Clinical Biochemistry 45 (2012) 243–248

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

HDL-associated enzymes and proteins in hemodialysis patients

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ARTICLE INFO

Article history:
Received 21 July 2011
Received in revised form 4 November 2011
Accepted 10 December 2011
Available online 20 December 2011

Keywords:
Cholesteryl-ester-transfer-protein
HDL
Hemodialysis
Hepatic lipase
Lipoprotein-associated phospholipase A_2
Paraoxonase

ABSTRACT

Objectives: To evaluate HDL-associated proteins and enzymes and their relation with lipoprotein profile and inflammatory markers in chronic renal patients on hemodialysis.

Design and methods: We studied 53 patients under hemodialysis and 32 healthy subjects as controls. We compared plasma lipids, Apoprotein-AI and hs-CRP, as a marker of chronic inflammation. We evaluated proteins and enzymes associated to HDL, involved in several points of lipoprotein metabolism: CETP, paraoxonase and LpPLA2 activities. Hepatic lipase was measured in postheparin plasma.

Results: Patients showed higher triglycerides and lower LDL-, HDL- and total-cholesterol than controls ($p<0.05$). Also, in comparison with controls, Apoprotein-AI, paraoxonase and hepatic lipase were lower, while CETP was higher ($p<0.03$). LpPLA2 did not show changes between groups.

Conclusion: Beyond plasma lipid-lipoprotein profile, other factors could contribute to induce a pro-oxidative and pro-inflammatory status. The protective role of HDL does not only depend on its concentration, but also on its functionality.

1. Introduction

It is known that patients with chronic renal failure present accelerated atherosclerosis and a higher incidence of death due to cardiovascular disease, even after receiving renal replacement therapy, such as hemodialysis. It is noteworthy that the increased cardiovascular risk cannot be entirely explained by traditional atherogenic risk factors.

Dyslipidemia is one of the involved factors, even though frequent low levels of LDL-cholesterol and a moderate hypertriglyceridemia are observed in hemodialyzed (HD) patients [1,2]. Furthermore, in HD patients HDL should be specially considered beyond its plasma concentration, which is already known to be decreased. In addition to its function in the reverse cholesterol transport, HDL plays an antioxidant role, in part, by means of the action of paraoxonase enzyme [3]. This HDL-associated enzyme might also provide protection against the induction of inflammatory responses in artery wall cells by neutralizing biologically active lipids in mildly oxidized LDL [4]. Moreover, Apo A-I, HDL's major apolipoprotein, also appeared to have an important anti-inflammatory effect, in addition to its action in mediating efflux of cholesterol from peripheral tissues [5]. It was already described that HD patients usually present a high degree of inflammation, and perhaps, Apo-AI reduction may contribute to this condition.

HDL efficiency in its atheroprotective actions depends on the maintenance of its structure and composition. In particular, the cholesteryl-ester-transfer-protein (CETP) and phospholipid-transfer-protein (PLTP) play a central role because these proteins are responsible for lipids interchange between lipoproteins, contributing to their remodeling [6,7]. Furthermore, hepatic lipase (HL), apart from its function in ApoB-containing lipoprotein metabolism, acts on HDL catabolism as phospholipase and triglyceride hydrolase, and it would constitute one of the last steps of the reverse cholesterol transport [8]. In fact, the higher the HL activity, the lower the HDL concentration [9].

Another factor is the lipoprotein-associated phospholipase A2 (LpPLA2), produced and released by inflammatory cells, which is assigned a pro or anti-atherogenic role [10,11]. LpPLA2 is linked to both LDL and HDL particles and it could protect lipoproteins from oxidation or, on the other hand, its presence in the vascular lumen could represent a biomarker of artery disease [12]. A small number of studies have evaluated this enzyme in chronic end-stage renal disease.

Abbreviations: HD, hemodialyzed; CETP, cholesteryl-ester-transfer-protein; HL, hepatic lipase; hs-CRP, high sensitivity C reactive protein; LpPLA2, lipoprotein-associated phospholipase A2; PON, paraoxonase.

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doi:10.1016/j.clinbiochem.2011.12.008

Alterations in enzymes and proteins associated to HDL would contribute to atherogenic risk, as well as the reduction in HDL-cholesterol concentration. Following this idea our aim was to evaluate HDL-associated proteins and enzymes and their relationship with lipoprotein profile and inflammatory markers in chronic renal patients under hemodialysis.

2. Material and methods

2.1. Subjects

We studied 53 patients with end-stage renal disease receiving maintenance hemodialysis who attended the dialysis Centre CEREHA (Kidney Disease Center and Arterial Hypertension) placed in Buenos Aires, Argentina. The recruitment period was between March and May 2010.

Nine of them suffered from type 2 diabetes; 10 had glomerulopathies; 7 polycystic kidney disease; 8 nephrosclerosis secondary to hypertension; 2 obstructive nephropathy; 2 tubulointerstitial nephropathy; 2 lupus nephritis and 13 unknown aetiology.

Thirty-two nondiabetic subjects without renal disease were studied as controls. The clinical characteristics of patients and controls are shown in Table 1.

Exclusion criteria for both patients and controls were liver dysfunction, thyroid disorders or acute infectious diseases. None of the subjects received corticosteroids, immunosuppressive agents or drugs known to influence lipid metabolism. In no case did alcohol consumption surpass 15 g/day; 5 patients and 4 controls consumed from 3 to 8 cigarettes per day, and the remaining subjects had been non-smokers for the last 10 years. Patients and controls did not follow any regular exercise training program. Patients were treated with antihypertensive drugs (angiotensin receptor blockers or calcium channel blockers). Diabetic patients were receiving insulin once or twice daily (doses from 10 to 35 IU) and none of them were treated with oral hypoglycaemic agents.

Patients followed a standardized diet for hemodialysis treatment, containing 1.2 g proteins/kg body weight/day and 35 cal/kg/day, appropriately administered with phosphate binders and vitamins. Controls followed a varied diet with calorie intake according to individual body weight.

Patients were dialyzed with conventional low-flux hemodialysis treatment, for at least 4 hours, 3 times per week using bicarbonate-containing dialysis fluid. The blood flow, dialysate flow rate, dialyzer model, and treatment time were tailored to individual patients to achieve the target equilibrated urea KT/V of 1.25.

Informed consent was obtained from all participants. This study was approved by the Ethical Committee of School of Pharmacy and Biochemistry of Buenos Aires University.

2.2. Blood sampling

Blood samples were drawn after 12-hour overnight fast by antecubital venipuncture. In patients, blood was obtained before the middle week day dialysis, prior to the initiation of hemodialysis procedure. Serum was kept at 4 °C until its processing within 48 hours. For paraoxonase and CETP determinations, serum aliquots were stored at −70 °C.

On the other hand, Heparin (60 IU/kg body weight) was administered intravenously for the determination of HL activity. Ten minutes later, blood obtained by venipuncture of the contralateral arm was collected in tubes placed in ice, and postheparin plasma was kept at −70 °C until its processing within 30 days.

Table 1
Clinical characteristics of hemodialyzed patients and controls. Mean ± SD and median (range) for age.

Feature	HD Patients (n = 53)	Controls (n = 32)
N (M/F)	26/27	16/16
Age (years)	50 (25–67)	46 (21–67)
BMI (kg/m^2)	24.2 ± 0.61	23.5 ± 0.69
Duration of Hemodialysis treatment (years)	4.83 ± 0.47	-

p = ns. HD: hemodialyzed, M: male, F: female, BMI: Body Mass Index.

2.3. Analytical procedures

Total cholesterol, triglycerides and glucose were determined in serum in a Hitachi 917 autoanalyzer by standardized enzymatic methods (Roche Diagnostics GmbH, Mannheim, Germany) under good quality control (routinely intra and inter CVs<3%). LDL- and HDL-cholesterol were determined by standardized selective precipitation methods and automatized enzymatic measurement of cholesterol in the supernatants.

Serum high sensitivity (hs)-CRP and Apo A-I were evaluated by immunoturbidimetry (Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Hepatic lipase activity

HL activity in postheparin plasma was determined by measuring the oleic acid produced by the enzyme-catalyzed hydrolysis of an emulsion containing [^{3}H]-triolein (Amersham TRA 191; Amersham, Buckinghamshire, UK) as previously published [1]. Results were expressed as μmol of free fatty acids /ml h of postheparin plasma. Using triplicate analysis, the intra-assay CV was 4% and the inter-assay CV 9%.

2.5. CETP activity

CETP activity was determined in serum samples as described previously [13]. Briefly, it consists in the evaluation of the ability of serum to promote the transfer of tritiated cholesteryl esters from a tracer amount of biosynthetically labeled HDL_3 (^{3}H–CE–HDL_3) (NEN-Life Science Products, Boston MA) towards serum apo B-containing lipoproteins. Results were expressed as the percentage of ^{3}H-cholesteryl esters transferred from HDL_3 to apo B-containing lipoproteins, per ml, per h. Measurements were all carried out in duplicate within the same assay. The intra-assay CV was 4.9%.

2.6. Paraoxonase (PON) activity

The PON enzyme was evaluated in serum samples employing two different substrates: paraoxon (*O,O*-diethyl-*O*-p-nitrophenylphosphate, Sigma Chemical Co St Louis Mo USA) for PON activity and phenylacetate (Sigma Chemical Co St Louis Mo USA) for its arylesterase (ARE) activity. As was previously described [14] for the PON activity the rate of generation of *p*-nitrophenol was determined at 405 nm and 25 °C, in a V-530 spectrophotometer (Jasco Co; Japan). On the other hand ARE activity was determined measuring the rate of generation of phenol at 270 nm and 25 °C, in the same spectrophotometer. Enzymatic activity was calculated from molar extinction coefficient (1310 mol^{-1} l cm^{-1}) and results were expressed as μmol/mL·min. Measurements were all carried out within the same assay, within-run precision (CV) was 5.5% for PON and 4.8% for ARE assay.

PON phenotypic distribution was estimated by dual substrate method [15]. This consists of plotting PON activity toward paraoxon vs. PON activity toward phenylacetate. According to this method, individuals may be assigned to one of the following three possible phenotypes: QQ (homozygous low activity), QR (heterozygous), or RR (homozygous high activity). A contingency table was constructed and data were analyzed by $\chi 2$ test.

2.7. Lp-PLA$_2$ activity

Lp-PLA$_2$ activity was measured following the radiometric assay described by Blank et al. [16] with few modifications reported in previous publications [17]. The tritiated substrate 1-hexadecyl-2-[3H]-acetyl-glicero-3-phosphocholine (13.5 μCi μmol^{-1}) was obtained from New England Nucleotides, Boston, Ma, USA, and the non-tritiated one was obtained from Cayman Chemical Co, Ann Arbor, Mi, USA. Results were expressed as μmol/ml·h. Measurements were all carried out within the same assay. Intra-assay CV was 5.1%.

2.8. Statistical analysis

Results are expressed as mean ± SD for normally distributed data and as median and range for skewed data. 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. Correlations between variables were assessed using the Pearson or Spearman correlation tests. The $\chi 2$ test was used to compare proportions. Differences were considered significant at $p<0.05$.

3. Results

HD patients showed higher plasma triglycerides and lower total, LDL- and HDL-cholesterol ($p<0.05$) than the control group (Table 2).

HDL-associated proteins and enzymes are shown in Table 3. Apo A-I levels decreased in HD patients ($p<0.05$), while CETP increased ($p<0.05$). CETP activity correlated negatively with HDL-cholesterol ($r=-0.57$, $p<0.0001$) (Fig. 1) and also with Apo A-I ($r=-0.39$, $p=0.004$). PON activity decreased in HD patients, $p<0.05$ and correlation between PON activity and HDL-cholesterol or apo A-I were not observed, neither with the whole population ($r=0.17$, $p=0.34$ and $r=0.04$, $p=0.88$ respectively), nor when only HD patients were considered ($r=0.18$, $p=0.21$ and $r=0.03$, $p=0.82$ respectively). ARE activity showed similar results between groups, however these results allowed the plot PON activity toward paraoxon vs. PON activity toward phenylacetate in order to estimate PON activity phenotypes. HD patients and controls had a similar phenotype distribution, Controls: QQ 15%, QR 19% vs HD: QQ 29% and QR 37%, $p=0.93$. These results permitted comparison of the enzyme activity of the two groups of subjects independently of PON phenotypic distribution.

Conversely, Lp-PLA$_2$ activity did not show differences between HD patients and controls (Table 3) and did not present significant correlation with HDL-cholesterol ($r=-0.17$, $p=0.25$). However, this enzyme correlated positively and significantly with LDL-cholesterol, considering both groups ($r=0.39$, $p=0.007$). This association indicates that Lp-PLA$_2$ is mainly linked to LDL.

As plasma LDL-cholesterol was lower in HD patients than in the control group, the ratio Lp-PLA2/LDL-cholesterol was calculated, revealing a higher ratio in HD patients than in controls (Fig. 2).

The inflammatory biomarker hs-CRP was increased in HD patients compared with controls (Fig. 3). Those subjects who presented overt inflammation with hs-CRP >10 mg/L were excluded for this evaluation.

The increase in hs-CRP correlated negatively with PON activity ($r=-0.32$, $p=0.043$), and with Apo A-I, ($r=-0.26$, $p=0.05$) as it is shown in Fig. 4, panel A and B, respectively.

Table 2
Serum lipid in patients in hemodialysis and controls. Mean ± SD.

	HD Patients	Controls	p<
Triglycerides (mmol/L)	1.88 ± 1.20	1.23 ± 0.57	0.01
Cholesterol (mmol/L)	4.39 ± 1.07	4.94 ± 1.01	0.05
HDL-cholesterol (mmol/L)	1.07 ± 0.34	1.33 ± 0.26	0.001
LDL-cholesterol (mmol/L)	2.29 ± 0.83	2.99 ± 0.94	0.001

Table 3
HDL-associated enzymes and proteins in hemodialyzed (HD) patients and controls. Mean ± SD and median (range) for PON and ARE activity.

	HD patients	Controls	p=
PON (nmol/ mL min)	276 (43–889)	357 (75–833)	0.017
ARE (umol/mL min)	95 (134–50)	98 (32–198)	0.71
Lp-PLA2 (μmol/mL h)	6.6 ± 2.4	6.3 ± 3.1	0.67
CETP (EC % mL^{-1} h^{-1})	257 ± 30	241 ± 26	0.022
Apo A-I (mg/dL)	123 ± 23	143 ± 27	0.007

Hepatic lipase activity was significantly decreased in HD patients ($p<0.002$), (Fig. 5). According to the role of HL on HDL catabolism, control subjects showed a significant inverse correlation between HL activity and HDL-cholesterol ($r=-0.85$, $p=0.0034$). However, HD group did not show significant correlation between this enzyme and HDL-cholesterol ($r=-0.22$, $p=0.14$).

4. Discussion

Despite the frequent low LDL-cholesterol levels in chronic renal patients undergoing hemodialysis, the atherogenic risk is still high. In this study, we have evaluated HDL-associated proteins and enzymes involved in several aspects of lipoprotein metabolism. From results, it is deduced that the anti-atherogenic and anti-inflammatory properties of HDL, would be reduced. HD patients showed a decrease in the antioxidant enzyme PON, in Apo A-I, and in hepatic lipase in comparison to healthy controls. CETP activity, responsible for lipoprotein remodeling in plasma, increased in the patient group and correlated inversely with HDL-cholesterol. The increase in hs-CRP confirmed the inflammatory status, which was associated to the lower PON activity and Apo A-I levels. In addition, Lp-PLA2/LDL-cholesterol ratio was increased in HD patients. Overall, this evidences that, beyond plasma lipid and lipoprotein profile, other factors could contribute to induce a pro-oxidative and pro-inflammatory situation. HDL particles contain PON, which plays an important role in LDL protection against oxidation, by neutralizing oxidized phospholipids in LDL [4]. Patients presenting high risk of atherosclerotic cardiovascular disease [3,18] as well as type 2 diabetic patients show low PON activity [19,20]. In the present study, PON activity was reduced in patients undergoing hemodialysis treatment, in agreement with other authors [21,22]. Because the enzyme is in close relationship to HDL particles, significant correlations between PON activity and components of HDL were already reported by

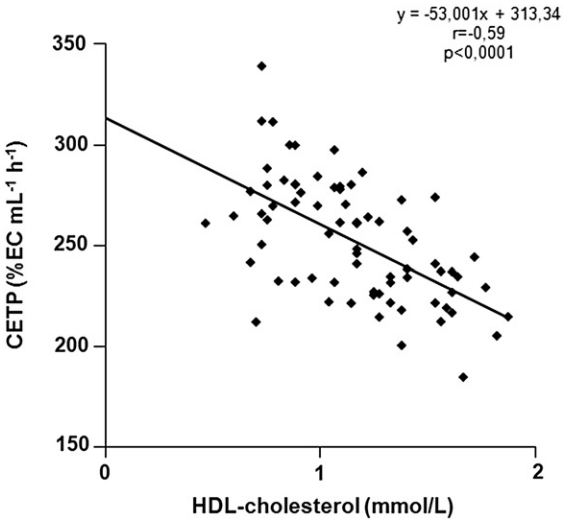

Fig. 1. Correlation between CETP activity and HDL-cholesterol in hemodialyzed patients and controls.

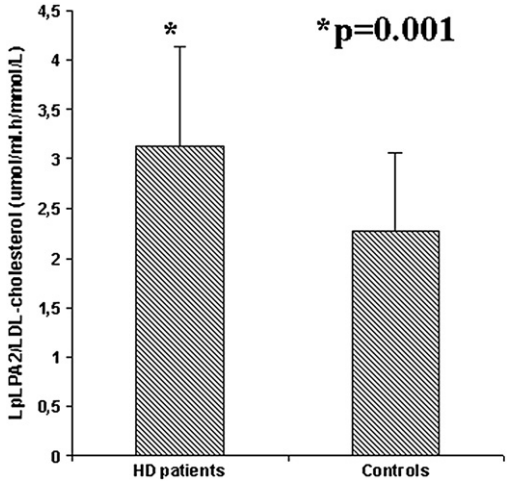

Fig. 2. Lp-PLA2/LDL-cholesterol ratio in hemodialyzed (HD) patients and controls.

several authors, including ourselves [14,23,24]. However, herein, we did not find an association between PON activity and HDL-cholesterol or Apo A-I concentration. Other previous studies showed that HD patients present an altered association between PON and HDL, suggesting that PON catabolism may be accelerated by alterations in the formation of HDL or by metabolic changes in the uremic milieu [23,25]. Given that paraoxonase exhibits a polymorphism in its activity it is important to note that estimation of phenotypes distribution did not show differences between groups. Therefore the decrease in PON activity in HD is not linked to the phenotypic distribution. In future studies it would be necessary to study the mechanism of PON activity reduction which might contribute to explain the absence of association with HDL.

The Lp-PLA2 could be considered a marker of inflammation and many studies emphasize the association between an increase of this enzyme and the incidence of cardiovascular disease [26,27]. In chronic kidney disease, there are conflicting results regarding the activity of Lp-PLA2 in patients with HD [28–30]. In this study, no differences in Lp-PLA2 activity were found between patients and controls. However, when analyzing the ratio of Lp-PLA2/LDL-cholesterol, a higher value was observed in HD patients, thus suggesting that every particle of LDL contains more Lp-PLA2. Despite lower plasma LDL-cholesterol levels, a predominance of small and dense LDL particles was reported in HD patients. It is known that LpPLA2's affinity is increased for these LDL [31,32]. This could explain the increase in Lp-PLA2 in relation to

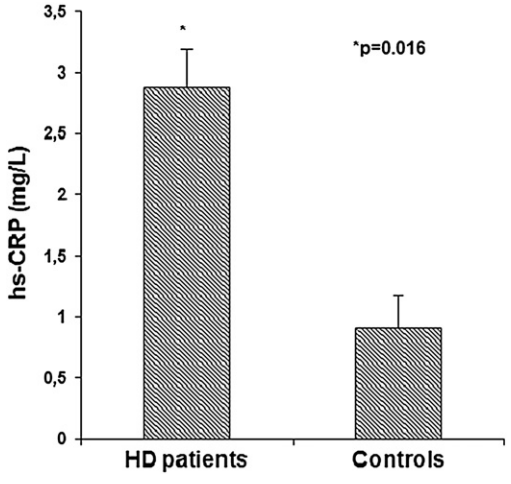

Fig. 3. hs-CRP in hemodialyzed (HD) patients and controls. Data was obtained excluding 9 patients and 2 controls whose presented hs-CRP over 10 mg/l.

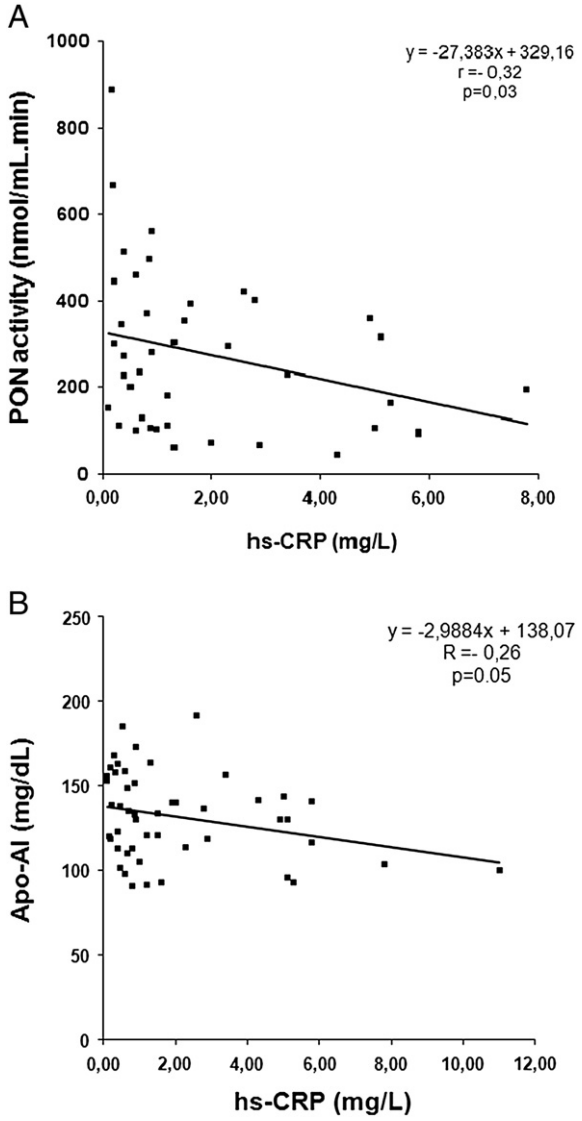

Fig. 4. Correlation between hs-CRP and PON activity according to Spearman (A) and Apo A-I, according to Pearson (B), in hemodialyzed (HD) patients and controls.

LDL particles. Measurement of Lp-PLA2 mass would clarify this result. While a positive association between LDL-cholesterol and Lp-PLA2 was observed, no correlation with HDL was found, suggesting that

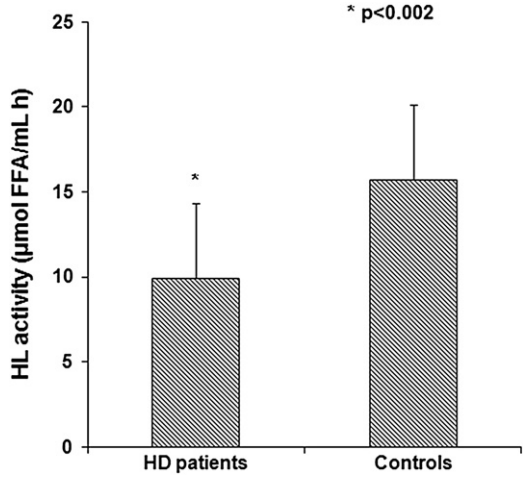

Fig. 5. Hepatic lipase (HL) activity in hemodialyzed (HD) patients and controls.

Lp-PLA2 is linked primarily to Apo B-containing lipoproteins and in a small proportion to HDL, according to Serruys et al. [33]. Our results provide data of Lp-PLA2 since there are scarce studies involving this enzyme in chronic renal failure. The CRP, measured by high sensitivity method, is considered a strong biomarker of chronic inflammation and a predictor of cardiovascular diseases, even when plasma lipid profile is within normal levels [34]. HD patients presented a genuine increase in CRP in comparison to controls, after excluding subjects with CRP over 10 mg/L, corresponding to an overt inflammation state. Other authors have described increased CRP in end-stage renal disease patients under hemodialysis and postulated CRP as a powerful predictor of cardiovascular disease and other causes linked to inflammation [35,36]. Therefore, the increase in CRP confirms that chronic renal disease, especially in hemodialysis, constitutes a chronic inflammatory status.

It was known that inflammation reduces HDL levels by decreasing the expression of ATP binding cassette transporter A-1 (ABCA-1) in macrophages, reducing, in turn, the efflux of cholesterol to the nascent HDL (preβ-HDL) [37]. Furthermore, inflammation was also associated with reduced Apo A-I levels, which is displaced from the HDL particle by serum amyloid A, that in inflammatory conditions, is synthesized by the liver as an acute phase reactant [38]. These processes impact on plasma concentrations of HDL-cholesterol and Apo A-I.

In contrast to serum amyloid A, Apo A-I is a negative acute phase protein and its mRNA decreases as a result of inflammation [39]. This concept is consistent with the significant negative correlation between Apo A-I and CRP observed in this work. The reduction in Apo A-I concentration and the increase in inflammatory status suggest that HDL may not fulfill its anti-inflammatory role and could even become a pro-inflammatory lipoprotein in patients with chronic renal disease [40,41].

As we observed in a previous report [42], in the present study we found again that the increase in CRP was significantly associated to the reduction in PON activity, reflecting a link between inflammatory and oxidative processes. So far, this finding has not been reported by others. Even though several authors found a decrease in PON activity in different groups of renal disease, they did not associate it with inflammatory biomarkers [43,44].

The measurement of CETP was relevant from the point of view of its role contributing to lipoproteins remodeling and the possibility to generate particles with increased atherogenic potential [45]. HD patients showed increased CETP activity which, in turn, it correlated inversely with the HDL-cholesterol. This correlation is in accordance with the fact that HDL and CETP are involved in the transfer and exchange of cholesterol and triglycerides among lipoproteins. It is known that HDL particles rich in triglycerides do not exert efficiently their antiatherogenic effect [46]. In preliminary studies carried out in another group of HD patients we observed an increase in HDL triglycerides content (data not shown); further studies are necessary to confirm this finding. In parallel, as a consequence of the CETP increase, LDL particles also become poor in cholesterol and rich in triglycerides, according to other reports [47,1]. It is important to note that if PLTP was measured, the evaluation of HDL-associated enzymes and proteins would be completed, since increased PLTP activity is associated to cardiovascular complications [48].

Finally hepatic lipase is another enzyme involved in the metabolism of HDL, acting in the last steps of the reverse cholesterol transport, even though it is not attached to HDL particles. As in previous studies, we have again observed a decrease in hepatic lipase activity in HD patients, whose causes are not yet clear [1]. While, as expected, an inverse correlation between hepatic lipase and HDL-cholesterol was significant among healthy controls, the association in HD patients was lost, indicating an alteration in HDL metabolism and function. Although hepatic lipase showed lower activity, a reduction in HDL-cholesterol was also observed. Several causes might contribute to the reduction of this lipoprotein, such as a decrease in apo A-I/A-II, in lecithin cholesterol acyl transferase (LCAT) and in the ABCA-I expression, among other factors [2].

Overall, the protective role of HDL does not only depend on its concentration, but also on its functionality. Its behavior would depend on factors related to its composition, some of which have been studied in this work. The results obtained will contribute to understanding atherogenic mechanisms and consider the evaluation of emerging biomarkers in HD patients.

Acknowledgments

This study was supported by grants from the University of Buenos Aires (B069 and B070) and ANPCyT (PICT 195).

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