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HDL Subclasses and the common CETP TaqIB variant predict the incidence of microangiopatic complications in type 2 diabetic women: a 9 years follow-up study

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INTRODUCTION

The prevalence of type 2 diabetes is globally increasing, with more than 400 million people affected around the world. This prevalence is expected to double within the next 20 years, with a significant increase in cardiovascular disease and in all the diabetes-related complications that are a major cause of disability, reduced quality of life and premature death in affected subjects (1).

Type 2 diabetes is associated with several microvascular complications including retinopathy, neuropathy, and nephropathy, all of which contribute to diabetes associated morbidity and mortality. Diabetic kidney disease (DKD) and diabetic retinopathy (DR) are the most common microvascular complications of diabetes. According to recent data, in 2005-2008 over 4 million patients (28.5%) with diabetes developed DR and 4.4% had a progression towards advanced stages of the disease (macular edema and proliferative retinopathy) potentially leading to vision loss (2).

Accordingly, diabetes is recognized as the primary cause of kidney failure. DKD, resulting in albuminuria, reduction of the glomerular filtration rate (GFR) or both, is one of the main causes of end-stage renal failure (ESRD) and its prevalence is increasing (3,4).

Hypertension and hyperglycemia play a major role in the progression of these two complications of diabetes. Up to date, hyperglycaemia and hypertension management is the gold standard of therapy to prevent microvascular complications, and epidemiological data suggest that the combined effects of blood pressure and glucose control may be even greater than the benefit of either intervention alone (5-7).

However, DKD and DR progress in many patients despite the achievement of the recommended targets for glycaemia and blood pressure (7). Thus, after the correction of these major risk factors, due to the better prevention and therapeutic options available, the spectrum of alterations associated with microangiopathy has enormously enlarged.

In particular diabetic dyslipidemia has been related to both DKD and DR (8,9), but to date there is no final agreement on considering these conditions as a relevant risk factor for microvascular complications.

Diabetic dyslipidemia, also defined as atherogenic dyslipidemia, recognizes as hallmark decreased levels of high density lipoprotein cholesterol (HDL-C) and elevated triglycerides (TG).

Growing evidence links diabetic dyslipidemia in type 2 diabetic subjectsnot only to cardiovascular risk and macrovascular events, but also to residual risk for new-onset or progression of microangiopatic complications (10).

A systematic review of association and intervention studies investigating the potential link between lipid subfractions and microvascular complications in diabetes indicated that dyslipidemia may cause the development or progression of microangiopatic disease (11).

However, not all available data are in accordance with this finding. Thus, several recent studies indicated an association of high TG/low HDL-C levels with the development of DKD (4,5), whereas other Authors did not find any association (12,13).

Diabetic dyslipidemia has also been associated with DR (9,14), although with controversial findings (15,16), or demonstrating an association between higher HDL-C levels and DR (17). Furthermore, other reports suggest that the impact of hypertriglyceridemia and low HDL-C levels may be different for DKD and RD, being high TG levels an independent risk factor for RD (18) and low-HDL-C significantly associated with DKD (16).

These controversial findings on the role of diabetic dyslipidemia on microvascular complications may depend upon several factors.

First, most studies investigating the potential associations between dyslipidemia and microvascular complications were of short duration with relatively small sample sizes, then resulting underpowered and with limited possibilities to generalize their results (11).

In addition, the association of atherogenic dyslipidemia with microangiopahty may be modulated by gender. Thus, diabetic dyslipidemia has no impact on DKD and RD in women with

type 2 diabetes (12,13) and lower HDL-C levels seem to be associated with the progression of DKD in men but not in women (19).

In addition, genetic background may play an important role and several genes coding for factors implied in TG/HDL metabolism may modulate the effects of lipids traits on microvascular outcomes. It has been shown that more than 50% of circulating HDL-C is genetically determined and the common TaqIB variant in the gene coding for cholesteryl ester transfer protein (CETP), a key enzyme in reverse cholesterol transport, has been associated with lower CETP activity, higher HDL-C levels and with amore atheroprotective HDL subpopulations profile (20,21), especially in diabetic women (22).

Finally, lipid modifications associated to diabetes are not limited to quantitative variations of TG/HDL-C but they comprise also subtler modifications of lipid fractions. Thus, measuring HDL-C or TG levels may not be sufficient to let the association of diabetic dyslipidemia with DKD and/or DR emerge. Notably, HDL particles differ in size and composition and it has been demonstrated that type 2 diabetes determines a shift in the distribution of Apo-AI containing HDL particles, with a depletion in the large lipid-rich α-1, α-2, and a rise in pre-α 1 and enriched in the small, lipid-poor α-3 HDL subpopulations (22). These modifications result in smaller and cholesterol poor HDL particles compared with those of non-diabetic subjects.

In order to better clarify these issues, here we report our findings on the effect of diabetic dyslipidaemia, the distribution of the HDL LpA-I and LpA-I:A-II subclasses and the common CETP TaqIB variant on the incidence or the progression of DKD and DR in a group of women with type 2 diabetes, after a ~9 years of follow-up.

MATERIALS AND METHODS

Study population

Clinical and laboratory data from 97 women with type 2 diabetes (T2D) attending the Metabolic Disease Outpatient Clinic of Messina University Hospital, Italy, collected between 1st January 2002 and 31st January 2015 (mean follow-up: 8.84 ± 3.88 years) were analyzed.

At baseline, exclusion criteria for all participants were: pregnancy, hormonal replacement therapy, oral contraceptive use or multivitamin supplementation, current treatment with β blockers, fibrates, statins, omega 3 fatty acids, niacin, fasting serum creatinine >1.5 mg/dL (>132.7_mol/L), macroalbuminuria (Albustix positive), any major medical condition in the last 6 months preceding the study.

All participants gave their informed consent and the study was approved by the Local Ethical Committee.

Measurements and assays

Lifestyle and clinical data were collected through a standardized questionnaire. BMI and blood pressure (BP) were measured according to standard procedures. Hypertension was defined as systolic blood pressure levels ≥140 mm Hg and/or diastolic blood pressure levels ≥90 mmHg, or current use of antihypertensive drugs (23).

T2D was diagnosed according to ADA criteria (24), and at baseline study subjects were treated with hypoglycemic drugs available at the time. In particular, none of participants was on incretin-based therapy or SGLT-2 inhibitors at the time of enrollment.

Blood samples were collected after a 12-14 hours fasting. Fasting plasma glucose and serum creatinine levels were measured with standard automated methods (Roche Diagnostics, Milan, Italy). Glycated haemoglobin was measured by an automated high-performance liquid chromatography

(HPLC) analyzer (Diamat; Bio-Rad Laboratories, Milan, Italy); normal range values in our laboratory are 4-6% (20-42 mmol/mol). Fasting insulin concentration was measured by radioimmunoassay (Diagnostic Corporation, LA, CA). Creatinine clearance (eGFR) was calculated by the MDRD formula (ml/min/1.73 m^2).

Markers of systemic inflammation, plasma lipids, lipoprotein, and HDL subpopulation measurements

All inflammatory markers, lipid and lipoprotein measurements were performed at the Lipid Metabolism Laboratory, Tufts University, Boston, USA. Plasma levels of hsCRP were measured on a Hitachi 911 (Roche Diagnostics, Indianapolis, Indiana) using the hsCRP kit from Wako Chemicals. Within- and between-run coefficients of variation were <5%. Plasma levels of interleukin-6 (IL-6) were determined by an ELISA assay (R&D Systems, Minneapolis, Minnesota).

Plasma total cholesterol and triglycerides levels were measured by automated enzymatic assays. HDL cholesterol was measured with a kit from Roche Diagnostics (Indianapolis, IN). Direct low-density lipoprotein cholesterol (LDL-C) was measured with reagents from Equal Diagnostics (Exton, PA). Plasma remnant-like particle (RLP) cholesterol levels were measured using an immunoseparation technique (Polymedco, Cortlandt Manor, NY) (25). This technique utilizes a monoclonal anti-apo-A-I antibody to remove HDL particles and an anti-apo B antibody that does not recognize partially hydrolysed lipoprotein remnants to remove both large nascent VLDL and LDL particles. The cholesterol content of the remaining remnant lipoproteins was then measured.

Apo-A-I-containing HDL subpopulations in plasma were measured by non-denaturing two-dimensional gel electrophoresis, as previously described (26). Briefly, HDL lipoproteins were first separated by charge, on agarose gel, into preβ-, α-, and preα-mobility particles. In the second dimension, each of these three fractions of HDL was further separated according to size (into preβ-1 and 2, α-1, -2, and -3, and preα-1, -2, and -3 by non-denaturing polyacrylamide gel electrophoresis.

This was followed by transfer into a nitrocellulose membrane and immunoblotting with a monospecific anti-apo-A-I primary antibody and a ^{125}I-labelled secondary antibody. Signals were quantitated by image analysis using a Fluoro Imager (Molecular Dynamics, Sunnyvale, CA). Apo-A-I concentrations of the subpopulations were calculated by multiplying the percent of each subpopulation by the plasma total apo-A-I concentration. The CV was <10% for α particles, and was <15% for all other subpopulations (16,21).

***CETP TaqIB (rs708272, G>A)* genotype**

All participants were genotyped for the CETP TaqIB (rs708272, G>A) polymorphism. A fragment of 535bp in intron 1 of the CETP gene was amplified by polymerase chain reaction (PCR) with the use of oligonucleotide primers (forward5′-CACTAGCCCAGAGAGA-GGAGTGCC-3′,reverse5′-CTGAGCCCAGCCGCACACTAAC-3′). The PCR products were subjected to restriction enzyme analysis by digestion with 4 U of the restriction endonuclease TaqI for 16 μL of PCR sample at 65° C for 2 h in the buffer recommended by the manufacturer (GIBCO-BRL); the fragments were separated by electrophoresis on an 1.5% agarose gel, and DNA fragments were visualized by UV illumination, after staining with ethidium bromide. The resulting fragments were 174 and 361 bp for the wild type B1 allele and 535 bp for the uncut B2 allele (22).

Assessment of diabetes long-term complications

Diabetic micro-and macroangiopathy were screened according to current Guidelines (24). Coronary heart disease (CHD) was defined as a history of myocardial infarction, chronic ischemic heart disease, coronary heart by-pass, coronary angioplasty, documented by cardiologist medical records and/or hospital discharge.As part of the annual screening, all our patients underwent to a standard electrocardiogram to exclude asymptomatic myocardial infarction or arrhythmia.

Diabetic retinopathy (DR) was diagnosed or excluded on the basis of direct ophthalmoscopy (through a dilated pupil) performed by an expert ophthalmologist and/or by fluorescein angiography within 1 year before the start of study. DR was classified as: Non-proliferative Diabetic Retinopathy, Proliferative Diabetic Retinopathy and Pan-retinal Photocoagulation. Retinopathy progression was defined as progression from Non-proliferative to Proliferative Diabetic Retinopathy and/orto Pan-retinal Photocoagulation; or progression from Proliferative Diabetic Retinopathy to Pan-retinal Photocoagulation.

Renal function was evaluated by measuring serum creatinine and calculating estimated Glomerular Filtration Rate (eGFR) by the Modification of Diet in Renal Disease (MDRD) equation (27). Diabetic nephropathy (DKD) was defined at baseline as the presence of micro- or macro-albuminuria and/or eGFR<60 ml/min/1.73 m2.

Statistical analysis

Numerical data are expressed as mean and standard deviation (minimum and maximum) and categorical variables as number and percentage.

For data analysis, a non-parametric approach was used for the low sample size and the non-normal distribution of some of the evaluated variables, as verified by the Kolmogorov Smirnov test. The Wilcoxon test was applied in order to evaluate any significant difference for numerical parameters and the McNemar test for dichotomous variables.

Univariate logistic regression models were estimated to assess the possible dependence of DR or DKD on potential explicative variables such as age, systolic blood pressure, diastolic blood pressure, BMI, duration of diabetes, hypertension, glycaemia, creatinine clearance, levels of insulin, T-C LDL-C, HDL-C, triglycerides, ApoAI, ApoAII, RLP-C, IL-6 and subpopulations of HDL. Variables with p-value<0.07 in univariate analysis were included in the multivariate stepwise logistic model, to identify independent predictive factors of DR. The same approach was used to test the possible dependence of DR progression.

A univariate logistic regression was performed to quantify the risk (OR) of developing DR or DKD, related to all study variables. Then, the linear regression models were determined using a *stepwise* selection procedure for the factors significant at the univariate analysis.

Statistical analyses were performed using SPSS 17.0 for Window package. All statistical comparisons are considered significant at the $P < 0.05$ level.

RESULTS

Baseline clinical characteristics of T2D women participating to the study

Baseline clinical characteristics of 97 diabetic women completing the follow-up evaluation are shown in **Table 1**. Study participants (mean age 57 years) were obese (mean BMI 32kg/m^2; mean waist circumference 100 cm), insulin-resistant (mean HOMA-IR values >7), with an acceptable metabolic control (mean HbA_{1c} 7.43%, 57 mmol/mol; mean FBG 163mg/dl) despite the relatively long duration of diabetes (mean 7 years). A family history of cardiovascular disease was present in the 37% of participants; 61% were affected by hypertension, with overall acceptable systolic and diastolic blood pressure values.

As for renal function parameters, values creatinine values were within the normal range (0.88±0.16 mg/dl), and creatinine clearance indicated only a mild renal impairment (69.36±13.62 ml/min). Inflammatory markers showed mean hsCRP values of 5.50mg/L, and mean IL-6 serum levels of 2.88 pg/ml.

At baseline, 26 subjects (27%) were affected by CHD and 7% of subjects had a diagnosis of neuropathy, 15.5% of diabetic retinopathy (DR), diabetic kidney disease (DKD) was present in 19.6% of subjects (2.1% microalbuminuria and 18.5% eGFR <60 ml/min).

As shown in **Table 2**, diabetic women showed the typical lipid profile associated with insulin-resistant states, with overall lipid values close to recommended targets (mean LDL-C 116 mg/dl,

HDL-C 49 mg/dl and TG 123 mg/dl). Apo AI and AII levels, LDL/HDL ratio, sdLDL-C and RLP-C were also concordant with the overall lipid profile. Table 1 shows plasma levels of Apo AI HDL-C subpopulations as determined by 2-gradient gel electrophoresis. Mean levels of the large lipid rich α-1 were 16.34 mg/dl, α-2 were 33.39 mg/dl and pre-α1 were 4.81 mg/dl; and the smaller α-3 HDL subparticles were 14 mg/dl.

The distribution of CETP TaqIB genotype did not differ from the frequencies predicted from the Hardy-Weinberg equilibrium (data not shown), with a B2B2 homozigous frequency of 14.4 % (**Table 2**).

Diabetic microvascular complications in T2D women at follow-up

Over a mean follow-up of 8.84±3.88 years (**Figure 1**), there were 23 incident cases of DKD (9 incident cases of microalbuminuria and 16 of moderate renal impairment), and 12 incident cases of DR. Main clinical characteristics (BMI, HbA1c) at follow-up were did not significantly different from baseline, with the exception of the percentage of subjects with hypertension: 61% vs 84.5%, P<0.001 (**Supplemental Table 1**).

An intensification of hypoglycaemic treatment was observed at the follow-up evaluation, with a reduction in the percentage of subjects on diet (7.2 vs. 13.4%) and sulphonylureas (18.6 vs 45.4%) and an increment of those treated with incretin-based therapies (10.3% vs none) and insulin(38.1 vs 9.3%). As for lipid-lowering medications, because of study design, none of study subjects was on statins and/or ezetimibe at baseline, and only 4 subjects (4.1%) were on stable treatment with omega 3/fibrates; at the end-of follow-up observation, 60.8% of T2D women were on treatment with statins, 6 (6.18%) with omega 3 or fibrates and 7 (7.2%) with ezetimibe (**Supplemental table 1**).

Baseline characteristics of T2D women participating to the study, according to incident diabetic kidney disease and diabetic retinopathy

Baseline characteristics of diabetic women who developed or not DKD and DR at follow-up are shown in **Tables 3 and 4**. Study participants who developed DKD, as defined by either albuminuria and/or eGFR values <60 ml/min were more obese (mean BMI 36 kg/m^2 vs 31 kg/m^2, p=0.022; waist circumference 103 cm vs 98 cm, P=NS) and showed comparable baseline eGFR values (73 ml/min vs 76 ml/min, P=NS) to those who maintained normal eGFR and albuminuria values. diabetes duration, metabolic and blood pressure control were comparable between the two groups. Inflammatory markers were significantly higher in the DKD group, although only differences in IL-6 values were statistically significant (P=0.032).

As for lipid profile, DKD women shower lower HDL-C (P=0.08) and higher LDL/HDL ratio values (P=0.07), with slightly higher LDL-C, triglycerides, and RLP-C serum levels and lower ApoAI concentrations, although all these difference were not statistically significant. HDL subpopulation analysis revealed significantly lower levels of the larger HDL α-1 levels in DKD women than in controls (P=0.02), with trivial alterations of the overall HDL profile toward a decrease of larger and more atheroprotective fractions. CETP TaqIB genotype distribution was not different between the two groups.

As compared to those who did not show any degree of DR at follow-up, the DR positive group had higher body weight and BMI but, unlike the DKD group, metabolic control was worst compared to RD-free women (P<0.0001 for HbA1c and P=0.003 for FPG). Also circulating levels of inflammatory markers were higher in the DR positive group, with a trend for the increase of IL-6 levels (P=0.09). Baseline lipid profile in DR women showed higher levels of TG and remnants-associated cholesterol (P=0.002 and P=0.007, respectively) and ~10 mg lower levels of HDL-C (P=0.01) than women without DR at follow-up. Also LDL/HDL ratio and sdLDL-C were higher in the DR group (P=NS). As for HDL subpopulations, HDL α-1 and pre α-3 HDL levels were significantly higher and the smaller, less atheroprotective α-3 significantly lower in women who

developed DR at follow-up than those who did not develop this complication (P<0.05 all). The CETP TaqIB genotype distribution was also different between the two groups, with a higher percentage of B2 carriers (P=0.07) among T2D women developing DR.

Factors associated to the development of DKD and DR at univariate and stepwise multivariate regression analysis

In this cohort of T2D women, BMI, LDL/HDL ratio and HDL α-1 levels were the variables associated tothe incidence of DKD at univariate regression analysis**,** but BMI was the stronger predictor of DKD incidence at stepwise multivariate regression analysis **(Table 5)**.

HbA1c, tryglicerides, HDL-C and HDL subfractions α-1, α-3, Pre α-3and Pre-β2 levels and the CETP TaqIB polymorphism were the variables associated to incident DR at univariate regression analysis**,** in this population (**Table 5**). At stepwise multivariate regression analysis, HbA1c, tryglicerides and HDLα-3 levelswere the factors independently associated to the incidence of DR.

Similarly, levels of HbA1c, fasting plasma glucose, triglycerides, HDL-C, HDL particles α-1, α-3,α-4 and Pre α-3 and the CETP TaqIB polymorphism were the variables associated tothe progression of DR at univariate regression analysis **(Supplemental table 2)**. At stepwise multivariate regression analysis, levels of HbA1c (OR 2.515; 95% CI 1.405-4.501), triglycerides (OR 1.008; 95% CI 1.00-1.015) and HDL α-3 (OR 1.208; 95% CI 0.944-1.468; P=0.057), were the factors independently associated to the progression of DR in this population.

DISCUSSION

Our data show that baseline BMI, HbA1c, triglycerides, HDL-C levels and HDL subpopulations, and to a minor extent the common CETP TaqIB polymorphism, predict the occurrence of microvascular complications in women with type 2 diabetes followed-up for ~9 years.

The pathophysiologic role of glucose control, as documented by HbA1c values, on the incidence of DKD and RD is undisputed, both in subjects with type 1 and type 2 diabetes (28,29). Blood pressure control is the other fundamental step in the prevention of microangiopathy (24), but in our cohort, it did not emerge as an independent risk factor, likely because baseline BP values were already optimal, and up to 61% of study subjects were under anti-hypertensive medications.

Besides HbA1c and blood pressure, evidence indicating the association of plasma lipids with microangiopathy is increasingly robust (25). In particular, diabetic dyslipidemia has been associated to DR, DKD (8,18) and diabetic neuropathy (31).

In diabetic women participating to our study, higher triglycerides and lower HDL-C levels were associated with both DR and DKD, although the independent association with DKD was attenuated after multivariable adjustment, leaving only BMI as the stronger risk factor.

Sacks et al. recently demonstrated in a large multi-centric study that DKD is associated with higher levels of plasma triglycerides and lower levels of HDL-C among diabetic patients with good control of LDL-C, whereas RD was less robustly associated with lipid abnormalities (8). In the RIACE study odds ratio for microvascular disease increased 1.16 times for every 0.5 mmol/L increment in triglycerides or decreased by a factor of 0.92 for every 0.2 mmol/L increase in HDL-C (18). In another Italian study, individuals with type 2 diabetes with conserved baseline renal function and without RD and CVD, TG/HDL-C ratio was positively associated with an increased risk of the composite end-point of DKD and RD after fully adjustment for numerous confounders (30). Similar results were obtained in another study on a large cohort of T2D adults where higher plasma levels of HDL-C were

associated with a lower risk of incident DKD independently of numerous confounding factors after 5 years of follow-up (32).

However, these findings were not confirmed by other Authors (33) and the impact of serum lipid abnormalities on the incidence of microangiopathy remains conflicting. These contrasting results may depend upon several factors. First, besides HDL-C and triglycerides levels, diabetic dyslipidemia is far more complex and it involves subtler alteration of lipid profile, with modifications in lipoproteins, lipids and proteins composition and function, that may show a stronger association with DR and DKD. Accordingly, it has been demonstrated that ApoAI and apoB and the apoB-to-apoAI ratio were significantly and independently associated with DR, DR severity and improved the ability to discriminate DR by 8%, suggesting that serum apolipoprotein levels may be stronger biomarkers of DR than traditional lipid measures (34).

Furthermore, HDL is a heterogeneous class of particles, differing in apolipoprotein and lipid composition, size, charge, and density. HDL, separated according to their apolipoprotein composition into subclasses containing only apoA-I (LpA-I) or both apoA-I and apoA-II (LpA-I:A-II), have been shown to differently promote cholesterol efflux, suggesting that different subpopulations may have a different role in reverse cholesterol transport and cardiovascular risk protection (35). Our study is the first report investigating the potential association of HDL subclasses, as separated and quantified by two-dimensional gel electrophoresis and image analyses (26), with diabetes microvascular complications.

Our data showed that that DR incidence and progression were associated with lower levels of the larger and more athero-protective α-1 and pre-β2 HDL and higher levels of the lipid poor HDL α-3 and preα-3 HDL particles. DKD incidence was also independently associated with lower levels of α-1HDL, although this association did not emerged after multivariable adjustment.

Overall, these associations are concordant to what observed with CHD risk. Thus, Asztalos et al. demonstrated that this same HDL subclasses trend was more strongly associated with CHD risk than

HDL-C levels in men participating to the Framingham Offspring study and to the VA-HIT study (36). Furthermore, when a subgroup of our diabetic female cohort was compared to control women without diabetes, HDL subclasses profile was clearly shifted toward less protective particles (22).

These results overall suggest that micro-and macrovascular complications may share common pathways in diabetic subjects. In a cross-sectional study on diabetic subjects it has been demonstrated that DR was an independent risk marker for subclinical atherosclerosis in patients with newly diagnosed type 2 diabetes (37). In another cross-sectional study, the severity of microangiopathy correlated with severity of carotid atherosclerosis (38).

The association of DKD risk with dyslipidemia may also be influenced by gender. Thus, it has been demonstrated that gender differences exist in the prevalence of DKD clinical manifestations, with diabetic women showing more GFR reduction while micro- and macroalbuminuric DKD occurs more frequently in men. Furthermore, a single-center observational study found that lower HDL-C levels were associated with the progression of DKD in men but not in women (33). After a median follow-up of 8 years, a large Japanese study demonstrated that male gender was an independent predictor for the onset of albuminuria, whereas no gender-specific association was found for GFR decline (39); in another Japanese study, lower HDL cholesterol levels seemed to be associated with the progression of DKD in men but not in women (19). Our results are in contrast with these observations, since our data clearly demonstrate that diabetic dyslipidemia and its features are a risk factor for both DR and DKD in diabetic female outpatients. Genetic differences between Japanese cohorts and our Mediterranean population may partly explain these contrasting results. Thus, several genes-gender interactions have been reported to influence lipid traits and CHD risk (22,40,41).

As for microangiopathy, several genes (42), including genes involved in lipid metabolism, have been implied as risk factors for both DKD and RD. Among candidate genetic risk factors, the common CETP TaqIB polymorphism is a strong contributor of HDL-C variability (22) and thus it may potentially be one of this risk genes. To date, only few reports have examined the association of

this polymorphism with microvascular complication in subjects with either type 1 and type 2 diabetes. In 494 patients with proliferative RD and various stages of DKD participating to the GENEDIAB Study, distribution of LPL, CETP and Apo E genes polymorphisms did not differ in terms of renal complications, suggesting that genetic basis for lipid disturbances had only marginal effects on diabetic nephropathy (43).

The potential pathophysiological mechanisms underlying the association between dyslipidemia and diabetes microvascular complications are still poorly understood.

According some evidences, dyslipidemia may cause or exacerbate DR and DKD by alterations in the coagulation-fibrinolytic system, changes in membrane permeability, damage to endothelial cells and increased atherosclerosis. All these mechanisms may lead to a faster decline in GFR and progression of albuminuria or progression of retinal damage (11).

Notably, the role of lipid fractions in the microvascular disease may also be mediated by a modulation of inflammation. In our cohort, type 2 diabetic women developing both DKD and DR showed higher levels of the inflammation marker IL-6. Besides their role in reverse transport of cholesterol, HDL particles are known to exert their antiatherosclerotic role through several other mechanisms, including a modulation of inflammation (44).We recently described, in women with type 2 diabetes, that the more atheroprotective HDL subpopulations are associated with lower levels of inflammatory markers IL-6 and hsCRP, suggesting that different HDL particles may exert a different role in inflammation process (45). Thus, the role of HDL-C and HDL subfractions in inflammation modulation could be involved not only in macroangiopatic complications and cardiovascular risk but also in the development and progression of microangiopathy.

Our study has several limitations and strengths. The small sample size and being a single center study are among the limitations, whereas the genetic homogenous cohort, the well-controlled and long follow-up and the evaluation, for the first time, of HDL subpopulations and CETP polymorphism are to be taken into account.

In conclusions, our data demonstrate that in diabetic women, atherogenic dyslipidemia as well as subtler modifications in lipoprotein particles profile and to a minor extent the common CETP Taq1B polymorphism are associated with incidence and progression of microvascular disease.

Taken together, these results strengthen the rationale for focusing on dyslipidemia treatment and for controlling targets of triglycerides and HDL-C, beyond LDL-C control alone, to prevent both macrovascular and microvascular disease in subjects with type 2 diabetes.

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Table 1- Baseline clinical characteristics of type 2 diabetic women participating to the study.

n	97
Age (yrs)	56.6±11.62
Diabetes duration (years)	7.11±7.54
Cardiovascular disease family history n (%)	36 (37.1)
BMI (kg/m^2)	32.0±6.56
Waist circumference (cm)	100.03±12.38
Systolic blood pressure (mmHg)	134.02±17.06
Diastolic blood pressure (mmHg)	78.2±8.96
Hypertension n (%)	59 (61)
Fasting plasma glucose (mg/dl)	163.09±48.53
Fasting insulin (mU/l)	17.84±12.67
HbA_{1c} (%)	7.43±1.49
Creatinine (mg/dl)	0.88±0.16
Creatinine clearance MDRD (ml/min/1.73 m^2)	69.36±13.62
Inflammatory markers	
hsCRP (mg/L)	5.50±6.69
IL-6 (pg/ml)	2.88±3.06
Diabetes complications	
Coronary heart disease n (%)	26 (27)
Neuropathy n (%)	7 (7.2)
Retinopathy n (%)	15 (15.5)
Diabetic kidney disease n (%)	19 (19.6)
Microalbuminuria n (%)	2 (2.1)
eGFR<60 ml/min/1.73m^2 n (%)	18 (18.5)

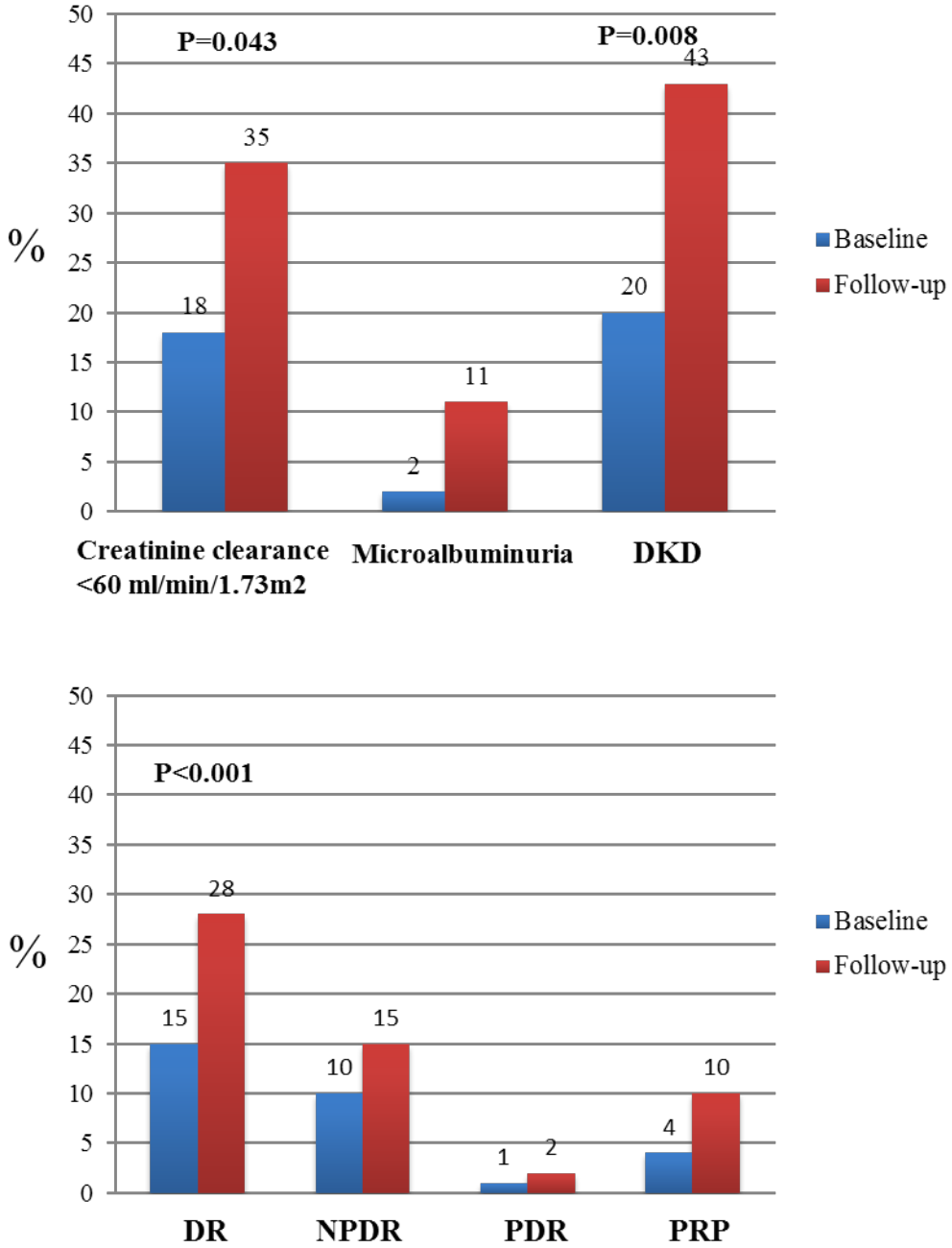
Data are n, %, means ± SD. hsCRP, C-reactive protein; IL-6, interleukin 6.

Table 2- Baseline lipid profile and CETP Taq1B Genotype distribution of type 2 diabetic women participating to the study.

Lipid profile	
T-C (mg/dl)	188.89±29.16
LDL-C (mg/dl)	115.59±29.21
HDL-C (mg/dl)	48.7±12.35
Triglycerides (mg/dl)	122.97±72.26
LDL/HDL	2.55±1.03
SdLDL-C (mg/dl)	10.35±7.45
RLP-C (mg/dl)	8.31±6.60
Apo AI (mg/dl)	124.53±18.24
Apo AII (mg/dl)	30.15±4.60
ApoAI containing HDL subpopulations	
α-1 (mg/dl)	16.34±6.15
α-2 (mg/dl)	33.39±4.99
α-3 (mg/dl)	14.47±3.90
Pre-α1 (mg/dl)	4.81±2.39
Pre-α3 (mg/dl)	1.97±0.83
Pre-β2(mg/dl)	1.51±0.77
CETP Taq1B Genotype distribution	
B1B1 n (%)	34 (35.1)
B2 *carriers* n (%)	63 (64.9)

Data are n, %, means ± SD. sdLDL-C, small dense LDL-cholesterol; RLP-C, Remnants associated cholesterol.

Figure 1- Incidence of diabetic kidney disease (DKD) and diabetic retinopathy (DR) at follow-up



DKD, diabetic kidney disease; DR, diabetic retinopathy, NPDR, Non-proliferative Diabetic Retinopathy; PDR, Proliferative Diabetic Retinopathy; PRP, Pan-retinal Photocoagulation.

Table 3- Baseline characteristics of diabetic women according to incident diabetic kidney disease (DKD) and diabetic retinopathy (DR) at follow-up.

	Diabetic kidney disease (DKD)		**Diabetic retinopathy (DR)**	
	Diabetic women not developing DKD	*Diabetic women developing DKD*	*Diabetic women not developing DR*	*Diabetic women developing DR*
n	40	24	60	12
Age (yrs)	52.20±10.61	52.71±8.02	55.27±11.98	53.58±9.33
Diabetes duration (years)	5.24±5.63	6.42±7.16	5.38±7.09	8.30±5.46
BMI (kg/m^2)	31.53±5.69	35.69±8.52*	31.45±6.28	34.96±10.25
Waist circumference (cm)	98.03±11.90	103.10±15.00	98.80±12.06	97.88±14.56
Systolic blood pressure (mmHg)	129.88±15.30	130.83±16.85	133.42±17.77	133.33±20.15
Diastolic blood pressure (mmHg)	77.38±8.16	77.08±8.06	77.58±9.04	79.58±9.64
Hypertension n (%)	19 (47.5)	13 (55.2)	33 (55)	7 (58.3)
Fasting plasma glucose (mg/dl)	165.73±51.54	167.79±51.35	152.08±42.53	192.92±43.27*
Fasting insulin (mU/l)	18.40±11.94	19.34±11.48	17.21±11.84	18.62±10.02
HbA_{1c} (%)	7.20±1.39	7.17±1.42	6.99±1.37	8.56±0.90*
Creatinine (mg/dl)	0.80±0.08	0.84±0.10	0.86±0.12	0.80±0.12
Creatinine clearance (ml/min/1.73 m^2)	76.46±10.12	72.81±12.31	70.49±12.59	77.64±14.47
Inflammatory markers				
hsCRP (mg/L)	5.52±6.88	8.06±8.82	5.27±6.39	8.47±10.77
IL-6 (pg/ml)	2.20±0.89	4.19±5.57*	2.66±2.12	4.50±6.92

Data are n, %, means ± SD. Only significant P values are shown.; hsCRP, C-reactive protein; IL-6, interlekin 6. * P<0.05

Table 4- Baseline lipid profile and CETP Taq1B Genotype distribution of diabetic women according to incident diabetic kidney disease (DKD) and diabetic retinopathy (DR) at follow-up.

Lipid profile				
T-C (mg/dl)	190.05±29.77	193.58±29.45	189.33±28.08	195.83±34.11
LDL -C(mg/dl)	115.34±31.63	122.03±30.05	116.67±28.61	118.44±40.55
HDL-C(mg/dl)	50.64±12.64	45.07±11.17	50.64±12.78	40.38±12.06*
Triglycerides (mg/dl)	120.40±96.44	132.42±49.54	110.15±47.11	185.00±155.26*
LDL/HDL	2.41±0.97	2.95±1.32	2.49±1.06	3.07±1.31
SdLDL-C (mg/dl)	9.08±4.76	9.08±6.15	9.58±7.36	12.54±6.09
RLP-C (mg/dl)	8.34±8.72	8.76±4.41	7.20±3.96	13.34±14.85*
Apo AI (mg/dl)	127.73±18.63	123.50±16.79	126.85±18.48	117.50±17.96
Apo AII (mg/dl)	30.78±5.54	30.08±3.80	30.05±4.92	30.92±3.82
ApoAI containing HDL subpopulations				
α-1 (mg/dl)	17.51±5.93	14.10±5.11*	17.88±5.79	12.03±5.40*
α-2 (mg/dl)	34.12±4.92	34.09±4.44	33.63±4.86	33.76±4.69
α-3 (mg/dl)	13.84±3.73	14.67±3.63	13.85±3.32	17.37±4.69*
Pre-α1 (mg/dl)	4.66±2.03	4.15±2.33	5.03±2.44	3.88±2.45
Pre-α3 (mg/dl)	1.82±0.83	2.01±0.72	2.33±0.87	2.57±1.31*
Pre-β2(mg/dl)	1.46±0.71	1.56±0.83	1.50±0.74	1.07±0.49
CETP TaqIB genotype distribution				
B1B1 n (%)	15 (37.5)	7 (29.2)	19 (32)	7 (58.3)
B2 *carriers* n (%)	25 (62.5)	17 (70.8)	41 (68)	5 (41.6)

Data are n, %, means ± SD. Only significant P values are shown.; sdLDL-C, small dense LDL-cholesterol; RLP-C, remnants associated cholesterol. * P<0.05

Table 5- Factors associated to incidence of diabetic kidney disease (DKD) and diabetic retinopathy

Factors associated to DKD incidence								
	Univariate regression analysis				***Stepwise* regression analysis**			
	B	**P**	**Exp(B)**	**95.0% CI**	**B**	**P**	**Exp(B)**	**95.0% CI**
BMI	0.108	0.011	1.114	1.025-1.210	0.102	0.02	1.107	1.016-1.207
LDL/HDL	0.457	0.048	1.579	0.999-2.495				
HDL α-1	-0.099	0.042	0.906	0.824-0.996	-	-	-	-
Factors associated to DR incidence								
	Univariate regression analysis				***Stepwise* regression analysis**			
	B	**P**	**Exp(B)**	**95.0% CI**	**B**	**P**	**Exp(B)**	**95.0% CI**
HbA1c	0.688	0.004	1.989	1.250-3.165	0.695	0.02	2.004	1.117-3.595
TG	0.014	0.021	1.04	1.002-1.026	0.009	0.033	1.009	1.001-1.017
HDL-C	-0.079	0.011	0.924	0.869-0.982				
HDL α-1	-0.203	0.006	0.817	0.707-0.944	-	-	-	-
HDL α-3	0.267	0.003	1.306	1.093-1.562	0.294	0.009	1.345	1.076-1.683
HDL Pre α-3	0.770	0.021	2.160	1.122-4.160	-	-	-	-
HDL Pre-β2	-1.060	0.053	0.346	0.118-1.016	-	-	-	-
CETP B1B1	1.199	0.028	0.301	0.103-0.878				

Only significant P values are shown. TG, triglycerides

Supplemental Table 1 - Clinical characteristics and current therapies of diabetic women at baseline and follow-up

	Baseline	**Follow-up**	**P**
Duration of follow-up (years)	-	8.84 ± 3.88	
BMI (kg/m^2)	32±6.56	31.35 ± 6.60	-
HbA_{1c} (%)	7.43±1.49	7.49±1.32	-
Hypertension (%)	59 (61)	82 (84.5)	<0.001
Creatinine (mg/dl)	0.88±0.16	0.91±0.32	-
eGFR (ml/min/1.73 m^2)	69.36±13.62	75.82±28.28	-
Hypoglycaemic drugs			
Only diet n (%)	13 (13.4)	7 (7.2)	-
Metformin users (%)	69 (71.1)	61 (62.9)	-
Sulfanylureas users n (%)	44 (45.4)	18 (18.6)	<0.001
Repaglinide users n (%)	7 (7.2)	17 (17.5)	0.03
Pioglitazone users n (%)	2 (2)	3 (3.09)	-
Acarbose users n (%)	0	3 (3.09)	-
Incretin-based therapies n (%)	0	10 (10.3)	0.001
Insulin users (%)	9 (9.3)	37 (38.1)	<0.001
Lipid-lowering medications			
Statin users n (%)	0	59 (60.8)	<0.001
omega 3/fibrates users n (%)	4 (4.1)	6 (6.18)	-
Ezetimibe users n (%)	0	7 (7.2)	0.007

Data are n, %, means ± SD.

Supplemental Table 2 - Factors associated to progression of diabetic retinopathy (DR) at follow-up at univariate and stepwise multivariate regression analysis.

Factors associated to DR progression								
	Univariate regression analysis				***Stepwise* regression analysis**			
	B	**P**	**Exp(B)**	**95.0% CI**	**B**	**P**	**Exp(B)**	**95.0% CI**
FPG	0.010	0.055	1.01	1.0-1.021	-	-	-	-
HbA1c	0.927	<0.001	2.527	1.507-4.238	0.922	0.002	2.515	1.405-4.501
TG	0.012	0.029	1.012	1.001-1.022	0.008	0.045	1.008	1.00-1.015
HDL-C	-0.062	0.02	0.939	0.891-0.990				
HDL α-1	-0.152	0.012	0.859	0.762-0.968	-	-	-	-
HDL α-3	0.197	0.013	1.218	1.042-1.424	0.189	0.057	1.208	0.994-1.468
HDL α-4	0.219	0.057	1.244	0.993-1.559	-	-	-	-
HDL Pre-α3	0.590	0.056	1.804	0.985-3.304	-	-	-	-
CETP B1B1	1.067	0.025	0.344	0.135-0.875				

Only significant P values are shown. FPG, fasting plasma glucose; TG, triglycerides.