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Increased cholesterol efflux capacity in metabolic syndrome: Relation with qualitative alterations in HDL and LCAT

Diego Lucero [a, b, *], Denis Svidirov [b], Lita Freeman [b], Graciela I. López [a], Eduardo Fassio [c], Alan T. Remaley [b], Laura Schreier [a]

[a] Laboratory of Lipids and Atherosclerosis, Department of Clinical Biochemistry, Faculty of Pharmacy and Biochemistry, INFIBIOC, University of Buenos Aires, Argentina
[b] Lipoprotein Metabolism Section, National Heart, Lung, Blood Institute, National Institutes of Health, Bethesda, Maryland, USA
[c] Department of Gastroenterology, National Hospital "Prof. A. Posadas", Buenos Aires, Argentina

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ABSTRACT

Background: Metabolic syndrome (MetS) is associated with changes in HDL levels, composition and sub-fraction profile. Whether these alterations affect HDL anti-atherogenic function, specifically measured as its capacity to perform cholesterol efflux, is not yet clearly known.
Objective: To evaluate the relation between serum cholesterol efflux capacity and the changes in HDL composition and sub-fraction profile in MetS.
Methods: In 35 non-treated MetS patients and 15 healthy controls, HDL mediated cholesterol efflux was measured as the ability of apoB-depleted serum to accept cholesterol from cholesterol-loaded BHK cells expressing either ABCA1 or ABCG1. Additionally we determined: lipid profile, HDL sub-fractions (NMR) and LCAT mass (ELISA). Isolated HDL (δ:1.063–1.210 g/mL) was chemically characterized. Pre-β1-HDL was determined by 2D-electrophoresis in a sub-group of MetS and controls (n = 6 each).
Results: Surprisingly, MetS patients presented higher ABCA1 mediated cholesterol efflux (10.4 ± 1.8 *vs.* 8.7 ± 0.3%; p = 0.0001), without differences in ABCG1 efflux. In MetS, HDL showed reduction in particle size and number (p < 0.02) and lower large/small HDL ratio (p = 0.05), as well as triglyceride enrichment (p = 0.0001). Pre-β1-HDL was increased in MetS (p = 0.048) and correlated with ABCA1-cholesterol efflux (r = 0.64; p = 0.042). LCAT mass showed a tendency to reduction in MetS (p = 0.08), and inversely correlated with ABCA1-cholesterol efflux (r = −0.51; p = 0.001), independently of obesity and insulin-resistance (β = −0.40, p = 0.034).
Conclusion: This is the first description of ABCA1 mediated cholesterol efflux in MetS. Regardless the reduced HDL-cholesterol, *in vitro* cholesterol efflux capacity by ABCA1 was enhanced, linked to increased pre-β1-HDL and slightly reduced in LCAT mass that would probably reflect a delay in reverse cholesterol transport occurring in MetS.

1. Introduction

Plasma high density lipoproteins (HDL) are a heterogeneous group of particle sub-fractions, differing in size, structure and biological functions [1]. Over the past few decades, observational and epidemiological studies have demonstrated that HDL cholesterol (HDL-C) levels are a negative cardiovascular disease risk factor [2]. The major mechanism by which HDL is believed to mediate atheroprotection is related to its central role in reverse cholesterol transport (RCT). Cholesterol efflux from cells, especially from macrophages in the arterial wall, is the first step in RCT [3]. Cholesterol efflux involves members of the ATP-binding cassette (ABC) family of transporters, ABCA1 and ABCG1, interacting with different sub-fractions of HDL; ABCA1 exports free cholesterol from cells to lipid poor apoA-I (pre-β1-HDL particles), whereas ABCG1 delivers cholesterol to mature HDL particles (α-HDL particles) [4].

The capacity of plasma to promote cholesterol efflux, by means of measuring the ability of apo B-depleted plasma to accept cholesterol from cholesterol-loaded macrophages *in vitro*, is a

* Corresponding author. Department of Clinical Biochemistry, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956 (C1113AAD), Buenos Aires, Argentina.
E-mail address: dmlucero@ffyb.uba.ar (D. Lucero).

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metric of the efficiency of the most important anti-atherogenic function of HDL. In this sense, it has been demonstrated that the decrease in cholesterol efflux capacity was an excellent predictor of carotid artery intima-media thickness in patients with coronary artery disease beyond HDL-C levels [5]. More recently, Rohatgi A et al. demonstrated an inverse association between cholesterol efflux capacity and the incidence of cardiovascular events, also independently of HDL-C levels and traditional risk factors, in a nine year longitudinal study [6]. This suggests that measuring HDL functionality would reflect the degree of progression of the atherosclerosis process. On the other hand, other authors showed a paradoxical positive association between high cholesterol efflux and more prevalent cardiovascular events in a three year longitudinal study [7]. These reports represent controversial results regarding the significance of plasma cholesterol efflux capacity, reflecting the necessity to advance the knowledge of the mechanisms involved, which would allow a better interpretation of results.

In recent years, there has been a worldwide increase in the prevalence of metabolic syndrome (MetS), leading to a rise in the risk of type 2 diabetes and cardiovascular disease [8]. It is well known that MetS is a constellation of metabolic disorders, including -among others features-the characteristic atherogenic dyslipidemic phenotype of high triglyceride levels, and a predominance of small dense LDL and low HDL-C levels. The mechanisms leading to reduced HDL-C in MetS are most likely related to changes in the sub-fractional distribution of HDL and alterations in composition and structure of HDL particles [9]. Whether these modifications in HDL composition and concentration affect its capacity to promote cholesterol efflux is not clearly understood.

Lecithin:cholesterol acyl transferase (LCAT) is a lipoprotein associated enzyme that esterifies the free cholesterol present in HDL particles, a key step in the process of HDL particle maturation [10]. Thus, this enzyme has a central role in the process of RCT. The evaluation of LCAT in relation to MetS and its implication in the cholesterol efflux in insulin-resistant states is not yet completely understood.

Within this context, our objective was to evaluate the principal anti-atherogenic function of HDL in MS, as measured by its cholesterol efflux capacity, and its relation with the changes in composition and sub-fractions of HDL.

2. Materials and methods

2.1. Subjects

From April 2013 to February 2014, a total of 35 metabolic syndrome patients (female/male: 20/15; age: 43 ± 16 years), diagnosed according to the ATPIII criteria [8], were enrolled from the Gastroenterology Service from the National Hospital Professor Alejandro Posadas, Argentina. In parallel, 15 clinically and biochemically healthy subjects were recruited, among hospital employees, and were matched by gender and age (female/male: 9/6; age: 37 ± 11 years, $p = 0.76$ and $p = 0.18$ respectively).

Subjects were excluded when they presented diabetes mellitus, kidney disease, cardiovascular disease, neoplasia, hypothyroidism, or other endocrine diseases, and when receiving lipid-lowering drugs, hormonal or insulin-sensitizing treatments. Those who consumed more than 20 g alcohol/day were also excluded. Written informed consent was required of all the participants to be included in the study, which had the approval of the Ethics Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

Waist circumference, weight and height were measured and body mass index (BMI) calculated.

2.2. Samples

After a 12-h overnight fast blood samples were drawn. Serum was kept at 4 °C within 48 h for the evaluation of glucose, lipids and lipoproteins, or stored at −70 °C for further determination of insulin levels and LCAT mass, HDL isolation by ultracentrifugation, determination of HDL sub-fractions by NMR and evaluation of HDL functionality by means of the cholesterol efflux capacity assay.

2.2.1. Biochemical determinations

Total cholesterol, triglycerides and glucose were measured in serum, using commercial kits (Roche Diagnostics, Mannheim, Germany) in a Hitachi 917 autoanalyser, CV intra-assay <1.9%, CV inter-assay <2.4%, averaging CV values of these parameters. HDL and LDL cholesterol were determined by standardized selective precipitation methods [11,12], CV intra-assay <2.0% and CV inter-assay < 3.0%. Apo A-I and apo B were determined by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany), CV intra-assay <1.9% and inter-assay <2.5%, for both parameters. Insulin was measured with Immulite/Immulite 1000 Insulin (Siemens, USA). In order to estimate insulin-resistance, the HOMA-IR index was calculated [13].

2.2.2. Cholesterol efflux assay

The capacity of plasma to accept cholesterol efflux through the transporter ABCA1 or ABCG1 was tested *in vitro* in a stably transfected baby hamster kidney (BHK) cell line expressing either ABCA1 or ABCG1, under Mifepristone switch [14,15]. For each cholesterol efflux assay, a mock-transfected BHK cell line was used as the control cell line. Cholesterol efflux was conducted at 37 °C in cells labelled with ^{3}H-cholesterol for 24 h, washed, and incubated for 4 h with the subject's serum previously depleted of apoB lipoproteins by precipitation with polyethylene glycol (MW 8000; Sigma–Aldrich, St Louis, MO). The equivalent of 1% serum was used in each efflux reaction, and each sample was tested in triplicates. The percentage of efflux specific through the ABCA1 or ABCG1 transporter was calculated by subtracting the radioactive counts in the blank medium (minimal essential medium with 0.1% bovine serum albumin) from the radioactive counts in the presence of serum and then dividing the result by the sum of the radioactive counts in the medium plus the cell fraction**.** Two pools of sera, of high and low HDL-C, were fractioned and each respective apoB depleted sera was used as controls in every cholesterol efflux assay at a concentration equivalent to 1% of serum. Besides, an aliquot of lipid free Apo AI (10 μg/ml) was also used as control for each assay. An acceptable range for quality control material was established as ±2SD. Any run in which this value was exceeded for either control material was repeated. The calculation of coefficient of variation% showed an average within-assay CV% of 6.2% and between-assay CV% of 7.0%.

2.2.3. HDL analysis by nuclear magnetic resonance

The HDL sub-fraction profile was measured by NMR spectroscopy, using a Vantera Clinical Analyzer (LipoScience, Inc, Raleigh, USA) on frozen serum specimens of patients and controls. Briefly, HDL sub-fractions were quantified from the measurement of the amplitudes of their spectroscopically distinct lipid methyl group NMR signals. Average particle size was derived from the sum of the diameter of each sub-fraction multiplied by its relative mass percentage. Thus, it was possible to determine HDL average size (nm) and total HDL particle number (nmol/L). Besides the number of particles, in nmol/L, of three HDL subclasses were determined: large HDL (defined as particles between 14 and 9.4 nm), medium HDL (diameter: 9.4–8.2 nm) and small HDL (diameter: 8.2–7.3 nm).

2.2.4. Pre-β1-HDL particle determination

In a randomly selected sub-group of MetS patients (n = 6) and healthy controls (n = 6), pre-β1-HDL particles were determined by native–native 2-dimensional gel electrophoresis, followed by Western-blotting with anti-apoA-I antibodies [16,17], as modified in reference [18]. Briefly, a volume of 7.5 μL of serum was separated in the first dimension of a 0.7% agarose gels, and then in the second dimension by electrophoresis on a gradient (3–25%) polyacrylamide gel (Jule, Inc. Milford, USA). HDL sub-fractions were transferred to PVDF membranes, which were incubated with HRP-labelled anti-apoA-I antibody (Meridian Life Science, USA). After washing, membranes were incubated with Western-blot Lightning Plus –ECL Enhanced Chemiluminescent Substrate (Perkin Elmer) - and exposed to Carestream Biomax MR2 film. Spot intensity was quantified using Fluorchem specific software (Alpha Innotech Corp, USA). The relative concentration of the pre-β1-HDL migrating subfraction was calculated as a percentage of the apoA-I total signal. The absolute pre-β1-HDL concentration was calculated by multiplying its relative concentration by the plasma apoA-I level, measured by the immunoturbidimetric method previously described.

2.2.5. HDL isolation

High density lipoprotein (δ:1.063–1.210 g/ml) was isolated by sequential preparative ultracentrifugation method at 105,000 × g for 18 h at 10 °C, in a XL-90 Beckman ultracentrifuge, with a type 90 Ti rotor [19]. Addition of EDTA (100 mg/l) in the salt solution used for the adjustment of density was done to minimize lipid peroxidation that may occur during the long centrifugation time. The supernatant was separated and washed once at the same density in order to minimize albumin contamination. Lipoprotein purity was tested by agarose gel electrophoresis (Hydragel 15 Lipoprotein E, Sebia, USA). Isolated HDL composition was characterized by the following parameters: cholesterol, triglycerides and apo A-I using the methods previously described, phospholipids were assessed by measuring the phosphorous in the dry residue after lipid extraction and proteins by the Lowry method. Data was expressed as the percentage of each component. Circulating HDL total mass -as an estimator of the whole circulating HDL level-was calculated as the sum of triglycerides, cholesterol, phospholipids and protein in mg/dl [20].

2.2.6. Lecitin:cholesterol acyl transferase mass measurement

Serum protein mass levels of LCAT were determined by monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) (ALPCO Diagnostics, USA).

2.3. Statistical analysis

Data is presented as mean ± SD or median (range) according to normal or skewed distribution, respectively. Differences between groups were tested using the unpaired Student's T test for normally distributed data and the Mann–Whitney U-Test for skewed data. Pearson or Spearman analysis, for parametric or non parametric variables, was used to determine correlations between parameters. A multivariate stepwise regression model was developed in order to assess associations between parameters. All analyses were performed using SPSS 17.0 software. P values < 0.05 were considered significant.

3. Results

Table 1 shows the general clinical and biochemical characteristics of both the MetS and age and sex matched control group. As expected, patients with MetS presented higher waist circumference and incremented body mass index, insulin levels and HOMA-IR index than controls ($p < 0.005$).

Lipid and lipoprotein profile is also presented in Table 1. MetS patients showed higher triglyceride levels and lower HDL-C levels ($p < 0.0007$), as well as higher apolipoprotein B and lower apolipoprotein A-I ($p < 0.05$). Additionally, patients with MetS presented higher total cholesterol, LDL cholesterol and non-HDL cholesterol ($p < 0.05$).

The anti-atherogenic function of HDL, was evaluated as the rate of cholesterol efflux through ABCA1 and ABCG1. Paradoxically, cholesterol efflux by apo B-depleted serum from ABCA1 was increased in MS patients [MetS patients: 10.36 ± 1.80% and healthy controls: 8.69 ± 0.32%; $p = 0.0001$]. Cholesterol efflux by ABCG1 did not differ between groups [MetS patients: 8.34 ± 1.22% and healthy controls: 8.08 ± 0.93%; $p = 0.52$].

When pre-β1-HDL was assessed in a sub-group of MetS patients and healthy controls, a marked increase in pre-β1-HDL was observed in the MetS group [45.4 ± 21.3 *vs.* 22.6 ± 12.1 mg apoA-I/dL; $p = 0.048$], presenting a positive correlation with cholesterol efflux by ABCA1 ($r = 0.64$; $p = 0.042$).

Table 2 shows the plasma HDL particle analysis by NMR. The average size of HDL in MetS patients was significantly reduced ($p = 0.016$), with decreased total HDL particle number ($p = 0.0004$), at the expense of the reduction in the particle number of large and small HDL ($p = 0.0215$ and $p = 0.0005$, respectively), with no change in the number of medium HDL particles ($p = 0.891$). Moreover, the ratio of large HDL/small HDL was lower in MetS patients ($p = 0.05$).

Table 1
Clinical and biochemical characteristics and lipid and lipoprotein profile of studied subjects: Metabolic syndrome patients (ATPIII) and healthy controls.

	Metabolic syndrome	Healthy controls	p value
Age (years)	46 ± 13	38 ± 10	0.1023
Waist circumference (cm)	108.7 ± 11.8	74.7 ± 5.6	< 0.0001
BMI (Kg/m^2)	33.1 ± 3.8	21.8 ± 1.7	< 0.0001
Glucose (mg/dl)	134 ± 60	93 ± 5	0.0922
Insulin (μU/ml)	10.9 (2.1–34.4)	5.4 (2.0–12.0)	0.0044
HOMA-IR	3.57 (0.50–10.03)	1.18 (0.40–2.80)	0.0012
Total cholesterol (mg/dl)	201 ± 32	176 ± 30	0.0454
Triglycerides (mg/dl)	153 ± 55	65 ± 11	0.0001
HDL-cholesterol (mg/dl)	43 ± 10	59 ± 17	0.0007
LDL-cholesterol (mg/dl)	124 ± 34	97 ± 26	0.0361
Non-HDL-cholesterol (mg/dl)	158 ± 32	112 ± 24	0.0056
Apo B (mg/dl)	114 ± 22	79 ± 12	0.0011
Apo A-I (mg/dl)	155 ± 18	175 ± 16	0.0501

Data is expressed as mean ± SD or median (range) for parameters with skewed distribution. BMI: Body mass index; HOMA-IR: Homeostasis model assessment for insulin resistance index.

Table 2
HDL size, particle number and sub-fractions by NMR and pre-β1-HDL of studied subjects: Metabolic syndrome patients (ATPIII) and healthy controls.

	Metabolic syndrome	Healthy controls	p value
HDL size (nm)	9.0 ± 0.5	9.6 ± 0.8	0.0163
Total HDL particles (nmol/L)	26.1 ± 5.6	33.6 ± 3.9	0.0004
Large HDL particles (nmol/L)	2.9 ± 2.1	5.4 ± 3.3	0.0218
Medium HDL particles(nmol/L)	6.1 ± 2.9	6.3 ± 3.3	0.8941
Small HDL particles (nmol/L)	15.8 ± 5.2	20.8 ± 2.2	0.0005
Large HDL/Small HDL	0.18 ± 0.13	0.28 ± 0.16	0.0500

Data is expressed as mean ± SD.

Table 3
Correlations of ABCA1 mediated cholesterol efflux with HDL-cholesterol, HDL size, HDL particle number and small, medium and large HDL sub-fractions.

	Total group (r/p)	Metabolic syndrome (r/p)	Healthy controls (r/p)
HDL-cholesterol (mg/dl)	−0.26/0.102	0.14/0.542	0.63/0.038
HDL size (nm)	0.02/0.885	0.32/0.110	0.51/0.107
Number HDL particles (nmol/L)	−0.43/0.005	−0.36/0.05	0.61/0.05
Small HDL particles (nmol/L)	−0.51/0.001	−0.42/0.02	0.20/0.541
Medium HDL particles (nmol/L)	−0.03/0.8461	0.03/0.862	−0.22/0.488
Large HDL particles (nmol/L)	−0.10/0.592	0.10/0.581	0.62/0.042

Correlation coefficients obtained by Pearson's Test.

Table 4
HDL total mass and HDL chemical composition of studied subjects: Metabolic syndrome patients (ATPIII) and healthy controls.

	HDL total mass (mg/dl)	HDL chemical composition (%)				HDL (Cholesterol/TG)
		TG	Cholesterol	Phospholipids	Proteins	
Metabolic syndrome	101.3 ± 38.0	5.8 ± 1.4	17.7 ± 1.8	22.2 ± 3.9	54.2 ± 4.0	3.27 ± 1.21
Healthy controls	140.2 ± 51.6	3.5 ± 0.8	17.5 ± 2.6	18.8 ± 2.9	60.2 ± 2.8	5.12 ± 1.19
p value	0.0403	0.0001	0.7815	0.0211	0.0003	0.0007

Data is expressed as mean ± SD. TG: triglycerides. HDL total mass was assessed as the sum of TG, cholesterol, phospholipids and proteins in mg/dl.

Table 3 shows the correlations of ABCA1 mediated cholesterol efflux with HDL-C, HDL size, HDL particle number and HDL sub-fractions, in the whole population and in each group by separate. Interestingly, there was no correlation in the total population nor in MetS group between ABCA1 mediated cholesterol efflux and HDL-cholesterol; but in healthy controls both parameters were positively associated ($r = 0.63$; $p = 0.0038$). Moreover, ABCA1 cholesterol efflux and HDL particle number were negatively associated in the whole population ($r = -0.43$; $p = 0.005$) and in MetS patients ($r = -0.36$; $p = 0.05$), while on the other hand both positively correlated in healthy controls ($r = 0.61$; $p = 0.05$). Finally, cholesterol efflux by ABCA1 was negatively associated with small HDL particles (determined by NMR) in the whole population ($r = -0.51$; $p = 0.001$) and in the MetS patients ($r = -0.42$; $p = 0.02$), but this correlation was not observed when analyzing the healthy control group ($r = 0.20$; $p = 0.541$).

Isolated HDL analysis is shown in Table 4. HDL total mass was decreased in MetS patients ($p = 0.04$). Additionally, HDL chemical composition analysis showed that HDL particles in MetS were enriched in triglycerides ($p = 0.0001$) and had a higher phospholipid content ($p = 0.021$). Interestingly, triglyceride content in HDL positively correlated with ABCA1 cholesterol efflux ($r = 0.72$; $p = 0.002$), but significance was lost after adjusting by HOMA-IR, waist circumference and BMI ($\beta = 0.23$, $p = 0.125$).

Although LCAT mass did not differ between groups [MetS patients: 5.5 ± 0.9 and healthy controls: 5.9 ± 0.5 μg/ml; $p = 0.0861$], it was negatively and significantly associated with cholesterol efflux through ABCA1 ($r = -0.51$; $p = 0.001$) (Fig. 1), even after adjusting by waist circumference and HOMA-IR ($\beta = -0.40$, $p = 0.034$). Also, LCAT mass positively correlated with HDL particle number ($r = 0.38$; $p = 0.01$), but no significant association was found between LCAT levels and pre-β1-HDL ($r = -0.28$; $p = 0.41$).

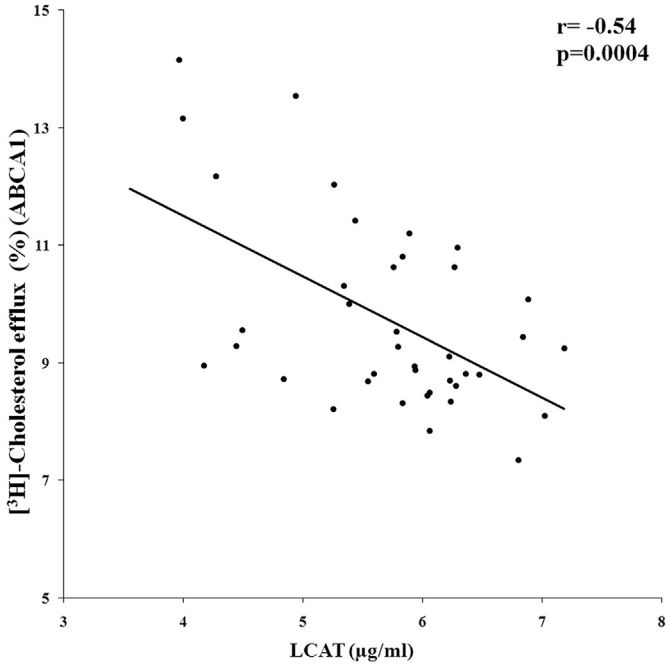

Fig. 1. Correlation between: LCAT and cholesterol efflux by ABCA1. Adjusted by waist circumference and homeostasis model assessment for insulin resistance index: $\beta = -0.40$; $p = 0.034$.

4. Discussion

In the present study, we have observed a higher anti-atherogenic HDL function -measured as serum cholesterol efflux capacity by ABCA1-in MetS patients, in comparison to healthy controls. This increase might be related to changes in the composition of HDL, its sub-fraction profile or to LCAT levels in MetS. In addition, the discoid nascent pre-β1-HDL, the most efficient cholesterol acceptor, was significantly increased in MetS and positively correlated with the cholesterol efflux.

Cholesterol content in HDL is largely recognized to be a negative risk factor for the development of cardiovascular disease [2]. But recently, controversies regarding the dissociation between HDL concentration and function have been raised [21], suggesting that the assessment of HDL function might be of high relevance. Among HDL functions, RCT constitutes the most important anti-atherogenic action and the cholesterol efflux is the first step of this process.

In order to assess HDL cholesterol efflux capacity, we have used BHK cells transfected with ABCA1. Notably, cholesterol efflux by the ABCA1 transporter using BHK cells should represent the cholesterol efflux from cholesterol loaded-macrophages, and indeed we have found a highly significant correlation between cholesterol efflux from BHK cells transfected with ABCA1 and efflux from THP-1 macrophages ($r = 0.67$, $p = 0.0002$). Nowadays, there are an increasing number of studies using transfected BHK cells for assessing cholesterol efflux [22–24], because unlike THP-1 cells it allows a direct assessment of the contribution of ABCA1 versus ABCG1 in the process.

It should be highlighted that there is controversial data in relation to oxidative modifications on apo A-I and their impact on the cholesterol efflux capacity of HDL. Some evidences show that oxidation of apo A-I could decrease HDL capacity to perform cholesterol efflux [25], while other authors suggest that oxidation of apo A-I, in certain residues, enhances the cholesterol efflux capacity of HDL [26]. Thus, further studies about the oxidative status of apo A-I could complement our results.

As mentioned above, we observed higher cholesterol efflux capacity by ABCA1 in MetS patients in comparison to healthy controls, without changes in the specific ABCG1 cholesterol efflux. In a recent publication, Nestel P et al. have found increased cholesterol efflux capacity in the whole plasma from insulin-resistant obese patients in comparison to non-insulin-resistant obese patients, with the same levels of HDL-C, using THP-1 macrophages [27]. The authors suggest that there may be a more active RCT in insulin-resistant states. The most remarkable finding in our study was the discordance between HDL-C levels and cholesterol efflux in our MetS patients, namely greater cholesterol efflux was observed in these patients despite their lower HDL-C levels. It is already known, however, that HDL-C only poorly correlates with cholesterol efflux [28].

In addition, we observed increased discoidal pre-β1-HDL levels in MetS compared to controls, which may explain the discrepancy. In a previous study, Tian L et al. also found that obese patients, in comparison to lean subjects, showed increased pre-β1-HDL levels [29]. Moreover, we obtained a positive association between pre-β1-HDL and ABCA1 cholesterol efflux. Thus, the higher cholesterol efflux by ABCA1 in MetS could be assumed to be mediated by the presence of an increase of pre-β1-HDL particles, which are known to be especially potent in mobilizing cholesterol by the ABCA1 transporter [4].

Interestingly, previous reports showed that the increase in circulating levels of pre-β1-HDL are positively associated with cardiovascular disease risk [30,31]. Accumulation of pre-β1-HDL fraction could be due to defects in the HDL maturation pathways promoting a delay in RCT. Another mechanism that would potentially favour the pre-β1-HDL increase is the action of lipolytic enzymes -hepatic lipase and endothelial lipase-which act on HDL catabolism. As a result, nascent HDL particles compatible with pre-β1-HDL are generated [32]. It is noteworthy that probably there are differences between the *ex vivo* cholesterol efflux assay, where we found apparently enhanced HDL function, and the actual *in vivo* situation in MetS with already known increased cardiovascular risk.

Several investigators believe that the measurement of HDL sub-fractions would allow a better evaluation of the cardio-protective status than the sole measurement of plasma HDL-C levels, because HDL sub-types may vary in their anti-atherogenic actions [33]. In the present study, implementing NMR analysis to determine HDL sub-fraction profile, we found that the presence of MetS was indeed linked to a reduction in the size and number of HDL particles. To date, there is almost no information on HDL sub-fractions measured by NMR in MetS and in relation to HDL function. The study carried out by Kathiresan S with the aim of evaluating small LDL particles measured by NMR in MetS, indirectly shows that MetS was related to a decrease in HDL number of particles and size [34], similar to our findings.

The NMR analysis showed a decrease in both small and large HDL particles. The lower large/small HDL ratio found in MetS suggests a shift to the predominance of small HDL particles. This is in line with other authors that found a reduction in large HDL_2b percentage and in the large/small HDL ratio in MetS patients, detected by gradient electrophoresis [35]. It must be taken into account that HDL sub-fraction levels is like a snapshot of a dynamic process. Our finding could be in accordance to the existence of a delay in HDL maturation and then a less efficient RCT.

The interesting correlations obtained between cholesterol efflux and HDL related parameters, in MetS patients and healthy controls, lead us to think that in normal conditions -as result of an efficient RCT-effluxed free cholesterol would be rapidly esterified and thus pre-β1-HDL particles transformed into larger spherical α-HDL particles, which finally transport excess of cholesterol to the liver for its elimination [36]. While in MetS patients, the negative associations with HDL particle number and small HDL particles –first HDL fractions formed after efflux step-suggest that there would be a slowing-down in HDL maturation, causing the accumulation of pre-β1-HDL particles, usually not reflected in the NMR analyses [37]. This would be linked to the enhanced cholesterol efflux by ABCA1 observed *in vitro*.

When HDL chemical composition was analysed, an expected increase in the triglyceride content was detected in patients with MetS which is likely a consequence of increased cholesteryl ester transfer protein (CETP) activity in insulin-resistant states, as well as in hypertriglyceridemic situation [38,39]. Recently, in another study carried out in type 2 diabetic patients divided into hypertriglyceridemic and with normal triglyceride levels, those with hypertriglyceridemia showed increased ABCA1 cholesterol efflux to plasma. The authors suggest that triglyceride enrichment in HDL by CETP would favour cholesterol efflux [22]. Indeed in the present study, we have found a positive association between triglyceride content in HDL and cholesterol efflux, however this association resulted to be strongly influenced by obesity and insulin-resistance after adjustments. Therefore, the insulin-resistant state would determine cholesterol efflux beyond triglyceride content in HDL.

Another critical factor related to RCT is LCAT. We observed a slight tendency to decrease in LCAT mass in MetS that did not reach statistical significance. There is no general consensus about LCAT in insulin-resistance, especially due to the different methodology used to measure it [40,27]. Moreover, more recently Sethi AA et al. observed low LCAT activity and high pre-β1-HDL levels in patients

with ischemic heart disease [30], comparable to our results. These observations suggest that the lack in cholesterol esterification by means of LCAT delays pre-β1-HDL maturation and then the RCT.

Herein, we did not find a significant correlation between LCAT mass and pre-β1-HDL, most likely due to the small number of subjects with the available measurement of pre-β1-HDL. But, an interesting result obtained in the present study was the independent and negative correlation between LCAT mass and cholesterol efflux by ABCA1, which to our knowledge has not been described so far. This finding suggests that the lower the LCAT the higher cholesterol efflux by ABCA1. In relation to this, Calabresi L et al. showed that serum of carriers of LCAT gene mutations −with characteristic low or null plasmatic LCAT activity-presented higher capacity for ABCA1-dependent cholesterol efflux than non-carrier controls, associated with increased pre-β1-HDL levels [41]. These observations suggest that reductions in LCAT would favour ABCA1 cholesterol efflux due to a delay in HDL maturation that would produce accumulation of functioning pre-β1-HDL particles.

The present study has some limitations. First, it must be considered that even though we evaluated the specific interaction of HDL particles with a particular cholesterol transporter, such as ABCA1 or ABCG1, this only constitutes a single step in the RCT pathway and does not necessarily relates to whole body cholesterol flux or cholesterol efflux from atherosclerotic plaque. In addition, even though we observed good correlation with the cholesterol efflux from BHK cells transfected with ABCA1 and from THP-1 macrophages, more studies will have to be done to better understand how cholesterol efflux from BHK cells relates to clinical cardiovascular events. It is important to also note that we only examined cholesterol efflux in a relatively small number of MetS patients. Larger studies will be needed to confirm our findings and to also assess whether our findings relate only to MetS or are also applicable to other disease states and/or the general population.

In summary, our results demonstrate that regardless of the reduced HDL-C level in MetS, cholesterol efflux capacity of plasma is enhanced, which is significantly correlated with the increase in pre-β1-HDL and a tendency of reduction in LCAT mass. Given the recent failure of Mendelian randomization and pharmacological interventions studies [42,43] to show the expected inverse relationship between HDL-C and cardiovascular events, we may need new measures of HDL function. Cholesterol efflux is an appealing functional test for HDL but the results described here suggest that additional studies will be needed to fully understand how it relates to cardiovascular disease and whether the relationship between cholesterol efflux and cardiovascular risk may differ between different disease states.

Conflict of interests

There are not conflicts of interest to disclose.

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