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Sclerostin and bone turnover in LADA

Serum sclerostin and bone turnover in latent autoimmune diabetes in adults

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Purpose: Bone formation is impaired in both type 1 and type 2 diabetes (T2D) while sclerostin, an antagonist of bone formation, is increased in T2D only. No data are available on latent autoimmune diabetes in adults (LADA), an autoimmune type of diabetes that may clinically resemble T2D at diagnosis. We evaluated serum sclerostin and bone turnover markers in LADA compared with T2D, and whether sclerostin is affected by metabolic syndrome (MetS) in T2D or LADA.

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

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

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

We evaluated serum sclerostin in LADA and in type 2 diabetes (T2D). LADA present lower bone resorption compared to controls, similarly to T2D. Sclerostin is increased in T2D but not in LADA.

Abbreviations

BMI: Body mass index;

CTX: C-terminal telopeptide of type I collagen;

GADA: Glutamic acid decarboxylase autoantibodies;

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

IQR: Interquartile range;

LADA: Latent autoimmune diabetes of adults;

MetS: Metabolic syndrome;

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

TRAP: Tartrate-resistant acid phosphatase.

Introduction

Both type 1 diabetes (T1D) and type 2 diabetes (T2D) are associated with impaired bone metabolism and increased risk of fractures (1). A large variety of mechanisms have been proposed including low bone turnover (2, 3).

The mechanism of reduced bone formation in diabetes is unclear, but emerging evidence implicates elevated levels of sclerostin. Sclerostin is a glycoprotein produced by osteocytes and is an antagonist of the osteoblastic bone formation through the inhibition of the Wnt/ β -catenin pathway (4, 5). Clinical studies have consistently shown raised sclerostin levels in T2D (6-9). Despite low bone formation being a feature of both T1D and T2D, clinical and experimental evidence have shown that sclerostin levels and/or expression are increased in T2D (10) but not in T1D (11). Furthermore, sclerostin is associated with clinical or biochemical surrogates of insulin resistance (a key feature of T2D), such as increased body weight or BMI (7) and the HOMA-IR index (12).

Latent autoimmune diabetes in adults (LADA) is an autoimmune type of adult-onset diabetes characterized by insulin independence at the time of diagnosis and positivity to circulating islet-autoantibodies, most commonly GAD-autoantibodies (GADA). (13). LADA patients may resemble T2D at the onset due to insulin independence and the increased prevalence of metabolic syndrome compared to T1D, representing about ten percent of adults initially misdiagnosed as having T2D. Many immunologic and genetic features in LADA are similar to those found in T1D so that this form of diabetes is classified as a subtype of T1D. Recent efforts by the Action LADA and the Non Insulin Requiring Autoimmune Diabetes (NIRAD) study projects have led to a better understanding of the biochemical, immunologic, metabolic and clinical characteristics associated with LADA (14-17). However, bone metabolic features of LADA patients, to our knowledge, have been not investigated.

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

Methods

Subjects.

This was a cross-sectional study involving 89 subjects with LADA and 98 subjects with T2D. Serum of patients with LADA and type 2 diabetes was obtained from the European Union Action LADA project (14) and the NIRAD study in Italy (18). Samples were selected where sufficient serum and data was available from four Action LADA centres in Spain, France, Belfast and London, and from the NIRAD group in Italy. The Action LADA multicentre study was performed to identify immune and clinical risk factors for adult-onset autoimmune diabetes, including its epidemiology, genetic susceptibility, metabolic characteristics and clinical progression (13). Diabetes was designated according to standard criteria, and LADA

was defined as patients aged 30–70 years with GADAs who did not require insulin treatment for at least 6 months after diagnosis (13, 19). Patients came from Europe and almost all of them were of Caucasian ethnicity (96% Caucasian, 3% Asian, 1% African, and 1% mixed race). The NIRAD Study is a nationwide survey sponsored by the Società Italiana di Diabetologia with the aim of assessing the prevalence and characteristics of autoimmune diabetes within adult patients attending diabetes clinics in Italy with a clinical diagnosis of non-insulin-requiring diabetes. Adult-onset autoimmune diabetic subjects were selected using the following inclusion criteria: 1) an initial diagnosis of T2D according to the American Diabetes Association, 2) documented antibody positivity for GADA and/or IA-2A (20), 3) no insulin requirement and no evidence of ketosis from diagnosis to screening time, and 4) time since diagnosis between 6 months and 5 years. All subjects from the NIRAD study were unrelated and of exclusively Italian origin (with parents and grandparents of Italian origin). Exclusion criteria included prior insulin therapy, pregnancy, renal disease with a raised creatinine level or proteinuria, and the presence of any other severe disease. MetS was assessed according to the NCEP criteria, as described below. T2D and LADA samples were selected where enough sample volume was available, aiming at ensuring a numeric balance between the following four groups, namely LADA with MetS; LADA without MetS; T2D with MetS; T2D without MetS. There was no other selection at all. We selected from the Action LADA cohort, LADA cases with MetS (n=31) and LADA cases without MetS (n=29); from the NIRAD group we selected LADA cases with MetS (n=11) and LADA cases without MetS (n=17). Overall, selected T2D cases were limited by availability of sufficient sera (n= 60 from the Action LADA, and n=38 from the NIRAD group), being of similar age, sex and disease duration to the LADA cases. Waist circumference and blood pressure, at least twice in the sitting position, were measured in each subject. Lipids and lipoproteins (total and HDL cholesterol, triglycerides) were determined by standardized assays at each center. Patients with T2D or LADA were divided into four groups according to the presence or absence of MetS: 1) type 2 diabetes with MetS (n=57); 2) type 2 diabetes without MetS (n=41); 3) LADA with MetS (n=42); 4) LADA without MetS (n=47). Sera from 53 individuals without diabetes (fasting blood glucose lower than 126 mg/dl) were used as control. Subjects treated with thiazolidinediones or sodium glucose transporter 2 inhibitors were not included in this study. Control subjects were recruited through the the Endocrinology outpatient clinics of Università Campus Bio-Medico di Roma. Subjects with diseases (e.g. osteoporosis, hyperparathyroidism, hyper or hypothyroidism, chronic kidney disease, etc.) or drugs (glucocorticoids, bisphosphonates, etc.) known to affect bone metabolism were excluded.

Diagnostic criteria for the metabolic syndrome (MetS).

MetS was assessed according to the NCEP criteria (21), with modifications by the AHA/NHLBI (22), as follows: waist circumference >102 cm in men and >88 cm in females, triglycerides ≥ 150 mg/dl, HDL cholesterol <40 mg/dl in men and <50 mg/dl in women, blood pressure $\geq 130/85$ mmHg or taking antihypertensive medication, and fasting glucose ≥ 100 mg/dl. All diabetic patients in this study were identified as fulfilling the criteria for hyperglycemia. MetS was defined by the presence of three of five criteria, including blood glucose.

Sclerostin and bone turnover markers.

Sclerostin serum levels were assessed by quantitative sandwich ELISA (Biomedica, Vienna, Austria). Bone turnover was evaluated analyzing serum levels of a bone formation marker, the total pro-collagen type 1 N-terminal propeptide (P1NP) by ELISA (Biomedica, Vienna, Austria) and a bone resorption marker, the CTX by ELISA (IDS, Boldon, UK). P1NP is a specific marker of bone formation, while CTX is an accurate marker of bone resorption. CTX

and P1NP have been suggested by the International Osteoporosis Foundation as the appropriate bone markers when exploring bone resorption and formation in clinical and research settings (23).

Sample size and power calculation:

Sample size was calculated for the primary aim on the hypothesis that circulating sclerostin would be different between T2D and LADA. The calculation was based on previous published data of increased circulating sclerostin in T2D compared to non-diabetic control subjects (8). Using a significance level of 0.05 and 80% power, the minimum sample size required was of 46 subjects per group (T2D, LADA and controls). To investigate the relationship between sclerostin and MetS status, the samples size was further increased on the base of available serum and clinical/biochemical information on the MetS status.

Statistical analysis.

Continuous variables are presented as mean \pm SD. Normality was tested with the Shapiro-Wilk test. When data were not normally distributed, logarithmic transformation was performed. Categorical variables are expressed as absolute frequencies. For continuous variables, mean differences across groups were compared by Generalized Linear Models/ANOVA for normally distributed variables. Homoscedasticity was tested with Levene and Brown-Forsythe test'. For *post-hoc* analyses, Tukey and Games-Howell tests were applied. Pearson (normal distribution) and Spearman (non-normal distribution) correlations were used to assess the correlations between serum sclerostin levels and other continuous variables. Multiple backward model linear regression analysis was performed to identify independent predictors of serum sclerostin levels (dependent variable, sqrt transformed) in the overall population and in LADA/ T2D groups. The models included time since diagnosis, BMI, HDL-cholesterol levels and triglycerides levels, while age and sex were not included in the multiple linear regression model as they were not correlated with serum sclerostin levels. Furthermore, after adjusting for age and sex, the results did not change. A two tailed p-value <0.05 was considered significant. A multiplicity adjusted p value was reported when multiple comparisons were performed. Data were analyzed with SAS version 9.4 statistical software (SAS Institute Inc., Cary, NC).

Results

Features of the studied population.

Clinical and biochemical features of subjects with T2D, LADA and non-diabetic control subjects are shown in Table 1. Overall, patients with LADA were younger and leaner than those with T2D [median body mass index (BMI) (range) of 24.7 (17.6-41.8) vs. 28.3 (18.0-44.5) Kg/m², respectively; p<0.0001], while time since diagnosis was similar in patients with LADA and T2D [median years of disease with range 2.9 (0-7) vs. 2.0 (0-11) years; p=0.25]. However, when divided according to presence or absence of MetS, the four groups were comparable in terms of age and time since diagnosis (Table 1). The reference non-diabetic group consisted of 53 subjects (36 females) without diabetes (age 48.2 \pm 21.1 years; median BMI 26.2, range 18.5-41.0, kg/m²). Non-diabetic subjects were of similar age compared to LADA but younger than T2D (p<0.001); the non-diabetic control group consisted of more female than the LADA and T2D groups (p<0.01). BMI of controls was not significantly different compared to LADA or T2D. Among control subjects, 5/53 (9.4%) had impaired fasting glucose, 9/53 (17%) had a diagnosis of primary hypertension, 5/53 (9.4%) had dyslipidemia and 9/53 (17%) were obese (BMI>30 kg/m²).

Circulating sclerostin in LADA and T2D.

Patients with T2D had higher serum sclerostin than those with LADA or controls (29.8 \pm 11.9 vs. 23.0 \pm 11.8 vs. 24.3 \pm 5.7 pmol/l, p=0.0001; p \leq 0.002 adjusted for sex and BMI) (Figure 1).

In the combined group of diabetic subjects, sclerostin tended to be higher in the group with MetS but this was not significant (25.0 ± 12.7 vs. 28.3 ± 12.8 pmol/l; $p=0.08$). Within MetS patients, serum sclerostin was higher in T2D than LADA ($p=0.01$; $p=0.03$ adjusted for sex and BMI). When analyzing separately T2D and LADA, in both groups serum sclerostin was similar between subjects with and those without MetS ($p \geq 0.15$). However, when all diabetic individuals were included in the analysis and divided in four groups according to diabetes type and MetS (namely LADA without MetS, LADA with MetS, T2D without MetS, T2D with MetS), we found a trend for increased sclerostin from LADA without MetS towards T2D with MetS ($p < 0.0001$ for trend) (Figure 2). In the whole group of diabetic subjects, sclerostin progressively increased with the number of MetS features ($p=0.002$ for trend); when analysis was performed according to diabetes type, sclerostin increased with the number of MetS features in T2D ($p=0.001$ for trend) but not in LADA.

Bone turnover markers in LADA and T2D.

Subjects with T2D had 11% and 13% lower P1NP compared with LADA or non-diabetic subjects, respectively, although the differences were not significant (57.3 ± 16.6 vs. 64.2 ± 19.5 vs. 66.5 ± 25.2 pg/ml, respectively). The bone resorption marker CTX was 43% lower in subjects with diabetes, either T2D or LADA, compared with non diabetic subjects (0.16 ± 0.06 vs. 0.16 ± 0.10 vs. 0.28 ± 0.16 ng/ml, respectively; $p=0.0003$) (Figure 3). When LADA and T2D were divided according to the presence of MetS we found no significant differences in either P1NP or CTX levels across the four groups. Levels of bone turnover markers were unrelated to age, time since diagnosis, BMI or other clinical and biochemical parameters in all study groups. Bone turnover markers were not correlated with serum sclerostin ($0.006 < \rho < 0.04$; $p \geq 0.67$) in all study groups.

Relationship of sclerostin with clinical and biochemical features.

In the control group, serum sclerostin were unrelated with age ($\rho=0.08$; $p=0.63$), BMI ($\rho=-0.06$; $p=0.73$) or blood glucose ($\rho=0.21$; $p=0.22$). In the overall cohort of subjects with diabetes, serum sclerostin was similar between males and females ($p=0.92$) and unrelated with age ($\rho=0.05$; $p=0.49$), HbA1c ($\rho=0.27$; $p=0.053$) or creatinine ($\rho=-0.18$; $p=0.16$), but increased significantly with BMI ($\rho=0.29$; $p=0.0001$), time since diagnosis ($\rho=0.32$, $p < 0.0001$), triglycerides ($\rho=0.19$; $p=0.02$), and was inversely correlated with HDL cholesterol ($\rho=-0.23$; $p=0.003$). In multiple regression analysis of the overall population with diabetes, we found that time since diagnosis ($\beta=0.19$; $p=0.002$) and triglycerides ($\beta=0.003$; $p=0.03$), but not BMI and HDL-cholesterol, were independent predictors of sclerostin levels (Supplementary Table 1). Altogether, time since diagnosis and triglycerides explained 11% of sclerostin variance.

Discussion

To our knowledge, this is the first study reporting sclerostin and bone turnover in patients affected with LADA. We found that sclerostin is increased in T2D but not in LADA, while the bone resorption marker CTX was equally reduced compared to control subjects in both types of diabetes. These data indicate that low bone resorption is a feature of both LADA and T2D. MetS did not affect bone turnover markers in either LADA or T2D. In contrast, sclerostin was positively associated with the number of MetS features in patients with T2D, suggesting a relationship between MetS severity and T2D.

The finding that bone resorption is reduced in LADA compared to controls is novel. Previous studies have shown consistently lower levels of CTX and the bone formation marker osteocalcin in T1D and T2D compared with controls, regardless of diabetes type, suggesting that both bone resorption and formation are reduced in these types of diabetes (24, 25). Our data follow a similar trend showing that also patients with LADA, as well as patients

with T2D, have lower CTX than controls. As highlighted by a recent metanalysis, most of the studies have reported reduced P1NP levels in T2D compared to non-diabetic controls (24). In our study, we found a non significant reduction of P1NP in T2D compared to non-diabetic and LADA subjects. However, the magnitude of P1NP reduction (over 10%) was similar to that reported by the metanalysis of Hygum et al. when comparing T2D to non-diabetic controls (24). This may suggest that the P1NP difference found in our study did not reach statistical significance, probably due to increased variance among the groups or the small sample size. Despite the similar reduction in bone turnover, we found that serum sclerostin was increased in T2D but not in LADA. The role of sclerostin in diabetic bone turnover is controversial and there are no data in LADA so far. According to the literature, our data clearly mirror those reported by other groups in T1D. T1D patients have sclerostin levels similar to (7, 26, 27) or only slightly higher than controls (28). Conversely, increased sclerostin has been consistently found in individuals with T2D (8, 29, 30). In a recent metanalysis, the magnitude of sclerostin increase in comparison to controls was four times higher than that reported for T1D (24). In the study by Gennari et al. sclerostin was increased in patients with T2D but not in those with T1D despite the similar reduction of bone turnover in both groups (7). This is consistent with the experimental evidence that sclerostin expression is down-regulated in a mouse model of T1D (11), while the SOST/sclerostin gene is up-regulated in T2D rats (10). Taken together, these findings may support a different role for sclerostin in the impairment of bone metabolism associated to T2D or autoimmune diabetes (including LADA and T1D). Drake et al. have shown that peripheral serum sclerostin correlates with bone marrow plasma levels (31). Although sclerostin is a locally active molecule, we may speculate that circulating levels could reflect activity in the bone microenvironment. In T2D, the increased sclerostin release by osteocyte may attenuate osteoblastogenesis via inhibition of the canonical Wnt pathway. On the other hand, this pathway may not be significantly affected by the autoimmune types of diabetes (LADA and T1D), where other T1D/LADA-specific elements, such as insulin deficiency (32), autoimmunity (33, 34) or an intrinsic osteoblast defect, may be the primary cause. This is plausible considering that T1D, T2D and LADA present a number of differences in genetic, metabolic and immunologic profile. It follows that bone metabolism/turnover is an additional element that can differentiate these forms of diabetes.

MetS is more common in T2D than autoimmune diabetes, and its features are clearly linked to insulin resistance (35). In this study, sclerostin and bone turnover markers were similar between subjects with MetS and those without MetS, regardless of diabetes type. However, when all diabetic individuals were included in the analysis and divided in four groups according to diabetes type and MetS (namely LADA without MetS, LADA with MetS, T2D without MetS, T2D with MetS), we found a trend for increased sclerostin from LADA without MetS towards T2D with MetS. Such increase may reflect an association with the progressive increase in insulin resistance across the four groups analyzed, where LADA without MetS is the group with the lowest degree of insulin resistance, while T2D with MetS is the group characterized by the highest degree of insulin resistance, respectively. Of note, in patients with T2D we found a correlation between sclerostin and the number of MetS features. These observations may partially resemble those provided by Daniele et al., who showed a correlation between sclerostin and insulin resistance in skeletal muscle, liver, and adipose tissue (12). Sclerostin has been studied in association with other features of MetS, such as body weight, providing mixed results. A positive correlation with body weight or BMI has been shown in a report (7), while others have reported no association (6, 9) such as in the present study, or even increased levels in response to weight loss, as also recently shown by our group (36).

According to multivariate analysis, time since diagnosis (which is an estimate of disease duration) was the strongest predictor of sclerostin levels. This may imply that when diabetes progresses, inhibition of Wnt/ β -catenin pathway by sclerostin becomes more significant leading to impaired bone turnover. Although bone turnover markers did not correlate with disease duration in our study, others have shown a negative correlation between diabetes duration and markers of bone formation (37) and resorption (38).

Our study has several strengths and limitations. To our knowledge, this is the first analysis of bone turnover and sclerostin in a large and well characterized population of patients with LADA. The study population consisted of subjects with relatively short time since diagnosis without chronic complications. The significance of our findings may be limited by the lack of bone mineral density measurements and by the cross-sectional nature of the study. An additional limitation is the lack of data on post-menopausal status, vitamin D status or treatment with bisphosphonates or other bone-active drugs in subjects with T2D and LADA, all factors that may potential alter bone turnover and/or sclerostin levels. Additional studies with larger cohorts (cross-sectional and longitudinal and at-risk individuals) are required to assess the role of sclerostin and the Wnt pathway on bone turnover and fragility in diabetes.

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

Contribution statement: NN and RS were responsible for the conception and design of the study, data acquisition, contributed to the analysis and were responsible for the interpretation of data, writing the manuscript and revising it critically for important intellectual content. GD and AP contributed to interpretation of data, and writing the manuscript and revising it critically for important intellectual content. GL, CM, SZ, LD, VG, SM contributed to the acquisition of the data and revised it critically for important intellectual content. GC was responsible for the analysis of the data and revised the manuscript for important intellectual content. MIH, RDGL, PP and RB contributed to the design of the study, data acquisition and revised the manuscript critically for important intellectual content.

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Disclosure statement:

The authors have nothing to disclose.

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Figure 1 – Sclerostin in patients with type 2 diabetes, LADA and non-diabetic control subjects. Patients with type 2 diabetes (T2D) had higher sclerostin than LADA ($p=0.0007$) or control subjects ($p=0.002$). Box plots show the 25th and 75th percentile, and the horizontal line shows the median (50th percentile). Bars outside the box indicate the minimum and maximum value. Diamond symbol represents mean.

Figure 2 – Sclerostin in T2D and LADA according to metabolic syndrome (MetS) status. When analyzing separately T2D and LADA, in both groups the presence of MetS did not influence significantly serum sclerostin ($p \geq 0.15$); however, within MetS patients, serum sclerostin was higher in T2D than LADA ($p=0.01$). T2D with MetS had higher sclerostin

than LADA without MetS ($p=0.001$). A trend for increased sclerostin across the four groups shown in the figure, from LADA without MetS to T2D with MetS, was found ($p<0.0001$ for trend). Box plots show the 25th and 75th percentile, and the horizontal line shows the median (50th percentile). Bars outside the box indicate the minimum and maximum value. Circle and diamond symbols represent outliers and mean, respectively.

Figure 3 – Bone turnover markers in T2D, LADA and non-diabetic control subjects. (a) Subjects with T2D had 11% and 13% lower P1NP compared with LADA or non-diabetic subjects, respectively, although the differences were not significant; (b) the bone reabsorption marker CTX was significantly reduced in both types of diabetes compared with control subjects ($p\leq 0.006$). Data are presented as mean \pm standard deviation.

Table 1 – Clinical characteristics of LADA (Latent Autoimmune Diabetes in Adults) and T2D (Type 2 Diabetes) patients categorized according to the Metabolic Syndrome (MetS) status, and non-diabetic control subjects.

	1	2	3	4	5		
	T2D		LADA		Non-diabetic controls	p between T2D and LADA	p vs. group 5
Variables	With MetS	Without MetS	With MetS	Without MetS			
N	57	41	42	47	53		
Sex (Male/Female) ^a	35/22	24/17	22/20	27/20	17/36	ns	<0.05
Age (years) ^b	52.7 \pm 9.6	52.4 \pm 9.2	51.5 \pm 11.1	47.5 \pm 11.2	48.2 \pm 21.1	ns	ns
Time since diagnosis (years) ^b	2.9 \pm 1.7	2.5 \pm 1.8	2.7 \pm 1.9	2.4 \pm 2.3	NA	ns	NA
Waist Circumference (cm) ^b	104.9 \pm 9.1	90.2 \pm 12.8	98.7 \pm 12.4	82.3 \pm 11.4	90.0 (24.0)	<0.0001	\leq 0.01
BMI (Kg/m ²) ^c	30.4 (6.3)	25.6 (5.9)	27.6 (7.7)	22.6 (4.6)	26.2 (6.7)	<0.0001	<0.0001
HbA1c (%) ^b	6.7 \pm 1.4	6.5 \pm 1.2	7.9 \pm 1.5	7.2 \pm 1.5	NA	ns	NA
Creatinine (mg/dl) ^b	0.79 \pm 0.13	0.80 \pm 0.08	0.95 \pm 0.26	0.85 \pm 0.20	0.81 \pm 0.17	ns	ns
Triglycerides (mg/dl) ^c	157.0 (139.0)	101.9 (35.4)	165.0 (124.0)	70.0 (31.3)	97.0 (53.0)	<0.0001	<0.0001
HDL-cholesterol (mg/dl) ^c	37.1 (13.0)	52.6 (19.4)	46.4 (20.9)	65.0 (22.6)	54.5 (22.5)	<0.0001	<0.0001
Systolic Blood Pressure (mmHg) ^b	134.9 \pm 12.0	123.1 \pm 13.4	134.3 \pm 16.9	120.0 \pm 11.1	123.7 \pm 10.9	<0.0001	<0.0001
Diastolic Blood Pressure (mmHg) ^c	80.0 (10.0)	80.0 (11.0)	77.0 (20.0)	75.5 (10.0)	75.0 (10.0)	0.005	ns

^aData are expressed as absolute frequencies; ^bData are expressed as mean \pm SD; ^cData log transformed and are expressed as median (IQR); NA: not available/not applicable

Waist Circumference: 1 vs. 2, 1 vs. 4., 2 vs. 3, 2 vs. 4, 3 vs. 4, $p\leq 0.03$

BMI: 1 vs. 2, 1 vs. 3, 1 vs. 4, 2 vs. 4 and 3 vs. 4, $p\leq 0.019$;

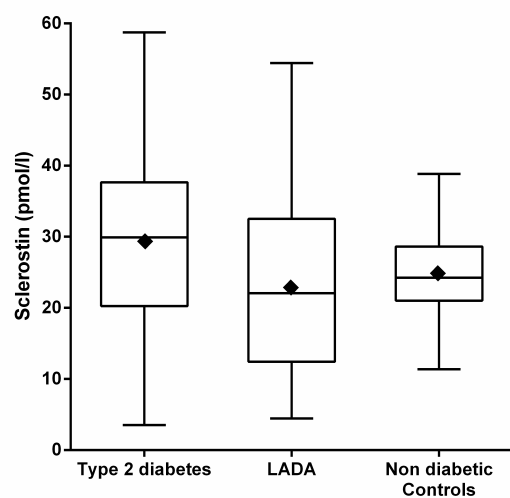
Triglycerides: 1 vs. 2, 1 vs. 3, 2 vs. 3, 2 vs. 4 and 3 vs. 4, $p\leq 0.01$;

HDL-cholesterol: 1 vs. 2, 1 vs. 4, 2 vs. 4 and 3 vs. 4, $p\leq 0.007$;

Systolic Pressure: 1 vs. 2, 1 vs. 4, 2 vs. 3 and 3 vs. 4, $p\leq 0.007$;

Diastolic Pressure: 1 vs. 2 and 1 vs. 4, $p\leq 0.048$.

Figure 1



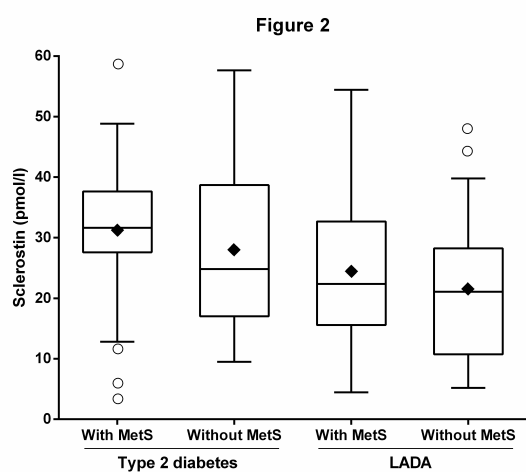


Figure 3

