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ORIGINAL ARTICLE

High risk of cardiovascular disease in iron overload patients

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ABSTRACT

Introduction Iron overload (IO) is defined as an increase in storage iron, regardless of the presence or absence of tissue damage. Whether increased iron stores are involved in the pathogenesis of cardiovascular disease remains controversial.

Objectives To study insulin resistance markers, lipoprotein profile, activities of anti and prooxidant enzymes and cholesteryl ester transfer protein (CETP) in patients with IO.

Methods Twenty male patients with IO were compared with 20 sex- and age-matched controls. General biochemical parameters, lipoprotein profile, and activities of paraoxonase 1, employing two substrates, paraoxon (PON) and phenylacetate (ARE), lipoprotein-associated phospholipase A_2 (Lp-PLA_2) and CETP were determined.

Results IO patients showed higher levels of HOMA-IR and triglycerides [median (Q1–Q3)] [128 (93–193) vs. 79(51–91) mg dL^{-1}, $P < 0{\cdot}0005$] while lower high-density lipoprotein (HDL) cholesterol (mean ± SD) (41 ± 9 vs. 52 ± 10 mg dL^{-1}, $P < 0{\cdot}0005$) in comparison with controls. Moreover, the triglycerides/HDL-cholesterol [3·2 (2·0–5·1) vs. 1·5 (1·0–1·9), $P < 0{\cdot}0005$] ratio and oxidized low-density lipoprotein levels [94 (64–103) vs. 68 (59–70) IU L^{-1}, $P < 0{\cdot}05$] were increased in the patient group. Although no difference was observed in ARE activity, PON activity was decreased in IO patients [246 (127–410) vs. 428 (263–516) nmol mL^{-1} min^{-1}, $P < 0{\cdot}05$]. In addition, CETP and Lp-PLA_2 activities were also increased in the patients (189 ± 31 vs. 155 ± 36% ml^{-1} h^{-1}, $P < 0{\cdot}005$; and 10·1 ± 2·9 vs. 8·2 ± 2·4 μmol mL^{-1} h^{-1}, $P < 0{\cdot}05$, respectively). Associations between ferritin concentration and the alterations in lipid metabolism were also found. Multiple regression analyses identified HOMA-IR as independent predictor of CETP activity (B = 65·9, $P < 0{\cdot}0001$, $r^2 = 0{\cdot}35$), as well as ferritin concentration of Lp-PLA_2 activity (B = 3·7, $P < 0{\cdot}0001$, $r^2 = 0{\cdot}40$) after adjusting for confounding variables.

Conclusions IO patients presented not only insulin resistance but also metabolic alterations that were related to elevated iron stores and are associated with high risk of cardiovascular disease.

Keywords Atherosclerosis, cholesteryl ester transfer protein, insulin resistance, iron, lipoprotein, lipoprotein-associated phospholipase A_2.

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Introduction

Iron overload (IO) is defined as an increase in storage iron, regardless of the presence of tissue damage [1]. It may occur as consequence of defects in genes involved in iron metabolism, or it may be secondary to acquired conditions including hazardous alcohol consumption and metabolic diseases, among others [1–3].

The 'iron hypothesis', postulated by Sullivan [4], brought insight into the possible role of increased iron stores in the pathogenesis of cardiovascular disease. Several cross-sectional, prospective and interventional studies were carried out; however, their results were not conclusive [5–10]. Furthermore, the existence of a synergistic interaction between high cholesterol levels and iron stores in the incidence of cardiovascular disease was analysed and yet, the results were also controversial [5–7, 11].

Nonetheless, different population-based studies reported several alterations in inflammatory, metabolic, and oxidative stress markers associated with high ferritin concentration, which is an estimator of body iron [12–14]. Moreover, increased ferritin concentration was positively associated with the risk to

develop metabolic syndrome and type 2 diabetes [15–18]. Actually, insulin resistance, a condition with known increased risk of cardiovascular disease, is quite prevalent in IO patients [19,20]. In addition, patients with hereditary hemochromatosis (HH), a primary IO disease caused by mutations in the HFE gene [21], exhibited an impaired vascular functionality that reverted with iron depletion [22]. Overall, these results provide evidence of increased oxidative stress, impaired glucose metabolism and endothelial dysfunction in IO patients. However, to our knowledge, lipid-related factors known to modulate inflammatory and oxidative processes, such as the activities of paraoxonase (PON) 1, lipoprotein-associated phospholipase A_2 (Lp-PLA_2) and cholesteryl ester transfer protein (CETP) [23], had not been evaluated.

The aims of the present work were to study lipid and lipoprotein metabolism and novel markers of cardiovascular disease in patients with IO in comparison with sex- and age-matched healthy controls as well as their relationship with ferritin concentration and insulin resistance.

Methods

Subjects

Twenty male patients with IO were enrolled from the Hematology Service of the Hospital Italiano de Buenos Aires, Argentina. IO was diagnosed based on: (i) transferrin saturation > 45%, (ii) ferritin concentration > 500 μg L^{-1} and (iii) homozygosity for HFE gene C282Y or H63D mutations or increased iron liver stores assessed by semi-quantitative grading in liver biopsy (Perl's staining technique) [24]. Ten of the 20 patients were already under phlebotomy sessions in the depletion phase of the treatment [24]. Patients were excluded if matched any of the following criteria: (i) diabetes mellitus, (ii) cardiomyopathy, (iii) any renal pathology, (iv) hazardous alcohol consumption (> 40 g ethanol per day), (v) smoking > 10 cigarettes per day, and (vi) current therapy with antioxidants or drugs that are known to affect glucose and lipid metabolism. IO patients were compared to 20 sex- and age-matched healthy controls. Control subjects were neither homozygous nor heterozygous for C282Y or H63D HFE mutations and presented normal glucose, lipid and iron metabolism parameters. Patients and control subjects were classified as overweight or obese according to the adult definition [25]. Reporting of the study conforms to STROBE [26,27].

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

Study protocol and samples

After a 12-h overnight fast, 1 day before phlebotomy session for IO patients, venous blood was drawn from the antecubital vein. Aliquots were placed in $EDTANa_2$ and serum collecting tubes. Serum was stored at 4 °C and used within 24 h for evaluation of lipid and lipoprotein profile and general biochemical and iron metabolism parameters. Serum aliquots were also stored at −70 °C for determination of oxidized low-density lipoprotein (LDL) levels and PON 1, CETP and Lp-PLA_2 activities. Whole blood was stored at 4 °C and immediately employed for complete blood count determination.

HFE genotyping by restriction enzyme digestion

HFE C282Y and H63D mutations were evaluated following the method previously described by Feder *et al.* [28].

Analytical procedures

Complete blood count was determined in a Coulter® GEN-S™ autoanalyser (Beckman Coulter, Fullerton, CA, USA). Plasma transferrin, apolipoprotein (apo) A-I and apo B concentrations were measured by immunonephelometry (IMMAGE®; Beckman Coulter). Ferritin concentration was assayed by an electrochemiluminiscence automatised assay (VITROS® ECiQ; Ortho-Clinical Diagnostics, Raritan City, NJ, USA). Serum levels of iron, glucose, urea, creatinine, uric acid, total bilirubin, triglycerides, and total cholesterol and the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase were quantified by standardized methods (Roche Diagnostics, Mannheim, Germany) in a Hitachi autoanalyser (Hitachi, Tokyo, Japan). Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) concentrations were determined by selective precipitation methods. Oxidized LDL levels were determined by ELISA (Mercodia AB, Uppsala, Sweden) in 10 patients randomly selected and their respective controls. Very low-density lipoprotein cholesterol (VLDL-C) and the ratio triglycerides/HDL-C were calculated.

PON 1 activity

The enzyme PON 1 was evaluated employing two different substrates: paraoxon and phenylacetate (Sigma Chemical Co, St Louis, MO, USA; PON and ARE activities, respectively). Both activities were measured in serum samples following the method of Furlong *et al.* [29]. Results were expressed as nmol ml^{-1} min^{-1} and μmol ml^{-1} min^{-1} for PON and ARE activities, respectively. Measurements were all carried out within the same assay. Within-run precision was 4·6% for PON activity and 4·2% for ARE activity. The ratios PON/ARE, PON/apo A-I and ARE/apo A-I were calculated. PON phenotype distribution was determined by a double substrate method [30].

CETP activity

Cholesteryl ester transfer protein (CETP) activity was determined in serum samples according to the general procedure

previously described by Lagrost *et al.* [31]. Results were expressed as percentage of ^{3}H-cholesteryl esters transferred from HDL_3 to apo B-containing lipoproteins, per mL, per h. Measurements were all carried out within the same assay. Within-run precision was 4·9%.

Lp-PLA_2 activity

Lp-PLA_2 activity was measured following the radiometric assay described by Blank *et al.* [32]. Results were expressed as μmol ml^{-1} h^{-1}. Measurements were all carried out within the same assay. Within-run precision for Lp-LPA2 activity was 5·1%.

Data and statistical analyses

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 (Q1–Q3) expression and the Mann–Whitney test (*U*-test) were employed for skewed data. Proportions and HFE allele-associations with insulin resistance and lipid metabolism parameters were assessed using the Fisher's exact test. Analysis of covariance was also performed after log-transforming skewed data using body mass index (BMI), HOMA-IR or both of them as covariates. Correlations were carried out by Pearson or Spearman test in accordance with data distribution in the total population ($n = 40$). Furthermore, partial correlations tests defining BMI and/or HOMA-IR as fixed variables were performed. Given the normal distribution of CETP and Lp-PLA_2 activities, untransformed values were employed and linear regression analyses were performed. For these analyses, CETP and Lp-PLA_2 activities were established as dependent variables and age, BMI, apo B and log-transformed ferritin, ALT activity, HOMA-IR and triglycerides as independent variables. *P*-values less than 0·05 were considered significant in the bilateral situation. The software INFOSTAT (Grupo INFOSTAT, National University of Córdoba, Córdoba, Argentina) was used for all data and statistical analyses.

Results

HFE genotypes and associations with lipid metabolism

From the 20 IO patients, four were homozygous for the C282Y HFE gene mutation, two patients were compound C282Y/H63D heterozygous and five were homozygous for the H63D HFE mutation. From the rest, two were C282Y heterozygous, three H63D heterozygous and four had none of the mutations evaluated.

Regarding HFE genotypes association with lipid metabolism and insulin resistance markers in IO patients; the presence of at least one H63D allele was significantly associated with total cholesterol levels over 200 mg dL^{-1} ($P < 0·05$; OR=9·33, 95% CI, 1·4–62·2) and LDL-C concentration above 160 mg dL^{-1} ($P < 0·05$). On the other hand, the C282Y allele was not associated with the studied lipid and insulin resistance parameters.

Table 1 Clinical and biochemical characteristics from IO patients and control subjects

	IO patients	Control subjects
n	20	20
Age (years)	51 ± 13	51 ± 13
BMI (kg m^{-2})	29 ± 4	26 ± 3*
Glucose (mg dL^{-1})	98 ± 20	90 ± 7
Insulin (mU L^{-1})	12·6 (5·0–22·5)	5·1 (3·1–7·6)†
HOMA-IR	2·4 (1·2–5·2)	1·2 (0·6–1·8)†
Urea (mg dL^{-1})	30 ± 7	38 ± 10*
Creatinine (mg dL^{-1})	1·00 (0·90–1·11)	1·00 (0·89–1·15)
Uric Acid (mg dL^{-1})	5·7 ± 1·5	6·0 ± 1·2
Total bilirrubin (mg dL^{-1})	0·9 (0·6–1·1)	0·5 (0·3–0·6)†
ALT (IU L^{-1})	31 (22–45)	13 (9–23)†
AST (IU L^{-1})	29 (24–35)	21 (17–24)†
ALP (IU L^{-1})	66 (50–81)	75 (56–129)
Tfs (%)	52 (36–57)	24 (21–29)‡
Ferritin (μg L^{-1})	364 (125–503)	114 (67–159)‡

IO, iron overload; BMI, body mass index; HOMA, homeostasis model assessment; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Tfs, transferrin saturation. Results are expressed as mean ± SD or median (Q1–Q3) for normal or skewed data, respectively. *$P < 0·01$; †$P < 0·001$; ‡$P < 0·005$.

Clinical and general biochemical characteristics

Table 1 presents clinical and biochemical characteristics from IO patients and control subjects. Plasma levels of urea were decreased, while those of insulin, total bilirubin and activities of liver enzymes (ALT and AST) were increased in IO patients. Also, the patients presented higher BMI and HOMA-IR when compared to the controls. In spite of the higher BMI observed, the frequencies of overweight (10/20 vs. 10/20) and obesity (7/20 vs. 3/20) were not statistically different between patients and controls, respectively. In addition, no significant difference was observed in the number of smokers (3/20, IO patients vs. 0/20, control subjects). Furthermore, deriving from the inclusion criteria, IO patients exhibited higher transferrin saturation and ferritin concentration. All these differences remained significant even when adjusting by BMI, by HOMA-IR or by both of them simultaneously.

In accordance with a possible relationship between body iron stores and insulin resistance, ferritin concentration and transferrin saturation were positively correlated with insulin levels ($r = 0{\cdot}36$, $P < 0{\cdot}05$; $r = 0{\cdot}34$, $P < 0{\cdot}05$, respectively) and HOMA-IR ($r = 0{\cdot}37$, $P < 0{\cdot}05$; $r = 0{\cdot}34$, $P < 0{\cdot}05$, respectively) adjusted by BMI. Moreover, ferritin concentration and transferrin saturation were also directly associated with total bilirubin levels ($r = 0{\cdot}44$, $P < 0{\cdot}005$; $r = 0{\cdot}67$, $P < 0{\cdot}001$, respectively) and ALT activity ($r = 0{\cdot}49$, $P < 0{\cdot}005$; $r = 0{\cdot}40$, $P < 0{\cdot}01$, respectively), both adjusted by BMI and HOMA-IR. In addition, transferrin saturation was significantly correlated with AST activity even when adjustments were made ($r = 0{\cdot}61$, $P < 0{\cdot}001$). Overall, these correlations reflect the deleterious effect of increased iron stores within the liver.

Lipids and lipoproteins

Lipid and lipoprotein profile is shown in Table 2. IO patients showed higher triglyceride and lower HDL-C levels in comparison with control subjects (Table 2). In addition, triglycerides/HDL-C ratio, which has been postulated as a marker of insulin resistance and of the proportion of the highly atherogenic small and dense LDL particles [33], was higher in IO patients (Table 2). Differences between groups remained significant even when adjusting by BMI and/or HOMA-IR.

Furthermore, plasma levels of oxidized LDL were significantly increased in IO patients than in control subjects [94 (64–103) vs. 68 (59–70) IU L^{-1}, respectively; $P < 0{\cdot}05$], although this difference was missed when adjusting by BMI and/or HOMA-IR.

Table 2 Lipid and lipoprotein profile from IO patients and control subjects

	IO patients ($n = 20$)	Control subjects ($n = 20$)
TG (mg dL^{-1})	128 (93–193)	79 (51–91)*
TC (mg dL^{-1})	193 ± 48	193 ± 30
VLDL-C (mg dL^{-1})	19 ± 8	17 ± 6
LDL-C (mg dL^{-1})	133 ± 48	120 ± 24
HDL-C (mg dL^{-1})	41 ± 9	52 ± 10*
Apo B (mg dL^{-1})	92 ± 34	85 ± 21
Apo A-I (mg dL^{-1})	134 (123–149)	145 (121–161)
TG/HDL-C	3·2 (2·0–5·1)	1·5 (1·0–1·9)*

IO, iron overload; TG, triglycerides; TC, total cholesterol; VLDL, very low density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo, apolipoprotein. Results are expressed as mean ± SD or median (Q1–Q3) for normal or skewed data, respectively.
*$P < 0{\cdot}0005$.

Table 3 PON 1 activity and related ratios from IO patients and control subjects

	IO patients ($n = 20$)	Control subjects ($n = 20$)
PON (nmol ml^{-1} min^{-1})	246 (127–410)	428 (263–516)*
ARE (μmol ml^{-1} min^{-1})	148 ± 38	138 ± 38
PON/ARE	2·0 (0·8–2·7)	3·4 (1·0–4·7)*
PON/APO A-I	1·7 (1·0–2·9)	2·4 (1·1–3·8)
ARE/APO A-I	1·1 ± 0·3	0·9 ± 0·2*

IO, iron overload; PON, paraoxonase activity; ARE, arylesterase activity; apo, apolipoprotein.
*$P < 0{\cdot}05$.

Regarding iron stores, higher ferritin concentration was significantly associated with the increase in triglycerides ($r = 0{\cdot}42$, $P < 0{\cdot}01$) and oxidized LDL ($r = 0{\cdot}53$, $P < 0{\cdot}05$) and to the decrease in HDL-C plasma concentration ($r = -0{\cdot}39$, $P < 0{\cdot}01$). The aforementioned correlations remained significant when adjusting by BMI and/or HOMA-IR.

Lipoprotein-associated enzymes and proteins

PON 1 phenotype distribution was not different between patients and controls ($P > 0{\cdot}05$), which allowed the comparison between groups without any adjustment. IO patients presented lower PON activity and PON/ARE ratio, as well as higher ARE/apo A-I quotient in comparison with healthy controls (Table 3). Nonetheless, when adjusting for BMI and/or HOMA-IR, the statistically significant differences were missed. In the same way, PON activity was inversely correlated to transferrin saturation ($r = -0{\cdot}33$, $P < 0{\cdot}05$); however, the correlation did not remain significant when adjusting for BMI and/or HOMA-IR.

Further on, CETP and Lp-PLA_2 activities were found to be increased in IO patients in comparison with control subjects independently of BMI (Fig. 1). However, when further adjustment for HOMA-IR was made, the difference observed in CETP activity did not remain significant, while it persisted for Lp-PLA_2 activity.

Consistent with CETP function, its activity was directly associated with triglyceride levels ($r = 0{\cdot}60$, $P < 0{\cdot}0001$) and inversely with HDL-C ($r = -0{\cdot}74$, $P < 0{\cdot}0001$). Moreover, CETP activity also showed positive correlations with ferritin concentration ($r = 0{\cdot}36$, $P < 0{\cdot}05$), insulin levels ($r = 0{\cdot}54$, $P < 0{\cdot}001$), HOMA-IR ($r = 0{\cdot}57$, $P < 0{\cdot}001$) and liver function indicators: total bilirubin concentration ($r = 0{\cdot}47$, $P < 0{\cdot}005$), ALT ($r = 0{\cdot}44$, $P < 0{\cdot}01$) and AST activities ($r = 0{\cdot}39$, $P < 0{\cdot}05$). Additionally, CETP and PON activities were inversely related ($r = -0{\cdot}41$, $P < 0{\cdot}01$). When adjusting for BMI and/or HOMA-IR, the only correlations which persisted were those of CETP with HDL-C and total bilirubin levels.

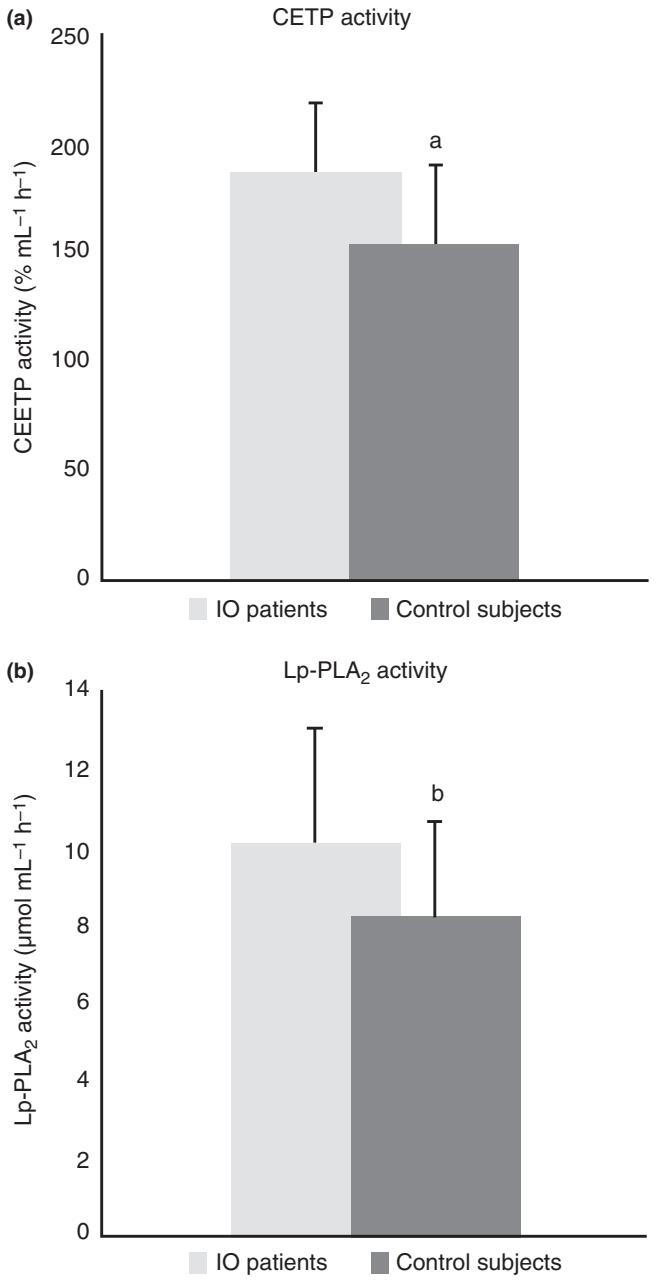

Figure 1 CETP (Panel a) and Lp-PLA_2 (Panel b) activities from IO patients (n = 20) and control subjects (n = 20). IO, iron overload; CETP, cholesteryl ester transfer protein; Lp-PLA_2, lipoprotein-associated phospholipase A_2. [a]$P < 0{\cdot}005$, [b]$P < 0{\cdot}05$ adjusted by body mass index.

Similarly, Lp-PLA_2 activity was significantly associated with plasma levels of ferritin (r = 0·52, $P < 0{\cdot}001$), triglycerides (r = 0·37, $P < 0{\cdot}05$), HDL-C (r = −0·39, $P < 0{\cdot}05$), LDL-C (r = 0·40, $P < 0{\cdot}01$), apo B (r = 0·34, $P < 0{\cdot}05$), oxidized LDL (r = 0·64, $P < 0{\cdot}001$) and ALT activity (r = 0·53, $P < 0{\cdot}001$), but neither to insulin concentration nor HOMA-IR. When the aforementioned correlations were adjusted by BMI and/or HOMA-IR, the ones which persisted were those with ferritin, HDL-C, oxidized LDL and ALT activity.

Multiple regression analysis showed that HOMA-IR was a significant predictor of CETP activity (B = 65·9, $P < 0{\cdot}0001$) independently of age, BMI, ALT activity, ferritin, triglycerides and apo B plasma levels (r^2 = 0·35). The same analysis performed with $LpPLA_2$, instead of CETP activity, identified ferritin concentration to be its only independent predictor (B = 3·7, $P < 0{\cdot}0001$, r^2 = 0·40).

Discussion

Several metabolic parameters associated with high risk of cardiovascular disease were found to be altered in IO patients. In particular, the main findings of the present study consisted of the presence of the so-called 'atherogenic dyslipemia' (high triglyceride and low HDL-C levels) in most patients with IO, apart from increased oxidized LDL concentration and higher CETP and Lp-PLA_2 activities in comparison with age- and sex-matched controls. Moreover, IO patients presented a reduction in the activity of the antioxidant enzyme PON 1. In addition, multiple regression analyses identified HOMA-IR as independent predictor of CETP activity, as well as ferritin concentration of Lp-PLA_2 activity.

Several lines of evidence support a relationship between increased body iron stores and insulin resistance [15–18]. In agreement with previous studies [34], IO patients also presented an impaired glucose metabolism, characterized by higher insulin concentration, HOMA-IR and triglycerides/HDL-C quotient than control subjects. In this context, the atherogenic lipoprotein profile described in IO patients could be attributed to insulin resistance. However, the observed differences and correlations remained significant even when adjustment for BMI and/or HOMA-IR was carried out, thus suggesting that IO might be further contributing to the development of patients' atherogenic dyslipidemia.

The assessment of CETP activity in this study allowed a deeper insight into the lipoprotein modifications observed in IO patients. In fact, higher CETP activity was found to be correlated with both the increase in plasma triglycerides and the decrease in HDL-C levels. Nonetheless, when CETP activity was adjusted for BMI and HOMA-IR, the difference between groups did not remain statistically significant. This result gave additional support to the leading role of insulin resistance on the atherogenic dislypidemia of IO. Actually, IO patients presented metabolic alterations comparable to those described in metabolic syndrome and type 2 diabetes [35–37]. It is

noteworthy that in the latter situations, CETP was reported to possess proatherogenic properties such as increasing the proportion of small and dense LDL particles and altering HDL chemical composition and antiatherogenic functions [35–37].

On line with a possible CETP-mediated triglyceride enrichment of HDL, impaired antioxidant capacity was to be expected [36]. Accordingly, when PON 1 activity was assessed, IO patients showed lower enzymatic activity and PON/ARE ratio but higher ARE/apo A-I quotient when compared to healthy controls. However, the significance of the differences was lost when adjusting for BMI and/or HOMA-IR. In a recent study PON 1 polymorphisms that modulate PON activity had been correlated with the risk of coronary artery disease incidence in secondary prevention [38]. To this regard, unlike PON activity which depends on polymorphisms that are determinant for PON 1 antioxidant capacity, ARE activity does not and its levels are dependent on PON 1 concentration. In consequence, while PON activity could be considered as an estimator of the antioxidant PON 1 capacity, ARE activity would reflect better the protein concentration [30]. Thus, in IO patients the reduced PON activity and the increased ARE activity per unit of apo A-I would suggest an inactivation of PON 1. Overall, in IO patients, this impairment might be related to an altered HDL chemical composition probably generated by the role of insulin resistance and the enhanced CETP activity [39].

Recently, Lp-PLA_2 has been recommended as an inflammatory marker for cardiovascular disease risk assessment [40]. In this study, IO patients exhibited higher enzymatic activity than healthy controls. Moreover, Lp-PLA_2 activity was significantly correlated with ALT activity and with ferritin, triglyceride, LDL-C, HDL-C and apo B plasma levels. The correlation between Lp-PLA_2 and ALT activity is noteworthy, as the latter constitutes a marker for liver injury and fat accumulation, apart from being recognized as an independent predictor of type 2 diabetes [41,42]. Furthermore, in this study, a 40% of Lp-PLA_2 activity total variance was attributed to ferritin concentration independently of age, BMI, ALT activity, HOMA-IR, triglyceride and apo B plasma levels. Then, the increase in Lp-PLA_2 activity resulted to be associated with iron stores and not with insulin resistance, which was in agreement with the results of a community-based study [43]. Ferritin concentration reflects not only body iron stores but also macrophage iron content. So, it could be plausible that an increased iron concentration in macrophages located in the subendothelial space could enhance the generation of oxidized phospholipids on LDL surface [44], which would trigger the activation of Lp-PLA_2. In fact, Lp-PLA_2 only acts on water-soluble polar phospholipids with oxidatively truncated *sn*-2 chains [45]. Accordingly, oxidized LDL levels were higher in IO patients than control subjects and were positively associated with Lp-PLA_2 activity and ferritin concentration, which extends the findings previously reported [14].

In the present study, the activities of PON 1, CETP and Lp-PLA_2 were analysed; however, the activities of other proteins related to lipoprotein metabolism like the lechitin:cholesterol acyl transferase (LCAT) and the phospholipid transfer protein (PLTP) have not been assessed yet. Furthermore, IO patients presented higher insulin concentration and HOMA-IR than control subjects and though these differences were considered for data analyses and proper adjusted statistical tests were performed, it might constitute a source of bias.

High CETP activity is known to increase triglyceride content of LDL and HDL particles, thus rendering LDL more susceptible to oxidation and HDL less competent in their antioxidant role [36,46,47], which would be further amplified by the reduction in PON activity. Then, in a context of IO characterized by the accumulation of prooxidant iron, LDL oxidation might be facilitated and the multiple deleterious effects of oxidized LDL would be amplified by oxidized fatty acids and lysophospholipids released by Lp-PLA_2 action [48]. In conclusion, IO patients presented not only insulin resistance but also other metabolic alterations which were related to elevated iron stores and are associated with high risk of cardiovascular disease.

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Conflict of interest

None of the authors have any conflict of interest.

Author contributions

Tomás Meroño and Leonardo Gómez Rosso have contributed equally in lipoprotein related analytical determinations and in manuscript writing. Patricia Sorroche and Laura Boero performed the general biochemical determinations. Jorge Arbelbide selected the patients and controls. Fernando Brites contributed with the result interpretation and the discussion.

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