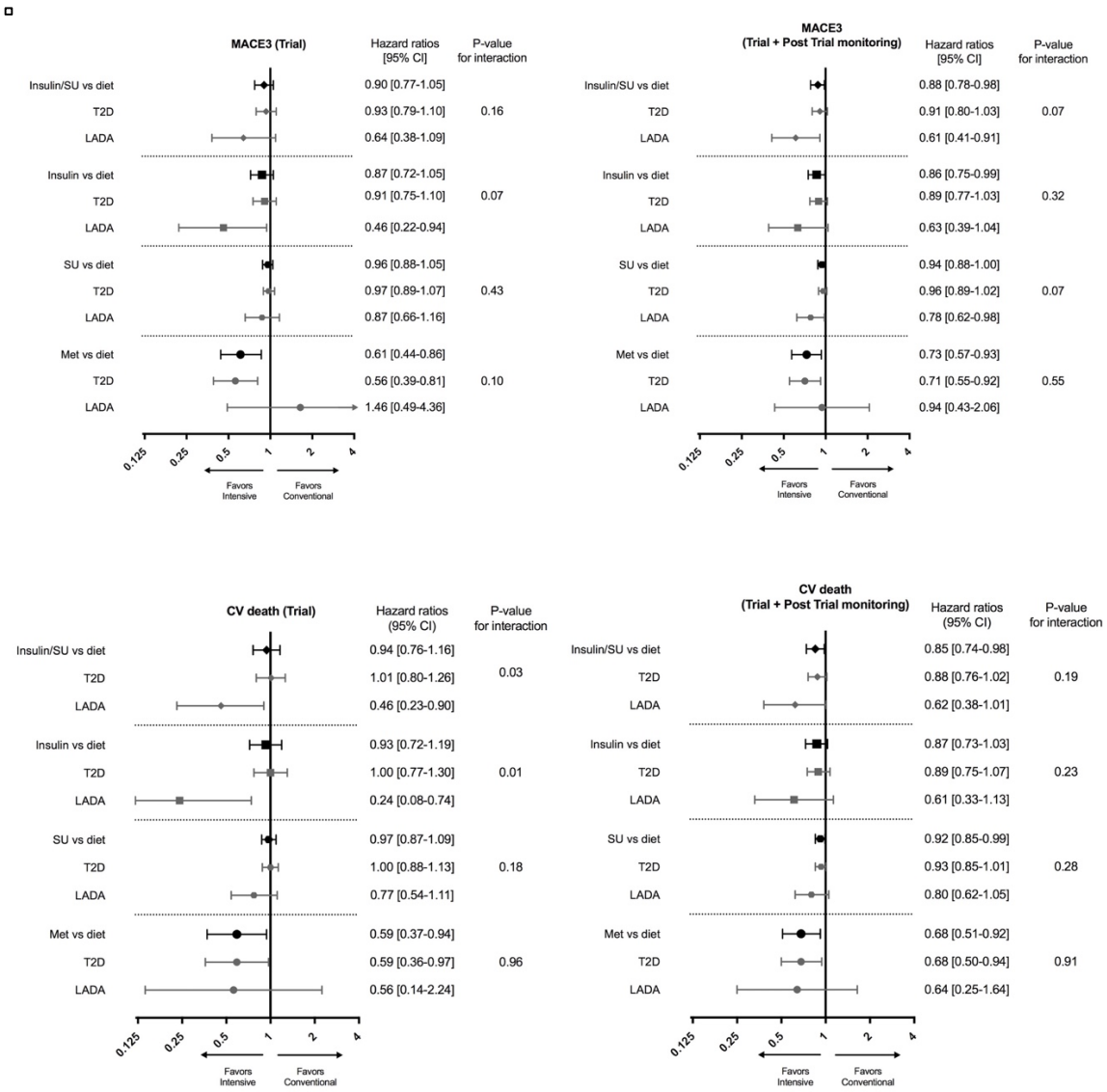


Figure 3.3. Post-hoc Cox regression analysis of LADA and T2D participants in the UKPDS with respect to MACE and CV death at the end of the trial and at the end of the post-trial monitoring period



4. Specific aim 2: Bone health in adults with autoimmune diabetes

4.1. Bone health in young adults with type 1 diabetes

Leanza G, Maddaloni E (First-author equal contribution), Pitocco D, Conte C, Palermo A, Maurizi AR, Lauria Pantano A, Suraci C, Altomare M, Strollo R, Pozzilli P, Schwartz AV, Napoli N. Risk Factors for Fragility Fractures in Type 1 Diabetes. Full manuscript currently under review..

4.1.1. Background

Diabetes-related complications are the main cause of morbidity and mortality in subjects with T1D and bone fragility is being recognized as a new complication of both T1D and T2D.[56,57] The association between diabetes and hip fracture is however stronger for T1D than T2D (hazard ratio [HR] 6.3 vs. 1.4)[58] and Weber *et al*. showed that the increased risk of hip fractures in subjects with T1D starts already in young adulthood.[22] While risk factors for fractures in T2D have been characterized,[59,60] risk factors for fractures in T1D have not yet been fully elucidated. Hypoinsulinemia, low levels of IGF-1 and vitamin D, poor metabolic control, vascular complications[61–63] have all been studied as possible contributors to poor bone health in T1D, with controversial results.[24,64] The majority of available clinical studies in this regard are limited in the number of subjects or by the lack of information about crucial data such as metabolic control, insulin exposure or hypoglycemic events.[23,26,65] The incidence of T1D is increasing worldwide.[66] Together with an increased life expectancy as a consequence of the improving quality of care, this is causing an exponential increase in the overall number of subjects with T1D in the age range at increased risk of fragility fractures. As bone fractures are associated with increased morbidity and mortality, a better understanding of factors related to bone fragility in T1D is crucial to identify risk factors to be tackled to decrease the incidence of fractures in this population. Goal of the present study was to investigate diabetes-related clinical factors associated with non-vertebral fragility fractures in adults with T1D.

4.1.2 Methods

Patients with T1D attending an outpatient clinic at one of three participating Institutions (Rome, Italy) who had been followed for at least 5 years were deemed eligible for this cross-sectional study. Diabetes diagnosis was based on the American Diabetes Association criteria.[67] Inclusion criteria for this analysis were: age ≥ 18 years, and eugonadal status. The exclusion criteria were: 1) history of secondary causes of osteoporosis (i.e., non-compensated hypothyroidism, hyperthyroidism, hyperparathyroidism, inflammatory bowel disease, malignancy, anorexia nervosa, rheumatoid arthritis, severe liver impairment or chronic obstructive pulmonary disease), 2) use of drugs that can affect bone metabolism (e.g. bisphosphonates, glucocorticoids, anticonvulsants, hormone replacement therapy). A total of 701 T1D patients were initially screened. After exclusion of 107 subjects according to the abovementioned criteria, 600 subjects (300 males and 300 females) were enrolled. All recruited participants gave their witnessed, informed consent before entering the study, which was approved by the ethical committee of Campus Bio-Medico University of Rome and conducted in accordance with Helsinki Declaration II.

Enrolled participants attended a study visit that included measurement of height and weight for body mass index (BMI) calculation and an interview during which the number of monthly hypoglycemic episodes and family history of fragility fractures in a blood relative (first or second degree, e.g., aunts and grandmothers) were recorded. Fractures were evaluated by a previously used questionnaire.[68] Participants were asked to report the occurrence and circumstances of any fractures after the diagnosis of T1D. Only non-vertebral fractures, including hip, wrist, and other non-vertebral sites (clavicle, upper arm, rib, pelvis, ankle, upper leg, lower leg, foot, hand, shoulder, knee, and elbow), and occurrence of single or multiple fractures were included in this analysis.[69] Also, the interview explored the circumstances of the fracture, in order to exclude fractures resulting from major trauma. Only low-trauma fractures caused by a fall from a standing height or less were included in this analysis.[70]

Furthermore, medical history of the enrolled participants was obtained from clinical electronic records. To assess long-term glucose control, we considered the average of at least 3 HbA1c measurements per year, for up to 5 years before enrollment. All clinical records included the physician's evaluation of macrovascular complications, neuropathy, and retinopathy, for which we included in the analysis the status at the most recent available visit. Specifically, the presence of cardiovascular disease (CVD) was assessed as recent history or evidence of coronary heart disease, cerebrovascular disease, or peripheral arterial disease. Diabetic neuropathy evaluation was based on symptoms, quantitative sensory testing (temperature, vibration, and pressure perception) and quantitative motor testing (patellar and ankle reflexes) as assessed by the physician in the medical history. Additionally, all patients underwent funduscopic examination to assess retinopathy as part of standard clinical care. In order to assess the presence of reduced kidney function in our cohort, we considered the latest available serum creatinine and urine analyses. In particular, microalbuminuria and macroalbuminuria were diagnosed on the basis of albumin excretion rate between 30 and 299 mg/day or ≥300 mg/day, respectively, using the last available measurement available in the medical record. Creatinine clearance (CCr) was calculated with the EPI-CKD formula. Nephropathy was defined as the presence of both albumin-to-creatinine ratio (ACR) ≥30 mg/g and CCr <60 mL/min. The last available cholesterol (total cholesterol, HDL, LDL) and triglycerides serum values were used to evaluate the lipid profile.

Statistical Analysis

Statistical analysis was performed using STATA Stata/IC 12.1 software (StataCorp, College Station, TX, USA). The distribution of variables was tested with the Kolmogorov-Smirnov test. The results are expressed as mean ± SD or median [interquartile ranges (IQRs)] as appropriate. Comparisons were done using Student's t-test, Kruskal–Wallis and Chi-square depending on distribution; Analysis of variance (ANOVA) was used for comparisons of continuous variables between more than two groups. Multinomial logistic regression was used to determine the contribution of the explanatory

variables to the occurrence of a single and multiple (≥ 2) fractures. Results are reported as relative risk ratios (RRR) and 95% confidence intervals. Each variable of interest was first assessed with minimal adjustment for age, sex and BMI. Variables listed in Table 1 were then tested to develop a final model using forward stepwise entry (p for retention <0.1). Age, sex and BMI were forced into the model. The effects of independent variables on the different categories of the dependent variable were tested for equality. Non-normally distributed continuous variables were ln-transformed before they were tested in the ANOVA and in the multinomial logistic regression model. Two-tailed p-value <0.05 was considered statistically significant

4.1.3 Results

The average age of patients was 41.9±12.8 years (range 18-79). Average BMI and disease duration were 24.4±3.7 kg/m^2 (15.6-44.8) and 19.9±12.0 years (1-63). Mean HbA1c over the previous 5 years was 7.6±1.0% (5.3-13.0). Mean insulin requirement was 0.56±0.19 IU/kg/day. A total of 111 subjects (18.5%) reported at least one fragility fracture after diabetes diagnosis, and 29 of the fractured subjects reported ≥2 fractures. The most common fracture sites were: hand (18.6%), foot (17.1%), tibia/fibula (10.5%), wrist (9.3%) and ribs (8%).

Clinical factors associated with single and multiple fractures.

Clinical features of T1D patients by fracture status (none, 1, 2+) are reported in Table 1. Variables that differed by fracture status included age (p=0.02), BMI (p=0.05), family history of fracture (p<0.01), disease duration (p<0.01) and CCr (p<0.01). Diabetic retinopathy (p<0.01), neuropathy (p<0.01) and CVD (p=0.01) were more prevalent in subjects with higher number of fractures. In particular, subjects who experienced 2+ fractures showed higher prevalence of CVD (p=0.004), retinopathy (p<0.001) and neuropathy (p=0.007) than subjects with no history of fractures. On the contrary, CVD and retinopathy did not differ between subjects with 1 fracture *vs* no fracture [Figure 1]. Only 4 (0.7%) patients had nephropathy, and only 20 (3.3%) had celiac disease. Because of this

low prevalence, mainly in the non-fractured group, these two variables were not considered in adjusted analysis.

In models minimally adjusted for age, sex, BMI and family history of fracture, none of the variables were associated with history of a single fracture (Table 2). Neuropathy, retinopathy, disease duration and earlier age at onset were each associated with history of multiple fractures. After full adjustment, HbA1c, disease duration, CCr and neuropathy were the independent variables retained in the final multivariate multinomial regression model (Table 2 and Figure 2). Subjects in the highest tertile of HbA1c (HbA1c ≥7.9%) had an increased risk of having ≥2 fractures (adjusted RRR: 3.50 [95%CI 1.04-11.73] compared to subjects in the lowest tertile (HbA1c <7.2%). However, HbA1c did not appear to influence the risk of a single fracture, even in the highest tertile of HbA1c (adjusted RRR 0.98 [95%CI 0.51-1.89]). Similarly, subjects in the highest tertile of disease duration (disease duration ≥26 years) had an adjusted RRR for multiple fractures of 7.59 [95%CI 1.60-35.98] when compared to subjects in the lowest tertile (disease duration <14 years), but no significant association was found with single fracture (adjusted RRR: 1.06 [95%CI: 0.52-2.18]). Testing for equality confirmed the differential association of HbA1c and disease duration with one and ≥2 bone fractures (p-values for the differences of the effects on outcome: p=0.044 for HbA1c; p=0.020 for disease duration).

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

4.1.4 Conclusions

In this population of well-characterized young adults with T1D and a high prevalence (18.5%) of non-vertebral fragility fractures, poor glycemic control and long disease duration were independent risk factors for multiple fractures. Family history of fragility fractures, and diabetic neuropathy were also associated with an increased risk of fracture. To our knowledge, this is the first study assessing fracture risk factors in a sub-population of multi-fractured T1D patients. This allowed us to show that poor glycemic control over the previous 5 years and long disease duration are associated with increased risk of multiple fractures. Although an association between poor glycemic control and increased fracture risk has been reported for individuals with T2D,[71–73] the evidence on glycemic control and fracture risk in T1D is less consistent.[22,74,75] Weber and colleagues found that each 1% (11 mmol/mol) greater average HbA1c level was associated with a 5% greater risk of incident fracture in males and an 11% greater risk of fracture in females participating in a large population-based cohort study.[22] Diabetes duration in this cohort was not reported. Conversely, Zhukouskaya and colleagues found no difference in either HbA1c levels or diabetes duration between T1D patients with and without vertebral fractures.[75] This may be due to lack of multiple HbA1c measurements, as reported in recent studies,[76] or different associations between glycemic control and fracture site. Also, these studies did not consider those with multiple fractures as a separate group. Our findings suggest that poor glycemic control and long disease duration associate with the presence of multiple fractures, identifying a "severe bone fragility" phenotype in T1D.

Here we show that subjects with longer disease duration and worse glycemic control have an increased prevalence of fragility fractures, independent of mean insulin dose.[77] This provides important new information as only one previous study has, to our knowledge, considered all of these factors in relation to fracture risk. Some,[22,74] but not all,[75] previous studies have reported an association between glycemic control and increased fracture risk in T1D. Of these previous studies, only Zhukouskaya et al considered insulin dose or disease duration, and these factors were not

associated with risk of prevalent vertebral fracture.[75] It is not surprising that both factors contribute independently to fracture risk as duration of disease provides a measure of how long the skeleton has been exposed to hyperglycemia, while mean HbA1c reflects the degree of hyperglycemia. This is in accordance with pre-clinical and clinical data showing that chronic hyperglycemia may impact osteoblast function [61,78] and bone quality [37] specifically in subjects with T1D.

Vascular complications have been suggested as possible contributors to the increased bone fragility by impacting bone mineral density,[63] bone quality,[25] final risk of fractures[65] and potentially as markers of reduced vascular function in bone. In this study we found a higher prevalence of retinopathy, and diabetic neuropathy, as well as significantly lower CCr among T1D patients with positive history of fractures. With adjustment for glycemic control, disease duration and each other, neuropathy, but not retinopathy, was retained as an independent risk factor for fracture. This is consistent with the findings of Weber and colleagues, who reported that diabetic neuropathy was a significant risk factor for incident fracture in males (HR 1.33; 95% CI 1.03–1.72) and females (HR 1.52; 95% CI 1.19–1.92) with T1D,[22] and with those of Miao and colleagues, who found that presence of diabetic complications (including neuropathy, nephropathy, retinopathy and CVD) increased hip fracture risk among individuals hospitalized for T1D.[65] Conversely, Vestergaard and colleagues found that, after adjustment for other complications and multiple confounders (e.g. drugs, CVD, working status etc.) presence of diabetic neuropathy or other complications – except for diabetic kidney disease - did not increase fracture risk in T1D.[79] Several studies have documented an increased risk of cardiac events in the presence of decreased skeletal health[80] but few studies have assessed the relationship between bone health and CVD in individuals with T1D. In our study, CVD was more prevalent in subjects with higher number of fractures, but the association was not significant after adjustment for confounders (age and disease duration in particular). In contrast, we have recently shown an association between BMD at the femoral neck and history of cardiovascular disease in older people with long-standing T1D who were homogeneous in terms of age, metabolic control and disease duration.[63]

We did not find evidence of an association between fracture risk and hypoglycemic episodes, possibly because there were relatively few hypoglycemic episodes. Finally, although an association between atherogenic lipid profile and low BMD has been reported in subjects with long-standing T1D,[63] we did not find evidence of an association between lipids and fracture risk.

Our study has several strengths. We were able to consider a full set of clinical risk factors, including glycemic control, insulin dose, disease duration, hypoglycemic episodes, lipid profile and complications. Differently from previous studies conducted on population-based registries, we analyzed glycemic control through frequent HbA1c measurements over 5 years, which better describes the long-term glucose control as compared with a single measurement. We also acknowledge that this study has limitations. The design is cross-sectional so we cannot determine the temporal relationship between glycemic control and fractures. Although diabetic complications were assessed by review of medical records, fractures were self-reported. Any resulting misclassification of fractures is unlikely to differ with respect to the risk factors considered and any bias of associations would tend towards the null.

In conclusion, our study identified diabetes-specific factors that can be used to evaluate fracture risk in T1D patients, namely disease duration, presence of neuropathy, HbA1c and CCr values. Importantly, glycemic control and kidney function are modifiable risk factors that could be targeted for prevention of fractures in diabetes. Prospective longitudinal studies of fracture risk in T1D that include diabetes-specific risk factors are needed to confirm our finding

4.1.5 Tables

Table 4.1.1 Population features. Values are median (25^{th}-75^{th} percentiles)

	0 fractures (n=489)	1 fracture (n=82)	≥ 2 fractures (n=29)	p-value
Age (years)	40 (32-49)	43 (34-54)	47 (37-54)	0.018
BMI (Kg/m^2)	23.7 (21.6-26.5)	24.4 (21.6-27.1)	25.6 (23.1-28.1)	0.052
Sex (Men)	240	46	14	0.492
Family history of fracture (Y)	162	41	15	0.003
Age at onset	21 (11-31)	19 (13-31)	15(11-18)	0.061
Disease duration (years)	18 (10-27)	20 (12-31)	31 (23-39)	<0.001
Insulin Unit (IU/kg)	0.54 (0.44-0.66)	0.55 (0.45-0.67)	0.53 (0.46-0.68)	0.774
Monthly Hypoglycemic episodes (n)	6 (4-10)	8 (4-12)	8 (4-15)	0.707
eGFR (ml/min)	105.8 (92.2-116.7)	101.6 (81.2-113.5)	96.9 (84.1-107.6)	0.002
Total Cholesterol (mg/dl)	178 (155-197)	176 (156-193)	173 (160-195)	0.840
HDL (mg/dl)	61 (51-75)	64 (50-74)	60.5 (52-73)	0.793
LDL (mg/dl)	97 (80-115)	95 (74-115)	95 (86-104)	0.279
Triglycerides (mg/dl)	66 (52-89)	72 (58-83)	72.5 (55-125)	0.252
A1C				0.181
- **Tertile 1 (n)**	164	25	4	
- **Tertile 2 (n)**	152	29	11	
- **Tertile 3 (n)**	155	24	13	
CVD (n)	31	8	6	0.012
Retinopathy (n)	97	20	14	0.001
Neuropathy (n)	47	15	8	0.002
Nephropathy (n)	3	0	1	0.263
Celiac disease (n)	19	1	0	0.440

Table 4.1.2. **Variables from Table 1 adjusted for age, sex, BMI, and family history of fractures.** Multinomial logistic regression models for 1 or 2+ fractures. Model 1 adjusted for age, sex, BMI. Model 2 adjusted for age, sex, BMI and other variables in table that were retained in the model. CVD = stroke, CHD, PAD, bypass or stent

VARIABLE	UNITS	Multinomial logistic model 1		Model 2	
		1 FX	≥2 FX	1 FX	≥2 FX
Ln CCr	1 unit	0.31 (0.09-1.08)	0.27 (0.05-1.50)	0.22 (0.06-0.83)	0.23 (0.04-1.48)
Neuropathy	Yes/no	1.86 (0.96-3.62)	2.60 (1.03-6.60)	2.57 (1.21-5.46)	2.57 (0.92-7.15)
A1C					
Tertile 1	≤7.17	Ref	Ref	Ref	Ref
Tertile 2	7.18-7.9	1.19 (0.66-2.15)	2.54 (0.78-8.27)	1.44 (0.76-2.72)	3.42 (0.97-12.05)
Tertile 3	>7.9	1.02 (0.55-1.88)	3.00 (0.94-9.58)	0.98 (0.51-1.89)	3.50 (1.04-11.73)
Disease duration					
Tertile 1	<14 yrs	Ref	Ref	Ref	Ref
Tertile 2	14-25 yrs	1.66 (0.92-3.01)	4.28 (0.89-20.59)	1.68 (0.89-3.19)	3.43 (0.68-17.24)
Tertile 3	≥26 yrs	1.23 (0.63-2.39)	10.10 (2.20-46.4)	1.06 (0.52-2.18)	7.59 (1.60-35.98)
Family history of fragility fractures	Yes/no			2.08 (1.23-3.50)	2.83 (1.21-6.59)
Lipid profile					
Ln (Total chol)		0.69 (0.18-2.69)	1.25 (0.15-10.44)	---	---
Ln (HDL)		1.83 (0.65-5.13)	1.18 (0.24-5.87)	---	---
Ln (LDL)		0.49 (0.21-1.16)	0.97 (0.24-3.87)	---	---
Ln (Triglycerides)		1.13 (0.62-2.05)	1.46 (0.58-3.67)	---	---
Ln (Age at onset)		0.86 (0.60-1.23)	0.42 (0.26-0.68)	---	---
Ln (Hypo/month)		1.08 (0.82-1.43)	1.20 (0.78-1.85)	---	---
Ln (Insulin dose)		1.27 (0.66-2.47)	1.47 (0.51-4.24)	---	---
CVD	Yes/no	1.22 (0.50-2.95)	2.46 (0.81-7.49)	---	---
Nephropathy	Yes/no	0.00	4.76 (0.44-51.42)	---	---
Celiac disease	Yes/no	0.29 (0.04-2.19)	---	---	---
Retinopathy	Yes/no	1.13 (0.64-2.01)	2.94 (1.32-6.57)	---	---

4.1.6 Figures

Figure 4.1.1. Complications by groups of number of fractures. Positive history of cardiovascular disease, nephropathy and neuropathy were more frequent with the increasing number of fractures. *p-value at univariate ANOVA; §p<0.05 *vs* no fracture; #p<0.05 *vs* 1 fracture.

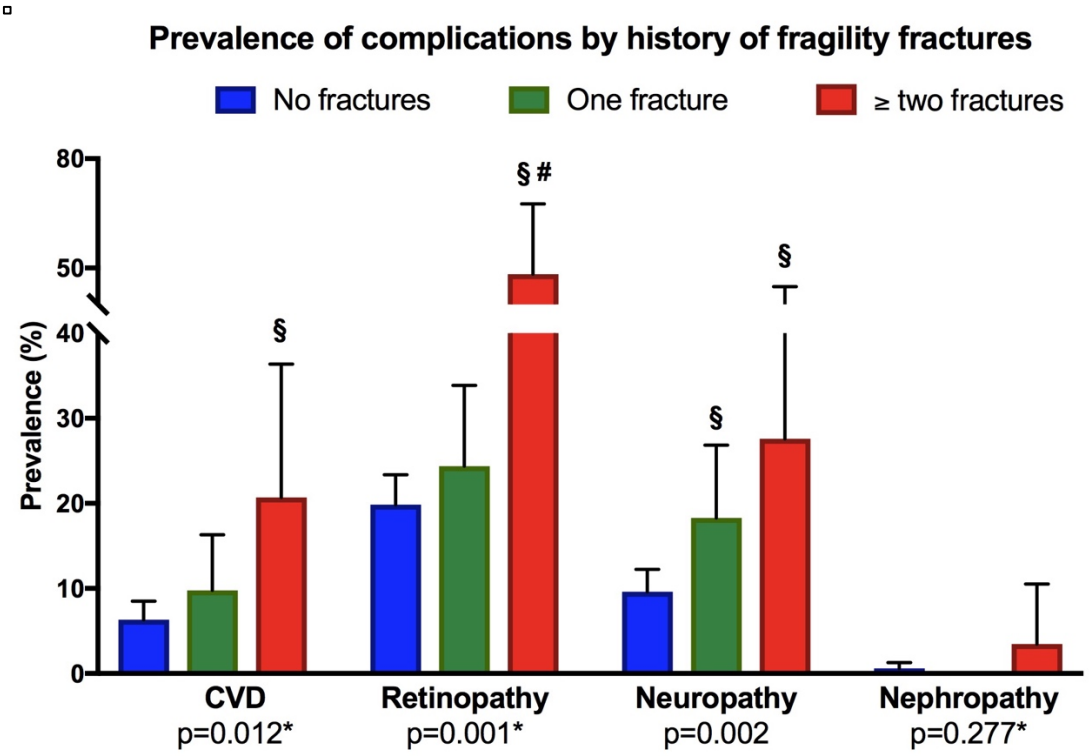
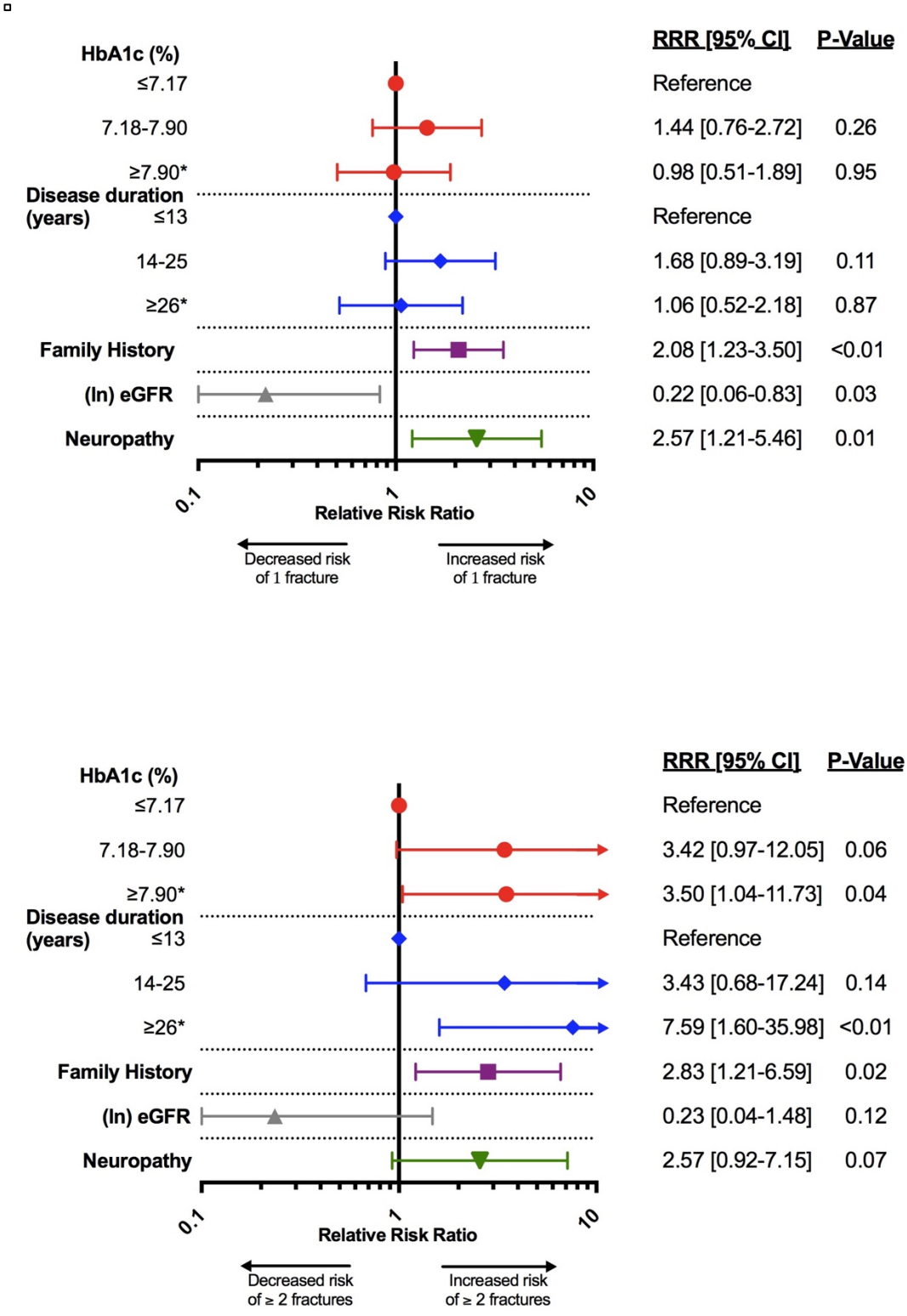


Figure 4.1.2. Graphical representation of the multinomial regression model with fractures [single (A) and ≥2 (B)] as dependent variable. Only variables significantly associated with the outcome in the fully adjusted model are represented. Values of HbA1c and disease duration are the respective tertiles' cut-offs. *p-value for the difference in the effects of the independent variable on single vs ≥2 fractures <0.05.



4.2 Bone health in elderly people with extreme duration type 1 diabetes

Maddaloni E, D'Eon S, Hastings S, Tinsley LJ, Napoli N, Khamaisi M, Bouxsein ML, Fouda SMR, Keenan HA. Bone health in subjects with type 1 diabetes for more than 50 years. Acta Diabetol. Vol. 54, no.5, pp: 479-488. May 2017. doi: 10.1007/s00592-017-0973-2; PMID: 28236093

4.2.2 Background

Despite declining skeletal health being thought of as an age-related condition, most studies in T1D have been conducted in subjects less than 40 years of age, with a primary focus on young adults.[77,81–84] Studies including the Scottish Linkage Study, the Pittsburgh Epidemiology of Diabetes Complications, the Swedish Registry and the Australian Registry[85–88] showed that rates of cardiovascular mortality are decreasing among those with T1D, leading to increases in longevity. This increase in the number of individuals aging with T1D represents a disproportionate increase in risk of fragility fractures due to the age-related decline of BMD and due to the documented pathological effects of T1D on bone.[24] The study presented here, despite being a pilot study, is a harbinger of what is to come as the number of individuals with T1D grows and their longevity increases. Indeed, we aim to provide insight on bone fragility, markers of bone turnover and factors associated with skeletal health in a subset of individuals with duration of T1D significantly longer than previously studied, and who are in the age range that is at highest risk for fragility fractures. To accomplish this, we enrolled individuals from the Joslin 50-Year Medalists, a unique cohort of subjects who have had insulin dependent diabetes since time of diagnosis 50 years or longer. Due to their age and extreme T1D duration this population provides a unique opportunity to study the effects of long term T1D on skeletal health in the context of age.

4.2.3 Methods

Study population and procedures

Details of the 50-Year Medalist Study and its methods have been extensively described elsewhere.[2,89,90] In brief, participants are Caucasian with 50 or more years of documented insulin dependence and are subsequently awarded the Joslin 50-Year Medal. The Medalist Study took place

between April 2005 and December 2015. Bone mineral density (BMD) testing by dual-energy X-ray absorptiometry (DXA) took place between October 2011 and June 2012, assessing each individual meeting inclusion criteria as they presented to the study to avoid sampling bias. To participate in the Medalist Study individuals must present three forms of documentation of insulin dependence since time of diagnosis or original medical record. All individuals were assessed at the Joslin Diabetes Center in Boston, MA by clinical exam, electrocardiogram, and standard laboratory measures. The Medalist Study has extensively characterized over 985 individuals (455 males and 530 females) with mean T1D duration of 55 years. Informed consent was obtained from all subjects prior to participation in the study. The Joslin Committee on Human Studies approved the study protocol.

Medalists were asked to report significant medical events including all fractures.[91] Data about all-cause hip and wrist fractures occurring at any age were collected by questionnaires. Between October 2011 and June 2012, 65 consecutive subjects meeting inclusion criteria (31 men and 34 post-menopausal women) presenting to the same study site received DXA scans. History of Cushing's disease, long-term (>6 months) steroid use, hypogonadism (premature menopause, anorexia, pituitary dysfunction), hypo- and hyperparathyroidism and/or alcoholism were considered as exclusion criteria for this study. Urine and blood specimens were collected on the same day of the DXA scan after an 8-hour fast. Cardiovascular disease was defined as self-reported as history of coronary artery bypass surgery, angioplasty, coronary stent placement, myocardial infarction, or angina.[2] Peripheral neuropathy was assessed using the Michigan Neuropathy Screening Index a score≥2 was considered positive.[92] An albumin-to-creatinine ratio (ACR) >30 mg/g and an estimated glomerular filtration rate (eGFR) <60 mL/min per 1.73 m^2 was considered positive for nephropathy [93]. Diabetic retinopathy was diagnosed using seven-standard field fundus photography and graded according the Early Treatment Diabetic Retinopathy Study.[94]

Biochemical assays

HbA1c was determined by high-performance liquid chromatography (Tosoh G7 and 2.2, Tokyo, Japan). Lipid profiles were determined by standard enzymatic methods (Roche Diagnostics, Indianapolis, IN; Denka Seiken, Tokyo, JP; and AsahiKasei, Tokyo, JP). High-sensitivity C-reactive protein (hsCRP) was determined by nephelometric methods; creatinine, calcium and albumin by spectrophotometry; intact parathyroid hormone (PTHi), 25-OH vitamin D, estradiol, follicle-stimulating hormone (FSH), luteinizing hormone (LH), sex hormone binding globulin (SHBG), testosterone by immunoassay; IGF1 by liquid chromatography; urine ACR by turbidimetric methods, (Quest Diagnostics, Wallingford, CT). The following serum bone turnover markers were evaluated: Collagen Type 1 C-Telopeptide (CTx) and total osteocalcin, by electrochemiluminescent immunoassay, Bone-Specific Alkaline Phosphatase by immunoenzymatic assay,

Areal bone mineral density (BMD)

Areal BMD of the lumbar spine, femoral neck, total hip and one-third distal radius was measured by DXA (Hologic QDR Elite Fan Beam X-Ray Densitometer; model number 4500A with a Delphi Upgrade, Waltham, MA). T- and Z-scores were automatically computed by the Hologic software on the basis of male and female reference values (NHANES reference set for hip and Hologic reference dataset for lumbar spine). A single trained technician performed all scans and analyses. According to the World Health Organization diagnostic criteria, normal BMD was defined as T-score values ≥ -1.0SD, "low bone mass" or "osteopenia" as T-score values < -1.0SD and > -2.5SD, and osteoporosis as T-score values ≤ -2.5SD.[95]

Statistical analysis

Two sets of analyses were done, one to examine protection from low bone mineral density (T-score values < -1.0SD), and a second to examine characteristics associated with lower bone mineral density

in regions known to have a higher cortical/cancellous bone ratio (one-third distal radius and femoral neck).

Variables were tested for normality using the Shapiro-Wilk test. Values are expressed as mean ± SD and medians [range] for continuous variables and as proportions for categorical variables (%) depending on distribution. Comparisons were done using Student's t-test, Kruskal–Wallis, and chi-square depending on distribution. Generalized linear models were used to calculate prevalence risk ratios adjusted for covariates. Those factors significant at $p < 0.1$ were tested in the final model with main effect and outcome. Effect modification was tested in the standard ways. Two-tailed p-value < 0.05 was considered statistically significant. All statistical analyses were performed using *Stata/IC 12.1* software (StataCorp, College Station, TX, USA).

Ethics

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

4.2.4 Results

Clinical population

Among the Medalists, mean (±SD) age was 66.0 ± 7.6 years (67.3 ± 7.9 in males and 64.9 ± 7.2 in females), disease duration was 54.7 ± 5.7 years and BMI 26.2 ±4.7 kg/m^2. The Medalists had a mean HbA1c of 7.2 ± 0.9% (55.2 ± xx mmol/mol), triglycerides of 75.0 ± 37 mg/dl, total cholesterol of 161.4 ± 32.7 mg/dl, HDL of 65.1 ± 19.9 mg/dl, LDL of 81.1 ± 23.8 mg/dL and eGFR of 69.6 ± 20.3 ml/min/1.73m^2. The study population had a CVD prevalence of 39.9% and proliferative diabetic retinopathy (PDR) was diagnosed in 46.4%. Diabetic nephropathy affected 12.5%, and 69.8% were found to have diabetic neuropathy. Data regarding bone fractures were available for all 985 subjects

enrolled in the 50-Year Joslin Medalist Study at the time of analysis. History of hip and wrist fractures was reported by 11 subjects (1.12%). More specifically, 9 subjects (0.91%; 4 males and 5 females) had a previous wrist fracture and 2 male subjects (0.20%) reported history of hip fracture.

Areal BMD

Clinical and biochemical features of the 65 subjects who underwent DXA scans are reported in Table 4.2.1. There were no significant differences in demographic, clinical features and medication use between the primary Medalist cohort and those who underwent the DXA scan. Overall, this subgroup has good glycemic control, HbA1c 7.1 ± 1.0% (54.1 ± 10.9 mmol/mol), total cholesterol 168.0 ± 36.2 mg/dl, triglycerides 75.4 ± 32.7 mg/dl, HDL 65.7 ± 20.8 mg/dl, LDL 86.8 ± 25.0 mg/dl, and vitamin D levels (35 ± 9 ng/ml). Only one subject had a detectable fasting serum c-peptide, the remaining 64 had had undetectable levels.

Results of the DXA scans are reported in Table 4.2.2. The cohort showed comparable or better BMD by Z-score to age, gender and race-matched population. Mean Z-scores were 1.15 ± 1.61 at the lumbar spine ($p<0.001$ for difference vs 0), 0.23 ± 0.87 at the total hip ($p=0.040$), -0.01 ± 0.85 at the femoral neck ($p=0.97$) and 0.26 ± 1.35 at one-third distal radius ($p=0.13$). The cumulative prevalence of osteoporosis using any of spine, total hip and femoral neck BMD measurements was 4.6%. When the one-third distal radius was considered in addition to the other diagnostic sites, the prevalence of osteoporosis increased to 16.9%. The proportion of subjects with a T-score ≤ -2.5 at the one-third distal radius was 15.4%, 3.1% at the femoral neck and 1.5% at the lumbar spine. The prevalence of low bone mineral density, osteopenia, was 66.2% considering all standard diagnostic sites (lumbar spine, total hip and femoral neck) (Table 4.2.2).

Features of Medalists with normal BMD

To identify factors associated with preservation of BMD we further compared clinical and biochemical features of those with normal BMD at all sites (n=10) to those with low bone mass in at

least one site (n=55). No differences in gender (50% vs 47% males, p=0.874), age (64.1 ± 6.1 vs 63.7 ± 7.1 years, p=0.865), age at diagnosis (11.2 ± 5.9 vs 10.0 ± 6.1 years, p=0.473) and disease duration (52.6 ± 2.5 vs 52.5 ± 3.2 years, p=0.592) were found between Medalists with normal and low BMD, respectively. Individuals with normal BMD showed a better lipid profile with lower total cholesterol, triglycerides and LDL levels, but no significant differences in HDL levels, this was independent of the use of anti-hyperlipidemics (adjusted p=0.008, p=0.049 and p=0.020, respectively) [Figure 4.2.1]. The presence of CVD, proliferative retinopathy, diabetic nephropathy and neuropathy did not differ between those with normal and low BMD [Table 4.2.3].

Those with normal BMD did not differ in IGF1 (97.0 ± 22.8 vs 98.3 ± 4.6 pg/ml, p=0.814), PTHi (25.7 ± 10.8 vs 28.6 ± 14.7 pg/ml, p=0.679), vitamin D (36.8 ± 9.1vs 34.4 ± 9.5 ng/ml, p=0.516), calcium (9.1 ± 0.2 vs 8.9 ± 0.6 mg/dl, p=0.158), bone alkaline phosphatase (11.6 ± 3.1 vs 10.8 ±4.2 mcg/L, p=0.300), total osteocalcin (15.1 ± 6.8 vs 17.2 ± 10.4 ng/ml, p= 0.666) and CTx levels (201.9 ± 58.1 vs 264.8 ± 178.7 pg/ml, p=0.364) from those with lower levels of BMD.

Features by low bone mass in cortical sites

Since the higher prevalence of osteoporosis at the one-third distal radius could suggest a greater effect of T1D at sites composed of a greater proportion of cortical bone, we compared traits between those with and without low bone mass at sites with higher cortical/cancellous bone ratio, specifically the femoral neck and one-third distal radius. Table 4 shows subjects with normal T-scores did not differ in clinical and biochemical features from those with T-score < -1.0 SD at both sites. However, as expected, subjects with low bone mass/osteoporosis at the femoral neck showed a trend towards higher level of bone turnover markers (total osteocalcin and CTx) than those with normal femoral neck T-score (Table 4.2.4).

Complications

No differences were found in the crude prevalence of the microvascular diseases retinopathy, nephropathy or neuropathy. A borderline difference was found in the prevalence of CVD between

those with and without low bone mass at the femoral neck (9.0% v 27.9%, p=0.07) [Table 4.2.3], but not at other skeletal sites. Adjusted models of CVD and BMD at the femoral neck demonstrated cholesterol as a significant confounder with the association climbing to a prevalence risk ratio (PRR) [95% CI] of 4.6 [1.2 – 18.1] of CVD in the presence of low BMD (p=0.03 with adjustment) [Figure 4.2.2]. Subjects with normal and low femoral neck T-score did not differ in presence of hypertension (p=0.60), smoking status (p=0.54), use of lipid lowering agents (p=0.77) or antiplatelet medications (p=0.41).

4.2.5 Conclusions

Our results found an unexpectedly low prevalence of fractures in a large cohort of aging people with T1D with greater than 50 years duration.[22,23] Accordingly, we showed an unexpectedly low prevalence of osteoporosis and BMD values comparable to non-diabetic peers, as shown by normal Z-scores, in a subgroup of subjects from the same cohort. Indeed, despite their median age of 63 years, only 4.6% of the Medalists were affected by osteoporosis, compared to 10.3% reported in US adults 50 years or older [96] and to 14% in individuals with between 9 to 20 years of T1D.[97] Despite a lower than expected prevalence of osteoporosis, osteopenia was present in 66.2% of the Medalists compared to 43.9% of US adults. While reports in young adults with shorter duration T1D showed they have lower BMD than their non-diabetic peers [98], studies in those with longer duration T1D have results consistent with our findings. Ingberg et al. did not find any significant reduction in terms of lumbar or femoral BMD in males and females [age range 33-55 years] affected by T1D for 33 years [range 28-37] [77]. Similarly, Lunt et al. measured lumbar spine and femoral neck BMD in 99 women (median age 42 years) with long-standing T1D (median disease duration 27 years) and found similar values to those of healthy volunteers.[99]

Several factors could explain our results. As we studied older adults having relatively few complications they may have been protected by endogenous factors or better glycemic control throughout their time with diabetes.[2,89,100] It is also possible that due to consistent care for their

diabetes, these individuals were more diligent regarding diet and other risk factors for skeletal health decline. BMI in the defined normal range and mean values of vitamin D above 30 ng/ml were seen in both those with lower and higher BMD. It has also been proposed that long term exogenous insulin therapy can recover the bone loss seen within the initial years of T1D diagnosis. These individuals with 50 or more years may have recovered initial bone deficit and benefited through exogenous insulin use. As several reports have showed islet transplantation to have positive effects on diabetes complications, especially macrovascular,[101,102] data regarding skeletal health are lacking. This restoration of endogenous insulin production in those T1D may ameliorate declines in skeletal health due to insulin deficiency. Moreover, other strategies to sustain beta-cell function in T1D, such as immunotherapies alone or in combination with other agents,[103] should also be studied in terms of bone protection.

Finally, the Joslin 50-Year Medalist cohort is documented to have low rates of diabetic vascular complications (45.6% PDR, 12.5% diabetic nephropathy, 40.5% CVD).[2,100] As shown by Miao et al. there is a strong association between vascular complications and rate of fractures in T1D. [65] Similarly, Shanbhogue et al. recently described an association between the presence of vascular complications of T1D and deficit in bone microarchitecture and volumetric BMD.[25] These findings show a similar pattern to that of the Medalists, protection from vascular complications and preservation of skeletal health. This is supported by our findings of a strong and independent association between CVD and low BMD at the femoral neck, which remained with adjustment. Indeed femoral neck could be particularly vulnerable to vessel occlusion because of the monolateral end-arterial blood supply.[104] Accordingly, a worse lipid profile was associated with low BMD in this group. Epidemiological studies have associated dyslipidemia with osteoporosis in postmenopausal women[105] and it has recently been shown that people with isolated high levels of LDL have lower BMD[106] Moreover, both *in vitro* and *in vivo* studies show dyslipidemia and products of lipid oxidation may decrease BMD by inhibiting osteoblast and stimulating osteoclast differentiation through the induction of macrophage-colony stimulating factor and tartrate resistant acid phosphatase.[107,108]

We also found the prevalence of osteoporosis increases by almost four times if the one-third radius is considered among the sites used for diagnosis together with the hip and the spine. This could suggest a preferential involvement of the cortical bone compartment, even though we cannot further speculate on this finding as we did not investigate bone microarchitecture. However, in line with these findings, it has recently been shown that cortical rather than trabecular bone microarchitecture is negatively affected by diabetes, particularly higher fasting glucose, especially at the radius.[25,109–111]

We acknowledge our study has some limitations. Self-report of fractures may result in underestimates of fragility fractures. Degenerative changes in spine anatomy and aortic calcification, which are common in older adults, may have overestimated BMD at the lumbar spine.[112] Additionally, as this is a cross-sectional study, causal associations cannot be made. However, for factors such as HbA1c, we have previously shown a strong correlation (r^2=0.51) between current and historical glycemic control in the 50-Year Medalists.[2] The lack of an internal control group did not allow the evaluation of the lipid-effect on BMD in subjects without diabetes compared to Medalists. As the exclusion of subjects with a previous diagnosis of osteoporosis would have introduced a selection bias, those currently using osteoporosis medications were included. Eight subjects were on medication for osteoporosis and the adjustment for this confounder did not modify our results. Finally, even with adjustment for known confounders, there may be unidentified factors influencing the bone health of this population. While the "extreme disease duration" of this population could limit the generalizability of our results, the study of people like Medalists is necessary to study the effects of long term T1D. This extraordinary population enables us to identify patterns and factors in a growing portion of the population, which could potentially heavily burden the healthcare system. As these individuals have escaped the primary causes of early death amongst those with diabetes, namely CVD,[2] this suggest a bias for better glycemic control, and other factors which protect against vascular disease.

In conclusion, this study provides the first data on fracture history and bone density in a large sample of aging individuals with T1D with more than 50 years of the disease. We found BMD is not on average lower in the Medalists, based on Z-scores. Thus, the 50-Year Medalists may serve as a unique model to identify factors which may protect T1D patients from the development of bone fragility, a common but under recognized complication of diabetes, particularly among those with life-long insulin dependence. In the Medalists, a good lipid profile is associated with favorable BMD values, suggesting that in T1D with reasonable glycemic control, there may be an association between cardiovascular disease and skeletal health decline. Additional prospective studies are needed to confirm these observations and to further elucidate potential mechanisms contributing to skeletal health in those with longstanding T1D.

4.2.6 Tables

Table 4.2.1. Study sample features.

	Overall (n=65)		**Males (n=31)**		**Females (n=34)**	
	Mean ± SD	**Median [range]**	**Mean ± SD**	**Median [range]**	**Mean ± SD**	**Median [range]**
Age, years	63.8 ± 6.9	63 [52-81]	65.9 ± 7.0	65 [52-81]	61.8 ± 6.4	61 [53-79]
Age at diagnosis, years	10.2 ± 6.0	10 [0-25]	10.9 ± 6.5	11 [2-25]	9.5 ± 5.6	9 [0-21]
Disease duration, years	52.5 ± 3.1	51 [50-67]	53.4 ± 3.9	52 [50-67]	51.6 ± 1.8	51 [50-56]
BMI, Kg/m^2	26.6 ± 5.0	25.8 [18.0-43.9]	27.8 ± 4.5	27.2 [20.6-42.5]	25.6 ± 5.3	24.6 [18.0-44.0]
Waist to hip ratio	0.9 ± 0.1	0.9 [0.8-1.1]	1.0 ± 0.1	1.0 [0.9-1.1]	0.9 ±0.1	0.9 [0.8-1.1]
Hba1c, % (mmol/mol)	7.1 ± 1.0 (54.1 ± 10.9)	7 [5.2-11.5] (53.0 [33.3-102.2])	7.0 ± 1.1 (53.0 ± 12.0)	6.9 [5.2-11.5] (51.9 [33.3-102.2])	7.2 ± 0.9 (55.2 ± 9.8)	7.2 [5.6-9.4] (55.2 [37.7-79.2])
Insulin dose, IU/kg	0.48 ± 0.20	0.45 [0.48-0.20]	0.56 ± 0.24	0.52 [0.23-1.30]	0.42 ± 0.14	0.42 [0.14-0.72]
eGFR, ml/min/1.73m^2	76.2 ± 19.2	79.8 [18.6-101.1]	78.2 ± 16.9	83.2 [40.6- 101.1]	74.4 ± 21.2	78.0 [18.6-100.8]
Total cholesterol, mg/dl	168.0 ± 36.2	163 [93-292]	156.6 ± 30.4	159 [93-219]	178.3 ± 38.4	168 [115-292]
Triglycerides, mg/dl	75.4 ± 32.7	70 [33-209]	74.8 ± 34.8	71 [33-209]	75.8 ± 31.1	68 [36-182]
HDL, mg/dl	65.7 ± 20.8	63 [23-136]	58.7 ± 18.7	58 [23-111]	72.0 ± 20.0	69.5 [31-136]
LDL, mg/dl	86.8 ± 25.0	85 [43-162]	82.9 ± 23.5	79 [51-154]	90.4 ± 26.1	87.5 [43-162]
hsCRP, mg/L	0.8 ± 1.3	0.2 [0.1-5.8]	1.0 ± 1.3	0.6 [0.1-5.5]	0.6 ± 1.3	0.2 [0.1-5.8]
Corrected calcium, mg/dl	8.9 ± 0.5	9.0 [6.9-10.3]	8.7 ± 0.6	8.8 [6.9-9.4]	9.1 ± 0.4	9.1 [8.4-10.3]
Vitamin D, ng/ml	35 ±9	32 [17-53]	33 ± 9	32 [17-53]	35.9 ± 9.6	33 [17-51]

Bone Alkaline Phosphatase, mcg/L	10.9 ± 4.0	9.9 [5.6-25.9]	10.0 ± 3.4	9.5 [5.6-23.3]	11.7 ± 4.3	10.9 [6.7-25.9]
Total osteocalcin, ng/ml	16.9 ± 9.9	14.0 [4.0-67]	15.6 ± 5.4	14.5 [7.0-29.0]	18.0 ±12.7	14.0 [4.0-67.0]
CTx, pg/ml	254.9 ±166.8	230 [14.0-1119.0]	226.3 ± 91.3	224.0 [73.0-412.0]	280.6 ± 211.6	241.5 [14-1119]
PTHi, pg/ml	28.1 ± 14.2	26 [7-71]	29.9 ± 12.2	28.5 [7- 57]	26.6 ± 15.8	23 [8-71]
IGF1, pg/ml	98.1 ± 32.5	91 [15-223]	100.4 ± 26.5	90 [64-156]	96.0 ± 37.5	91 [15-223]

Table 4.2.2. Bone mineral density in adults with longstanding Type 1 diabetes. Low bone mass and osteoporosis are defined as T-score <-1.0SD and >-2.5SD and T-score ≤-2.5SD respectively. *Low BMD at lumbar spine, total hip or femoral neck; ** Low BMD at lumbar spine, total hip, femoral neck or one-third distal radius.

		Overall (n=65)		Males (n=31)		Females (n=34)	
		Mean ± SD, %	**Median [range]**	**Mean ± SD,%**	**Median [range]**	**Mean ± SD,%**	**Median [range]**
Lumbar spine	**BMD (g/cm^2)**	1.068 ± 0.170	1.039 [0.788-1.693]	1.079 ± 0.200	1.070 [0.788-1.693]	1.057 ± 0.137	1.038 [0.800-1.367]
	Z-score (SD)	1.15 ± 1.61	1.2 [-1.8 - 6.5]	0.69 ± 1.88	0.6 [-1.8 – 6.5]	1.59 ± 1.17	1.65 [-0.7 – 4.1]
	T-score (SD)	-0.00 ± 1.542	-0.1 [-2.8 – 5.5]	-0.10 ± 1.82	-0.2 [-2.8 – 5.5]	0.093 ± 1.24	-0.1 [-2.2 – 2.9]
	T-score <-1.0 SD (%)	22.5		32.3		11.8	
	T-score ≤-2.5 SD (%)	1.5		3.2		0	
Total hip	**BMD (g/cm^2)**	0.908 ± 0.123	0.924 [0.658-1.258]	0.950 ± 0.134	0.962 [0.681-1.258]	0.867 ± 0.099	0.883 [0.658-1.061]
	Z-score (SD)	0.23 ± 0.87	0.3 [-1.9 – 2.2]	0.04 ± 0.89	0.2 [-1.9 – 1.9]	0.41 ± 0.83	0.6 [-1.2 – 2.2]
	T-score (SD)	-0.58 ± 0.84	-0.5 [-2.3 – 1.5]	-0.56 ± 0.88	-0.5 [-2.3 – 1.5]	-0.61 ± 0.82	-0.5 [-2.3 – 1.0]
	T-score <-1.0 SD (%)	33.9		32.3		35.3	
	T-score ≤-2.5 SD (%)	0		0		0	
	BMD (g/cm^2)	0.738 ± 0.110	0.731 [0.515-1.026]	0.770 ± 0.121	0.756 [0.586-1.026]	0.707 ± 0.089	0.702 [0.515-0.918]

Femoral neck	**Z-score (SD)**	-0.01 ± 0.85	-0.1 [-1.7 – 2.1]	-0.09 ± 0.91	-0.1 [-1.5 – 1.8]	0.08 ± 0.78	0 [-1.7 – 2.1]
	T-score (SD)	-1.23 ± 0.84	-1.3 [-3.0 – 0.7]	-1.17 ± 0.89	-1.3 [-2.5 – 0.7]	-1.28 ± 0.79	-1.3 [-3.0 – 0.6]
	T-score <-1.0 SD (%)	66.2		67.7		64.7	
	T-score ≤-2.5 SD (%)	3.1		0		5.9	
One-third distal radius	**BMD (g/cm^2)**	0.691 ± 0.110	0.694 [0.495-0.982]	0.771 ± 0.083	0.788 [0.562-0.982]	0.618 ± 0.076	0.615 [0.495-0.819]
	Z-score (SD)	0.26 ± 1.35	0.30 [-3.4 – 4.1]	0.14 ± 1.45	0.3 [-3.4 – 4.1]	0.37 ± 1.3	0.3 [-1.7 – 3.4]
	T-score (SD)	-1.06 ± 1.4	-0.9 [-4.6 – 2.7]	-0.97 ± 1.43	-0.7 [-4.6 – 2.7]	-1.14 ± 1.31	-1.2 [-3.3 – 2.3]
	T-score <-1.0 SD (%)	47.7		41.9		52.9	
	T-score ≤-2.5 SD (%)	15.4		9.7		20.6	
Three-points low bone mass*		66.2		71.0		61.8	
Three-points osteoporosis*		4.6		3.2		5.9	
Four-points low bone mass**		67.7		71.0		64.7	
Four-points osteoporosis**		16.9		12.9		20.6	

Table 4.2.3. Prevalence of chronic complications of diabetes by T-score overall and at cortical sites

	Subjects with normal T-score (n=10)	Subjects with osteoporosis / low bone mass (n= 55)	Femoral Neck T-score > -1.0 SD (n= 22)	Femoral Neck T-score < -1.0 SD (n= 43)	1/3 Radius T-score > -1.0 SD (n=34)	1/3 Radius T-score < -1.0 SD (n=31)
Cardiovascular diseases	20.0 %	21.8 %	9.0 %	27.9 %	20.6 %	22.6 %
Proliferative diabetic retinopathy	66.7 %	35.6 %	37.5 %	42.1 %	50.0 %	29.2 %
Diabetic nephropathy	10 %	17.4 %	4.6 %	20.9 %	20.6 %	9.7 %
Diabetic neuropathy	80.0 %	64.1 %	66.7 %	66.7 %	64.7 %	69.0 %

Table 4.2.4 Population features by T-score at cortical sites.

	Femoral Neck T-score ≥ -1.0 SD (n= 22)	Femoral Neck T-score < -1.0 SD (n= 43)	p-value	1/3 Radius T-score ≥ -1.0 SD (n=34)	1/3 Radius T-score <-1.0 SD (n=31)	p-value
Gender, % male	45.5	48.8	0.796	52.9	41.9	0.375
Age, years	63.9 ± 6.6	63.7 ± 7.2	0.928	63.1 ± 7.4	64.5 ± 6.4	0.421
Age at diagnosis, years	11.3 ± 6.0	9.6 ± 6.0	0.269	10.2 ± 6.4	10.2 ± 5.6	0.911
Duration, years	51.9 ± 1.9	52.8± 3.5	0.466	52.3 ± 3.2	52.7 ± 3.0	0.340
BMI, kg/m^2	26.4 ± 4.9	26.7 ± 5.2	0.954	27.7 ± 5.9	25.3 ± 3.6	0.145
Waist to hip ratio	0.90 ± 0.08	0.90 ± 0.08	0.970	0.92 ± 0.10	0.87 ± 0.06	0.086
Insulin dose, UI/Kg	0.47 ± 0.14	0.49 ± 0.23	0.691	0.47 ± 0.18	0.50 ± 0.22	0.927
HbA1c, % (mmol/mol)	7.1 ± 0.8 (54.1 ± 8.7)	7.1 ± 1.1 (54.1 ± 12.0)	0.921	7.0 ± 0.9 (53.0 ± 9.8)	7.3 ± 1.1 (56.3 ± 12.0)	0.346
eGFR, ml/min/1.73m^2	79.7 ± 15.9	74.4 ± 20.7	0.406	74.7 ± 18.3	77.9 ± 20.4	0.344
Total cholesterol, mg/dl	159.3 ± 37.1	172.4 ± 35.4	0.170	162.6 ± 32.7	173.9 ± 39.5	0.210
Triglycerides, mg/dl	71.4 ± 31.3	77.4 ± 33.6	0.305	72.6 ± 32.9	78.4 ± 32.9	0.454
HDL, mg/dl	63.2 ± 17.3	66.9 ± 22.5	0.505	60.9 ± 18.7	70.9 ± 22.1	0.054
LDL, mg/dl	81.7 ± 26.4	89.5 ± 24.1	0.142	87.1 ± 24.4	86.6 ± 26.0	0.655
hsCRP, mg/L	0.6 ± 0.9	0.9 ± 1.5	0.611	0.6 ± 1.1	1.0 ± 1.5	0.693
Corrected Calcium, mg/dl	9.0 ± 043	8.8 ±0.6	0.625	8.9 ± 0.5	8.8 ± 0.6	0.253
25-OH Vitamin D, ng/ml	33.8 ± 9.8	35.2 ± 9.1	0.604	35.6 ± 9.2	33.8 ± 9.5	0.468
Bone Alkaline Phosphatase, mcg/L	10.8 ± 2.9	11.0 ± 4.4	0.626	10.9 ± 3.1	11.0 ± 4.8	0.691
Total osteocalcin, ng/ml	13.6 ± 6.5	18.4 ± 10.9	0.053	17.1 ± 11.1	16.6 ± 8.8	1.0
C-Telopeptide, pg/ml	206.6 ± 123.6	277.2 ±180.4	0.093	255.0 ± 190.9	254.7 ± 141.1	0.707
PTHi, pg/ml	24.7 ± 8.6	29.7 ± 16.0	0.369	28.0 ± 16.7	28.3 ± 11.3	0.525
IGF1, pg/ml	95.5 ± 24.13	99.5 ± 36.2	0.821	97.2 ± 31.2	99.1 ± 34.2	0.601

4.2.7 Figures

Figure 4.2.1. Normal T-score is associated with better lipid profile. Subjects with T-score ≥ -1.0 SD at all sites showed lower levels of total cholesterol, triglycerides and LDL than subjects with T-score < -1.0 SD. No significant differences were found in HDL levels. Bars are for standard deviation.

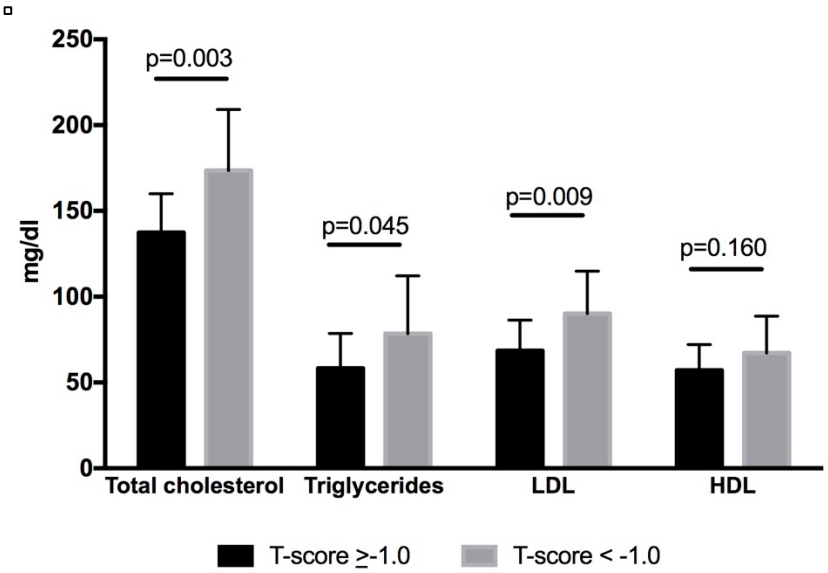
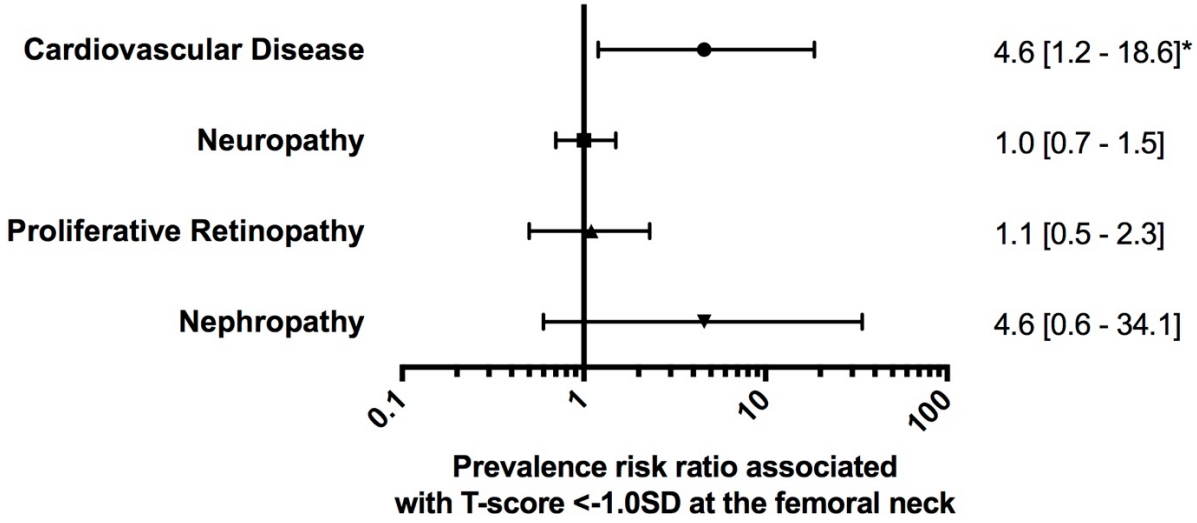


Figure 4.2.2. Prevalence risk ratio of diabetic complications associated with T-score < -1.0SD at femoral neck. *Adjusted for total cholesterol; other complication estimates not significant in bivariate or multivariate models



5 Specific aim 3: Circulating osteoprogenitors and cardiovascular disease in adults with autoimmune diabetes

Maddaloni E, Xia Y, Park K, D'Eon S, Tinsley LJ, St-Louis R, Khamaisi M, LI Q, King GL, Keenan HA. High Density Lipoprotein modulates osteocalcin expression in circulating monocytes: a potential protective mechanism for cardiovascular disease in type 1 diabetes. Cardiovasc Diabetol. Vol. 16, no. 1 pp:116. Sep 2017. doi: 10.1186/s12933-017-0599-2. PMID 28915881

5.1 Background

CVD is the major cause of decreased life expectancy in people with T1D.[113] Factors other than hyperglycemia also contribute to the pathogenesis of CVD in T1D. In particular inflammation and dyslipidemia play a pivotal role in the development of atherosclerotic diseases.[114–116] Overall, an imbalance between mechanisms of injury and protective factors contributes to vascular complications in diabetes [117]. The data previously discussed [see section 4.2] show a relationship between bone health and CVD in people with long-standing T1D, suggesting common pathways of disease may be involved.[63] Circulating osteoprogenitor cells, defined as circulating cells co-expressing osteocalcin (OCN) together with the progenitor stem cell antigen CD34, have been found increased in subjects with cardiovascular disease with and without diabetes,[118] and might be implicated in the bone vascular axis in T1D. Because of their pro-calcific phenotype, these cells are hypothesized to contribute to the development of vascular calcification and atherosclerosis [119]. Recently, the differentiation towards a pro-calcific phenotype of circulating monocytes has been related to vascular calcification and CVD in those with T2D.[120,121] The surface expression of the bone-related protein OCN is the first and essential marker of this drift.[120,122].Growing evidence demonstrates the ability of circulating OCN+ mononuclear cells to contribute to ectopic ossification.[123–125]

Some clinical and pathological features of CVD differ between T1D and T2D, and related vascular complications [8]. Following on this, OCN+ monocytes have not been explored yet in T1D. In addition, characterizing factors modulating the expression of OCN in monocytes, especially in diabetes could

be important. Oxidized low density lipoprotein (OxLDL) is a known activator of monocytes which are involved in the pathogenesis of atherosclerosis. OxLDL can also induce osteogenic differentiation of different cell types including smooth muscle and endothelial cells.[126,127] As monocytes are the main target cells of oxidized lipids, we hypothesized that OxLDL could induce OCN expression in these cells. Moreover, as high density lipoprotein (HDL) actively interacts with monocytes to protect from the development of CVD and vascular calcification, we hypothesized that HDL may counteract the pro-calcific effects of oxidized lipids. We have previously identified a cohort of subjects who after 50 years or more of T1D showed protection from clinical CVD significantly associated with elevated HDL-c levels.[100] Therefore we decided to test our hypothesis whether OCN+ monocytes were related to CVD and HDL-c in this cohort, the 50-Year Joslin Medalist, then characterize the action of OxLDL and HDL on OCN expression and possible mechanism *in vitro*

5.2 Methods

Study population. Details of the 50-Year Medalist Study and its methods have been extensively described elsewhere.[2,63,89,90] In brief, participants have 50 or more years of documented insulin dependence since time of diagnosis. All individuals were assessed at the Joslin Diabetes Center in Boston, MA, by clinical exam, electrocardiogram, and standard laboratory measures. Thirty-three consecutive Medalist Study participants were enrolled from March 2015 to November 2015 and screened for this sub-study. Subjects with chronic immobilization, hematologic or neoplastic diseases in progress, history of hyper- or hypoparathyroidism were excluded (n=3) from the study. Diabetic complications were assessed according to pre-specified criteria as follows: (I) positive cardiovascular history was defined as self-reported history of coronary angioplasty, cardiac bypass surgery, hospitalization for heart attack, leg bypass surgery, leg angioplasty or stroke;[2] (II) peripheral neuropathy was assessed using the Michigan Neuropathy Screening Index (MNSI; score≥2);[92] (III) nephropathy was defined as an albumin-to-creatinine ratio (ACR) >30 mg/g and an estimated glomerular filtration rate (eGFR) <60 mL/min per 1.73 m^2;[93] (IV) diabetic retinopathy was diagnosed

using a seven-standard field fundus photography and graded according the Early Treatment Diabetic Retinopathy Study (ETDRS >53).[94]

Urine and blood specimens were collected for biochemical assays. HbA1c was assessed by Immunoturbidimetry; high-sensitivity C-reactive protein (CRP) was determined by nephelometric methods; creatinine, calcium, albumin, total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides were assessed by spectrophotometry and LDL cholesterol calculated; urine ACR by turbidimetric method (Quest Diagnostics, Wallingford, CT). The Lipoprint system (Quantimetrix, Inc., Redondo Beach, CA) was used to assess HDL subfractions. Ten HDL subfractions were distinguished and grouped into 3 categories: large (HDL 1–3), intermediate (HDL 4–7), and small (HDL 8–10).[128]

Identification and quantification of circulating OCN+ monocytes by flow cytometry. Based on previous reports,[121,129] OCN+ monocytes were searched in the peripheral blood mononuclear cells (PBMCs) fraction. Blood samples were collected while subjects were fasting and peripheral blood monocluear cells (PBMCs) were isolated within 2 hours. Fasting blood samples were collected into cell preparation tubes with sodium citrate for the separation of mononuclear cells from whole blood (BD Vacutainer CPT). After collection, tubes were stored upright at room temperature until centrifugation. Blood samples were centrifuged within two hours from collection at room temperature (18-25ºC) in a horizontal rotor (swing-out head) for a minimum of 20 minutes at 2800 rpm (low brake). Immediately following centrifugation the mononuclear cell layer (buffy coat) was collected with a Pasteur pipette and transferred to a 15 mL size conical centrifuge tube for washing steps. Cell washing: cells were resuspended to 10mL with PBS+1%FBS and then centrifuged for 8 minutes at 1500rpm at 10°C with full brake and acceleration.

Freshly isolated PBMCs were washed 3 times in phosphate-buffered saline (PBS) with 1% fetal bovine serum (FBS) and then incubated for 45 min at 4°C in the dark with BrilliantViolet421-conjugated anti-human CD45 (BioLegend, San Diego, CA), PE/Dazzle594-conjugated anti-human CD14 (BioLegend, San Diego, CA) and AlexFluor488-conjugated anti-human osteocalcin antibodies

(R&D Systems, Minneapolis, MN), according to the manufacturers' instructions. All antibodies were titrated to achieve working concentrations. After incubation samples were washed other three times in PBS with 1% FBS and then assessed by flow cytometry. Ten minutes before cell counts, cells were stained for viability with 7-aminoactinomycin D (7AAD). Up to one million events were recorded for each sample. Data were analyzed with FlowJo software (Tree Star, Ashland, OR) according to the gating strategy described below Samples were processed in duplicate; the mean of two runs was used as levels of circulating OCN+ monocytes.

THP-1 and U937 culture and treatments. THP-1 and U937 cells (ATCC® TIB-202 and ATCC® CRL-1593.2 Manassas, VA) were cultured in low glucose RPMI-1640 containing 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin and treated with 40 µg/ml Oxidized-LDL (OxLDL) ± HDL ± 40 µg/ml LDL (AlfaAesar, ThermoFisher Scientific, Waltham, MA). The chemical inhibitor BLT-1 (SML0059, Sigma, St. Louis, MO) and scavenger receptor, class B, type I (SR-BI) specific blocking antibody (NB400-101, Novus Biologicals, Littleton, CO) were used to inhibit the SR-B1. Anti-rabbit IgG (Sigma, St. Louis, MO) was used as a control Ab. THP-1 cells were treated with 0.25 uM BLT-1 or SR-BI antibody (1:800 and 1:500, respectively) for 1 hr, and then OxLDL and/or HDL were added as described above.

Assessment of osteocalcin expression in THP-1 and U937 cells. Osteocalcin expression in THP-1 and U937 cells was assessed by immune-blot analysis and by flow cytometry as described below. OCN mRNA was assessed by quantitative real time polymerase chain reaction (qRT-PCR) as described below.

Gating strategy for the identification and quantification of circulating OCN+ monocytes by flow-cytometry [**Figure 5.1**]. Data were analyzed with FlowJo software (Tree Star, Ashland, OR) according to the following gating strategy. Doublets were excluded by a FSC-W vs FSC-A scatter. A side and forward scatter was used for a first identification of lymphocytes and monocytes by size and granularity. After exclusion of nonviable cells by gating cells negative for 7AAD, we gated CD45

bright cells and then examined the expression of CD14 and osteocalcin. Fluorescence minus one (FMO) controls were used to optimize the gating strategy. OCN+ monocytes were quantified as CD45_bright PBMCs positive for CD14 and OCN and are expressed as percentage of CD45_bright.

Flow cytometry of THP-1 cells. After the appropriate treatment cells were washed three times in PBS+1%FBS and then incubated for 45 minutes at 4°C in the dark with AF488-conjugated anti-human osteocalcin (R&D System, Minneapolis, MN, USA) and with APC-conjugated anti-human CD11b (BioLegend, San Diego, CA) according to manufacturer's instructions. After incubation samples were washed other three times in PBS+1%FBS and then assessed by flow cytometry. Ten minutes before cell counts, cells were stained for viability with 7-aminoactinomycin D (7AAD). Doublets and nonviable cells were excluded as previously described. Osteocalcin positive cells were gated on the morphologic mononuclear cell fraction according to FMO controls.

Immuno-blot analysis. Cells were harvested and lysed in RIPA lysis buffer. The protein concentration was quantified using the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific). Equivalent proteins were loaded onto 4-20% gradient native gel (criterion, Bio-Rad) and subsequently electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5 % skim milk and incubated overnight at 4℃ with anti-Osteocalcin antibody (1:1000; AB10911, EMD Millipore). After washing with TBS-T, the membranes incubated with secondary antibody (1:3000, Cell Signaling) for 1 h at room temperature. Finally, the membranes were visualized using enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific) and quantified using ImageJ.

qRT-PCR To evaluate mRNA expression level of human osteocalcin, RNA was extracted by using TRIreagent (Invitrogen, ThermoFisher Scientific, Waltham, MA) . cDNA was synthesized from RNA by using a commercially available cDNA synthesis kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA) according to manufacturer's instructions. Three steps cycling protocol (initial denaturation at 95°C for 10 min, 35 cycles of 15 s denaturation at 95°C, 30 s annealing at 60°C, and 30 s extension at 72°C) was used to amplify osteocalcin gene (forward primer: 5'-

TGACGAGTTGGCTGACCA-3'; reverse primer: 5'-AGGGTGCCTGGAGAGGAG-3'). Relative fold difference between an experimental and calibrator sample was calculated by using comparative Ct (2-ΔΔCt) method. 18S was used as internal standard to normalize the expression of the gene of interest.

Assessment of nuclear levels of Run-related Transcription Factor 2 in THP-1 and U937 cells. Nuclear and cytosolic fractions were isolated from THP-1 and U937 cells using the Compartment Protein Extraction Kit (Millipore, Cat#2145, Billerica, MA). Nuclear and cytosolic protein concentrations were measured using the Bradford assay. The proteins were blotted with an antibody specific for RUNX2 and Lamin B1 purchase from Abcam (Cambridge, MA) at 1:1,000 dilution.

Statistical analysis. Values are expressed as mean ± SD or as medians [25^{th}-75^{th} percentile range] for continuous variables and as proportions for categorical variables (%). Variables were tested for normality using the Shapiro-Wilk test. Comparisons were done using Student's t-test, Kruskal–Wallis, and chi-square or Fisher exact test depending on distribution and sample size. One-way, two-way or three-way analysis of variance (ANOVA) were used as appropriate. Correlations were tested by Pearson or Spearman test depending on distribution. Linear models were used for multivariable analyses to adjust for covariates, with $p < 0.05$ considered significant for testing in the final model with main effect and outcome. All statistical analyses were performed using Stata/IC 12.1 software (StataCorp, College Station, TX).

5.3 Results

Population features

Thirty-three subjects enrolled in the 50-Year Joslin Medalist Study were screened for participation in this study and three excluded according to the pre-specified exclusion criteria: two for hematologic diseases (chronic lymphocytic leukemia and lymphoma), and 1 due to hyperparathyroidism, leaving

14 males and 16 females eligible. Population features in the whole population and by CVD are summarized in Table 5.1. For complications, 14 (46.7%) had reported CVD, 18 (60.0%) had diabetic retinopathy, three (10.0%) had nephropathy and 15 (50.0%) had neuropathy. Twenty-three (76.7%) subjects were on lipid lowering agents, 20 (66.7%) on anti-hypertensive medications and 3 (10.0%) on anti-osteoporotic drugs. There were no differences in gender, age, disease duration and anthropometric parameters between subjects with and without CVD. Median [Q1-Q3] HbA1c was 6.9% [6.6-7.3], and similar between subjects with and without CVD (p=0.92). Those with and without CVD status did not have significant differences in eGFR (69.5 [53.0-90.4] vs 92.0 [72.0-96.4] ml/min/1.73m^2, p=0.093). HDL cholesterol levels trended higher (65 [55-85] vs 61 [45-71] mg/dl, p=0.09) while intermediate HDL sub-fractions were significantly higher in those without CVD (29.6 ± 7.1 vs 24.3 ± 4.6 mg/dl, p=0.03). Yet, no significant differences were found in large (32.1 ± 12.5 vs 27.0 ±11.5 mg/dl, p= 0.26) and small HDL sub-particles (8.6 ± 2.6 vs 7.6 ± 1.7 mg/dl, p= 0.20). Total cholesterol, triglycerides, LDL, 25-OH vitamin D, calcium, alkaline phosphatase and hs-CRP levels did not differ between those with and without CVD. Additionally, no differences in these markers were found by sex. A higher level of large particle HDL was found in females relative to males (34.1 ± 11.7 vs 24.8 ± 11.0 mg/dl, p=0.03). Yet, no differences by sex were found in intermediate (28.5 ± 6.6 vs 25.5 ± 6.3 mg/dl, p= 0.22) and small HDL (7.8 ± 2.5 vs 8.6 ± 1.9 mg/dl, p=0.32).

OCN+ monocytes levels differ by CVD and its risk factors.

Subjects without CVD showed significantly lower levels of circulating CD45_bright/CD14+/OCN+ cells than subjects with CVD (13.1 ± 8.4% vs 19.9 ± 6.4%, p=0.02) [**Figure 5.2**]. No significant differences were found in the overall levels of CD45_bright and CD45_bright/CD14+ cells between CVD groups indicating no bias in the overall number of cells [**Figure 5.3**]. Three subjects with overt diabetic nephropathy had reported a history of CVD and a corresponding higher levels of CD45_bright/CD14+/OCN+ cells compared to subjects without nephropathy (27.3 ± 3.1% vs 15.1 ±

7.6%, p=0.03). Additionally, circulating levels of CD45_bright/CD14+/OCN+ were neither associated with proliferative diabetic retinopathy (p=0.31) nor neuropathy (p=0.53).

As the clinical relationship of HDL and CVD was further explored with levels of circulating CD45_bright/CD14+/OCN+, we found these cells were significantly and inversely associated to total HDL cholesterol levels (r= -0.424, p=0.019) [**Figure 5.4A**]. Additionally, similarly to the analysis of CVD, examination of HDL sub-fractions showed that the levels of OCN+ cells were inversely related to the favorable large (r=-0.413, p=0.02) and intermediate (r=-0.445, p=0.01) subfractions, while no significant relationship was found with small subfraction levels [**Figure 5.4B-D**]. Differently from HDL, CD45_bright/CD14+/OCN+ cell levels were not related to total cholesterol, LDL cholesterol and triglycerides. However, the analysis of LDL subfractions showed a trend towards a positive association between small and dense LDL and CD45_bright/CD14+/OCN+ cell levels (r= 0.336, p=0.07).

In parallel with the above, CD45_bright/CD14+/OCN+ cell levels were not related to age, disease duration, glycemic control, renal function, total cholesterol, LDL, triglycerides, calcium, 25-OH Vitamin D, alkaline phosphatase or hs-CRP. The use of lipid lowering agents and anti-hypertensive drugs was also not associated with the levels of CD45_bright/CD14+/OCN+ cells.

OxLDL and HDL action on Osteocalcin expression in monocyte cell lines through SR-B1

To evaluate a possible direct interaction between HDL and the expression of OCN, we studied its expression by HDL and OxLDL in THP-1 cells, a human monocyte cell line. With the addition of 40 µg/ml OxLDL, the number of THP-1 cells expressing osteocalcin, as evaluated by flow cytometry, significantly increased 3 to 10 folds after 12, 24, 48 and 72 hrs. (p<0.001) [**Figure 5.5A-C**].

To determine whether HDL can directly modify the expression of OCN, we incubated THP1 cells with HDL at a starting concentration of 50 µg/ml and titrated up to 400 µg/ml with and without OxLDL [**Figure 5.5B**]. HDL alone had no effects on the number of OCN+/THP1 cells. A 30% reduction in OCN+/THP1 cells after 48 hours exposure to 50 and 100 µg/ml HDL, 40% reduction

with 200 μg/ml HDL and approximately 70% reduction with 400 μg/ml ($p<0.001$) were observed. [**Figure 5.5B**]. Time course and dose-dependent studies showed the effect increased with incubation time and dose of HDL [**Figure 5.5A-B**]. Henceforth, stimulation experiments were performed by incubating cells for 48 hrs. with 200 μg/ml of HDL, a condition which mimicked human physiology.[130]

To evaluate whether non-oxidized LDL may affect OCN expression, THP1 cells were incubated for 48 hours with 40 μg/ml LDL with and without OxLDL and HDL [**Figure 5.5C**]. Differently from OxLDL, non-oxidized LDL did not change the expression of OCN and did not interact with HDL.

To better characterize the effect of HDL on OCN+ cells, the effect of SR-B1 inhibition was studied. As shown in **Figure 5.6**, the number of OCN+ cells was increased by incubation with OxLDL by 9 (±2) fold and were reduced by 50.1% (± 9.3%) in the presence of HDL. The addition of SR-B1 Ab at 1:800 and 1:500 decreased the effect of OxLDL by 29.8% (± 9.9%) and 39.2% (± 15.7%), which was similar to HDL above. Presence of Ab to SR-B1 did not have consistent effect on the protein levels of OCN+ cells. Similar results were obtained using BLT-1, a small molecule inhibitor of HDL's actions via HDL receptor SR-B1 [**Figure 5.6**].

The increases in OCN expression on circulating monocytes induced by OxLDL appears to be at the protein level since *Ocn* mRNA levels were not changed by exposure to OxLDL, LDL or HDL [**Figure 5.7A**]. However, total protein levels of OCN in monocytes were significantly increased by OxLDL and returned to baseline by the addition of HDL, while LDL alone did not affect OCN protein in monocytes [**Figure 5.7C**]. Consistent with the absence of changes in gene expression, no significant differences in the expression of the Run-related transcription factor 2 (Runx2), a major regulator of the OCN gene, was observed both at the gene and protein levels [**Figure 5.7B-D**]. Experiments were repeated using another monocyte cell line (U937) confirming those obtained with THP-1 cells.

5.4 Conclusions

In this study a reduced number of OCN+ monocytes was associated with reduced prevalence of cardiovascular disease and higher HDL-c levels. In addition, data from cellular models support the clinical finding HDL significantly reduces the number of monocytes expressing OCN due to OxLDL by interacting with SR-B1, a receptor already known for interacting with HDL to effect cholesterol efflux.[131] Overall, this suggests a potential link between increased HDL-c and lowering the risk of cardiovascular disease by decreasing the expression of OCN in monocytes.

Eghbali-Fatourechi et al. reported circulating osteoblast progenitors as mononuclear circulating OCN^+ cells are able to form mineralized nodules when cultured in osteoblast-differentiating medium.[124] Subsequently other investigators have confirmed that circulating mesenchymal osteoprogenitors ability to cause ectopic vascular calcification.[132–135] Monocytes have recently been described as a source of mesenchymal progenitors which can differentiate into osteoblast-like cells[119,136] contributing to atherosclerotic calcification,[125] and some reports have suggested circulating myeloid cells with osteogenic potential may affect CVD in the general population.[120,121] Our studies extend the association of OCN+ monocytes and CVD in T1D which while having similarities with T2D also differs in that individuals with T1D develop CVD often without the typical insulin resistance hallmarks seen in T2D.[8]

OCN is the most abundant non-collagenous bone matrix protein, with several functions beyond skeletal health. In particular, circulating levels of serum osteocalcin have been associated with both glucose metabolism [61,137,138] and cardiovascular disease,[139,140] but with contrasting results. As a bone-related protein, OCN is mainly produced and secreted by osteoblasts. However, different cell types involved in CVD express OCN, such as platelets, monocytes and endothelial progenitor cells.[119,141] The presence in the bloodstream of circulating cells with an osteogenic phenotype linked to CVD supports the existence of a bone-vascular axis we have recently described also in the Medalists.[63] Therefore, further studies should be designed to clarify whether an association exists between markers of bone health (bone-turnover markers, bone mineral density, etc.) and circulating osteogenic cells.

Here we propose a mechanism which stimulates the pathological expression of OCN in circulating monocytes and a means to reverse it, with support from *in vitro* experiments. Accumulated OxLDL in the arterial walls may lead to vascular calcification by inducing the trans-differentiation of vascular smooth muscle cells into calcifying cells through the upregulation of Runx2, which is a major regulator of osteoblast differentiation.[126,142] Our data show OxLDL promotes the pro-calcific phenotypic drift of monocytes as well, demonstrated by an increased number of cells expressing OCN on the cell surface. However, this does not seem related to genetic regulation as neither changes in *Ocn* mRNA levels nor in the expression of Runx2, the main transcription factor regulating *Ocn* were seen; thus suggesting other mechanisms causing a change in OCN protein levels and surface expression not accompanied by gene expression change. One possibility is protein recycling which has been associated with HDL action on monocytes.[131,143] Alternatively, the changes observed in protein levels by immunoblot could also be due to post-translational processing of OCN regulated by HDL and OxLDL. Further studies should be performed to clarify this issue.

Consistently with the absence of relationships between LDL cholesterol levels and CD45_bright/CD14+/OCN+ cell levels in the Medalists, *in vitro* studies also showed non-oxidized LDL have no effect on OCN expression. However, we describe a trend towards a positive association between CD45_bright/CD14+/OCN+ circulating cell levels and small and dense LDL, but not with large LDL. This is consistent with the *in vitro* study as small and dense LDL represent the LDL fractions more prone to oxidation with higher atherogenic properties than LDL particles with higher size and lower density.[144,145]

Other mechanisms we did not investigate here may lead to the induction of osteogenic drift of monocytes and should be searched in future studies. Overall, it is evident that different stimuli such as hyperglycemia, dyslipidemia, chronic inflammation and hypoxia,[118–120] all of which are pathologically elevated in people affected by diabetes, combine leading to the pro-calcific milieu contributing to the high prevalence of vascular calcification observed in people with diabetes.

Importantly, our data show both *in vivo* and *in vitro,* HDL is a relevant factor which can counteract

the increased expression of OCN. The inverse relationship between OCN+ monocytes and HDL-c suggests a potential new mechanism for HDL to lower the risk for CVD. In particular the larger and intermediate sub-particles of HDL, which facilitate cholesterol efflux and are associated with lower cardiovascular risk,[146–148] are correlated with lower levels of OCN+ cells. This supports our previous epidemiologic work demonstrating protection from CVD in the 50-Year Medalist is associated with HDL-c levels.[100] This is consistent with several other studies reporting an inverse relationship between HDL cholesterol levels and CVD in those with type 2 diabetes and those with the disease.[149,150] However, interventional trials aimed at increasing HDL-c levels have not shown success in decreasing cardiovascular events,[151] highlighting the importance of a greater mechanistic understanding of HDL-mediated cardiovascular protection. The interaction between HDL and monocytes/macrophages has always been considered a key for the maintenance of healthy vessels. Previously, this relationship was confined to HDL's effect on cholesterol efflux and anti-inflammatory actions, preventing foam cell formation and inflammation.[152] Based on our results, we propose a new mechanism by which HDL and monocytes interact to protect from cardiovascular disease by counteracting monocytes differentiation into pro-calcific cells.

The surface receptors ATP-binding cassette A1 and C1 and SR-B1 are the main molecules mediating the action of HDL on monocytes/macrophages. Of these, SR-B1 appears to contribute both to the mechanisms of cholesterol diffusion and retro-endocytosis.[131] Our data suggests SR-B1 is also a key receptor in the regulation of OCN expression by both OxLDL and HDL, providing a potential target to reduce vascular stiffness and/or increase plaque stability.

A bias of the study may be that it was done in an older population with T1D who have a median age of 65 years. However, this is an advantage as it is a population who have reached their end-phenotype- those who will or will not develop significant CVD . The extreme phenotype provides more power with a smaller sample size. This provides added value as current knowledge about CVD in T1D is from studies conducted in the previous era of less intensive glycemic control, in those of lesser duration or extrapolated from studies in T2D.[8] Moreover, the changing epidemiology of T1D,

characterized by increased longevity and longer disease duration without complications increases the need for understanding of what makes survival possible, particularly for CVD, the largest cause of mortality among this group.[153] Yet, these findings were confirmed in via *in vitro* experiments performed on two different types of monocyte cell lines (THP-1 and U937) and not on primary monocytes from this selected population. THP-1 and U937 cells have been widely used to investigate monocytes/macrophages pathophysiology in the cardiovascular system and it has been shown that these cell lines have features of primary monocytes derived from control human donors.[154] The absence of a control group of healthy subjects without T1D may also limit our study. However, previous studies have already widely shown that non-diabetic people have lower levels of OCN+ monocytes.[118,120,121] While the main mechanism of vascular damage associated to OCN+ monocytes should be ectopic calcification in the vessel wall, we acknowledge that the lack of direct measurements of vascular calcification in the Medalist cohort should be considered as a limit of this study. However, this was not the primary aim of our study as previous studies already investigated the contribution of OCN+ monocytes to vascular calcification[124,125] and it has been widely demonstrated that vascular calcification is among the main pathological findings in T1D with CVD.[8] In conclusion, this study supports an association between CVD protection and lower levels of circulating osteogenic cells of myeloid origin in long duration T1D, along with higher HDL-c levels, particularly those of larger sub-particle size. Our data suggest a mechanism for the increased OCN+ monocytes due to oxidized lipids found in diabetes, and that this may be mitigated by HDL. These findings indicate that circulating OCN+ monocytes may be a marker for vascular disease in diabetic patients and may be modified by HDL elevation. Results regarding the regulation of OCN expression on monocytes by OxLDL and HDL through SR-B1 and its relationship with CVD in T1D provide new information on vascular pathophysiology. Indeed, these findings may provide new insights on the mechanism of HDL-mediated cardiovascular protection and promote advances in therapeutic strategies.

5.5 Tables

Table 5.1. Population features. Values are median [Q1-Q3] for continuous variables and numbers for categorical variables. Abbreviations used: CVD, Cardiovascular disease; BMI, Body Mass Index. eGFR, estimated glomerular filtration rate; HDL-c, High Density Lipoprotein cholesterol; LDL-c, Low Density Lipoprotein cholesterol; hsCRP, high sensitivity C-Reactive Protein

	Overall n=30	**No CVD n=16**	**CVD n=14**	**P value between CVD status**
Gender, males/females	14/16	6/10	8 / 6	0.210
Age, years	64 [59-71]	62 [59-68]	68 [64-73]	0.257
Disease duration, years	56 [51-62]	55 [51-60]	56 [52-64]	0.333
BMI, Kg/m^2	26.1 [22.9-28.9]	26.3 [22.4-28.1]	26.1 [23.9-30.1]	0.863
Waist to hip ratio	0.9 [0.8-0.9]	0.9 [0.8-0.9]	0.9 [0.8-0.9]	1.000
Insulin dose, IU/Kg	0.45 [0.38-0.58]	0.46 [0.41-0.58]	0.44 [0.36-0.54]	0.830
eGFR, ml/min/1.73m^2	86.8 [60.0-95.6]	92.0 [72.0-96.4]	69.5 [53.0-90.4]	0.093
HbA1c, %	6.9 [6.6-7.3]	6.9 [6.3-7.4]	6.9 [6.6-7.2]	0.922
Total cholesterol, mg/dl	152 [140-167]	160 [140-176]	146 [123-157]	0.275
Triglycerides, mg/dl	63 [46-80]	60 [45-75]	65 [50-86]	0.236
HDL-c, mg/dl	64 [52-76]	65 [55-85]	61 [45-71]	0.091
LDL-c, mg/dl	68 [61-86]	69 [63-90]	67 [60-85]	0.901
Triglycerides/HDL ratio	1.00 [0.64-1.34]	0.80 [0.56-1.33]	1.15 [0.72-1.43]	0.155
Correct calcium, mg/dl	9.0 [8.8-9.2]	9.0 [8.8-9.3]	9.0 [8.8-9.1]	0.956
25-OH Vitamin D, ng/ml	40 [31-44]	40 [29-46]	40 [32-44]	0.906
Alkaline Phosphatase, U/L	62 [46-81]	59 [52-83]	66 [45-78]	0.921
hsCRP, mg/dl	0.14 [0.10-0.26]	0.13 [0.10-0.37]	0.15 [0.10-0.24]	0.630

5.6 Figures

Figure 5.1 Gating strategy for the identification and quantification of circulating OCN+ monocytes by flow-cytometry. Details in the main text. FMO: Fluorescence Minus One

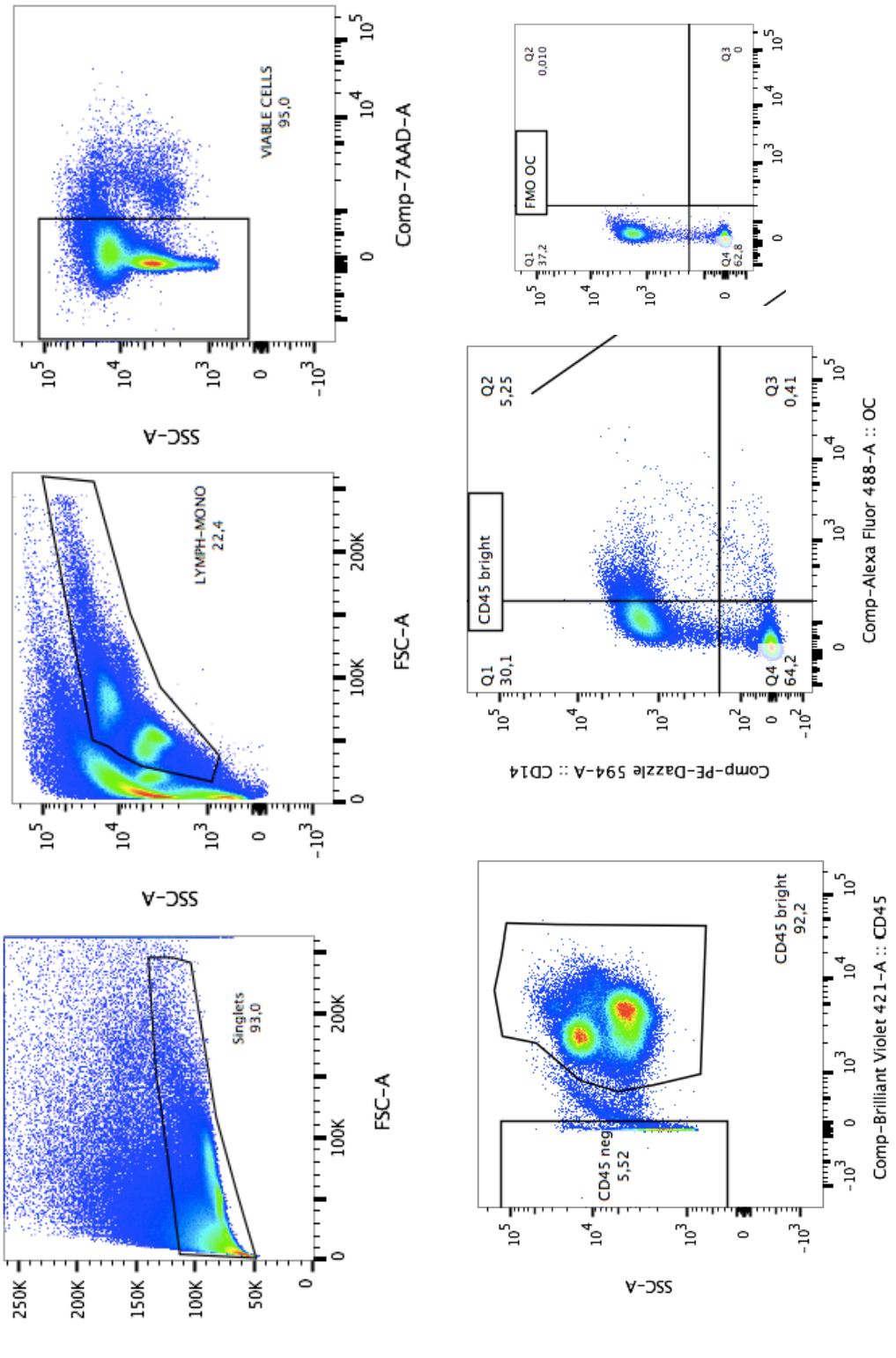


Figure 5.2. OCN+ monocytes by presence of cardiovascular disease. OCN+ monocytes are expressed as percentage of CD45_bright PBMCs. Subjects without history of CVD showed lower levels of circulating OCN+ monocytes.

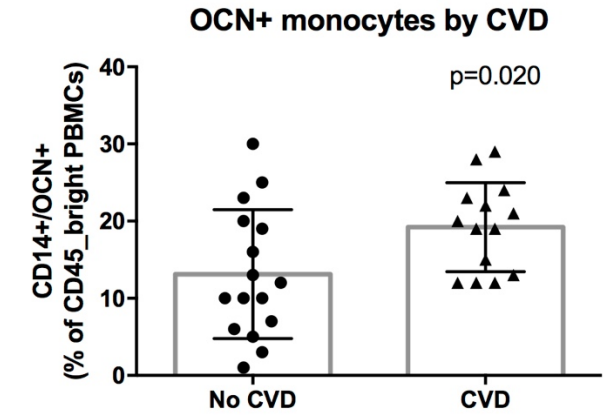


Figure 5.3 CD45_bright and CD45_bright/CD14+ PBMCs by presence of cardiovascular disease. No differences were found between Medalists with and without history of cardiovascular disease

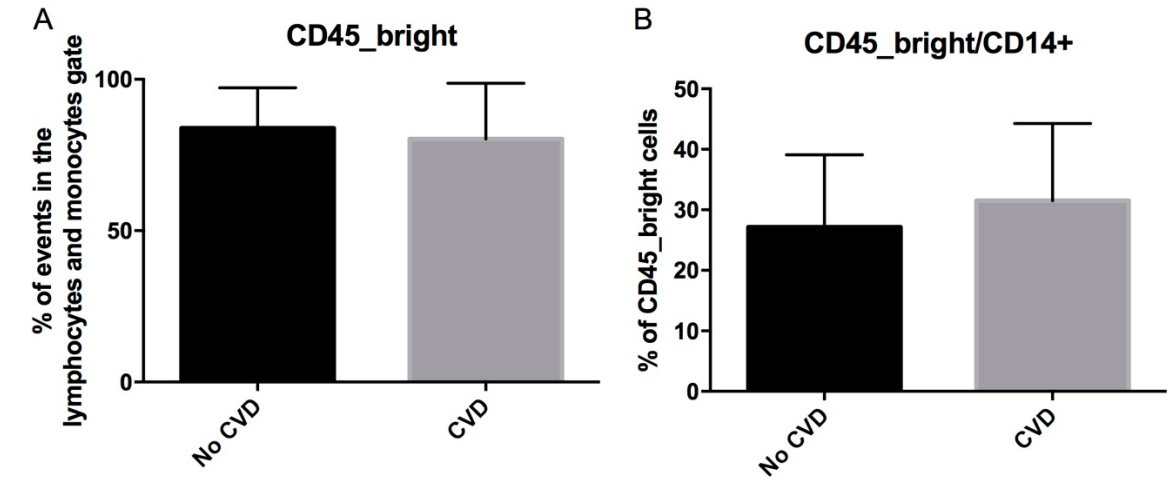
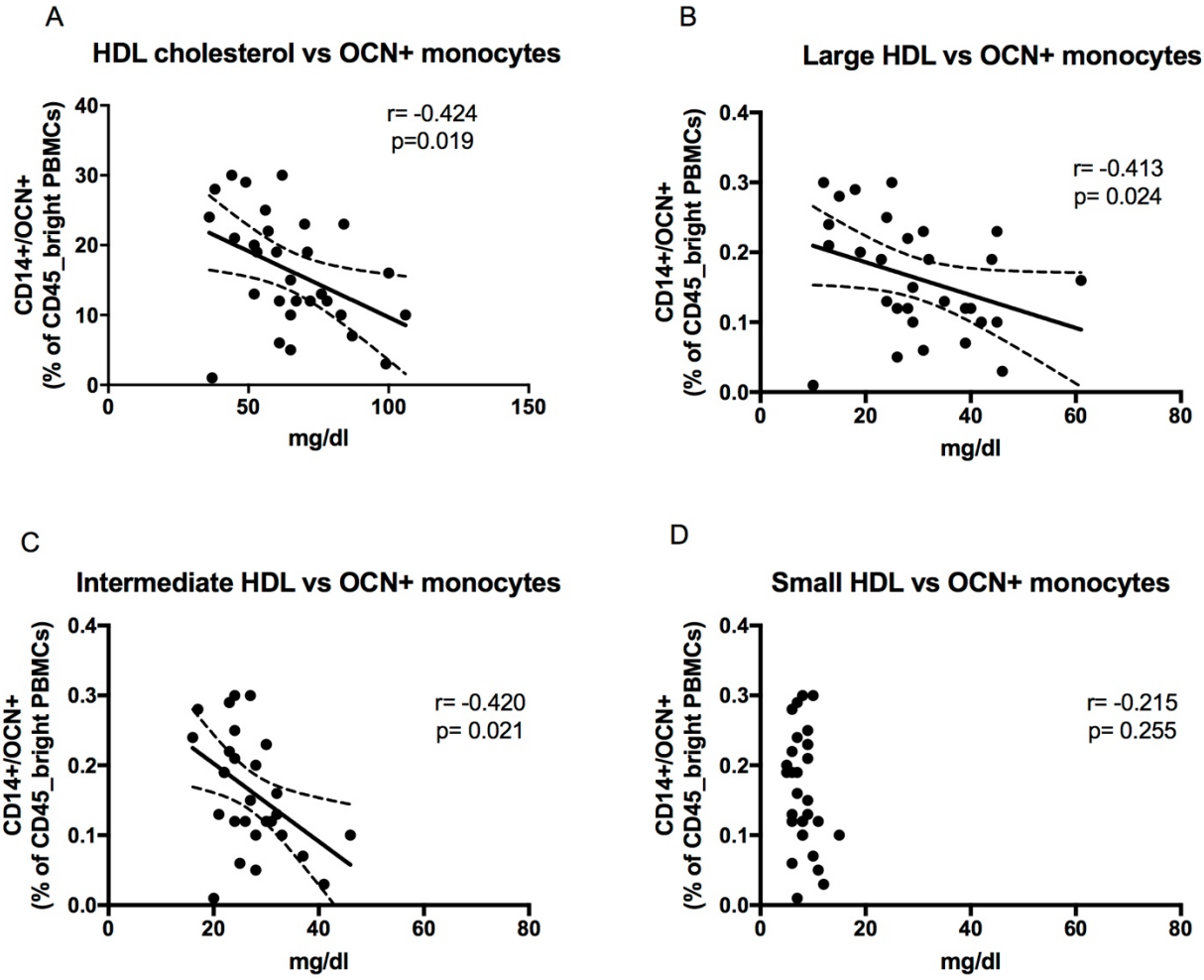


Figure 5.4. Correlation between HDL cholesterol and HDL sub-fractions with OCN+ monocytes. OCN+ monocytes are expressed as percentage of CD45_bright PBMCs. Total, large and intermediate, but not small HDL were inversely related to OCN+ monocytes



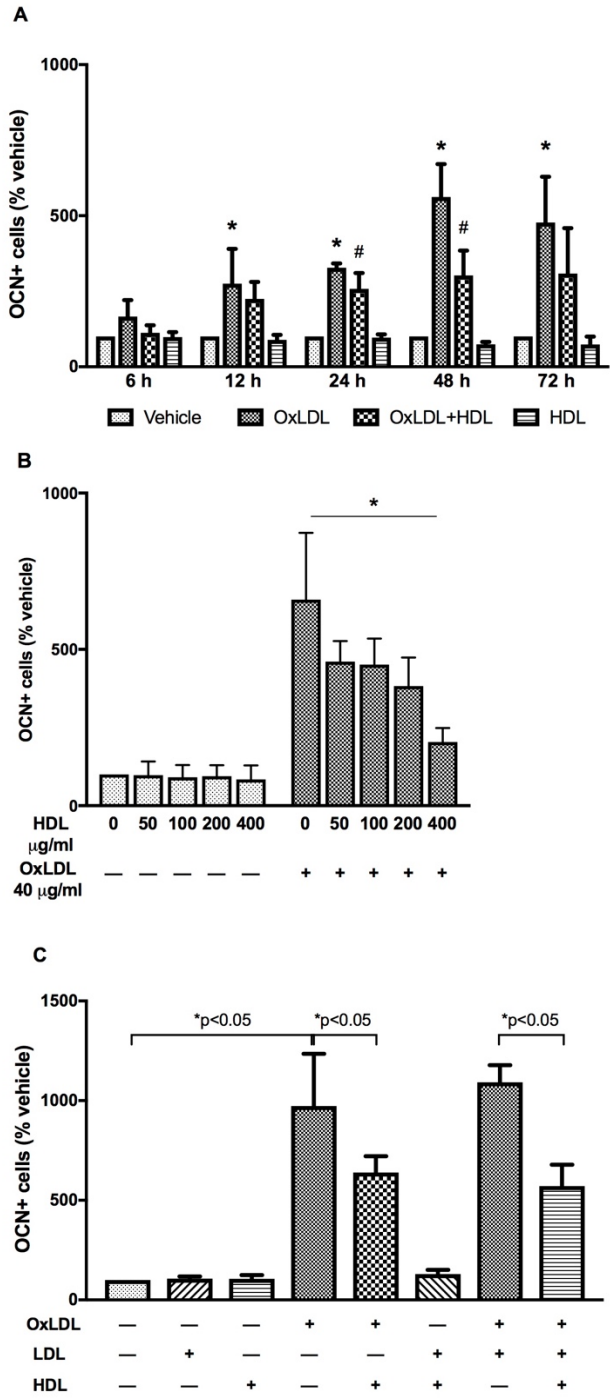


Figure 5.5. Effect of OxLDL, LDL and HDL on OCN surface expression in THP-1 cells.

A. OCN+ THP1 cells were measured by flow cytometry after treatment with 40 µg/ml OxLDL ± 200 µg/ml HDL at different time points (n=4 in each group). Three-way ANOVA for multiple comparisons: $p<0.001$. Kruskal-Wallis test for pairwise comparison: *$p<0.05$ vs untreated; $^{\#}p<0.05$ vs OxLDL treated.

B. OCN+ THP1 cells were measured by flow cytometry after treatment with 40 µg/ml OxLDL and different concentrations of HDL for 48 hours (n=5 in each group). Two-way ANOVA for multiple comparisons: *$p<0.001$.

C. OCN+ THP1 cells were measured by flow cytometry after 48 hours treatment with 40 µg/ml OxLDL ± 200 µg/ml HDL ± 40µg/ml LDL (n=3 in each group). Three-way ANOVA for multiple comparison: $p<0.001$. *p-values at the Kruskal-Wallis test for pairwise comparisons.

Figure 5.6. Inhibition of the scavenger receptor B1

Treatment of THP-1 cells with inhibitors of SR-B1 - SRB1-Ab (A) and BLT1 (B) - mitigates the effects of both OxLDL and of HDL in terms of OCN+ cell as evaluated by flow cytometry. Three-way ANOVA: $p<0.001$. *p-values at the Kruskal-Wallis test for pairwise comparisons.

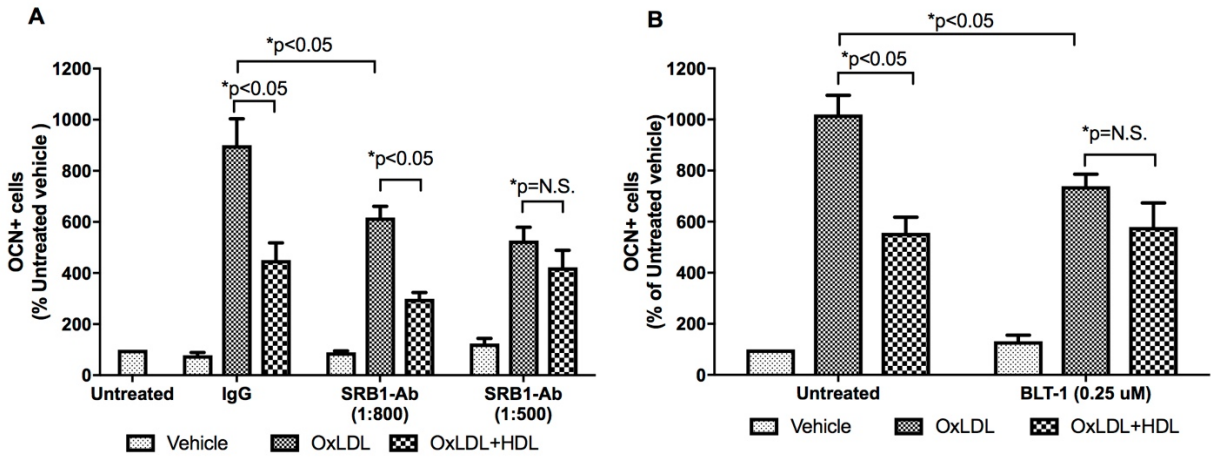
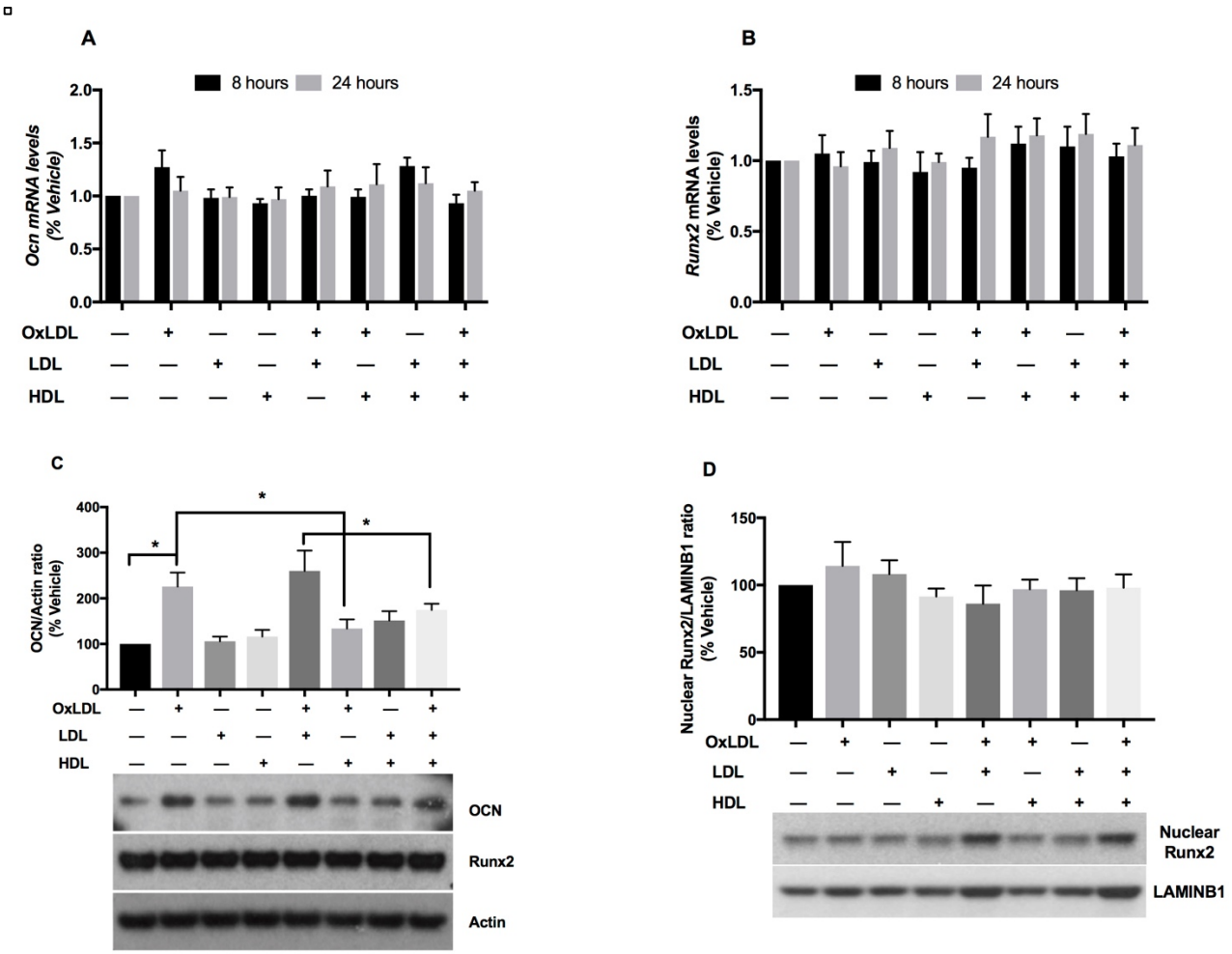


Figure 5.7 Effect of OxLDL, LDL and HDL on OCN and Runx2 gene and protein expression in THP1 cells. mRNA and protein levels were quantified after treatment with 40 µg/ml OxLDL ± 200 µg/ml HDL ± 40µg/ml LDL for 8 and 24 hours (RT-PCR) or for 48 hours (immunoblot).

OCN (A) and Runx2 (B) mRNA levels (n= 3 for each group) did not change after 8 or 24 hours incubation with OxLDL, LDL or HDL. Three-way ANOVA for multiple comparison: $p > 0.05$.

C. A representative immunoblot is shown for both OCN and Runx2 proteins. The bar graph shows the quantification of the immunoblot for OCN (n=3 in each group). OCN protein level significantly increased after incubation with OxLDL alone, but not after incubation with OxLDL+HDL. LDL did not affect OCN levels. Three-way ANOVA for multiple comparison: $p<0.001$. *$p<0.05$ at the Kruskal-Wallis test for pairwise comparisons. Quantification of Runx2 expression did not show significant changes between groups (data not shown).

D. Nuclear Runx2 protein levels did not change after incubation with OxLDL, HDL or LDL (n=3 in each group). Three-way ANOVA for multiple comparison: $p > 0.05$.



List of full publications during the three-year Ph.D. course

I. **E. Maddaloni,** N Lessan, A. Al Tikriti, R. Buzzetti, P.Pozzilli, MT Barakat. "Latent Autoimmune Diabetes in Adults in the United Arab Emirates: Clinical Features and Factors Related to Insulin-Requirement." Plos One. Vol. 10, no 8, pp: e0131837, eCollection 2015. PMID: 26252955.

II. **E. Maddaloni**, L. D'Onofrio, P. Pozzilli. "Frailty and Geography: should these two factors be added to the ABCDE contemporary guide to diabetes therapy?". Diabetes Metab Res Rev. Vol. 32, no 2, pp 169-75. Feb 2016. doi: 10.1002/dmrr.2762. PMID: 26484614.

III. K. Park, A. Mima, Q. Li, C. Rask-Madsen, P. He, K. Mizutani, S. Katagiri, Y. Maeda, IH. Wu, M. Khamaisi, S.R. Preil, **E. Maddaloni**, D. Sørensen, L.M. Rasmussen, P.L. Huang, G.L. King. "Insulin decreases atherosclerosis by inducing endothelin receptor B expression". JCI Insight. Vol 1, no. 6, pii: e86574. May 2016. PMID: 27200419.

IV. **Maddaloni E,** Pozzilli P. "Why China guidelines for type 2 diabetes represent an opportunity for treating this disease". Diabetes Metab Res Rev Vol. 32, no. 5, pp: 438-439. Jul 2016. doi: 10.1002/dmrr.2825. PMID: 27464263.

V. **Maddaloni E**, Cavallari I, De Pascalis M, Keenan H, Park K, Manfrini S, Buzzetti R, Patti G, Di Sciascio G, Pozzilli P. "Relation of Body Circumferences to Cardiometabolic Disease in Overweight-Obese subjects". Am J Cardiol. Vol. 118, no. 6, pp: 822-827. Jun 2016 doi: 10.1016/j.amjcard.2016.06.044. PMID: 27457430.

VI. **Maddaloni E**, Sabatino F. In vivo corneal confocal microscopy in diabetes: Where we are and where we can get. World J Diabetes. Vol. 7, no.17 pp: 406-41. Sep 2016. doi: 10.4239/wjd.v7.i17.406. PMID: 27660697.

VII. Keenan HA, **Maddaloni E**. Bone Microarchitecture in Type 1 Diabetes: It Is Complicated. Curr Osteoporos Rep. Vol. 14, no. 6, pp: 351-358, December 2016. PMID: 2770439.

VIII. Soare A, Khazrai MY, Fontana L, Del Toro R, Lazzaro MC, Di Rosa C, Buldo A, Fioriti E, **Maddaloni E**, Angeletti S, Di Mauro A, Gesuita R, Skrami E, Tuccinardi D, Fallucca S, Pianesi M, Pozzilli P. Treatment of reactive hypoglycemia with the macrobiotic Ma-pi 2 diet as assessed by continuous glucose monitoring: The MAHYP randomized crossover trial. Metabolism. Vol. 69, pp: 148-156. Apr 2017. doi: 10.1016/j.metabol.2017.01.023, PMID 28285645.

IX. **Maddaloni E**, D'Eon S, Hastings S, Tinsley LJ, Napoli N, Khamaisi M, Bouxsein ML, Fouda SMR, Keenan HA. Bone health in subjects with type 1 diabetes for more than 50 years. Acta Diabetol. Vol. 54, no. 5, pp: 479-488. May 2017 doi: 10.1007/s00592-017-0973-2; PMID: 28236093

X. Buzzetti R, Zampetti S, **Maddaloni E.** Adult-onset autoimmune diabetes: current knowledge and implication for management. Nat Rev Endocrinol. Vol. 13, no 11, pp: 674-686. Nov 2017 Doi: 10.1038/nrendo.2017.99; PMID: 28885622.

XI. **Maddaloni E,** Pastore G, Del Buono MG, Porcari A, Fittipaldi M, Garilli F, Tiberti C, Angeletti S, Pozzilli P, Mottini G, Napoli N. High prevalence of autoimmune diabetes and poor glycaemic control among adults in Madagascar: a brief report from a humanitarian health

campaign in Ambanja. J Diabetes Res. 2017; 3860674. Epub Sep 2017. doi: 10.1155/2017/3860674

XII. **Maddaloni E,** Xia Y, Park K, D'Eon S, Tinsley LJ, St-Louis R, Khamaisi M, LI Q, King GL, Keenan HA. High Density Lipoprotein modulates osteocalcin expression in circulating monocytes: a potential protective mechanism for cardiovascular disease in type 1 diabetes. Cardiovasc Diabetol. Vol. 16, no. 1 pp:116. Sep 2017. doi: 10.1186/s12933-017-0599-2. PMID 28915881.

XIII. **Maddaloni E**, Pozzilli P. Diabetes: the disease of the 10D. Endocrine. Oct 2017. Epub ahead of print. PMID: 29075975.

XIV. Park K, Li Q, Evcimen ND, Rask-Madsen C, Maeda Y, **Maddaloni E**, Yokomizo H, Shinjo T, St-Louis R, Fu J, Gordin D, Khamaisi M, Pober D, Keenan H, King GL. Exogenous Insulin Infusion Can Decrease Atherosclerosis in Diabetic Rodents by Improving Lipids, Inflammation, and Endothelial Function. Arterioscler Thromb Vasc Biol. Vol. 38, no.1, pp: 92-101, Jan 2018. doi: 10.1161/ATVBAHA.117.310291. PMID: 29162603

XV. **Maddaloni E,** Cavallari I, Napoli N, Conte C. Vitamin D and Diabetes Mellitus. Front Horm Res. vol. 50, pp: 161-176. Mar 2018 doi: 10.1159/000486083. PMID: 29597238.

XVI. Lauria A, **Maddaloni E (first author equal contribution),** Briganti SI, Beretta Anguissola G, Perrella E, Taffon C, Palermo A, Pozzilli P, Manfrini S, Crescenzi A. Differences between ATA, AACE/ACE/AME and ACR TI-RADS ultrasound classifications performance in identifying cytological high-risk thyroid nodules. Eur J Endocrinol. Vol. 178, no. 6, pp: 595-603. Jun 2018.doi: 10.1530/EJE-18-0083. PMID: 29626008.

XVII. **Maddaloni E**, Buzzetti R. Why only macro and not micro in type 2 diabetes? Time to change the goals of clinical trials in diabetes. Diabetes Metab Res Rev. Vol. 34, no. 6, pp: e3012. Sep 2018. doi: 10.1002/dmrr.3012. PMID: 29673094

XVIII. Pieralice S, Vigevano F, Del Toro R, Napoli N, **Maddaloni E.** Lifestyle Management of Diabetes: Implications for the Bone-Vascular Axis. Curr Diab Rep. Vol. 18, no. 10, pp: 84. Aug 2018. doi: 10.1007/s11892-018-1060-y. PMID: 30121859

XIX. Patti G, Cavallari I, Andreotti F, Calabrò P, Cirillo P, Denas G, Galli M, Golia E, **Maddaloni E,** Marcucci R, Parato VM, Pengo V, Prisco D, Ricottini E, Renda G, Santilli F, Simeone P, De Caterina R; Working Group on Thrombosis of the Italian Society of Cardiology. Prevention of atherothrombotic events in patients with diabetes mellitus: from antithrombotic therapies to new-generation glucose-lowering drugs. Nat Rev Cardiol. Vol 16, no. 2, pp: 113-130. Feb 2019. doi: 10.1038/s41569-018-0080-2. [Epub ahead of print]. PMID: 30250166

XX. **Maddaloni E**. New strategy to study the impact of ethnicity on diabetic neuropathy. Diabetes Metab Res Rev. Vol. 35, no. 1, pp: e3080. Jan 2019. doi: 10.1002/dmrr.3080. PMID: 30261130.

XXI. Cavallari I, Delli Veneri A, **Maddaloni E**, Melfi R, Patti G, Napoli N, Pozzilli P, Di Sciascio G. Comparison of Lipid-Lowering Medications and Risk for Cardiovascular Disease in Diabetes. Curr Diab Rep Vol. 18, n. 12, pp: 138. Oct 2018. doi: 10.1007/s11892-018-1117-y. PMID: 30370486

XXII. Cavallari I, **Maddaloni E**. Cardiovascular effects of SGLT-2 inhibitors: What we have learned from cardiovascular outcome trials and what we still need to understand. Diabetes Metab Res Rev. 2019 Jan 3:e3124 [Epub ahead of print] doi: 0.1002/dmrr.3124; PMID: 30604504

Abstracts as presenting author at national and international meetings

I. **Maddaloni E**, Keenan HA, Differential changes in bone geometry and microarchitecture in extreme duration type 1 diabetes compared to younger type 1's and controls. ASBMR 2015 Annual meeting. October 9-12 2015, Seattle, Washington, USA. – Poster Presentation

II. **Maddaloni E**, Tinsley LJ, D'Eon S, Bouxsein ML, Napoli N, King GL, Keenan HA. Age and LDL cholesterol differentially affect bone quality in subjects with extreme duration type 1 diabetes. ENDO2016, The Endocrine Society 98th Annual Meeting and Expo, April 1-4 2016, Boston, MA, USA – Poster and Poster Preview Presentation

III. **Maddaloni E**, Napoli N, Pozzilli P, King GL, Keenan HA. Fragilità e qualità dell'osso in soggetti anziani con >50 anni di diabete di tipo 1. 26th Congresso Nazionale SID (Società Italiana di Diabetologia), 4-7 Maggio 2016, Rimini, Italy. – Oral Presentation

IV. **Maddaloni E**, Park K, D'Eon S, Xia Y, Khamaisi M, King GL, Keenan HA. High density lipoprotein modulates osteocalcin expression in circulating monocytes: a potential marker of CVD in type 1 diabetes. 76th Scientific Sessions of the American Diabetes Association (ADA), June 10-14 2016, New Orleans, LA, USA. Oral Presentation

V. **Maddaloni E**, Bouxsein ML, Tinsley LJ, D'Eon S, Napoli N, King GL, Keenan HA. Protection from trabecular bone loss in elderly subjects with extreme duration type 1 diabetes. 76th Scientific Sessions of the American Diabetes Association (ADA), June 10-14 2016, New Orleans, LA, USA. Oral Presentation

VI. **Maddaloni E,** Del Toro R, Fioriti E, Tabacco G, Defeudis G, Caggiano C, Manfrini S, Pozzilli P. Cardiac autonomic neuropathy is efficiently diagnosed by Valsalva maneuver alone. 77th Scientific Sessions of the American Diabetes Association (ADA), June 9-13 2017, San Diego, CA, USA. Poster Presentation.

VII. **Maddaloni E**, Strollo R, Del Toro R, Pozzilli P, Napoli N. Effect of calcidiol on insulin resistance and beta cell function in subjects with pre-diabetes. 53rd EASD Annual Meeting, Sep 11-15 2017, Lisbon, Portugal.

VIII. **Maddaloni E**, Maurizi AR, Minutolo A, Fioriti E, Del Toro R, Naciu AM, Pieralice S, Rosati MM, Manfrini S, Pozzilli P. Palmitoylthanolamide in add-on to alpha ipoic acid for control of symptoms of diabetic peripheral neuropathy. 78th Scientific Sessions of the American Diabetes Association (ADA), June 22-26 2018, Orlando, FL, USA. Poster Presentation.

IX. **Maddaloni E,** Coleman RL, Pozzilli P, Holman RR. Risk of cardiovascular disease in individuals with latent autoimmune diabetes of adults: results from the UKPDS. 54th EASD Annual Meeting, Oct 1-5 2018, Berlin, Germany.

Acknowledgments

Firstly, I would like to express my sincere gratitude to my mentor Prof. Paolo Pozzilli, for the continuous support of my education, career and related research, for his enthusiasm, motivation, and knowledge.

My gratitude also goes to the co-supervisor of this research project, Prof. Raffaella Buzzetti. She generously gave her time to offer me valuable comments toward improving my work.

Their guidance helped me in all the time of research. I could not have imagined having better advisors and mentors.

My sincere thanks to all the following collaborators contributing to this research project:

- Prof. George L King and Prof Hillary A Keenan, who supervised my research fellowship at the Laboratory of Vascular Cell Biology of the Joslin Diabetes Center (Harvard Medical School); all my lab-mates and to the staff of the core facilities at the JDC. There is no way to express how much it meant to me to have been a member of the lab. A special mention to Min Park, for his friendship and for teaching the fundaments of lab research.
- Prof. Rury R Holman, for welcoming me at the Diabetes Trial Unit, University of Oxford, as a Mentee of the European Foundation for the Study of Diabetes. His mentorship has contributed to my professional growth in the field clinical research in diabetes. Sincere thanks to all the DTU staff for supporting my research activities in Oxford.
- Prof. Nicola Napoli, a friendly and thoughtful guide for my career; the clinical staff of the Endocrinology and Diabetes Unit of the University Hospital Campus Bio-Medico, Rome; my lab-mates in the laboratory of Endocrinology and Diabetes at Campus Bio-Medico University of Rome. A special mention to Dr Luciana Valente, for her unfailing support and assistance in the lab.

I'm grateful to all the organizations supporting my work with research grants: the "Campus Bio-Medico Alumni Associations", the "European Foundation for the Study of Diabetes", the "National Institute of Diabetes and Digestive and Kidney Diseases".

Finally, I would like to thank my family. My parents and my brother, for their unrestricted support, because I owe all to them. My beloved wife: muse, greatest fan and my reason to go beyond my limits.

References

1. Maddaloni E, Pozzilli P. Tipo 1 e LADA in crescita: prevenzione e assistenza ottimale. In: Bonora E, Sesti G, editors. Il diabete in Italia. Bononia University Press; 2016. p. 157–63.
2. Sun JK, Keenan HA, Cavallerano JD, et al. Protection from retinopathy and other complications in patients with type 1 diabetes of extreme duration: the joslin 50-year medalist study. Diabetes Care 2011;34(4):968–74.
3. Maddaloni E, Lessan N, Al Tikriti A, Buzzetti R, Pozzilli P, Barakat MT. Latent Autoimmune Diabetes in Adults in the United Arab Emirates: Clinical Features and Factors Related to Insulin-Requirement. PLoS One 2015;10(8):e0131837.
4. Buzzetti R, Di Pietro S, Giaccari A, et al. High titer of autoantibodies to GAD identifies a specific phenotype of adult-onset autoimmune diabetes. Diabetes Care 2007;30(4):932–8.
5. Cousminer DL, Ahlqvist E, Mishra R, et al. First Genome-Wide Association Study of Latent Autoimmune Diabetes in Adults Reveals Novel Insights Linking Immune and Metabolic Diabetes. Diabetes Care 2018;41(11):2396–403.
6. Fourlanos S, Perry C, Stein MS, Stankovich J, Harrison LC, Colman PG. A clinical screening tool identifies autoimmune diabetes in adults. Diabetes Care 2006;
7. Buzzetti R, Zampetti S, Maddaloni E. Adult-onset autoimmune diabetes: current knowledge and implications for management. Nat Rev Endocrinol 2017;13(11):674–86.
8. de Ferranti SD, de Boer IH, Fonseca V, et al. Type 1 Diabetes Mellitus and Cardiovascular Disease: A Scientific Statement From the American Heart Association and American Diabetes Association. Diabetes Care 2014;37(10):2843–63.
9. Secrest AM, Becker DJ, Kelsey SF, Laporte RE, Orchard TJ. Cause-specific mortality trends in a large population-based cohort with long-standing childhood-onset type 1 diabetes. Diabetes 2010;59(12):3216–22.
10. Libby P, Nathan DM, Abraham K, et al. Report of the National Heart, Lung, and Blood Institute-National Institute of Diabetes and Digestive and Kidney Diseases Working Group on Cardiovascular Complications of Type 1 Diabetes Mellitus. Circulation 2005;111(25):3489–93.

11. Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM. High risk of cardiovascular disease in patients with type 1 diabetes in the U.K.: a cohort study using the general practice research database. Diabetes Care 2006;29(4):798–804.

12. Huxley RR, Peters SAE, Mishra GD, Woodward M. Risk of all-cause mortality and vascular events in women versus men with type 1 diabetes: a systematic review and meta-analysis. lancet Diabetes Endocrinol 2015;3(3):198–206.

13. Lind M, Svensson A-M, Kosiborod M, et al. Glycemic control and excess mortality in type 1 diabetes. N Engl J Med 2014;371(21):1972–82.

14. Rawshani A, Rawshani A, Franzén S, et al. Range of Risk Factor Levels: Control, Mortality, and Cardiovascular Outcomes in Type 1 Diabetes Mellitus. Circulation 2017;135(16):1522–31.

15. Isomaa B, Almgren P, Henricsson M, et al. Chronic complications in patients with slowly progressing autoimmune type 1 diabetes (LADA). Diabetes Care 1999;22(8):1347–53.

16. Myhill P, Davis WA, Bruce DG, Mackay IR, Zimmet P, Davis TME. Chronic complications and mortality in community-based patients with latent autoimmune diabetes in adults: the Fremantle Diabetes Study. Diabet Med 2008;25(10):1245–50.

17. Hawa MI, Buchan AP, Ola T, et al. LADA and CARDS: a prospective study of clinical outcome in established adult-onset autoimmune diabetes. Diabetes Care 2014;37(6):1643–9.

18. Mautner SL, Lin F, Roberts WC. Composition of atherosclerotic plaques in the epicardial coronary arteries in juvenile (type I) diabetes mellitus. Am J Cardiol 1992;70(15):1264–8.

19. Djaberi R, Schuijf JD, Boersma E, et al. Differences in atherosclerotic plaque burden and morphology between type 1 and 2 diabetes as assessed by multislice computed tomography. Diabetes Care 2009;32(8):1507–12.

20. Center JR, Nguyen T V, Schneider D, Sambrook PN, Eisman JA. Mortality after all major types of osteoporotic fracture in men and women: an observational study. Lancet (London, England) 1999;353(9156):878–82.

21. Cameron ID, Chen JS, March LM, et al. Hip fracture causes excess mortality owing to cardiovascular and infectious disease in institutionalized older people: a prospective 5-year study. J Bone Miner Res 2010;25(4):866–72.

22. Weber DR, Haynes K, Leonard MB, Willi SM, Denburg MR. Type 1 Diabetes Is Associated With an Increased Risk of Fracture Across the Life Span: A Population-Based Cohort Study Using The Health Improvement Network (THIN). Diabetes Care 2015;

23. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol 2007;166(5):495–505.

24. Keenan HA, Maddaloni E. Bone Microarchitecture in Type 1 Diabetes: It Is Complicated. Curr Osteoporos Rep 2016;14(6).

25. Shanbhogue V V, Hansen S, Frost M, et al. Bone Geometry, Volumetric Density, Microarchitecture, and Estimated Bone Strength Assessed by HR-pQCT in Adult Patients With Type 1 Diabetes Mellitus. J Bone Miner Res 2015;30(12):2188–99.

26. Starup-Linde J, Lykkeboe S, Gregersen S, et al. Bone structure and predictors of fracture in type 1 and type 2 diabetes. J Clin Endocrinol Metab 2016;101(3):jc20153882.

27. Armas LAG, Akhter MP, Drincic A, Recker RR. Trabecular bone histomorphometry in humans with Type 1 Diabetes Mellitus. Bone 2012;50(1):91–6.

28. Sennerby U, Farahmand B, Ahlbom A, Ljunghall S, Michaëlsson K. Cardiovascular diseases and future risk of hip fracture in women. Osteoporos Int 2007;18(10):1355–62.

29. Bagger YZ, Tankó LB, Alexandersen P, Qin G, Christiansen C. Radiographic measure of aorta calcification is a site-specific predictor of bone loss and fracture risk at the hip. J Intern Med 2006;259(6):598–605.

30. Arnett TR, Gibbons DC, Utting JC, et al. Hypoxia is a major stimulator of osteoclast formation and bone resorption. J Cell Physiol 2003;196(1):2–8.

31. Arnett TR. Acidosis, hypoxia and bone. Arch Biochem Biophys 2010;503(1):103–9.

32. Reeve J, Arlot M, Wootton R, et al. Skeletal blood flow, iliac histomorphometry, and strontium kinetics in osteoporosis: a relationship between blood flow and corrected apposition rate. J Clin Endocrinol Metab 1988;66(6):1124–31.

33. Marenzana M, Arnett TR. The Key Role of the Blood Supply to Bone. Bone Res 2013;1(3):203–15.

34. Collins TC, Ewing SK, Diem SJ, et al. Peripheral arterial disease is associated with higher rates of hip bone loss and increased fracture risk in older men. Circulation 2009;119(17):2305–12.

35. Pieralice S, Vigevano F, Del Toro R, Napoli N, Maddaloni E. Lifestyle Management of Diabetes: Implications for the Bone-Vascular Axis. Curr Diab Rep 2018;18(10):84.

36. Unger WWJ, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. Eur J Immunol 2009;39(11):3147–59.

37. Lettgen B, Hauffa B, Möhlmann C, Jeken C, Reiners C. Bone mineral density in children and adolescents with juvenile diabetes: selective measurement of bone mineral density of trabecular and cortical bone using peripheral quantitative computed tomography. Horm Res 1995;43(5):173–5.

38. Moyer-Mileur LJ, Dixon SB, Quick JL, Askew EW, Murray MA. Bone mineral acquisition in adolescents with type 1 diabetes. J Pediatr 2004;145(5):662–9.

39. Bechtold S, Putzker S, Bonfig W, Fuchs O, Dirlenbach I, Schwarz HP. Bone size normalizes with age in children and adolescents with type 1 diabetes. Diabetes Care 2007;30(8):2046–50.

40. Heap J, Murray MA, Miller SC, Jalili T, Moyer-Mileur LJ. Alterations in bone characteristics associated with glycemic control in adolescents with type 1 diabetes mellitus. J Pediatr 2004;144(1):56–62.

41. Saha MT, Sievänen H, Salo MK, Tulokas S, Saha HH. Bone mass and structure in adolescents with type 1 diabetes compared to healthy peers. Osteoporos Int 2009;20(8):1401–6.

42. Roggen I, Gies I, Vanbesien J, Louis O, De Schepper J. Trabecular bone mineral density and bone geometry of the distal radius at completion of pubertal growth in childhood type 1 diabetes. Horm Res pædiatrics 2013;79(2):68–74.

43. Abdalrahaman N, McComb C, Foster JE, et al. Deficits in Trabecular Bone Microarchitecture in Young Women With Type 1 Diabetes Mellitus. J Bone Miner Res 2015;30(8):1386–93.

44. Sarwar N, Gao P, Seshasai SRK, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet (London, England) 2010;375(9733):2215–22.

45. Turner R, Stratton I, Horton V, et al. UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. UK Prospective Diabetes Study Group. Lancet (London, England) 1997;350(9087):1288–93.

46. Maddaloni E, Pastore G, Del Buono MG, et al. High Prevalence of Autoimmune Diabetes and Poor Glycaemic Control among Adults in Madagascar: A Brief Report from a Humanitarian Health Campaign in Ambanja. J Diabetes Res 2017;2017.

47. Davis TME, Wright AD, Mehta ZM, et al. Islet autoantibodies in clinically diagnosed type 2 diabetes: prevalence and relationship with metabolic control (UKPDS 70). Diabetologia 2005;48(4):695–702.

48. UK Prospective Diabetes Study (UKPDS). VIII. Study design, progress and performance. Diabetologia 1991;34(12):877–90.

49. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HAW. 10-Year Follow-up of Intensive Glucose Control in Type 2 Diabetes. N Engl J Med 2008;359(15):1577–89.

50. Bottazzo GF, Bosi E, Cull CA, et al. IA-2 antibody prevalence and risk assessment of early insulin requirement in subjects presenting with type 2 diabetes (UKPDS 71). Diabetologia 2005;48(4):703–8.

51. Rubin DB. Inference and missing data. Biometrika 1976;63:581–92.

52. Wod M, Thomsen RW, Pedersen L, Yderstraede KB, Beck-Nielsen H, Højlund K. Lower mortality and cardiovascular event rates in patients with Latent Autoimmune Diabetes In Adults (LADA) as compared with type 2 diabetes and insulin deficient diabetes: A cohort study of 4368 patients. Diabetes Res Clin Pract 2018;139:107–13.

53. Olsson L, Grill V, Midthjell K, Ahlbom A, Andersson T, Carlsson S. Mortality in adult-onset autoimmune diabetes is associated with poor glycemic control: results from the HUNT Study. Diabetes Care 2013;36(12):3971–8.

54. American Diabetes Association. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes—2018. Diabetes Care 2018;41(Supplement 1):S73–85.

55. Patti G, Cavallari I, Andreotti F, et al. Prevention of atherothrombotic events in patients with diabetes mellitus: from antithrombotic therapies to new-generation glucose-lowering drugs. Nat Rev Cardiol 2018;

56. Napoli N, Chandran M, Pierroz DD, et al. Mechanisms of diabetes mellitus-induced bone fragility. Nat Rev Endocrinol 2017;13(4):208–19.

57. Hough FS, Pierroz DD, Cooper C, Ferrari SL. Mechanisms and evaluation of bone fragility in type 1 diabetes mellitus. Eur. J. Endocrinol. 2016;

58. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes--a meta-analysis. Osteoporos Int 2007;18(4):427–44.

59. Napoli N, Strotmeyer ES, Ensrud KE, et al. Fracture risk in diabetic elderly men: the MrOS study. Diabetologia 2014;57(10):2057–65.

60. Napoli N, Schwartz A V., Schafer AL, et al. Vertebral Fracture Risk in Diabetic Elderly Men: The MrOS Study. J Bone Miner Res 2018;

61. Maddaloni E, D'Onofrio L, Lauria A, et al. Osteocalcin levels are inversely associated with Hba1c and BMI in adult subjects with long-standing type 1 diabetes. J Endocrinol Invest 2014;37(7):661–6.

62. McCabe LR. Understanding the pathology and mechanisms of type I diabetic bone loss. J Cell Biochem 2007;102(6):1343–57.

63. Maddaloni E, D'Eon S, Hastings S, et al. Bone health in subjects with type 1 diabetes for more than 50 years. Acta Diabetol 2017;54(5):1–10.

64. Hamann C, Kirschner S, Günther K-P, Hofbauer LC. Bone, sweet bone--osteoporotic fractures in diabetes mellitus. Nat Rev Endocrinol 2012;8(5):297–305.

65. Miao J, Brismar K, Nyrén O, Ugarph-Morawski A, Ye W. Elevated hip fracture risk in type 1 diabetic patients: a population-based cohort study in Sweden. Diabetes Care 2005;28(12):2850–5.

66. Mayer-Davis EJ, Lawrence JM, Dabelea D, et al. Incidence Trends of Type 1 and Type 2 Diabetes among Youths, 2002–2012. N Engl J Med 2017;

67. Care D, Suppl SS. Classification and diagnosis of diabetes: Standards of medical care in Diabetesd2018. Diabetes Care 2018;41(January):S13–27.

68. Napoli N, Schwartz A V., Black DM. Vertebral Fracture Risk in Diabetic Elderly Men: The MrOS Study. J Bone Miner Res 2017;57(10):2057–65.

69. Pucci F, Garris C, Lai CP, et al. HHS Public Access. 2016;352(6282):242–6.

70. Center JR, Bliuc D, Nguyen T V., Eisman JA. Risk of subsequent fracture after low-trauma fracture in men and women. J Am Med Assoc 2007;

71. Oei L, Zillikens MC, Dehghan A, et al. High bone mineral density and fracture risk in type 2 diabetes as skeletal complications of inadequate glucose control: the Rotterdam Study. Diabetes Care 2013;36(6):1619–28.

72. Li C-I, Liu C-S, Lin W-Y, et al. Glycated Hemoglobin Level and Risk of Hip Fracture in Older People with Type 2 Diabetes: A Competing Risk Analysis of Taiwan Diabetes Cohort Study. J Bone Miner Res 2015;30(7):1338–46.

73. Schneider ALC, Williams EK, Brancati FL, Blecker S, Coresh J, Selvin E. Diabetes and risk of fracture-related hospitalization: The atherosclerosis risk in communities study. Diabetes Care 2013;36(5):1153–8.

74. Neumann T, Sämann a, Lodes S, et al. Glycaemic control is positively associated with prevalent fractures but not with bone mineral density in patients with Type 1 diabetes. Diabet Med 2011;

75. Zhukouskaya V V., Eller-Vainicher C, Vadzianava V V., et al. Prevalence of morphometric vertebral fractures in patients with type 1 diabetes. Diabetes Care 2013;36(6):1635–40.

76. Schwartz A V, Vittinghoff E, Bauer DC, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA 2011;305(21):2184–92.

77. Ingberg C-M, Palmér M, Aman J, Arvidsson B, Schvarcz E, Berne C. Body composition and bone mineral density in long-standing type 1 diabetes. J Intern Med 2004;255(3):392–8.

78. de Paula FJA, Horowitz MC, Rosen CJ. Novel insights into the relationship between diabetes and osteoporosis. Diabetes Metab Res Rev 2010;26(8):622–30.

79. Vestergaard P, Rejnmark L, Mosekilde L. Diabetes and its complications and their relationship with risk of fractures in type 1 and 2 diabetes. Calcif Tissue Int 2009;84(1):45–55.

80. Anagnostis P, Karagiannis A, Kakafika AI, Tziomalos K, Athyros VG, Mikhailidis DP. Atherosclerosis and osteoporosis: age-dependent degenerative processes or related entities? Osteoporos Int 2009;20(2):197–207.

81. Onder A, Cetinkaya S, Tunc O, Aycan Z. Evaluation of bone mineral density in children with type 1 diabetes mellitus. J Pediatr Endocrinol Metab 2013;26(11–12):1077–81.

82. Joshi A, Varthakavi P, Chadha M, Bhagwat N. A study of bone mineral density and its determinants in type 1 diabetes mellitus. J Osteoporos 2013;2013:397814.

83. Miazgowski T, Czekalski S. A 2-year follow-up study on bone mineral density and markers of bone turnover in patients with long-standing insulin-dependent diabetes mellitus. Osteoporos Int 1998;8(5):399–403.

84. Strotmeyer ES, Cauley JA, Orchard TJ, Steenkiste AR, Dorman JS. Middle-aged premenopausal women with type 1 diabetes have lower bone mineral density and calcaneal quantitative ultrasound than nondiabetic women. Diabetes Care 2006;29(2):306–11.

85. Livingstone SJ, Levin D, Looker HC, et al. Estimated life expectancy in a Scottish cohort with type 1 diabetes, 2008-2010. JAMA 2015;313(1):37–44.

86. Miller RG, Secrest AM, Sharma RK, Songer TJ, Orchard TJ. Improvements in the life expectancy of type 1 diabetes: the Pittsburgh Epidemiology of Diabetes Complications study cohort. Diabetes 2012;61(11):2987–92.

87. Petrie D, Lung TWC, Rawshani A, et al. Recent trends in life expectancy for people with type 1 diabetes in Sweden. Diabetologia 2016;59(6):1167–76.

88. Huo L, Shaw JE, Wong E, Harding JL, Peeters A, Magliano DJ. Burden of diabetes in Australia: life expectancy and disability-free life expectancy in adults with diabetes. Diabetologia 2016;59(7):1437–45.

89. Keenan HA, Costacou T, Sun JK, et al. Clinical factors associated with resistance to microvascular complications in diabetic patients of extreme disease duration: the 50-year medalist study. Diabetes Care 2007;30(8):1995–7.

90. Keenan HA, Sun JK, Levine J, et al. Residual insulin production and pancreatic ß-cell turnover after 50 years of diabetes: Joslin Medalist Study. Diabetes 2010;59(11):2846–53.

91. Martin LM, Leff M, Calonge N, Garrett C, Nelson DE. Validation of self-reported chronic conditions and health services in a managed care population. Am J Prev Med 2000;18(3):215–8.

92. Feldman EL, Stevens MJ, Thomas PK, Brown MB, Canal N, Greene DA. A practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy. Diabetes Care 1994;17(11):1281–9.

93. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150(9):604–12.

94. Fundus photographic risk factors for progression of diabetic retinopathy. ETDRS report number 12. Early Treatment Diabetic Retinopathy Study Research Group. Ophthalmology 1991;98(5 Suppl):823–33.

95. Kanis JA, Melton LJ, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. J Bone Miner Res 1994;9(8):1137–41.

96. Wright NC, Looker AC, Saag KG, et al. The recent prevalence of osteoporosis and low bone mass in the United States based on bone mineral density at the femoral neck or lumbar spine. J Bone Miner Res 2014;29(11):2520–6.

97. Kemink SA, Hermus AR, Swinkels LM, Lutterman JA, Smals AG. Osteopenia in insulin-dependent diabetes mellitus; prevalence and aspects of pathophysiology. J Endocrinol Invest 2000;23(5):295–303.

98. Bridges MJ, Moochhala SH, Barbour J, Kelly CA. Influence of diabetes on peripheral bone mineral density in men: a controlled study. Acta Diabetol 2005;42(2):82–6.

99. Lunt H, Florkowski CM, Cundy T, et al. A population-based study of bone mineral density in women with longstanding type 1 (insulin dependent) diabetes. Diabetes Res Clin Pract 1998;40(1):31–8.

100. He ZH, D'Eon SA, Tinsley LJ, et al. Cardiovascular disease protection in long-duration type 1 diabetes and sex differences. Diabetes Care 2015;38(5):e73-4.

101. Fiorina P, Folli F, Maffi P, et al. Islet transplantation improves vascular diabetic complications in patients with diabetes who underwent kidney transplantation: a comparison between kidney-pancreas and kidney-alone transplantation. Transplantation 2003;75(8):1296–301.

102. Bassi R, Fiorina P. Impact of Islet Transplantation on Diabetes Complications and Quality of Life. Curr Diab Rep 2011;11(5):355–63.

103. Pozzilli P, Maddaloni E, Buzzetti R. Combination immunotherapies for type 1 diabetes mellitus. Nat Rev Endocrinol 2015;11(5):289–97.

104. Bagger YZ, Rasmussen HB, Alexandersen P, Werge T, Christiansen C, Tankó LB. Links between cardiovascular disease and osteoporosis in postmenopausal women: serum lipids or atherosclerosis per se? Osteoporos Int 2007;18(4):505–12.

105. Makovey J, Chen JS, Hayward C, Williams FMK, Sambrook PN. Association between serum cholesterol and bone mineral density. Bone 2009;44(2):208–13.

106. Yerges-Armstrong LM, Shen H, Ryan KA, Streeten EA, Shuldiner AR, Mitchell BD. Decreased bone mineral density in subjects carrying familial defective apolipoprotein B-100. J Clin Endocrinol Metab

2013;98(12):E1999-2005.

107. Parhami F, Garfinkel A, Demer LL. Role of lipids in osteoporosis. Arterioscler Thromb Vasc Biol 2000;20(11):2346–8.

108. Parhami F, Jackson SM, Tintut Y, et al. Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. J Bone Miner Res 1999;14(12):2067–78.

109. Patsch JM, Burghardt AJ, Yap SP, et al. Increased cortical porosity in type 2 diabetic postmenopausal women with fragility fractures. J Bone Miner Res 2013;28(2):313–24.

110. Yu EW, Putman MS, Derrico N, Abrishamanian-Garcia G, Finkelstein JS, Bouxsein ML. Defects in cortical microarchitecture among African-American women with type 2 diabetes. Osteoporos Int 2015;26:673–9.

111. Farr JN, Drake MT, Amin S, Melton LJ, McCready LK, Khosla S. In vivo assessment of bone quality in postmenopausal women with type 2 diabetes. J Bone Miner Res 2014;29(4):787–95.

112. Kinoshita H, Tamaki T, Hashimoto T, Kasagi F. Factors influencing lumbar spine bone mineral density assessment by dual-energy X-ray absorptiometry: comparison with lumbar spinal radiogram. J Orthop Sci 1998;3(1):3–9.

113. Morrish NJ, Wang SL, Stevens LK, Fuller JH, Keen H. Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. Diabetologia 2001;44 Suppl 2:S14-21.

114. Wadwa RP, Kinney GL, Ogden L, et al. Soluble interleukin-2 receptor as a marker for progression of coronary artery calcification in type 1 diabetes. Int J Biochem Cell Biol 2006;38(5–6):996–1003.

115. Lopes-Virella MF, Carter RE, Gilbert GE, et al. Risk factors related to inflammation and endothelial dysfunction in the DCCT/EDIC cohort and their relationship with nephropathy and macrovascular complications. Diabetes Care 2008;31(10):2006–12.

116. Pérez A, Wägner AM, Carreras G, et al. Prevalence and phenotypic distribution of dyslipidemia in type 1 diabetes mellitus: effect of glycemic control. Arch Intern Med 2000;160(18):2756–62.

117. Rask-Madsen C, King GL. Vascular Complications of Diabetes: Mechanisms of Injury and Protective Factors. Cell Metab 2013;17(1):20–33.

118. Fadini GP, Albiero M, Menegazzo L, et al. Procalcific phenotypic drift of circulating progenitor cells

in type 2 diabetes with coronary artery disease. Exp Diabetes Res 2012;2012:921685.

119. Fadini GP. A reappraisal of the role of circulating (progenitor) cells in the pathobiology of diabetic complications. Diabetologia 2014;57(1):4–15.

120. Fadini GP, Albiero M, Menegazzo L, et al. Widespread increase in myeloid calcifying cells contributes to ectopic vascular calcification in type 2 diabetes. Circ Res 2011;108(9):1112–21.

121. Collin J, Gössl M, Matsuo Y, et al. Osteogenic monocytes within the coronary circulation and their association with plaque vulnerability in patients with early atherosclerosis. Int J Cardiol 2015;181:57–64.

122. Long MW, Williams JL, Mann KG. Expression of human bone-related proteins in the hematopoietic microenvironment. J Clin Invest 1990;86(5):1387–95.

123. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Bone marrow-derived osteoblast progenitor cells in circulating blood contribute to ectopic bone formation in mice. Biochem Biophys Res Commun 2007;354(2):453–8.

124. Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating osteoblast-lineage cells in humans. N Engl J Med 2005;352(19):1959–66.

125. Albiero M, Rattazzi M, Menegazzo L, et al. Myeloid calcifying cells promote atherosclerotic calcification via paracrine activity and allograft inflammatory factor-1 overexpression. Basic Res Cardiol 2013;108(4):368.

126. Yan J, Stringer SE, Hamilton A, et al. Decorin GAG synthesis and TGF-β signaling mediate Ox-LDL-induced mineralization of human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2011;31(3):608–15.

127. Liu L, Liu Z, Chen H, Zhang G, Kong Y, Kang X. Oxidized low-density lipoprotein and β-glycerophosphate synergistically induce endothelial progenitor cell ossification. Acta Pharmacol Sin 2011;32(12):1491–7.

128. Berthold HK, Rizzo M, Spenrath N, Montalto G, Krone W, Gouni-Berthold I. Effects of lipid-lowering drugs on high-density lipoprotein subclasses in healthy men-a randomized trial. PLoS One 2014;9(3):e91565.

129. Menegazzo L, Albiero M, Millioni R, et al. Circulating myeloid calcifying cells have antiangiogenic

activity via thrombospondin-1 overexpression. FASEB J 2013;27(11):4355–65.

130. Sloop CH, Dory L, Roheim PS. Interstitial fluid lipoproteins. J Lipid Res 1987;28(3):225–37.

131. Röhrl C, Stangl H. HDL endocytosis and resecretion. Biochim Biophys Acta - Mol Cell Biol Lipids 2013;1831(11):1626–33.

132. Gössl M, Mödder UI, Atkinson EJ, Lerman A, Khosla S. Osteocalcin expression by circulating endothelial progenitor cells in patients with coronary atherosclerosis. J Am Coll Cardiol 2008;52(16):1314–25.

133. Flammer AJ, Gössl M, Widmer RJ, et al. Osteocalcin positive CD133+/CD34-/KDR+ progenitor cells as an independent marker for unstable atherosclerosis. Eur Heart J 2012;33(23):2963–9.

134. Pignolo RJ, Kassem M. Circulating osteogenic cells: implications for injury, repair, and regeneration. J Bone Miner Res 2011;26(8):1685–93.

135. Fadini GP, Rattazzi M, Matsumoto T, Asahara T, Khosla S. Emerging role of circulating calcifying cells in the bone-vascular axis. Circulation 2012;125(22):2772–81.

136. Kuwana M, Okazaki Y, Kodama H, et al. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. J Leukoc Biol 2003;74(5):833–45.

137. Booth SL, Centi A, Smith SR, Gundberg C. The role of osteocalcin in human glucose metabolism: marker or mediator? Nat Rev Endocrinol 2012;9(1):43–55.

138. Hwang Y-C, Jeong I-K, Ahn K-J, Chung H-Y. Circulating osteocalcin level is associated with improved glucose tolerance, insulin secretion and sensitivity independent of the plasma adiponectin level. Osteoporos Int 2012;23(4):1337–42.

139. Luo Y, Ma X, Hao Y, et al. Relationship between serum osteocalcin level and carotid intima-media thickness in a metabolically healthy Chinese population. Cardiovasc Diabetol 2015;14:82.

140. Sheng L, Cao W, Cha B, Chen Z, Wang F, Liu J. Serum osteocalcin level and its association with carotid atherosclerosis in patients with type 2 diabetes. Cardiovasc Diabetol 2013;12(1):22.

141. Foresta C, Strapazzon G, De Toni L, et al. Platelets express and release osteocalcin and co-localize in human calcified atherosclerotic plaques. J Thromb Haemost 2013;11(2):357–65.

142. Taylor J, Butcher M, Zeadin M, Politano A, Shaughnessy SG. Oxidized low-density lipoprotein promotes osteoblast differentiation in primary cultures of vascular smooth muscle cells by up-

regulating Osterix expression in an Msx2-dependent manner. J Cell Biochem 2011;112(2):581–8.

143. Pagler TA, Rhode S, Neuhofer A, et al. SR-BI-mediated high density lipoprotein (HDL) endocytosis leads to HDL resecretion facilitating cholesterol efflux. J Biol Chem 2006;281(16):11193–204.

144. Berneis KK, Krauss RM. Metabolic origins and clinical significance of LDL heterogeneity. J Lipid Res 2002;43(9):1363–79.

145. de Graaf J, Hak-Lemmers HL, Hectors MP, Demacker PN, Hendriks JC, Stalenhoef AF. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. Arterioscler Thromb Vasc Biol 1991;11(2).

146. Asztalos BF, Cupples LA, Demissie S, et al. High-density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study. Arterioscler Thromb Vasc Biol 2004;24(11):2181–7.

147. Asztalos BF, Collins D, Cupples LA, et al. Value of high-density lipoprotein (HDL) subpopulations in predicting recurrent cardiovascular events in the Veterans Affairs HDL Intervention Trial. Arterioscler Thromb Vasc Biol 2005;25(10):2185–91.

148. Lamon-Fava S, Herrington DM, Reboussin DM, et al. Plasma levels of HDL subpopulations and remnant lipoproteins predict the extent of angiographically-defined coronary artery disease in postmenopausal women. Arterioscler Thromb Vasc Biol 2008;28(3):575–9.

149. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 1977;62(5):707–14.

150. Gordon DJ, Probstfield JL, Garrison RJ, et al. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation 1989;79(1):8–15.

151. Singh IM, Shishehbor MH, Ansell BJ. High-density lipoprotein as a therapeutic target: a systematic review. JAMA 2007;298(7):786–98.

152. Yamamoto S, Narita I, Kotani K. The macrophage and its related cholesterol efflux as a HDL function index in atherosclerosis. Clin Chim Acta 2016;457:117–22.

153. Tinsley LJ, Kupelian V, D'Eon SA, et al. Association of glycemic control with reduced risk of large vessel disease after more than 50 years of type 1 diabetes. J Clin Endocrinol Metab 2017;

154. Qin Z. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. Atherosclerosis 2012;221(1):2–11.