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Proatherogenic disturbances in lipoprotein profile, associated enzymes and transfer proteins in women with iron deficiency anaemia

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Abstract

Objective: To characterize the lipid-related atherogenic risk factors in iron deficiency anaemia (IDA) patients.

Design and methods: Twenty IDA women were compared to healthy age-matched controls. Lipoprotein profile, cholesteryl ester transfer protein (CETP), paraoxonase (PON) 1 and lipoprotein-associated phospholipase A_2 ($LpPLA_2$) activities and plasma levels of oxidized-LDL were evaluated.

Results: Triglycerides were higher (median [range]) (1.0 [0.5–1.9] *vs.* 0.7 [0.5–1.5] mmol/L, $p<0.05$) and HDL-C lower (mean±SD) (1.3±0.3 *vs.* 1.6±0.4 mmol/L, $p<0.01$) in the patients group. CETP (197±29% *vs.* 151±29% mL^{-1} h^{-1}, $p<0.001$), PON 1 (122±17 *vs.* 140±33 μmol mL^{-1} min^{-1}, $p<0.05$) and $LpPLA_2$ (9.6±2.0 *vs.* 8.1±1.7 μmol mL^{-1} h^{-1}, $p<0.05$) activities were different in IDA women. No difference was observed in oxidized-LDL. Haemoglobin correlated negatively with triglycerides ($r=-0.35$, $p<0.05$), CETP ($r=-0.62$, $p<0.001$) and $LpPLA_2$ ($r=-0.34$, $p<0.05$), while ferritin was positively associated with HDL-C ($r=0.39$, $p<0.05$) and inversely with CETP ($r=-0.49$, $p<0.005$).

Conclusion: The alterations in lipoprotein profile, CETP, PON 1 and $LpPLA_2$ activities described in the present study indicate that non-treated IDA might represent a proatherogenic state.

Keywords: Iron; Iron deficiency anaemia; Atherosclerosis; Cholesteryl ester transfer protein; Paraoxonase 1; Lipoprotein-associated phospholipase A_2; Oxidized LDL

Introduction

Iron deficiency is the leading cause of anaemia worldwide and is considered one of the most common nutritional deficiencies nowadays. Iron deficiency anaemia (IDA) results when body iron demands are not met by iron absorption and is commonly associated to inadequate iron intake, impaired absorption or transport and physiologic or pathologic blood loss [1]. Iron deficiency is a silent process that impairs not only the synthesis of haemoglobin but also a large variety of iron-containing proteins, such as cytochromes, peroxidase and myoglobin, among others [2]. Anaemia is defined by the World Health Organization (WHO) as the decrease in haemoglobin concentration below 120 g/L for women and 130 g/L for men [3]. It is interesting to note that IDA is more frequent in women than men due to the iron loss through menstrual bleeding.

Recent interest has arisen on the relationship between iron metabolism and the development of atherosclerosis. Different population-based studies identified anaemia as an independent cardiovascular risk factor and as a predictor of bad prognosis in patients with coronary artery disease [4–6].

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Studies carried out in IDA patients evaluated the influence of iron levels on oxidative stress and showed lower activities of the antioxidant enzymes, superoxide dismutase and catalase, when compared to controls [7,8]. Consistently, Sundaram et al. [9] reported higher concentrations of a marker of oxidative stress, malondialdehyde, in IDA patients which was normalised after iron supplementation. It is known that oxidative stress is strongly associated to low density lipoprotein (LDL) oxidation and endothelial dysfunction, both primary events in the development of atherosclerosis [10].

Furthermore, Aslan et al. [11] found that IDA patients presented lower activity of the high density lipoprotein (HDL)-associated antioxidant enzyme paraoxonase (PON) 1 and higher concentration of lipid hydroperoxides (another marker of oxidative stress) when compared to controls. Moreover, the authors also observed a positive correlation between PON 1 activity and ferritin concentration, highlighting a relationship between the enzymatic activity and iron status. PON 1 is an antioxidant enzyme, synthesized by the liver and transported along the plasma bound to HDL. This calcium-dependent esterase has three known activities, paraoxonase, arylesterase and diazoxonase [12], and is implied in the antiatherogenic properties of HDL, by preventing LDL oxidation [13].

However, PON 1 alone cannot explain all the antiatherogenic properties of HDL particles. In fact, HDL is a multienzymatic complex, comprising antioxidant enzymes and also transfer proteins that modulate HDL lipid composition, such as the cholesteryl ester transfer protein (CETP) and the phospholipid transfer protein (PLTP) [14]. CETP mediates cholesteryl ester—triglyceride interchange between HDL and apolipoprotein (apo) B-containing lipoproteins [14]. Thus, CETP activity is considered a determinant factor influencing HDL-C levels. Consistently, epidemiologic studies confirmed the association between high CETP activity, decreased HDL-C concentration and higher risk of cardiovascular disease [15,16]. Moreover, high CETP activity has been described in different known metabolic affections like metabolic syndrome [17], diabetes mellitus [18] and primary hypertriglyceridemia [19] among others.

Another enzyme closely linked to lipoproteins is the lipoprotein-associated phospholipase A_2 ($LpPLA_2$), which is produced and secreted by inflammatory cells and circulates through plasma mainly bound to LDL [20]. This calcium-independent phospholipase acts preferentially on water-soluble polar phospholipids with oxidatively truncated *sn-2* chains and lacks enzymatic activity on naturally occurring long-chain fatty acids of normal phospholipids [20]. $LpPLA_2$ is thought to play a proatherogenic role based on the biological effects of its reaction products (oxidized nonesterified fatty acids and lysophosphatidylcholine), such as upregulation of cell-adhesion molecules (VCAM-1/ICAM-1) and inflammatory mediators [21], induction of endothelial cell apoptosis [22], etc. Supporting this proatherogenic function, results from epidemiologic studies found a positive association between $LpPLA_2$ mass and activity with increased risk of cardiovascular disease [23,24]. To our knowledge, CETP and $LpPLA_2$ activities have not yet been evaluated in IDA patients.

The aim of the present study was to characterize the spectrum of lipid-related atherogenic risk factors, through the evaluation of the lipoprotein profile, CETP activity, plasma levels of oxidized LDL and the activity of the enzymes PON 1 and $LpPLA_2$ in women with IDA in comparison with sex- and age-matched healthy controls.

Materials and methods

Subjects

Subjects were consecutively recruited from the Haematology Service of Hospital Italiano de Buenos Aires, Argentina, between 2007 and 2008. Twenty women with IDA and 20 sex- and age-matched healthy controls were included in the present study. Samples from IDA patients were drawn before they received therapy with intravenous iron, which was indicated by the haematologist. All patients presented IDA typical clinical signs and symptoms plus 4 of the following criteria: (1) haemoglobin concentration <120 g/L, (2) haematocrit <35%, (3) mean corpuscular volume (MCV) <80 fL, (4) red blood cell distribution width (RDW) >15%, (5) transferrin saturation (Tfs) <15% and (6) ferritin <20 μg/L, which represents depleted body iron stores. Most patients met the 6 criteria abovementioned. Subjects were excluded from the study if they matched any of the following criteria: (1) diabetes mellitus or other endocrine disorders, (2) hepatic or renal pathologies, (3) chronic inflammatory diseases such as rheumatoid arthritis or celiac disease, (4) excessive tobacco (>10 cigarettes/day) or ethanol (>30 g/day) consumption, (5) hypertension and (6) therapy with drugs that could affect lipid or carbohydrate metabolism or with antioxidants.

Informed consent was obtained from all participants and the protocol was approved by the Ethical Committees from Hospital Italiano de Buenos Aires and Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

Study protocol and samples

After a 12-h overnight fast, venous blood was drawn from the antecubital vein. Aliquots were collected in serum-separating and $EDTANa_2$-containing tubes. Serum-separating tubes were centrifuged at 1500×*g*, for 15 min, at 4 °C, and serum was stored at 4 °C and used within 24 h for evaluation of lipid profile and general biochemical and iron metabolism parameters. Serum aliquots were also stored at −70 °C for the determination of pro-hepcidin and oxidized LDL levels and for CETP, PON 1 and $LpPLA_2$ activities. Whole blood was stored at 4 °C and employed for complete blood count determination.

Analytical procedures

Complete blood count was determined in a Coulter GEN S autoanalyser (Beckman Coulter, Fullerton, CA, USA). Transferrin concentration was measured using an automatised nephelometry assay (IMMAGE®, Beckman Coulter, Fullerton, CA, USA) and ferritin by an electrochemiluminescence

automatised assay (VITROS® ECiQ, Ortho-Clinical Diagnostics, Raritan City, NJ, USA). Serum levels of iron, glucose, urea, creatinine, uric acid, triglycerides and total cholesterol and the activities of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and gammaglutamyl transferase were quantified by standardized methods (Roche Diagnostics, Mannheim, Germany) in a Hitachi autoanalyser. Tfs was calculated according to the formula (serum iron/transferrin)× 80.5. Pro-hepcidin (DRG diagnostics, Mountainside, NJ, USA) and oxidized LDL (Mercodia AB, Uppsala, Sweden) levels were determined by ELISA. LDL-C and HDL-C concentrations were determined by selective precipitation methods employing polyvinylsulphate [25] and phosphotungstic acid in the presence of magnesium ions [26], respectively. Apo B and apo A-I were evaluated by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Very low density lipoprotein cholesterol (VLDL-C) and non-HDL-C and the ratios triglycerides/HDL-C, total cholesterol/HDL-C, VLDL-C/triglycerides and apo B/apo A-I were also calculated.

CETP activity

CETP activity was determined in serum samples following the general procedure previously described with few modifications [14]. Briefly, the ability of serum to promote the transfer of tritiated cholesteryl esters from a tracer amount of biosynthetically labelled HDL_3 (^{3}H-CE-HDL_3) (NEN Life Science Products, Boston, USA) towards serum apo B-containing lipoproteins was evaluated. Samples were incubated with ^{3}H-CE-HDL_3 (50 μmol/L cholesterol) and 1.5 mmol/L iodoacetate for 3 h, at 37 °C. After incubations, lipoproteins were separated by selective precipitation method employing 0.44 mmol/L phosphotungstic acid in the presence of magnesium ions [26]. Radioactivity was measured in the incubation mixture and in the supernatant containing the HDL fraction in a liquid scintillation analyser (Packard 210TR; Packard Instruments, Meridian, CT). Results were expressed as percentage of ^{3}H-cholesteryl esters transferred from HDL_3 to apo B-containing lipoproteins, per mL, per hour. Measurements were all carried out within the same assay. Within-run precision (CV) was 4.9%.

Paraoxonase 1 activity

The enzyme PON 1 was evaluated employing phenylacetate (Sigma Chemical Co, St. Louis, MO, USA) as substrate. The activity was measured in serum samples following the method of Furlong et al. [27]. PON 1 activity was measured by adding serum samples (20 μL of 1/20 dilution in distilled water) to 2 mL Tris/acetate buffer (50 mmol/L, pH 7.8) containing 20 mmol/L $CaCl_2$ and 4.4 mmol/L phenylacetate. The rate of generation of phenol was determined at 270 nm and 25 °C, in a Hitachi U-1100 spectrophotometer. Increases in the absorbance were recorded at 45-s intervals during 5 min, after 30 s of initial pre-incubation. Blanks were included to correct for the spontaneous hydrolysis of phenylacetate. Enzymatic activity was calculated from the molar extinction coefficient (1310 mol^{-1} cm^{-1}) and results were expressed as μmol mL^{-1} min^{-1}. Measurements were all carried out within the same assay. Within-run precision (CV) was 4.8%.

Lipoprotein-associated phospholipase A_2 activity

$LpPLA_2$ activity was measured following the radiometric assay described by Blank et al. [28] with few modifications. The separation of the released radiolabelled acetate from the lipid substrate was carried out by phase–phase partitioning and measurement of the radioactivity in the aqueous phase. Briefly, each incubation mixture contained 50 μL of 1/50 diluted serum and 10 μmol/L 1-hexadecyl-2-[^{3}H]acetyl-glicero-3-phosphocholine (specific activity=25 μCi/μmol) in a total volume of 0.5 mL of phosphate-buffered saline (pH 7.4). The tritiated substrate 1-hexadecyl-2-[^{3}H] acetyl-glicero-3-phosphocholine (13.5 Ci/mmol) was obtained from New England Nucleotides, and the non-tritiated one was obtained from Cayman Chemical. Once the substrates were mixed, the solvents were evaporated under a stream of nitrogen and redisolved in phosphate-buffered saline. There was a sonication step consisting of one cycle of 5 min. Incubation was carried out for 5 min at 37 °C and the enzymatic reaction was stopped by placing the tubes in an ice bath and by the addition of 1.5 mL of chloroform. Then, 0.5 mL of saturated sodium bicarbonate solution was added. After centrifugation, the aqueous phase was washed twice with 1.5 mL of chloroform. The radioactivity of the aqueous phase of each sample and sample-blanks was measured by liquid scintillation using a Liquid Scintillation Analyzer (Packard 210TR; Packard Instruments, Meridian, CT). Radioactivity of the substrate buffer was also measured. Results were expressed as μmol mL^{-1} h^{-1}. Measurements were all carried out within the same assay. Within-run precision (CV) for Lp-LPA_2 activity was 5.1%.

Data and statistical analysis

Data distribution was tested using the modified Shapiro–Wilks method. Parameters following Gaussian distribution were presented as the mean±standard deviation and Student parametric test (*t* test) was used to compare the different groups, while the median (range) expression and the Mann–Whitney non-parametric test (*U* test) were employed for data that did not follow the Gaussian distribution. Correlations were carried out by Pearson or Spearman tests depending on parameter distribution. Differences were considered significant at $p<0.05$ in the bilateral situation.

Results

Table 1 shows clinical characteristics and general biochemical parameters from IDA patients and control subjects. There were no differences in age, menopausal state, BMI and the different biochemical parameters evaluated except for alkaline phosphatase which was lower in IDA patients (Table 1).

According to the inclusion criteria, haemoglobin concentration, haematocrit, MCV, Tfs and ferritin levels were significantly lower, while red blood cell width significantly higher in

Table 1
Clinical characteristics and general biochemical parameters from IDA patients and control subjects.

	IDA patients	Control subjects
N	20	20
Age (years)	45±13	45±13
Premenopausal/postmenopausal women	14/6	14/6
BMI (kg/m^2)	25±5	24±3
Glucose (mmol/L)	4.7±0.9	4.6±0.8
Urea (mmol/L)	5.5±2.3	6.5±2.0
Creatinine (μmol/L)	67.8 (35.2–140.8)	70.4 (46.6–46.8)
Uric Acid (mmol/L)	0.22±0.09	0.27±0.08
ALAT (IU/L)	16 (9–53)	16 (12–28)
ASAT (IU/L)	18 (12–55)	20 (12–29)
ALP (IU/L)	53 (9–232)	138 (48–229)*
GGT (IU/L)	15 (7–69)	15 (8–74)

IDA, iron deficiency anaemia; BMI, body mass index; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gammaglutamyl transferase. Values are expressed as mean±SD or median (range) for data following parametric or non-parametric distribution, respectively.
* $p<0.05$ *vs.* IDA patients by Mann–Whitney *U* test.

IDA patients than in control subjects (Table 2). No difference was observed in serum pro-hepcidin concentration ($p>0.05$) (Table 2).

Regarding lipid and lipoprotein metabolism, IDA patients exhibited higher triglyceride and lower HDL-C concentrations than controls (Table 3). Furthermore, VLDL-C/triglycerides ratio, an indicator of VLDL composition, was reduced and triglycerides/HDL-C ratio, an indicator of insulin resistance and of the proportion of small and dense LDL particles, was increased in the patient group. The alterations described in the lipoprotein profile might be related to the higher CETP activity observed in IDA patients (Fig. 1). Accordingly, CETP activity was positively associated with triglyceride ($r=0.38$, $p<0.05$) and negatively with HDL-C ($r=-0.38$, $p<0.05$) levels. In relation to haematological parameters, haemoglobin concentration correlated significantly and negatively with triglyceride levels ($r=-0.35$, $p<0.05$) and CETP activity ($r=-0.62$, $p<0.001$), while ferritin exhibited a positive association with HDL-C concentration ($r=0.39$, $p<0.05$) and a negative one with CETP activity ($r=-0.49$, $p<0.005$).

Table 2
Haematological parameters from IDA patients and control subjects.

	IDA patients ($n=20$)	Control subjects ($n=20$)
Hb (g/L)	99 (62–130)	132 (125–151)*
Hct (%)	30 (21–35)	40 (36–46)*
MCV (fL)	74 (60–96)	89 (85–96)*
RDW (%)	17 (12–30)	13 (12–16)*
Tfs (%)	6 (2–30)	31 (14–53)*
Ferritin (μg/L)	7 (3–116)	43 (13–173)*
Pro-hepcidin (μg/L)	108 (62–208)	98 (65–177)

IDA, iron deficiency anaemia; Hb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; RDW, red blood cell distribution width; Tfs, transferrin saturation. Values are expressed as mean±SD or median (range) for data following parametric or non-parametric distribution, respectively.
* $p<0.001$ *vs.* IDA patients by Mann–Whitney *U* test.

Table 3
Lipid and lipoprotein profile from IDA patients and control subjects.

	IDA patients ($n=20$)	Control subjects ($n=20$)
TG (mmol/L)	1.0 (0.5–1.9)	0.7 (0.5–1.5)*
TC (mmol/L)	4.4±0.9	4.9±0.9
VLDL-C (mmol/L)	0.4±0.2	0.4±0.2
LDL-C (mmol/L)	2.5 (1.6–4.5)	2.7 (1.9–4.3)
HDL-C (mmol/L)	1.3±0.3	1.6±0.4†
Non HDL-C (mmol/L)	3.1 (2.0–4.6)	2.8 (2.2–4.8)
Apo B (g/L)	0.78±0.25	0.77±0.16
Apo A-I (g/L)	1.50±0.31	1.65±0.33
TG/HDL-C	1.7 (0.7–4.63)	1.1 (0.5–2.46)‡
TC/HDL-C	3.3 (2.4–5.6)	3.0 (2.1–4.4)
VLDL-C/TG	0.14 (0.05–0.30)	0.21 (0.08–0.60)‡
Apo B/Apo A-I	0.51 (0.26–0.81)	0.48 (0.29–0.73)

IDA, iron deficiency anaemia; TG, triglycerides; TC, total cholesterol; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein. Values are expressed as mean±SD or median (range) for data following parametric or non-parametric distribution, respectively.
* $p<0.05$ *vs.* IDA patients by Mann–Whitney *U* test.
† $p<0.01$ *vs.* IDA patients by Student *t* test.
‡ $p<0.005$ *vs.* IDA patients by Mann–Whitney *U* test.

Further on, the activities of the lipoprotein-associated enzymes PON 1 and $LpPLA_2$ were assessed. PON 1 activity was lower and that of $LpPLA_2$ higher in IDA patients when compared to controls (Fig. 2), being $LpPLA_2$ inversely related with haemoglobin concentration ($r=-0.34$, $p<0.05$).

Oxidized LDL levels were also determined in plasma from patients and controls and no statistically significant difference was found between both groups (63±20 *vs.* 71±22 IU/L, $p>0.05$).

Discussion

The present study shows that IDA is a condition characterized by the presence of alterations due to iron deficiency and/or

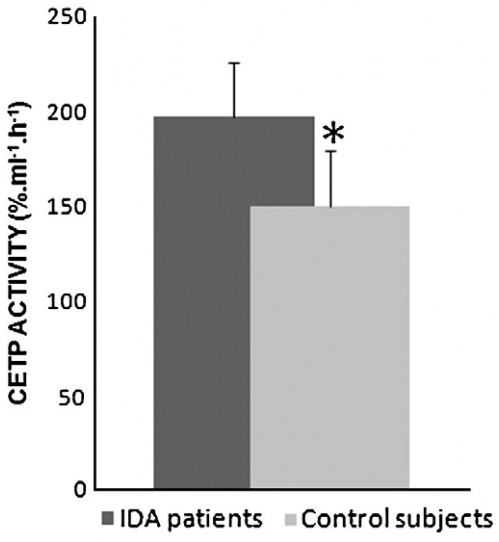

Fig. 1. CETP activity from IDA patients ($n=20$) and control subjects ($n=20$). IDA, iron deficiency anaemia; CETP, cholesteryl ester transfer protein. *$p<0.001$ *vs.* IDA patients by Student *t* test.

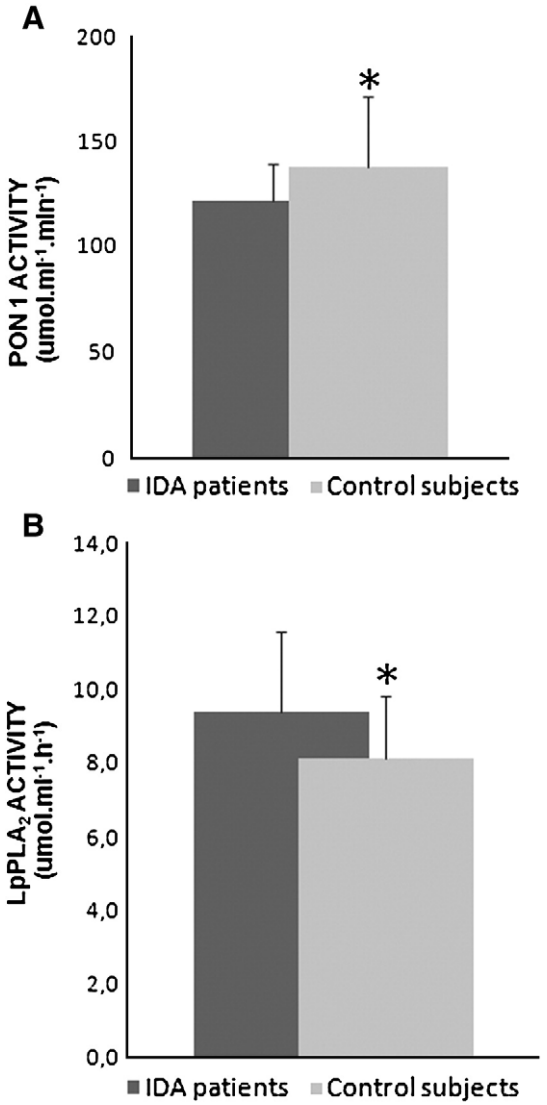

Fig. 2. PON 1 (A) and $LpPLA_2$ (B) activities from IDA patients ($n=20$) and control subjects ($n=20$). IDA, iron deficiency anaemia; PON, paraoxonase; $LpPLA_2$, lipoprotein-associated phospholipase A_2. *$p<0.05$ *vs*. IDA patients by Student *t* test.

to anaemia which are translated into abnormalities of lipoprotein profile and of the activity of lipoprotein-associated enzymes. More precisely, in IDA women, we observed higher triglyceride and lower HDL-C concentrations, higher CETP, decreased PON 1 and increased $LpPLA_2$ activities, when compared to sex- and age-matched healthy controls, all abnormalities closely related to higher risk of cardiovascular disease.

Actually, different studies have identified anaemia as a cardiovascular risk factor in the general population. In an observational study, Sarnak et al. [4] noticed that anaemia defined by haemoglobin concentration < 130 g/L in men and < 120 g/L in women was independently associated with an increased risk of cardiovascular disease. Another study carried out in an older population of patients from Veterans Affairs medical centres also pointed out anaemia as an independent risk factor [5]. Furthermore, Go et al. [29] noticed that both very high (≥ 170 g/L) and reduced (< 130 g/L) haemoglobin levels were strong predictors of death and hospitalization among adults with heart failure. Prior reports had already suggested that the association between haemoglobin levels and cardiac events in the general population was not linear and could be better described as a U-shaped relationship [30–32], indicating that patients with both low and high haemoglobin levels had an increased risk of developing new cardiac events. Moreover, it was also shown that anaemia could interact in synergy with risk factors for cardiovascular disease, such as chronic kidney disease, increasing the hazard ratio to an even higher extent than when considering each factor independently [33]. The postulated mechanisms explaining the association between anaemia and the increased cardiovascular disease risk were by means of increased cardiac output, vascular remodelling processes, activation of the renin–angiotensin axis, higher levels of proinflammatory cytokines, etc. [5]. The present study shows that dyslipidemia and altered activity of lipoprotein-associated enzymes would constitute an additional link between anaemia and cardiovascular diseases.

In fact, the lipid and lipoprotein profile was more atherogenic in the group of IDA patients. However, this finding might have been underestimated if we had only analyzed the results form each individual patient in comparison to normal values. In effect, most patients showed data from lipid and lipoprotein profile within the reference range, suggesting the existence of subtle modifications in IDA. Accordingly, some markers of anaemia and iron deficiency, such as haemoglobin and ferritin, showed negative and positive associations with triglyceride and HDL-C levels, respectively. The simultaneous increase in triglyceride levels and the decrease in HDL-C concentration are in agreement with the well-known relationship between the metabolism of triglyceride-rich lipoproteins (chylomicrons and VLDL) and the maturation of HDL particles [34]. Furthermore, triglyceride/HDL-C ratio, proposed by Mc Laughlin et al. [35] as a predictor of the proportion of the highly atherogenic small and dense LDL particles, was higher in IDA patients, which increase is frequently associated to hypertriglyceridemia [19].

Accordingly, the abovementioned alterations have been already recognized in different studies carried out both in animal models [36–39] and in humans [11,40,41]. Among the latter, Skrede et al. [40] have already described higher triglyceride levels and lower HDL-C concentration in a small group of anemic children. Tanzer et al. [41] studied 70 children of about 14 months old suffering from IDA and found significantly increased triglyceride levels due to higher VLDL concentration in comparison to 20 healthy controls. Given the close relationship between triglyceride metabolism and carnitine and based on the hypothesis that iron deficiency could cause a reduction in carnitine synthesis [42], the authors evaluated carnitine levels which were lower in IDA children. Based on this observation, it was suggested that carnitine would not be available for fatty acid transportation into mitochondria for oxidation. Therefore, fatty acid metabolism would shift towards glyceride synthesis which would result in an increase in serum and tissue triglyceride levels [43]. Low carnitine

concentrations were also reported by Citak et al. [44] who studied another group of IDA children ($n=60$) as compared to healthy controls ($n=60$).

The increment in triglyceride levels and the decrease in HDL-C concentration were also associated to the higher CETP activity observed in IDA patients. The enhancement in triglyceride concentration, which was due to VLDL accumulation, could be inducing CETP activity. The mechanism by which VLDL-triglycerides are a determinant factor for CETP activity has been already reported in previous studies carried out in patients with primary hypertriglyceridemia, increased CETP activity and unchanged CETP concentration [45,46] and also in diabetic patients with hypertriglyceridemia [47]. Furthermore, this is consistent with *in vitro* experiments showing that the addition of increasing amounts of VLDL to normal plasma enhances the net mass transfer of cholesteryl esters out of HDL [48]. Moreover, hypertriglyceridemic VLDL are generally resistant to lipolysis by lipoprotein lipase, and the reduced lipolytic efficiency may prevent the accumulation of CETP inhibitory molecules on the HDL surface [49].

In fact, CETP is responsible for triglyceride—cholesteryl ester interchange between circulating lipoproteins; hence, CETP might be amplifying the alterations originally caused by impaired fatty acid catabolism described in IDA patients. The relationship between CETP activity and haematological disturbances was also pointed out by the existence of strong inverse correlations between this transfer protein and both haemoglobin and ferritin levels. High CETP activity could lead to triglyceride depletion and cholesteryl ester enrichment of apo B-containing lipoproteins and the opposite in HDL fraction, thereby increasing the atherogenic potential of the first ones and decreasing HDL antiatherogenic properties. Both lipoprotein modifications were already described in diabetic and hypertriglyceridemic patients with increased risk of cardiovascular disease [18,19]. In IDA rats [38], the presence of an increase in VLDL- and LDL-triglycerides with no change in HDL-C concentration cannot be attributed to neutral lipid interchange because, unlike humans, rats lack CETP [50]. As a result, in rats, hepatic synthesis would be a determinant factor for the altered VLDL lipid composition. In humans, atypical VLDL particles could be also generated by the liver due to a hyperinsulinemic insulin resistant-state associated to higher free fatty acid influx to the liver, situation already described by Özdemir et al. [51] in a group of anemic premenopausal non-diabetic women. Once in circulation, triglyceride-enriched VLDL fraction, as suggested by the lower VLDL-C/triglycerides ratio, would turn patients VLDL into better acceptors of cholesteryl esters coming from HDL, so contributing to the reduction in HDL-C concentration [52]. Moreover, this alteration in VLDL composition in conjunction with our finding of higher CETP activity could be assigned to the fact that newly synthesized VLDL particles might have highly increased triglyceride content and, after CETP modulation, they still remained triglyceride enriched in comparison with control VLDL. Another possibility could be that CETP action on VLDL particles with high triglyceride content would generate smaller VLDL remnants, easily cleared from the circulation [53]. Similar abnormalities in VLDL fraction and high CETP activity are also found in patients with metabolic syndrome or type 2 diabetes (17,47). Nevertheless, further research is needed to exactly confirm this hypothesis in IDA patients.

Another lipid transfer protein responsible for modulating lipoprotein composition is PLTP [54]. Abnormalities in plasma PLTP levels have been reported in patients with obesity, type 2 diabetes mellitus and cardiovascular disease [55,56]. PLTP is expressed at relatively high levels in adipose tissue and it binds to and transfers a number of amphipathic compounds, including phospholipids, diacylglycerides, unesterified cholesterol and lipopolysacharides when secreted within the bloodstream. Up to our knowledge, PLTP has not been studied in IDA patients or in relation with iron metabolism, which would constitute an interesting field of research.

Further on, when lipoprotein-associated enzymes were assessed, IDA patients presented lower PON 1 and higher $LpPLA_2$ activities in comparison to healthy controls. Aslan et al. [11] also found diminished PON 1 activity in young IDA women, possibly due to the diminution in HDL concentration, PON 1 unique plasma carrier. PON 1 would play a crucial role by preventing LDL oxidation in the artery wall. Hence, decreased PON 1 activity could lead to higher oxidized LDL particles, most of which are internalized by resident macrophages, a recognized primary event in atherogenesis [10]. Regarding $LpPLA_2$ activity, it is important to note that its use as an inflammatory marker is based on its hydrolytic activity on oxidized phospholipids present in LDL, consequently generating proinflammatory molecules. Hence, this increased enzymatic activity in IDA patients and the negative association observed with haemoglobin concentration could reflect the activation of inflammatory pathways occurring in response to oxidative stress induced by the decrease in PON 1 and other antioxidant enzymes present in iron deficiency and/or anaemia [7,8,11].

In the context of diminished PON 1 and increased $LpPLA_2$ activities, an increment in oxidized LDL levels was expected in IDA patients. Nevertheless, no statistically significant difference was observed between patients and controls. The lack of capacity to evidence LDL oxidative modification in IDA patients could be related to the fact that oxidized LDL was measured in plasma due to the impossibility to directly analyze the artery wall. In agreement, it has been stated that data showing unchanged oxidized LDL levels in plasma are not enough to discard the occurrence of oxidative processes in the intima [10]. LDL oxidation may take place in the artery wall due to several factors inherent to lipoproteins such as LDL triglyceride-enrichment, an increase in small and dense LDL proportion, an increment in LDL associated-$LpPLA_2$ and a reduction in PON 1 activity, among others. Physiologically, HDL particles are able to go through the endothelium and into the intima, where they exert their antioxidant capacity, mainly through PON 1 activity, and then return into plasma circulation. A decrease in PON 1 activity would contribute to LDL oxidation and then oxidatively modified LDL particles would

be retained in the subendothelial space, thus turning more difficult the detection of higher oxidized LDL levels in plasma. Therefore, the determination of oxidized LDL levels in plasma by immunological assays would constitute a very specific technique but of poor sensibility.

In conclusion, IDA patients showed the so called "atherogenic dyslipidemia", increased CETP and $LpPLA_2$ activities and diminished PON 1 function. The alterations described in the present study clearly indicate that non-treated IDA might represent a proatherogenic state.

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