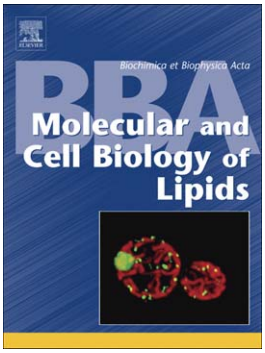

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Poor glycemic control in Type 2 diabetes enhances functional and compositional alterations of small, dense HDL3c.

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Abstract

High-density lipoprotein (HDL) possesses multiple biological activities; small, dense HDL3c particles displaying distinct lipidomic composition exert potent antiatherogenic activities which can be compromised in dyslipidemic, hyperglycemic insulin-resistant states. However, it remains indeterminate (i) whether such functional HDL deficiency is related to altered HDL composition, and (ii) whether it originates from atherogenic dyslpidemia, dysglycemia, or both.

In the present work we analysed compositional characteristics of HDL subpopulations and functional activity of small, dense HDL3c particles in treatment-naïve patients with well-controlled (n=10) and poorly-controlled (n=8) Type 2 diabetes (T2D) and in normolipidemic age- and sex-matched controls (n=11).

Our data reveal that patients with both well- and poorly-controlled T2D displayed dyslipidemia and low-grade inflammation associated with altered HDL composition. Such compositional alterations in small, dense HDL subfractions were specifically correlated with plasma HbA1c levels. Further analysis using a lipidomic approach revealed that small, dense HDL3c particles from T2D patients with poor glycemic control displayed additional modifications of their chemical composition. In parallel, antioxidative activity of HDL3c towards oxidation of low-density lipoprotein was diminished.

These findings indicate that defective functionality of small, dense HDL particles in patients with T2D is not only affected by the presence of atherogenic dyslipidemia, but also by the level of glycemic control, reflecting compositional alterations of HDL.

Keywords: Type 2 diabetes; HDL; antioxidative activity; lipidomics; functionality

Abbreviations:

ANOVA: Analysis of Variance

ApoAI: Apolipoprotein AI

ApoB: Apolipoprotein B

BMI: Body Mass Index

CVD: Cardiovascular Disease

CE: Cholesteryl Ester

CETP: Cholesteryl Ester Transfer Protein

EDTA: Ethylene Diamine Tetra acetic acid

EGF: Endothelial Growth Factor

FC: Free Cholesterol

GP: Generalized Polarization

HbA1c: Glycated Hemoglobin

HDL: High-Density Lipoprotein

HDL-C: HDL-Cholesterol

HsCRP: High-Sensitive C-Reactive Protein

ICAM-1: Intercellular Cell Adhesion Molecule-1

IDL: Intermediate Density Lipoprotein

IL-6: Interleukin 6

LCAT: Lecithin Cholesterol Acyl Transferase

LDL: Low-Density Lipoprotein

LDL-C: LDL-Cholesterol

Lp-PLA$_2$: Lipoprotein-Associated Phospholipase A_2

LPL: Lipoprotein Lipase

PBS: Phosphate-Buffered Saline

PC: Phosphatidylcholine

PL: Phospholipids

PON1: Paraoxonase 1

SM: Sphingomyelin

TC: Total Cholesterol

TG: Triglycerides

TP: Total Protein

VEGF: Vascular Endothelial Growth Factor

VCAM-1: Vascular Cell Adhesion Molecule 1

VLDL: Very Low-Density Lipoprotein.

1. Introduction

Insulin-resistant states are associated with elevated risk of cardiovascular disease (CVD); indeed, Type 2 diabetes (T2D) represented a major CVD risk factor in the INTERHEART study across 52 countries worldwide (1). Atherogenic dyslipidemia, involving hypertriglyceridemia and low circulating levels of high-density lipoprotein-cholesterol (HDL-C), is a key component of excess CVD risk in insulin-resistant states, and notably in T2D (2-4). In addition, diabetic dyslipidemia results in qualitative modifications of HDL particles that are thought to diminish their antiatherogenic capacities, which include effluxing cellular cholesterol, protecting low-density lipoprotein (LDL) against oxidative stress, diminishing cellular death, decreasing vascular constriction, reducing inflammatory response, and improving glucose metabolism, among others (5). Importantly, such biological functions directly rely on HDL composition and structure, with phospholipids (PLs) being of critical importance (6).

Plasma HDL is markedly heterogeneous in composition, structure, metabolism and function, comprising different particles which range from large, cholesteryl ester-rich, protein-poor HDL2 to small, dense, protein-rich HDL3 subfractions (7, 8). Our previous studies have revealed that HDL3c particles exhibit more potent antiatherogenic properties as compared to other HDL subpopulations (7). Indeed, cholesterol efflux capacity, antioxidative activity toward LDL oxidation, antithrombotic activity in human platelets, anti-inflammatory activity, and antiapoptotic activity in endothelial cells were all enriched in the small, dense, protein-rich HDL3c relative to other HDL subfractions (6, 9). Importantly, biological activities of small, dense HDL have been reported to be markedly altered in patients with insulin resistance and inflammation (10-12).

However, since the majority of earlier studies do not distinguish between patients with different levels of glycemic control, relative roles of atherogenic dyslipidemia and

dysglycemia for compositional and functional HDL alterations in T2D still remain indeterminate. To provide further insight into this question, we studied compositional and functional characteristics of HDL particles in two groups of treatment-naïve patients with well- and poorly-controlled T2D and in a group of healthy age- and sex-matched normolipemic controls.

Our data revealed that glycemic status preferentially affected small, dense HDL3c particles which displayed distinct compositional alterations closely related to increased cholesteryl ester transfer protein (CETP) activity. Moreover, antioxidative activity of small, dense HDL3c was specifically reduced in T2D patients with poor glycemic control. Lipidomic analyses showed that HDL3c from poorly-controlled patients displayed additional alterations that were associated with functional HDL deficiency.

Taken together, our findings indicate that antioxidative deficiency of small, dense HDL particles in patients with T2D is not only affected by the presence of atherogenic dyslipidemia, but also by the level of glycemic control, reflecting compositional alterations of HDL.

2. Materials and Methods

2.1 Subjects

Eighteen treatment-naïve adult patients with T2D (males and postmenopausal women) diagnosed according to the American Diabetes Association (ADA) criteria (13) were recruited from the Ramon Carrillo Centre, La Matanza, Buenos Aires, Argentina. Subjects presenting any of the following criteria were excluded: a) history of cardiovascular disease, b) concomitant kidney, thyroid or liver disease, c) excessive alcohol consumption, d) smoking, and e) treatment with drugs affecting lipid or carbohydrates metabolism and/or with antioxidants during the last 6 months. Diabetic patients were in turn divided into 2 groups, well- and poorly-controlled, following the ADA glycemic recommendations for patients with diabetes (13). Accordingly, poorly-controlled patients were defined as those presenting HbA1c > 7% and fasting glucose > 130 mg/dl (T2DPC, n=8), while the remaining 10 diabetic patients constituted the well-controlled (T2WC) group. Eleven healthy non-smoking normolipidemic subjects were recruited from the same geographical area and with similar socio-demographic characteristics to constitute the control group. Written informed consent was obtained from all patients and control subjects and the study was approved by the Ethics Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires, in accordance with guidelines conformed to the Declaration of Helsinki.

2.2 Blood Samples

Blood samples were withdrawn from the antecubital vein of each participant after a 12 h overnight fast. Serum and Na_2EDTA plasma (final Na_2EDTA concentration, 1 mg/ml) were prepared from venous blood collected into sterile, evacuated tubes (Vacutainer). Plasma was immediately separated by low-speed centrifugation at 4°C; serum and plasma were each mixed with sucrose (final concentration, 0.6%), as a cryoprotectant for lipoproteins (14),

aliquoted and frozen at -80°C under nitrogen. Each aliquot was thawed only once directly before analyses.

2.3 Clinical and Biological Parameters

Circulating levels of glucose, HbA1c, total cholesterol (TC), triglyceride.s (TG), low-density lipoprotein-cholesterol (LDL-C) and HDL-C were measured using commercially available enzymatic kits (Wiener Lab, Argentina). Non-HDL-C was calculated as a difference between TC and HDL-C. Very low-density lipoprotein-cholesterol (VLDL-C) was calculated as a difference between TC, LDL-C and HDL-C. Plasma apolipoprotein (apo) A-I and apoB were quantitated by immunoturbidimetry (Diasys, France). High-sensitivity C-reactive protein (hsCRP) was measured by immunoassay (Roche Diagnostics, Mannheim, Germany). Plasma levels of interleukin 6 (IL-6), vascular cell adhesion molecule (VCAM)-1, intercellular cell adhesion molecule (ICAM)-1, E-selectin, endothelial growth factor (EGF), and vascular endothelial growth factor (VEGF) were determined using a Biochip Array Technology analyzer (Evidence Investigator, Randox, Ireland).

2.4 Activities of Lipoprotein-Associated Proteins and Enzymes

CETP Activity. CETP activity was determined in serum samples according to the endogenous radiometric procedure of Lagrost et al. (15). Results were expressed as a percentage of ^{3}H-cholesteryl esters transferred from HDL3 to apoB-containing lipoproteins, per ml, per hour. Measurements were all carried out within the same assay. Within-run assay precision was 4.9%.

Lipoprotein-Associated Phospholipase A_2 Activity. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) activity was measured using the radiometric assay described by Blank et al.(16). Results were expressed as µmol per ml, per h. Measurements were all carried out within the same assay. Within-run precision for the Lp-PLA_2 activity assay was 5.1 %.

2.5 Lipoprotein Isolation

Plasma lipoproteins were isolated from serum and plasma by a single step, isopycnic non-denaturing density gradient ultracentrifugation using a Beckman SW41 Ti rotor at 40,000 rpm for 44 hours in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al. (17) as previously described (18). Briefly, plasma density was increased to 1.21 g/mL by adding dry solid KBr. A discontinuous density gradient was then constructed as follows: 2 mL of 1.24 g/mL NaCl/KBr solution, 3 mL plasma (*d* 1.21 g/mL), 2 mL of 1.063 g/mL NaCl/KBr solution, 2.5 mL of 1.019 g/mL NaCl/KBr solution, and 2.5 mL of NaCl solution (*d* 1.006 g/mL). All density solutions contained sodium azide (0.01%), EDTA (0.01%), and gentamicin (0.005%), pH 7.4. After centrifugation, gradients were collected from the top of the tube in 12 fractions corresponding to VLDL (*d* <1.017 g/mL), IDL (*d* 1.018 to 1.019 g/mL), LDL1 (*d* 1.019 to 1.023 g/mL), LDL2 (*d* 1.023 to 1.0239 g/mL), LDL3 (*d* 1.029 to 1.039 g/mL), LDL4 (*d* 1.039 to 1.050 g/mL), LDL5 (*d* 1.050 to 1.063 g/mL), HDL2b (*d* 1.063 to 1.091 g/mL), HDL2a (*d* 1.091 to 1.110 g/mL), HDL3a (*d* 1.110 to 1.133 g/mL), HDL3b (*d* 1.133 to 1.156 g/mL), and HDL3c (*d* 1.156 to 1.179 g/mL). Density limits were defined using a calibration curve of density versus volume derived from control gradients containing only salt solutions.

All lipoprotein fractions were dialysed against phosphate-buffered saline (PBS; pH 7.4) at 4°C in the dark and analyzed for their lipid and protein content. Recovery was 98% for cholesterol and between 90% and 95% for all other lipids. Comparison of lipoprotein mass profiles as a function of density, computed from chemical analyses of lipoprotein subfractions isolated from aliquots of the same plasma fractionated in separate tubes during the same ultracentrifugal run, revealed a high degree of reproducibility; indeed, mass profiles were indistinguishable, with coefficients of variation of <5% for the masses of individual

subfractions. Importantly, this density gradient procedure allows fractionation of HDL particle subspecies in a non-oxidised, native state (17, 19).

2.6 Chemical Analysis of Llipoproteins

Total protein (TP), TC, free cholesterol (FC), PL and TG contents of isolated HDL subfractions were determined using commercially available assays (Diasys, France). Cholesteryl ester (CE) was calculated by multiplying the difference between total and free cholesterol concentrations by 1.67. Total lipoprotein mass was calculated as the sum of TP, CE, FC, PL and TG. ApoA-I and apoA-II contents in HDL were quantitated by immunoturbidimetry (Diasys, France).

2.7 Antioxidative Activity of HDL3c

Antioxidative activity of serum-derived HDL3c subfraction (final concentration, 10 mg total mass/dl) was assessed towards reference LDL (10 mg TC/dl) isolated from a single plasma sample obtained from a reference healthy normolipidemic donor. The sample was aliquoted, stored at -80°C under nitrogen and used for all the experiments. LDL was oxidized, alone or in the presence of individual HDL3c isolated from each of T2DWC (n=10), T2DPC (n=8) and control (n=11) subjects, by 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH), an azo-initiator of lipid peroxidation (1 mM) (11, 20, 21). The HDL3c subfraction was added to LDL directly before oxidation. Serum was used as a source of HDL for this assay to ensure intact paraoxonase 1 (PON1) activity, which is inhibited by EDTA (22). Accumulation of conjugated dienes was measured as the increment in absorbance at 234 nm. Absorbance kinetics was corrected for the absorbance of AAPH itself run in parallel as a blank. The kinetics of diene accumulation revealed two characteristic phases, the lag (initiation) and propagation phases. For each curve, the duration of each phase, average oxidation rates within each phase and amount of dienes formed at the end of the propagation phase (maximal

amount of dienes) were calculated. The coefficient of variation (CV) remained below 10% for all the parameters.

2.8 HDL3c lipidomic analysis

Ten PL and sphingolipid (SL) subclasses, specifically phosphatidylcholine (PC), sphingomyelin (SM), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), PC-based plasmalogen (PC-pl), ceramide (Cer), phosphatidic acid (PA) and phosphatidylglycerol (PG), comprising together >160 individual lipid species were assayed by LC/MS/MS. The PL subclasses were divided into major (those whose content was >1% of total PL, i.e. PC, SM, LPC, PE and PI) and minor (those whose content was <1% of total PL, i.e. PS, PC-pl, Cer, PA and PG).

Extraction: The HDL3c subpopulation (30µg of enzymatically-quantified PLs) was extracted with 4ml of cold $CHCl_3$/acidified CH_3OH (5/2, v/v) containing 4µg of PC d9, 400ng of LPC 15:0, 100ng of PI 25:0, 80ng of PE 25:0, 80ng of PA 25:0, 40ng of PS 25:0, 20ng of PG 25:0 and 20ng of Cer 17:0. Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). A blank and a control samples were extracted in parallel with each batch to ensure for quality control; each sample was corrected for blank readings. K_4EDTA (200mM) solution was added (1:5 v/v) and the mixture was vortexed for 1 min and centrifuged at 3600 g for 10 min at 4°C. The organic phase was transferred into 5ml chromacol glass tubes and dried under nitrogen. PLs were reconstituted into 150µl isopropanol/hexane/water (10/5/2, v/v), transferred into LC/MS amber vials with inserts and dried under nitrogen. Lipids were reconstituted in 40µl of isopropanol/hexane/water (10/5/2, v/v) and lipids measured by LC/MS/MS.

LC/MS analysis: Ten PL and SL subclasses were quantified by LC-ESI/MS/MS using a QTrap 4000 mass spectrometer (AB Sciex, Framingham, MA, USA): Lipid species were detected using multiple reaction monitoring reflecting the headgroup fragmentation of each

lipid class. PC, LPC and SM species were detected as product ions of m/z 184, PE, PS, PG and PA as neutral losses of respectively m/z 141, 185, 189 and 115, PI molecular species as product ions of m/z -241. N_2 was used as a collision gas. PE, PS, PG, PI, PA and ceramide species were monitored for 18 ms, PC, LPC and SM species were monitored for 30 ms at a unit resolution (0.7 atomic mass unit at half peak height).

Quantification**:** Lipids were quantified using calibration curves specific for the nine individual lipid classes and up to 12 fatty acid moieties. Twenty-three calibration curves were generated in non-diluted and 10-fold diluted matrices to correct for matrix-induced ion suppression effects. More abundant lipid species which displayed non-linear response in non-diluted extracts were quantified from a 10- or 100-fold diluted sample. An in-house developed Excel Macro was used to compile the data from the three injections.

Limits of detection (LOD) and limits of quantitation (LOQ) were assessed using at least two individual lipid species per each PL or SL subclass and equaled 0.5 nM for Cer, 0.25 nM for LPC, 25 nM for LPE, between 50 and 200 nM for PA, between 60 and 250 pM for PC, between 5 and 10 nM for PE, 1 nM for PG, 10 nM for PI, between 1 and 2 nM for PS and 250 pM for SM.

Repeatability of lipidomic analysis was tested in nine quality control samples extracted on three different occasions. The average coefficient of variation (CV) for individual lipid species was 11.6%; importantly, 90% of all the species revealed CV below 20%. Lipid species displaying CV greater than 20% were exclusively present in minor abundance close to LOD.

2.9 Statistical Analysis

Distributions of all variables were analysed for normality using the Shapiro Wilk test. Normally distributed variables are expressed as means ± SD and non-Gaussian distributed variables are expressed as median (interquartile range). Between-groups differences in

normally-distributed variables were analyzed using Student's t-test. For non-Gaussian distributed variables, the non-parametric Mann-Whitney U-test was employed. When comparing more than two groups, variables were first normalized by log or reciprocal transformation and then differences were evaluated using Bonferroni's post-hoc test. Differences in dichotomous variables were analyzed by Fisher's exact test. Spearman's correlation coefficients were calculated to evaluate relationships between variables. Differences were considered statistically significant at $p<0.05$.

Power analysis using G*Power software (Dusseldorf University, Dusseldorf, Germany) was performed to estimate the sample size required to detect differences between groups in accordance with previous studies performed in patients with Metabolic Syndrome and T2D (11) (23).

The statistical software employed was INFOSTAT (University of Córdoba, Argentina) and SPSS 17.0 (Chicago, Illinois, USA).

Principal component analysis and heatmap generation were performed using R (http://www.r-project.org/) version 3.2.2, including gplots, ggbiplot and Cairo packages.

3. Results

3.1 Clinical Parameters, Lipoprotein Profile and Inflammatory Biomarkers

Age and sex distribution were similar across the three study groups (Table 1). As expected, both T2D groups exhibited increased BMI as well as elevated plasma TG, VLDL-C, and reduced plasma HDL-C concentrations relative to controls, reflecting dyslipidemia of insulin-resistant states. Glucose and HbA1c concentrations were progressively increased from the control group to T2DWC and further to T2DPC groups (Table 1). By contrast, there was no significant difference in TC, LDL-C, apoB and apoA-I concentrations between the groups; non-HDL-C levels were only elevated in poorly-controlled patients. Noteworthy, CETP activity, which may induce HDL enrichment in TG and depletion in cholesteryl esters, was increased in T2DPC patients compared to control subjects.

The presence of a chronic inflammatory state in poorly-controlled patients was evidenced by elevated plasma concentrations of hsCRP, ICAM-1, E-selectin, EGF and VEGF (Table 2). Furthermore, the novel vascular inflammatory biomarker, plasma Lp-PLA_2 activity, was significantly increased in the T2DPC group. By contrast, only E-selectin levels and Lp-PLA_2 activity were elevated in well-controlled patients. Circulating concentrations of IL-6 and VCAM-1 did not differ between the groups. Overall, diabetic patients featured classical atherogenic dyslipidemia of T2D in a coexistence with a chronic proinflammatory state which was more pronounced in patients with poor glycemic control.

3.2 Total Chemical Composition of HDL subfractions.

All five HDL subpopulations isolated by density gradient ultracentrifugation from the both groups of diabetic patients were depleted in CE and enriched in TG (with the exception of T2DWC HDL3b and 3c) as compared to control subjects (Table 3). In parallel, FC was reduced in HDL2a and 3a particles from the both T2D groups relative to controls. Additionally, PL content was significantly increased in diabetic patients across the whole spectrum of HDL, except the buoyant HDL2b subpopulation, relative to controls. Noteworthy, HDL3c PL content was increased in the T2DPC compared to the other groups. Furthermore, the content of TP was specifically decreased in T2DPC HDL3c vs. controls. Finally, large, light HDL2b and HDL2a particles from the both T2D groups were characterised by reduced apoA-I/apoA-II ratio, consistent with altered properties of HDL surface.

Interestingly, TG and apolipoprotein content of HDL were altered in association with plasma CETP activity. This observation is consistent with the role of CETP as a key factor accounting for the altered lipid core composition of HDL in T2D (24). Indeed, CETP activity was positively correlated with HDL3b content of TG ($r=0.45$, $p<0.05$) and negatively correlated with the apoA-I/apoA-II ratio in HDL2b, HDL3b and HDL3c ($r=-0.58$, $p<0.05$; $r=-0.56$, $p<0.05$; $r=-0.69$, $p<0.01$; respectively).

In addition, CETP activity was directly correlated with plasma HbA1c levels ($r=0.44$, $p<0.05$). As a consequence, several modifications in the chemical composition of HDL subpopulations were correlated with HbA1c. Importantly, HbA1c was positively correlated with TG content of small, dense HDL3b and HDL3c subfractions ($r=0.48$, $p<0.01$; $r=0.40$, $p<0.05$; respectively), while negatively correlated with CE content of HDL3b ($r=-0.45$, $p<0.05$) and the HDL3c apoA-I/apoA-II ratio ($r=-0.53$, $p<0.05$). Thus, glycemic control

appeared to specifically affect chemical composition of small, dense HDL particles in the present study, with the HDL3c subfraction displaying the greatest alterations (Table 3).

3.3 Role of glycemic control for HDL characteristics

Our earlier studies revealed that altered lipid core composition can contribute to the attenuation of antioxidative activity of HDL3c particles in T2D (11). Therefore, the capacity of HDL3c to protect LDL from oxidative stress was analysed in the present study. To characterise LDL oxidation kinetics, two characteristic oxidation phases, the initial lag phase and the ensuing propagation phase, as well as the maximal amount of conjugated dienes formed were analysed. Interestingly, the maximal diene concentration observed in LDL incubated with HDL3c particles from the T2DPC group was significantly increased as compared to that measured in the presence of control HDL3c, evidencing diminished capacity of HDL3c to prevent LDL oxidation in vitro (Figure 1). By contrast, no difference in other oxidation parameters was detected between the groups.

3.4 Phosphosphingolipid species of HDL3c

Using a lipidomic approach, ten PL and SL subclasses (PC, LPC, SM, PE, PI, PS, PC, Cer, PG and PA) were quantitatively assayed in small, dense HDL3c by LC/MS/MS and compared between the study groups (Figure 2). Among the major PL species (defined as those present at >1 wt% of total PL), PC and PI were elevated in T2DPC HDL3c as compared to its counterpart from control subjects. Interestingly, HDL3c contents of PC and PI were directly correlated with HbA1c (r=0.40, p=0.05; r=0.55, p=0.004, respectively).

Regarding minor lipid classes, Cer content was increased in T2DPC HDL3c in comparison with its counterparts from T2DWC and control subjects. Furthermore, HDL3c Cer content tended to be associated with HbA1c (r=0.39, p=0.06). Finally, PC and Cer contents of HDL3c were positively associated with the maximal diene concentration measured during LDL incubation with HDL3c (r=0.40, p=0.048; r=0.42, p=0.040, respectively). Between-group distributions of individual lipid species were largely consistent with those observed for the lipid subclasses (Suppl. Figure 1). Indeed, numerous species of PC, PI and Cer were significantly elevated in the T2DPC group relative to controls.

Lipidomic data projected onto the plane space depicting maximal inertia (total variance) of the dataset were utilized during principal component analysis. The first, second, third and fourth principal components explained 53% of the data variance, which allowed certain distinguishing between diabetic and control subjects (Suppl. Figure 2). Lipidomic alterations associated with T2D were visualized as a heat map presenting ratios of each lipid concentration measured in individual T2DWC or T2DPC subjects to the corresponding mean concentrations measured in control subjects, thereby revealing a large number of altered lipid species in the T2DWC and T2DPC groups (Suppl. Figure 3). A dendrogram with a leaf structure constructed using these data revealed two clusters of subjects. Each of the clusters involved both T2DPC and T2DWC patients, indicating that lipidomic alterations observed in

the two groups were qualitatively similar, although those found in T2DPC were more pronounced (Figure 2).

3.5 Fatty Acid Composition of Phospho- and Sphingolipids in HDL3c

Phospho- and sphingolipid species classified according to the level of unsaturation of their esterified fatty acid moieties were also evaluated in HDL3c. Importantly, HDL content of highly oxidable polyunsaturated fatty acids (PUFA) residues was significantly elevated in T2DPC patients in the PC subclass which predominates in plasma HDL, accounting for 74% to 80% of the total phospho- and sphingolipidome (7). In addition, the content of PUFAs in less abundant PI, PG and Cer subclasses was also increased in the T2DPC group as compared to controls (Table 4). By contrast, there was no significant difference between the groups in the HDL3c content of saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) in the PC subclass (data not shown). HDL3c SFAs were only elevated in low-abundant PE, PI and Cer subclasses, whereas MUFAs were increased in PE, PI, PG and Cer (Table 4).

Finally, PC-PUFA and Cer-PUFA contents in HDL3c were directly correlated with HbA1c ($r=0.43$, $p=0.034$; $r=0.51$, $p=0.01$, respectively) and with the maximal diene concentration measured in LDL incubated with HDL3c ($r=0.48$, $p=0.018$; $r=0.44$, $p=0.03$, respectively).

4. Discussion

Patients with T2D display a three-fold increase in CV mortality and a two-fold increase in overall mortality compared to age-matched patients without diabetes (25). Although T2D has been well established to lead to modifications in HDL particles, the underpinnings of this phenomenon remain obscure. Patients with T2D typically present dyslipemia characterized by high TG and low HDL-C levels. However, such patients also display qualitative HDL alterations which are associated with the increased risk of CVD. In the present study we show that several molecular characteristics of small, dense HDL particles were altered in parallel to HbA1c levels in patients with T2D. As a consequence, HDL3c particles from T2D patients featuring poor glycemic control not only showed an altered chemical composition, but also displayed reduced capacity to prevent LDL oxidation.

Antioxidative capacity of HDL3c was impaired in the T2DPC group. According to the oxidative hypothesis of atherosclerosis, LDL can be retained in the arterial wall and oxidatively modified. By contrast, HDL can protect LDL from oxidative stress, potentially contributing to the prevention of atherosclerosis. Removal of oxidized lipids from LDL represents the first step of HDL-mediated protection from oxidative damage. Inactivation of oxidized lipids associated with HDL particles represents the second step in this antioxidative pathway. Depending on their structure, oxidized lipids can be reduced by apoA-I and other redox-active HDL components (5). As expected, T2DPC patients presented HDL particles which were TG-enriched and CE-depleted. This is a classic compositional alteration found in insulin-resistant states, which is primarily linked to enhanced CETP activity, a finding confirmed in the present study. Notably, replacement of CE by TG in the HDL lipid core have been reported to result in a reduced penetration of the central and C-terminal regions of apoA-I into the lipid phase (26), a structural alteration which may contribute to the functional deficiency of small, dense HDL in T2D (11, 20, 27). Consistent with this notion, we observed

that increased CETP activity was associated with enrichment in TG and loss of ApoA-I from small, dense HDL particles, as the former was inversely correlated with the ApoA-I/ApoA-II ratio in HDL3b and 3c.

Interestingly, our previous work showed that composition of small, dense HDL3 particles was markedly affected in T2D patients and that these modifications were associated with both glycaemia and hypertriglyceridemia (11). In the present work, correlational analysis revealed that several compositional alterations in small, dense HDL3 were only associated with HbA1c levels. Moreover, HDL3c particles from T2DPC were the only HDL subfraction that was enriched in TG and PL, depleted in CE and TP, and exhibited defective functionality, suggesting that HDL3c represents the preferential target for poor glycemic control across the HDL particle profile. Potential clinical implications of this observation are highlighted by the fact that small, dense HDL3 exert potent atheroprotective activities and that reduced plasma levels of HDL3 can enhance cardiovascular risk. Indeed, the VA-HIT trial reported that plasma levels of HDL3-C were a powerful predictor of cardiovascular risk in insulin-resistant subjects, even more strong than total HDL-C (28). In addition, these studies found that HDL3-C, but not HDL2-C, at baseline and as a percentage change with fibrates therapy was significantly related to the incidence of coronary heart disease (28). Moreover, a recent work from our laboratory showed that small, dense HDL3c displayed more potent capacity to efflux cellular cholesterol from lipid-loaded human macrophages as compared to other HDL subpopulations (9). These data suggest that alterations in small, dense HDL particles can play a role in the development of atherosclerosis in T2D.

Additional alterations observed in the lipidomic composition of small, dense HDL3c in T2DPC were the enrichment in PC, PI and Cer. Of note, PC represents the key structural PL of cell membranes and lipoproteins, accounting for 33-45 wt% of total lipid in HDL (5). In addition, PC is the major carrier of highly oxidable PUFA, which can increase oxidative

susceptibility of HDL and lower its capacity to prevent oxidation of LDL particles. Our previous studies confirmed deleterious effects of PC enrichment on antioxidative activity of HDL using in vitro experiments (29). Moreover, specific increase in PUFA-enriched PC has previously been reported to be associated with insulin levels (30). In agreement with this observation, HDL3c from T2DPC displayed increased content of PC-PUFA accompanied by impaired antioxidative capacity in our study, suggesting that HDL content of PC-PUFA can serve as a negative biomarker of antiatherogenic functionality of HDL. Interestingly, the lipidomic analysis revealed that alterations observed in T2DPC and T2DWC groups were qualitatively similar, although those found in T2DPC were more pronounced, consistent with the role of the glycemic control.

Among the major PL species, PI is a negatively-charged phospholipid regulating intracellular signalling cascades that was previously reported to be enriched in small, dense HDL3 (7). Interestingly, we observed that HDL3c content of PI-PUFA was also increased in T2DPC, potentially further contributing to the overall reduction in the antioxidative capacity. In addition, HDL3c content of Cer, a proinflammatory lipid implicated in the development of skeletal muscle insulin resistance and macrophage inflammation (30), directly correlated with impaired antioxidative capacity. Consistent with these data, previous reports have shown Cer levels to be associated with the development of T2D and prediabetes (31-33). Moreover, Cer has been demonstrated to be detrimental to pancreatic beta cells (34-36) and may therefore play an important role in the pathogenesis of T2D.

Similar phospholipidomic-based studies carried out in diabetic patients also reported alterations in the HDL PL content (30). Thus, Stahlman and colleagues observed that dyslipidemia predominated relative to hyperglycemia for the occurrence of alterations in the HDL lipidome, in contrast to what we describe for the small, dense HDL3c particles in the present study. Such apparently divergent results can be attributed to the fact that Stahlman et

al. studied total HDL (30), while we focused on the small, dense HDL3c subfraction, which appears more likely to be affected by the glycemic imbalance. The contribution of HDL3c to total HDL lipid mass does not exceed 10%, rendering undetectable alterations in the HDL3c lipidome upon analysing total HDL. In addition, Stahlman et al. evaluated three groups of women, a control one together with two groups featuring T2D and insulin resistance. Importantly, the both T2D groups displayed similar HbA1c levels in this study (30), complicating evaluation of the impact of the glycemic status on the HDL lipidome.

In summary, the present study for the first time identifies PL subclasses altered in small, dense HDL3 from patients with T2D in association with impaired HDL functionality. The study is not free of limitations, the major of which involves the small number of included subjects. Importantly however, essentially treatment-naïve patients and controls recruited in order to avoid a drug-treatment bias renders this cohort unique for modern T2D research.

The prevalence of diabetes worldwide is increasing; by 2030, 439 million people are expected to be living with T2D (37). This number highlights the need of novel therapeutic strategies for this disease. Lipid parameters presently evaluated in T2D, including TG, TC, HDL-C and LDL-C, provide only a narrow snapshot of the dynamic processes of lipid metabolism. Functional evaluation of HDL particles combined with lipidomic approaches bear a potential to improve current CV risk prediction tools. It is important in this regard that the present study not only documents the presence of profound molecular alterations in small, dense HDL in poorly-controlled T2D, but equally introduces the notion of the glycemic status as a determinant of altered HDL composition and functionality in this metabolic condition.

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Figure legends

Figure 1: Influence of small, dense HDL3c (10 mg total mass/dl) on AAPH-induced oxidation of reference LDL in T2DPC patients (n=8), T2DWC patients (n=10), and control subjects (n=11). Reference LDL (10 mg TC/dl) was incubated at 37°C in PBS in the presence of AAPH (1 mmol/l). Data are shown as % of values measured in reference LDL incubated without added HDL. For each curve oxidation rates of initiation phase and propagation phase, and the amount of dienes formed at the end of propagation phase (maximal amount of dienes concentration) of LDL oxidation are shown. T2DPC, poorly-controlled Type 2 diabetes; T2DWC, well-controlled Type 2 diabetes. p-value obtained by ANCOVA. †, significant difference between T2DPC *vs.* control subjects.

Figure 2: Phospho- and sphingolipidome of small, dense HDL3c in T2DPC (n=8), T2DWC (n=10) and control subjects (n=11). A, major lipid subclasses: PC, phosphatidylcholine; SM sphingomyelin; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine and PI, phosphatidylinositol; and B, minor lipid subclasses (logarithmic scale): PG, phosphatidylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; Cer, ceramide. T2DPC, poorly-controlled Type 2 diabetes; T2DWC, well-controlled Type 2 diabetes. $^{\dagger}p<0.01$, significant difference between T2DPC *vs.* control subjects. $^{\bullet}p<0.01$, significant difference between T2DPC *vs.* T2DWC and control subjects.

Supplementary Figure 1: Individual phospho- and sphingolipid species in small, dense HDL3c from T2DPC (n=8), T2DWC (n=10) and control subjects (n=11). T2DPC, poorly-controlled Type 2 diabetes; T2DWC, well-controlled Type 2 diabetes.

Supplementary Figure 2: Principal component analysis of HDL3c lipidomic data from T2DPC (n=8), T2DWC (n=10) and control subjects (n=11). For simplicity, two-dimensional covariance matrix is shown for the principal components 1 and 3. T2DPC, poorly-controlled Type 2 diabetes; T2DWC, well-controlled Type 2 diabetes.

Supplementary Figure 3: Heat map of the ratios of the content of individual lipid species in HDL3c from T2DPC (n=8) and T2DWC (n=10) patients relative to control subjects (n=11). Rows indicate T2DPC and T2DWC patients; columns represent individual lipid siècles grouped into their classes (LPE, PE, PS, PG, PA, Cer, PI, LPC, PC, SM). A dendrogram was constructed using the weights calculated by heatmap.2 function from gplots package; the branches of the dendrogram were reordered according to the weights. At each node, the branches are ordered in the order of increasing weight; the weight of a branch is defined by the aforementioned function.

TABLE 1: Clinical and Biochemical Parameters in T2DPC, T2DWC and Control Subjects.

	T2DPC (n=8)	T2DWC (n=10)	Control (n=11)	p<
Age (Years)	59±11	57±10	56±9	NS
Sex (Females/Males)	7/1	7/3	8/3	NS
Body Mass Index (kg/m^2)	29.5±1.4	31.9±5.7	24.2±3.4*	0.01
Glucose (mg/dl)	157 (113-225)	133 (89-139)	78 (67-87)*	0.001
HbA1c (%)	9.5 (8.9-10.5)	6.2 (5.8-6.4)	5.6 (3.9-5.7)$^{\Phi}$	0.001
TG (mg/dl)	234 (185-257)	195 (176-221)	73 (58-80)*	0.001
TC (mg/dl)	218±35	202±44	199±22	NS
VLDL-C (mg/dl)	47 (26-48)	37 (22-44)	19 (15-21)*	0.001
LDL-C (mg/dl)	137±33	120±43	115±21	NS
HDL-C (mg/dl)	37±8	38±8	65±10*	0.001
Non-HDL-C (mg/dl)	182±33	163±44	134±21†	0.01
ApoB (mg/dl)	135 (107-173)	138 (66-173)	86 (69-125)	NS
ApoA-I (mg/dl)	134 (120-182)	139 (108-153)	150 (140-254)	NS
CETP Activity (%/ml.h)	193±43	176±42	139±38†	0.05

T2DPC, poorly controlled type 2 diabetes; T2DWC, well controlled type 2 diabetes; HbA1c, glycated haemoglobin; TC, total cholesterol; TG, triglycerides; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; apo, apolipoprotein. Data are expressed as mean ± S.D. or median (interquartile range), depending on data distribution. Shown p-values were obtained using parametric or non-parametric ANOVA tests; *, significant difference between T2DPC

and T2DWC *vs.* control subjects. †, significant difference between T2DPC *vs.* control subjects. ❖, significant difference between T2DPC *vs.* T2DWC and control subjects.

TABLE 2: Inflammatory Biomarkers in T2DPC (n=8), T2DWC (n=10) and Control Subjects (n=11).

	T2DPC (n=8)	T2DWC (n=10)	Control (n=11)	p<
hsCRP (mg/l)	1.4 (1.0-5.4)	1.4 (0.9-3.2)	0.5 (0.3-1.4)†	0.05
IL-6 (pg/ml)	1.6 (1.4-3.3)	1.7 (1.4-2.3)	0.9 (0.7-1.1)	NS
VCAM-1 (ng/ml)	691 ± 90	639 ± 84	671 ± 67	NS
ICAM-1 (ng/ml)	315 (304-330)	262 (238-307)	219 (207-225)†	0.01
E-Selectin (ng/ml)	22 ± 6	22 ± 6	13 ± 5*	0.05
EGF (pg/ml)	41 (40-42)	23 (10-50)	5 (4-16)†	0.05
VEGF (pg/ml)	64 ± 30	26 ± 18	47 ± 29$^{\Delta}$	0.05
Lp-PLA$_2$ Activity (µmol/ml.h)	7.3± 1.5	7.3± 2.2	5.3± 1.1*	0.01

T2DPC, poorly controlled type 2 diabetes; T2DWC, well controlled type 2 diabetes; hsCRP, high sensitivity C reactive protein; IL, interleuquin; VCAM, vascular cell adhesion molecule; ICAM, intercellular cell adhesion molecule; E-selectin, endothelial-selectin; EGF, endothelial growth factor; VEGF, vascular endothelial growth factor. Data are expressed as mean ± S.D. or median (interquartile range), depending on data distribution. Shown p corresponds to that obtained from parametric or non-parametric ANOVA tests. *, significant difference between T2DPC and T2DWC *vs.* control subjects. †, significant difference between T2DPC *vs.* control subjects. $^{\Delta}$, significant difference between T2DPC *vs.* T2DWC subjects.

TABLE 3: Total Mass and Total Lipid and Protein Composition of HDL Subpopulations from T2DPC (n=8), T2DWC (n=10) and Control Subjects (n=11)

	Group	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
CE (wt %)	T2DPC	20±3	18±2	18±2	17±2	15±2
	T2DWC	21±3	19±3	18±2	17±3	15±3
	Control	26±4*	24±2*	23±2*	21±3*	18±3*
FC (wt %)	T2DPC	3.1±0.5	2.5±0.2	2.0±0.1	1.4±0.4	1.2±0.4
	T2DWC	3.4±0.6	2.5±0.2	1.9±0.3	1.4±0.6	1.0±0.6
	Control	4.0±1.4	3.2±0.3*	2.4±0.3*	1.8±0.8	1.6±0.9
TG (wt %)	T2DPC	9±4	6±1	6±2	8±4	8±5
	T2DWC	9±4	5±1	5±1	6±4	5±3
	Control	4±1*	3±1*	3±1*	4±2†	4±2†
PL (wt %)	T2DPC	35±7	37±5	35±5	30±5	25±6
	T2DWC	36±4	36±3	34±3	29±4	23±6
	Control	35±4	32±4*	30±3*	26±3*	21±4$^{\bullet}$
Total protein (wt %)	T2DPC	32±4	36±4	40±4	45±5	51±7
	T2DWC	31±5	37±4	41±4	47±6	55±9
	Control	31±2	38±3	42±2	48±2	56±4†
ApoA-I/apoA-II	T2DPC	3.8±0.8	2.6±0.3	2.7±0.3	3.7±1.1	5.6±0.7
	T2DWC	3.3±1.2	2.6±0.3	2.7±0.3	3.9±1.0	5.7±0.8
	Control	4.9±1.4*	2.9±0.4*	2.8±0.4	3.7±1.1	5.9±0.9

HDL, high density lipoprotein; T2DPC, poorly controlled type 2 diabetes; T2DWC, well controlled type 2 diabetes; CE, cholesteryl esters; FC, free cholesterol; TG, triglycerides; PL, phospholipids; TP, total protein; apo, apolipoprotein. Data are expressed as mean ± S.D. * $p<0.05$, significant difference between T2DPC and T2DWC *vs.* control subjects. † $p<0.05$, significant difference between T2DPC *vs.* control subjects, ° $p<0.05$, significant difference between T2DPC *vs.* T2DWC and control subjects.

TABLE 4. Significant different phospho- and sphingolipid species in HDL3c classified according to the level of unsaturation of esterified fatty acid moieties in T2DPC, T2DWC and control subjects

	T2DPC (n=8)	T2DWC (n=10)	Control (n=11)	p<
PC-PUFA (wt%)	18.8 (18.0-19.1)	16.2 (11.2-19.0)	12.3 (11.1-14.6)†	0.05
PE-SFA ($x10^{-4}$, wt%)	17.2 (10.7-100.2)	13.0 (11.1-21.3)	8.6 (6.6-10.3)†	0.05
PE-MUFA(wt%)	0.08 (0.04-0.09)	0.05 (0.04-0.05)	0.03 (0.02-0.05)†	0.05
PI-SFA ($x10^{-3}$, wt%)	10.4 (10.1-10.7)	2.0 (1.7-3.1)	2.0 (1.5-2.5)Φ	0.0001
PI-PUFA (wt%)	0.74±0.18	0.55±0.19	0.47±0.17†	0.05
PI-MUFA (wt%)	0.08 (0.04-0.14)	0.04 (0.02-0.12)	0.05 (0.03-0.07)Φ	0.05
PG-PUFA ($x10^{-3}$, wt%)	4.8 (1.8-7.5)	2.9 (1.0-4.9)	1.7 (0.6-4.9)†	0.05
PG-MUFA ($x10^{-3}$, wt%)	4.0±1.5	2.6±1.5	1.8±1.5†	0.01
Cer-SFA (wt%)	0.07 (0.02-0.11)	0.02 (0.01-0.02)	0.01 (0.01-0.02)Φ	0.05
Cer-MUFA ($x10^{-3}$, wt%)	10.3 (4.9-20.5)	4.1 (3.1-10.0)	3.4 (3.0-3.9)Φ	0.01
Cer-PUFA ($x10^{-5}$, wt%)	18.6 (12.3-24.7)	12.0 (8.2-17.0)	7.3 (6.1-8.7)†	0.01

T2DPC, poorly-controlled Type 2 diabetes; T2DWC, well-controlled Type 2 diabetes; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyinsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; Cer, ceramide; wt%, weight % of total HDL3c mass. Data are expressed as mean ± S.D. or median (interquartile range), depending on data distribution. p value obtained by ANCOVA. Only PL fatty acid classes that revealed significant between-group differences are included in the table.†, significant difference between T2DPC *vs.* control subjects, Φ , significant difference between T2DPC *vs.* T2DWC and control subjects.

Fig. 1

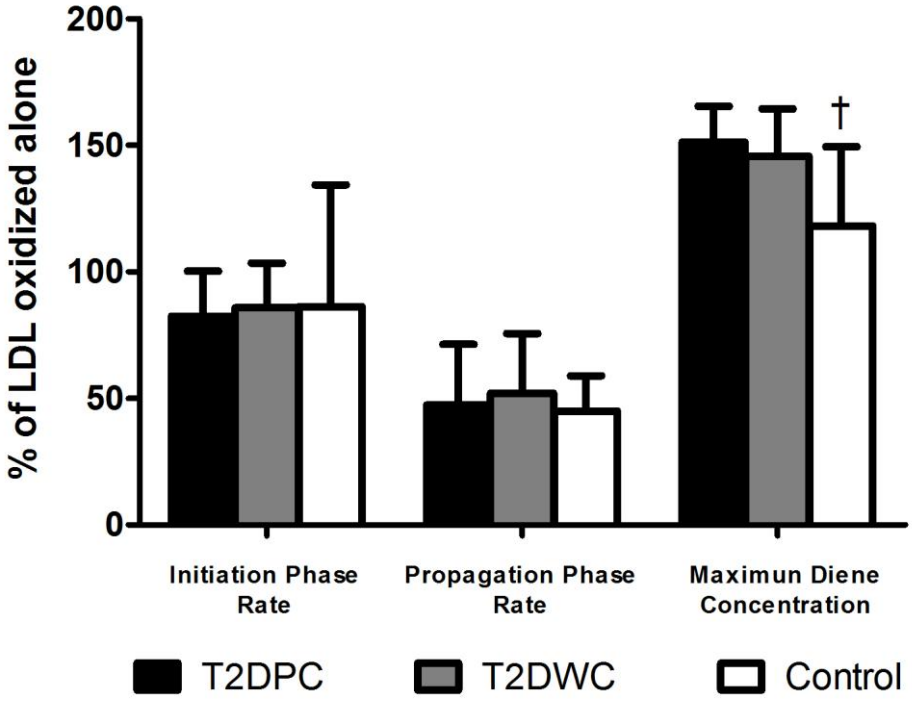

Fig. 2