Lipid Transfer to HDL in Type-2 Diabetic Patients: Associations with Microalbuminuria, Statin, and Insulin

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Summary

Background: Type-2 diabetes mellitus (T2DM) is an isolated risk factor for coronary artery disease, especially when associated with microalbuminuria (MA). Structural and functional changes in lipoproteins have not yet been fully elucidated in this context.

Objective: To assess lipid transfer (T) to HDL in type-2 diabetic patients and its association with microalbuminuria and treatment with statins or insulin.

Methods: Thirty-three patients with type-2 diabetes mellitus and 34 age-matched control subjects were studied. A synthetic cholesterol-rich nanoemulsion radiolabeled with ^{3}H- triglycerides (TG) and ^{14}C-free cholesterol (FC) or ^{3}H-cholesteryl ester (CE) and ^{14}C-phospholipids (PL) was incubated with plasma. Both the nanoemulsion and lipoproteins were precipitated, except for HDL, which was counted for radioactivity.

Results: PLT (%) was higher in the T2DM group than in the control group (25.2 ± 3.2 and 19.7 ± 3.2 respectively; p < 0.001), as was free cholesterol (% FC): 9.1 ± 2.7 and 6.3 ± 1.5 respectively; p < 0.001. The diagnosis of microalbuminuria (MA) was not associated with changes in lipid transfers. Insulin therapy was associated with lower PLT rates: 23.5 ± 2.1 *versus* 26.1 ± 3.3; p = 0.018. Statin therapy, in turn, was associated with a drop in all lipid transfers - CET 3.5 ± 0.9; PLT: 23.8 ± 2.0; TGT: 3.9 ± 0.8; FCT: 7.4 ± 1.3 - as compared to the group that was not on statin therapy (CET: 5.9 ± 2.4; PLT: 26.9 ± 3.6; TGT: 6.4 ± 2.2; FCT: 11.1 ± 2.6).

Conclusion: Type-2 diabetes mellitus increased lipid transfer to HDL particles, whereas statin therapy decreased all lipid transfers. The presence of MA was not associated with changes in lipid transfer. (Arq Bras Cardiol 2009;92(2):94-100)

Key words: Cholesterol, HDL; lipoproteins; diabetes mellitus, type 2; insulin; hydroxymethylglutaryl-CoA reductase inhibitors; albuminuria; diabetic nephropathies.

Introduction

Type-2 diabetes mellitus (T2DM) is a major cardiovascular risk factor, and atherosclerosis is the most common cause of death in diabetic patients. The dyslipidemia of T2DM is characterized by hypertriglyceridemia, decreased HDL-cholesterol, and increased small dense LDL particles[1,2]. Statins significantly decrease the risk of cardiovascular events in T2DM pacients[3-5]. The presence of microalbuminuria increases the risk of coronary artery disease[6,7].

Lipoprotein are composed of different amounts of triglycerides and esterified cholesterol in their hydrophobic core and phospholipids and free cholesterol on their amphipatic surface, where apolipoproteins are located. HDL has antiatherosclerotic activity, the mechanism of which is not entirely understood. The most likely hypothesis is reverse cholesterol transport. Nevertheless, other mechanisms, such as anti-inflammatory, antithrombotic, and antioxidant properties[8,9], seem to play a role in the protective effect of HDL.

Reverse cholesterol transport (RCT) is the process by which excess cholesterol is removed from peripheral tissues to the liver for subsequent excretion. Lecithin:cholesterol acyltransferase (LCAT) contributes to this transport by catalyzing the esterification of cholesterol, thus allowing it to be transferred to the hydrophobic core of the HDL particle.

Plasma lipoproteins exchange lipids constantly, a process facilitated by transfer proteins, such as cholesteryl ester transfer protein (CETP), which mediates the exchange of cholesterol esters, triglycerides, and phospholipids between lipoproteins[10], and phospholipid transfer protein (PLTP), which mediates phospholipid and cholesterol transfer from other triglyceride-rich lipoproteins to HDL[11,12].

Plasma PLTP[13,14] and CETP[15,16] activities seem to be higher in type-2 diabetic patients. The role of microalbuminuria

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in transfer protein activities has yet to be defined[17]. Some studies, however, show that the use of statin[18-21] and insulin[22-26] decreases plasma CETP and PLTP.

Lipid transfers depend on several other factors as well. Plasma concentration of each lipoprotein subclass influences these exchanges, since they are brought about by collisions between lipoprotein particles. The lipid and protein content of the particle and concentrations of several other plasma proteins may play a role in this process.

The complex relationship between lipid transfer and atherogenesis remains unclear. Lipid transfer plays a critical role in plasma HDL metabolism, by constantly remodeling lipoprotein particles and, thereby, interfering with their many functional aspects.

The purpose of this study was twofold: first, to assess, in T2DM patients, the simultaneous transfer to HDL of the four major lipids that make up its structure, namely, free cholesterol, cholesterol ester, triglycerides, and phospholipids; and second, to ascertain the influence of microalbuminuria, as well as that of statin or insulin therapy, on lipid transfer.

Methods

Thirty-three volunteer patients with type-2 diabetes mellitus were recruited from the outpatient services of the Hospital das Clínicas Heart Institute of the University of São Paulo Medical School (InCor - HCFMUSP) and the Diabetes Outpatient Clinic of the Division of Diabetes of the Discipline of Endocrinology and Metabolism of the *Hospital das Clínicas* of the University of São Paulo Medical School. Patients taking protease inhibitors, immunosuppressive agents, or corticosteroids were excluded from the study, as were transplant recipients and patients with liver cirrhosis, neoplasias, thyroid disease, or serum creatinine > 1.5 mg/dL. All patients signed an informed consent form. Blood and urine samples were collected at the Laboratory of Lipid Metabolism of InCor. HDL functional assays (lipid transfer) were performed at the same laboratory. Urine samples were tested at *Hospital das Clínicas* Central Laboratory, and blood samples at the InCor laboratory.

Thirty-four age-matched subjects were selected from the database of the Laboratory of Lipid Metabolism to serve as control group. No subject in this group had any comorbidity or was taking any medication. Those with fasting plasma glucose equal to or greater than 100 mg/dl were excluded.

All participants were interviewed about their medical history, with particular emphasis on past conditions and current medications. Laboratory tests were performed on a blood sample and two or three urine samples collected on different days to measure the urinary albumin/creatinine ratio.

Serial laboratory tests

The following parameters were measured in diabetic and control subjects: total cholesterol (TC), lipoprotein fractions (VLDL, LDL, and HDL), and fasting plasma glucose - in serum samples obtained after 12 hours of fasting - using the enzymatic colorimetric method (COD-PAD; Labtest). LDL-cholesterol was quantified using the Friedewald formula[27]: LDL-C = TC-(VLDL-C+HDL-C), in mg/dL, where VLDL-C is estimated by dividing triglyceride value by 5. Glycated hemoglobin (HbA1c) was measured in whole blood by immunoturbidimetric assay at the laboratory of InCor-HCFMUSP. The presence or absence of microalbuminuria was determined in first-morning spot urine samples by the nephelometric method at the *Hospital das Clínicas* Central Laboratory. Urine creatinine was measured in the same sample, and the albumin-creatinine ratio was expressed in mcg/mg of creatinine. Patients with urinary albumin-creatinine ratio less than 30 mcg/mg were considered normoalbuminuric, whereas those with urinary albumin-creatinine ratio between 30 and 300 mcg/mg were considered microalbuminuric, according to the American Diabetes Association criteria[28].

Urine samples were collected on different days, outside of menses for women, from patients with no symptoms of urinary tract infection. If the results of the two tests did not agree, a third sample was requested.

LDE as an investigative tool

In earlier studies, the Laboratory of Lipid Metabolism of the Heart Institute (InCor-HCFMUSP) reproduced LDL metabolism using a synthetic emulsion with a lipid composition resembling that of the native LDL (LDE)[29]. The primary purpose of these studies was to use LDE to gain further insight into dyslipidemias. LDE is protein-free, but when it comes into contact with native lipoproteins it acquires apo E, which may be recognized by LDL receptors and, thereby, taken up by the cell[29]. This emulsion was prepared according to the technique described by Ginsburg et al[30] and modified by Maranhão et al[29].

Transfer of free cholesterol, cholesterol ester, triglycerides, and phospholipids from lde to hdl

Blood samples were collected after a 12-hour fast in EDTA-containing tubes (1.5 g/l), and plasma was obtained after centrifugation at 3.000 rpm for 10 minutes in a Sorval RT7 centrifuge. Two 200-μ plasma samples were incubated with 50 μl of LDE each for 60 minutes at 37°C in a Gyromax 706R orbital shaker and agitated at 40 rpm. Each 50 μl of LDE was radiolabeled with ^{3}H- cholesteryl ester (^{3}H-CE) and ^{14}C-phospholipids (^{14}C-PL) or ^{3}H- triglycerides (^{3}H-TG) and ^{14}C-free cholesterol (^{14}C-FC). After incubation, 250 μl of precipitating reagent for apo-B-containing lipoproteins (dextran sulfate 0.2%/ $MgCl_2$ 3M, v/v) was added to the tubes. The resulting mixtures were vortexed for 30 seconds and centrifuged for 10 minutes at 3.000 rpm. The HDL fraction was obtained after precipitation of the nanoemulsion, together with apo B-containing lipoproteins, with 250 μl of dextran/$MgCl_2$ (0.2% Dextran and 0.3 mol/L $MgCl_2$). Aliquots (250-μl) of supernatant containing HDL were transferred with a pipette to scintillation vials containing 5.0 of scintillation fluid (Ultima Gold, PerkinElmer, Boston, USA), and radioactivity was measured on a beta counter (Liquid Scintillation Analyzer, Packard 1600 TR, Palo Alto, CA) using the Plus 5.01 software (Diamond Computers) to detect ^{14}C and ^{3}H in the samples. Sample blank was a mixture of 200 μl TRIS-HCl buffer and 50 μl of LDE, which was incubated and precipitaded as described previously. Total radioactivity in each sample was

measured in 200 μl of plasma with 50 μl of LDE, followed by incubation, but without precipitating reagent. Lipid transfer from LDE to plasma HDL was expressed as percentage (%) of total radioactivity.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows (Statistical Package for Social Sciences). Continuous variables were expressed as means and standard deviations, and categorical variables as percentages and absolute values.

The Kolmogorov-Smirnov test was performed for normal distribution. As variables were non-normally distributed, nonparametric tests were used. Differences between categorical variables were assessed by Fisher's exact test or the chi-square test.

Diabetic patients were compared with their controls, in addition to subgroups of diabetic patients, regarding the presence of microalbuminuria and use of statin and insulin. The Mann-Whitney test was performed to compare independent continuous variables, and Spearman's nonparametric test was used for correlations. Two tailed p values < 0.05 were considered statistically significant.

Results

Table 1 summarizes clinical data, plasma lipid composition, and glucose levels in T2DM patients and control subjects. Mean age of the T2DM group (55 ± 9) did not differ significantly from that of the control group.

Table 1 - Clinical data and serum lipid and glucose levels of T2DM patients

Variables	Diabetic patients (n = 33)	Control subjects (n = 34)	P
Age (years)	55±9	54±9	0.327
Male, n (%)	18 (54.5%)	16 (47.1%)	0.540
TC (mg/dl)	183±40	174±21	0.455
LDL-C (mg/dl)	103±37	108±22	0.547
HDL-C (mg/dl)	50±13	49±13	0.890
TG (mg/dl)	149±71	92±36	<0.001
FG (mg/dl)	155±66	87±8	<0.001
HbA1c	7.5±1.4	-	-
BMI (kg/m^2)	31±7	25±3	<0.001
Duration of DM (years)	11±7	-	-
Hypertensive (%)	22 (66.7%)	0	<0.001
Using statins, n (%)	18 (54.5%)	0	<0.001
Using insulin, n (%)	10 (30.3%)	0	<0.001
Microalbuminuria, n (%)	9 (27.3%)	-	-

TC - total cholesterol; LDL-C - low-density lipoprotein cholesterol; HDL-C - high-density lipoprotein cholesterol; TG - triglycerides; FG - fasting plasma glucose; HbA1c - glycated hemoglobin; BMI - body mass index.

No difference was found in plasma total cholesterol, LDL-C, and HDL-C between the T2DM group and the control group, $p > 0.05$. Triglycerides levels were 38% higher ($p < 0.01$) in the T2DM group than in the control group. Fasting plasma glucose was 44% higher in the T2DM group ($p < 0.001$). Body mass index was 19% higher among T2DM patients ($p < 0.001$).

Table 2 shows lipid values (cholesterol ester, phospholipids, triglycerides, and free cholesterol) transferred from the nanoemulsion to HDL in diabetic patients and control subjects.

There was no difference in cholesterol ester and triglyceride transfer from the nanoemulsion to HDL between diabetic patients and control subjects. As for free cholesterol and phospholipid transfers, these were approximately 30% and 22% higher in the diabetic group than in the control group, respectively ($p < 0.001$).

Among diabetic patients, no significant differences were found regarding the presence or not of microalbuminuria. However, mean age was 13% higher in the microalbuminuric than in the non-microalbuminuric group (p = 0.008). Age, gender, hypertension prevalence, body mass index, use of statins or insulin, plus lipid and glucose levels did not differ between both groups.

Comparing the subgroups of microalbuminuric and normoalbuminuric diabetic patients aged over 55, it was possible to compare groups within the same age range. Once more, there was no significant difference in lipid transfer to HDL between these groups (Table 3).

Table 2 - Lipid transfer from LDE to HDL in T2DM patients and control subjects

Variables	Diabetic patients (n = 33)	Control subjects (n = 34)	P
CET (%)	4.6±2.1	3.8±1.5	0.074
PLT (%)	25.2±3.2	19.7±3.2	<0.001
TGT (%)	5.1±2.1	4.5±1.5	0.314
FCT (%)	9.1±2.7	6.3±1.5	<0.001

CET - cholesterol ester transfer from LDE to HDL; PLT - phospholipid transfer from LDE to HDL; TGT - triglyceride transfer from LDE to HDL; FCT - free cholesterol transfer from LDE to HDL.

Table 3 - Lipid transfer from LDE to HDL in T2DM according to the diagnosis of microalbuminuria, after adjustment for age

	DM with microalbuminuria (n = 9)	DM without microalbuminuria (n = 13)	P
CET (%)	4.5±1.6	4.4±2.5	0.521
PLT (%)	25.2±3.3	25.8±4.1	0.887
TGT (%)	4.7±1.7	5.0±2.5	0.887
FCT (%)	8.4±2.6	9.1±2.9	0.594

CET - cholesterol ester transfer from LDE to HDL; PLT - phospholipid transfer from LDE to HDL; TGT - triglyceride transfer from LDE to HDL; FCT - free cholesterol transfer from LDE to HDL.

Using the urinary albumin-to-creatinine ratio as a continuous variable, a relationship was established between all variables by the Spearman correlation test. A higher urinary albumin/creatinine ratio was positively correlated with age and glycated hemoglobin and negatively correlated with free cholesterol transfer (Table 4).

Table 4 - Associations (Spearman's correlation coefficients) of microalbuminuria with age, lipid levels, glucose levels, anthropometric data, and lipid transfer from LDE to HDL

Variables	R	P
Age (years)	0.45	0.040
TC (mg/dl)	-0.02	0.918
LDL-C (mg/dl)	-0.08	0.716
HDL-C (mg/dl)	-0.19	0.413
TG (mg/dl)	0.26	0.260
FG (mg/dl)	0.20	0.388
HbA1c (%)	0.53	0.013
CET (%)	-0.005	0.982
PLT (%)	-0.08	0.710
TGT (%)	-0.12	0.609
FCT (%)	-0.50	0.024
BMI (kg/m^2)	0.29	0.204
WC (cm)	0.15	0.582
Duration of diabetes (years)	0.32	0.160

TC - total cholesterol; LDL-C - low-density lipoprotein cholesterol; HDL-C - high-density lipoprotein cholesterol; TG - triglycerides; FG - fasting plasma glucose; BMI - body mass index; HbA1c - glycated hemoglobin; CET - cholesterol ester transfer from LDE to HDL; PLT - phospholipid transfer from LDE to HDL; TGT - triglyceride transfer from LDE to HDL; FCT - free cholesterol transfer from LDE to HDL; WC - waist circumference.

Regarding insulin therapy, age, gender, body mass index, hypertension prevalence, plus lipid and glucose levels were similar in the insulin and non-insulin treated groups. The frequency of statin use was also similar in both groups. Insulin therapy was associated with a 10% decrease in phospholipid transfer to HDL (p = 0.018) (Figure 1).

With respect to statin therapy, transfer of all lipids to HDL was significantly lower in the group receiving this drug class than in the group that was not (Figure 2). Among T2DM patients, transfer of cholesterol ester, phospholipids, triglycerides, and free cholesterol was, respectively, 41%, 12%, 39%, and 33% lower in those who were on statins than in those who were not.

Discussion

The contribution of HDL-associated proteins to the generation, maturation, and reverse transport of cholesterol has been extensively studied. Cholesteryl ester transfer protein (CETP) modulates HDL levels and composition by mediating the exchange of neutral lipids (esterified cholesterol and triglycerides) between HDL and triglyceride-rich lipoproteins, in addition to promote phospholipid transfer to HDL. This protein also releases apolipoproteins from HDL in the presence of fatty acids[31,32]. Plasma phospholipid transfer protein (PLTP) itself can release apolipoproteins from HDL[33]. Cholesterol ester in HDL particles may originate from two sources: 1) esterification of HDL-free cholesterol, and 2) cholesterol ester of other lipoprotein[34,35].

PLTP acts by transferring surface phospholipids to HDL and displacing apo AI from the particle's surface[36,37]. An important PLTP-mediated product of HDL particles are the small, lipid-poor pre-β-HDLs that act as initial acceptors of free cholesterol[38,39]. Apo E (but not apo AI) is capable of converting inactive PLTP into its active form[40]. This is a major

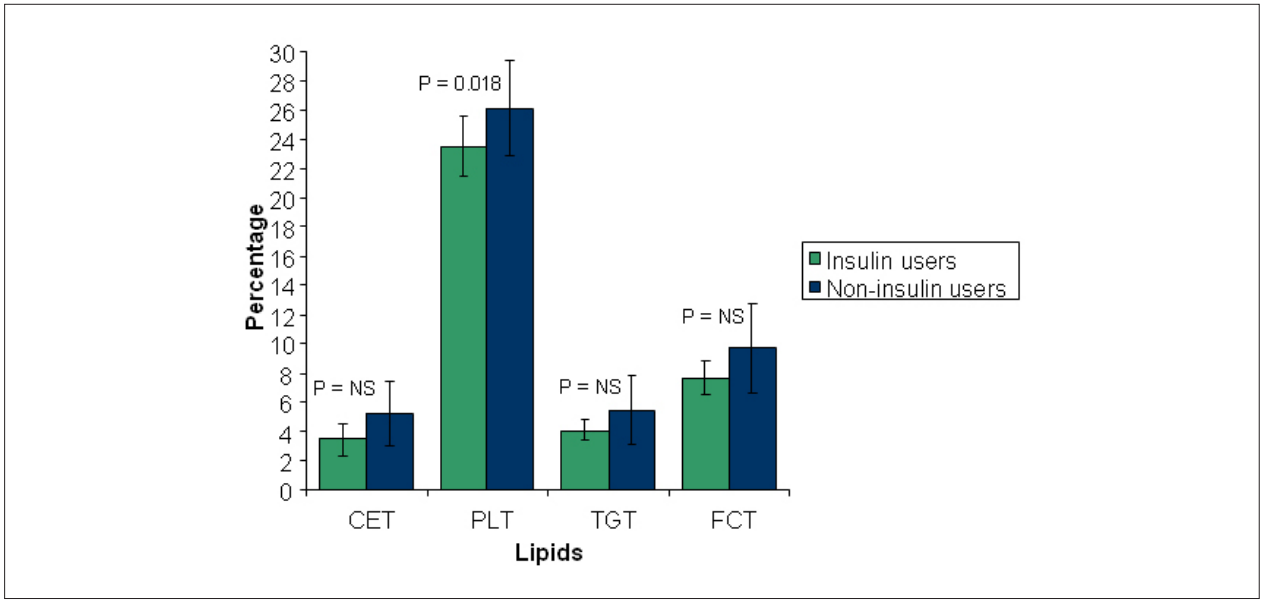

Figure 1 - *Comparison of lipid transfer between insulin-dependent and non-insulin-dependent diabetic patients. CET - cholesterol ester transfer from LDE to HDL; PLT - phospholipid transfer from LDE to HDL; TGT - triglyceride transfer from LDE to HDL; FCT - free cholesterol transfer from LDE to HDL; NS - non-significant.*

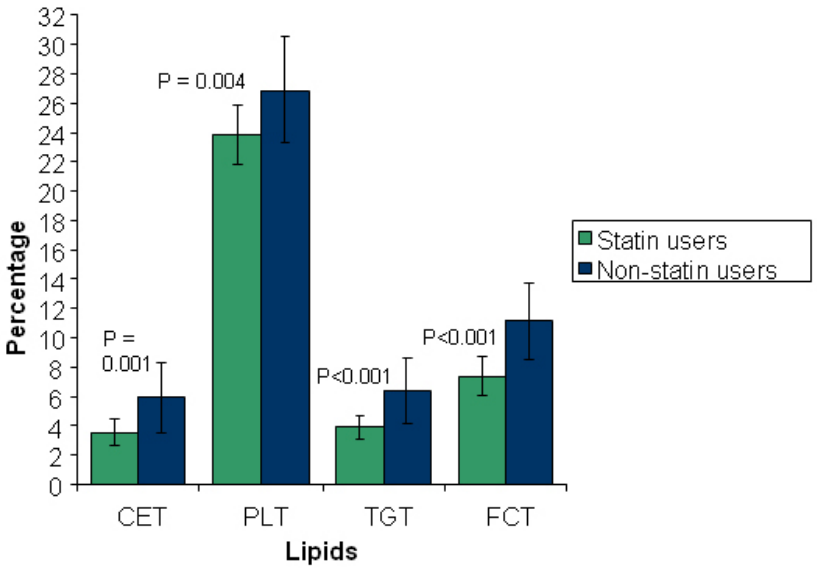

Figure 2 - Comparison of lipid transfer between diabetic patients with and without statin therapy. CET - cholesterol ester transfer from LDE to HDL; PLT - phospholipid transfer from LDE to HDL; TGT - triglyceride transfer from LDE to HDL; FCT - free cholesterol transfer from LDE to HDL.

characteristic that must be kept in mind, since LDE is devoid of apolipoproteins and acquires apo E when comes into contact with the blood.

This study provides a simple and fast manner to estimate HDL acceptor activity of all lipids using a single methodology. It allowed us to demonstrate that the uptake of phospholipids and free cholesterol by HDL is greater in diabetic patients, as compared to control subjects.

The finding of increased phospholipid transfer in diabetic patients, suggesting higher PLTP activity, is consistent with some reports in the literature[13,14]. Earlier studies have also shown an increase in plasma cholesteryl ester transfer[15,16]

The relationship between microalbuminuria and dyslipidemia in diabetic patients has been well explored. In this study, however, no differences were found in lipid transfer to HDL between normoalbuminuric and microalbuminuric T2DM patients. In the literature, no change in CETP activity was reported in microalbuminuric patients[17].

Phospholipid transfer to HDL was lower in insulin-treated than in non-insulin-treated patients, with no changes in the other three lipids. PLTP is responsible for most phospholipid transfer to HDL and, to a lesser extent, facilitates the influx of free cholesterol to this lipoprotein. The present study suggests a possible correlation between insulin and PLTP activity. Most studies in the literature, though not all, that sought to investigate the relationship between insulin and PLTP activity have shown inhibition of the latter[22-26].

Statin therapy was found to reduce the uptake of all lipids by HDL. Other studies have demonstrated that it decreases plasma CETP activity by three different mechanisms: 1) reducing plasma CETP mass[18], 2) reducing lipoproteins with which HDL interacts[19], and 3) possibly reducing CETP gene expression[20]. In a substudy of the DALI trial, statin therapy reduced plasma PLTP activity, despite increasing PLTP mass[21]. In the present study, the lipid acceptor activity of HDL was assessed. The other direction of lipid exchange between lipoproteins was not measured: the amount of lipids from HDL that is donated to the other lipoproteins is not known. Paradoxically, HDL acceptor activity was directly associated with risk factors of atherosclerosis, and statin therapy decreased all lipid transfer to HDL. This may be explained by the fact that the HDL with transfer characteristics that are more compatible with good markers is associated with less acquisition and loss of lipids and, therefore, would be a more stable HDL.

This study provides additional data regarding the complex and still incompletely understood mechanism of lipid exchange between lipoproteins. It also demonstrates that the use of insulin and statins is associated with functional aspects of HDL, as well as with its transfer proteins.

Study limitations

Diabetes mellitus is a condition that may have a broad clinical spectrum. An attempt was made to standardize the study population, excluding diabetic patients who were receiving exclusively non-pharmacological therapy, as well as those with evidence of renal failure or macroalbuminúria.

In spite of this, there was a degree of heterogeneity in the diabetic sample, in that diabetes mellitus was more advanced or more associated with comorbidities in some patients than in others. Moreover, part of the study sample was using insulin, statins, or anti-hypertensive drugs.

Ideally, any medication that might interfere with lipid metabolism or microalbuminuria should have been discontinued. That was not the case. Lipid measurements following discontinuation of statin therapy, therefore, were

not performed, because we believe that such discontinuation would lead to a transient change in lipid levels in a population with cardiovascular risk factors without conferring any benefit. The same is true regarding anti-hypertensive and hypoglycemic drugs.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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