Clinical Science (2015) **128**, 609–618 (Printed in Great Britain) doi: 10.1042/CS20140300

Metabolic alterations, HFE gene mutations and atherogenic lipoprotein modifications in patients with primary iron overload

Tomás Meroño*†, Fernando Brites*, Carolane Dauteuille†, Marie Lhomme†, Martín Menafra*, Alejandra Arteaga‡, Marcelo Castro§, María Soledad Saez‖, Esteban González Ballerga‡, Patricia Sorroche‖, Jorge Rey§, Philippe Lesnik†, Juan Andrés Sordá‡, M. John Chapman†, Anatol Kontush† and Jorge Daruich‡¶

*Laboratory of Lipids and Lipoproteins, School of Pharmacy and Biochemistry, INFIBIOC, University of Buenos Aires, CONICET, Junín 956, 1113 Buenos Aires, Argentina
†National Institute for Health and Medical Research (INSERM), UMR ICAN 1166, University of Pierre et Marie Curie – Paris 6, AP-HP, Groupe hospitalier Pitié-Salpétrière, ICAN, Paris F-75013, France
‡Hepatology Division, Hospital "José de San Martín", University of Buenos Aires, Buenos Aires, Argentina
§Department of Hemotherapy and Immunohematology, Hospital "José de San Martín", University of Buenos Aires, Buenos Aires, Argentina
‖Central Laboratory, Italian Hospital of Buenos Aires, Buenos Aires, Argentina
¶Diagnostic and Therapeutical Gastroenterology (GEDYT), Buenos Aires, Argentina

Abstract

Iron overload (IO) has been associated with glucose metabolism alterations and increased risk of cardiovascular disease (CVD). Primary IO is associated with mutations in the HFE gene. To which extent HFE gene mutations and metabolic alterations contribute to the presence of atherogenic lipoprotein modifications in primary IO remains undetermined. The present study aimed to assess small, dense low-density lipoprotein (LDL) levels, chemical composition of LDL and high-density lipoprotein (HDL) particles, and HDL functionality in IO patients. Eighteen male patients with primary IO and 16 sex- and age-matched controls were recruited. HFE mutations (C282Y, H63D and S65C), measures of insulin sensitivity and secretion (calculated from the oral glucose tolerance test), chemical composition and distribution profile of LDL and HDL subfractions (isolated by gradient density ultracentrifugation) and HDL functionality (as cholesterol efflux and antioxidative activity) were studied. IO patients compared with controls exhibited insulin resistance (HOMA-IR (homoeostasis model assessment-estimated insulin resistance): +93%, $P < 0.001$). Metabolic profiles differed across HFE genotypes. C282Y homozygotes ($n = 7$) presented a reduced β-cell function and insulin secretion compared with non-C282Y patients ($n = 11$) (−58% and −73%, respectively, $P < 0.05$). In addition, C282Y homozygotes featured a predominance of large, buoyant LDL particles (C282Y: 43 ± 5; non-C282Y: 25 ± 8; controls: 32 ± 7%; $P < 0.001$), whereas non-C282Y patients presented higher amounts of small, dense LDL (C282Y: 23 ± 5; non-C282Y: 39 ± 10; controls: 26 ± 4%; $P < 0.01$). HDL particles were altered in C282Y homozygotes. However, HDL functionality was conserved. In conclusion, metabolic alterations and HFE gene mutations are involved in the presence of atherogenic lipoprotein modifications in primary IO. To what extent such alterations could account for an increase in CVD risk remains to be determined.

Key words: atherosclerosis, cardiovascular disease, hereditary haemochromatosis, high-density lipoprotein (HDL), insulin resistance, iron overload, low-density lipoprotein (LDL)

INTRODUCTION

Iron overload (IO) is defined as an increase in storage iron regardless of the presence of tissue damage [1]. Primary causes of IO are hereditary conditions in which the expression or the regulation of iron metabolism-related proteins or hormones is affected [2]. The most common of these conditions is hereditary haemochromatosis (HH), an affliction caused by mutations in

Abbreviations: AAPH, 2,2′-azobis-(2-amidinopropane) hydrochloride; ALP, alkaline phosphatase; ALT, alanine aminotransferase; apo, apolipoprotein; AST, aspartate aminotransferase; BMI, body mass index; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CVD, cardiovascular disease; FC, free cholesterol; HH, hereditary haemochromatosis; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HFE, human haemochromatosis protein; HOMA-IR, homoeostasis model assessment-estimated insulin resistance; hsCRP, high-sensitivity C-reactive protein; IO, iron overload; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; OGTT, oral glucose tolerance test; PL, phospholipids; PMA, phorbol 12-myristate 13-acetate; TC, total cholesterol; TG, triacylglycerol; TP, total protein; VLDL, very-low-density lipoprotein; VLDL-C, VLDL-cholesterol

Correspondence: Dr. Tomás Meroño (email tomasmero@yahoo.com.ar).

the gene encoding the human haemochromatosis protein (HFE). The most frequent mutation in the HFE gene leading to HH in the European population encodes the amino acid substitution C282Y [3]. However, there are also other HFE variants that are associated with a similar phenotype, mainly characterized by hepatocellular IO [4]. In Hispanic and Latin American patients with abnormal iron biomarkers, the H63D variant of the HFE gene is more frequent than the C282Y [5]. Nonetheless, it has been reported that the HFE genotype scarcely affects the appearance of hepatic and some extrahepatic manifestations of IO [4,6].

IO has been increasingly proposed as a conditioning factor for the development of atherosclerotic cardiovascular disease (CVD) [7,8], and patients with primary IO have been reported to present early signs of atherosclerosis [9]. In this regard, it seems that HFE mutations can influence CVD risk [10,11]. Recent meta-analyses have identified a significant association of CVD risk with the H63D variant but not with the C282Y [10,11]. Such differences suggest a genotype–phenotype interaction which remains largely undetermined.

Current insights into atherosclerosis highlight the role of atherogenic lipoprotein alterations that can trigger and/or exacerbate the vascular inflammatory response [12]. Such alterations include increased levels of small, dense low-density lipoprotein (LDL), triacylglycerol (TG)-enriched LDL and TG-enriched high-density lipoprotein (HDL), together with impaired HDL functionality [13]. Metabolic alterations capable of promoting atherogenic modifications of circulating lipoproteins have been previously documented in IO [14–17].

Previous studies carried out in patients with primary IO have shown a high prevalence of glucose metabolism alterations [14,16,17]. Although iron-driven oxidative damage of β-cells has been associated with these alterations [16,18], insulin resistance has equally been observed [14,17]. Insulin resistance is known to be associated with increased levels of small, dense LDL particles and dysfunctional HDL [19,20]. In addition, iron levels have been shown to have a role in lipid metabolism. Indeed, iron depletion was associated with a reduction in TG levels in IO patients [15]. A recent study has revealed that IO patients feature low HDL-cholesterol (HDL-C) concentration and increased cholesteryl ester transfer protein (CETP) activity [14], resembling lipid profiles of patients with metabolic syndrome or Type 2 diabetes [21,22]. In this context, the proportion of small, dense LDL particles and the chemical composition and functionality of HDL have been scarcely explored in IO.

Since conditions responsible for atherogenic lipoprotein modifications are present in IO, this study was conducted to assess the levels of small, dense LDL, the chemical composition of LDL and HDL particles, and the functionality of HDL in male patients with IO.

MATERIALS AND METHODS

Patients and study protocol

Adult male patients with IO were recruited from the Hepatology Division of the University Hospital "José de San Martín" (Buenos Aires, Argentina) between 2010 and 2012 ($n = 38$). IO was diagnosed when patients presented transferrin saturation $>45\%$ or ferritin concentration >300 μg/l, and liver histology compatible with primary iron overload (iron deposition preferentially within hepatocytes in the periportal region of the hepatic lobule). Patients with previous diagnosis of Type 2 diabetes, hypothyroidism, cirrhosis, viral hepatitis, HIV infection or cancer, patients under treatment with statins, fibrates and/or antioxidant supplementation, patients with excessive alcohol consumption (>20 g ethanol/day) or tobacco abuse ($n = 6$), and patients who were in the maintenance phase of an iron-depletion treatment (ferritin concentration <100 μg/l, $n = 14$) were excluded from the study. After a 12-h overnight fast, the patients selected ($n = 18$) performed a 75 g oral glucose tolerance test (OGTT) and samples were withdrawn at 0, 30, 60, 90 and 120 min. Sixteen age-matched male normolipidaemic healthy subjects from a pool of blood donors without clinical or biochemical signs of IO and wild-type for mutations in the HFE gene were also recruited. A single blood sample from each control subject was drawn from the antecubital vein after a 12-h overnight fast. Whole blood was used to determine the complete blood count and an aliquot was stored at $-20\,^{\circ}$C to perform the HFE gene mutation testing. Serum samples were immediately used for general biochemical determinations and aliquots were stored at $-70\,^{\circ}$C. Baseline serum samples were used to determine high-sensitivity C-reactive protein (hsCRP) levels and CETP activity, as well as to subfractionate lipoproteins by density gradient ultracentrifugation. For this assay, sucrose (final concentration 0.6% w/v) was added to the samples as a cryoprotectant. Each aliquot was thawed only once before analyses.

The present study was carried out in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the University Hospital "José de San Martín" and from the School of Pharmacy and Biochemistry, University of Buenos Aires. All patients provided informed consent to participate in the study.

Anthropometric, analytical and genetic procedures

Height and weight were measured with the subject wearing light clothes and without shoes. Body mass index (BMI) was calculated and BMI categories were defined according to the World Health Organization (WHO) adult definition [23]. Blood pressure was determined with a random-zero sphygmomanometer after 5 min of rest and the average of two measurements was recorded. Hypertension was defined in accordance to international guidelines [24].

Complete blood count was determined in a Coulter® GEN-STM autoanalyser (Beckman Coulter). Plasma transferrin, apolipoprotein (apo) A-I and apo B concentrations were measured by immunonephelometry (IMMAGE®, Beckman Coulter). Ferritin concentration was determined by an electrochemiluminescence assay (VITROS® ECiQ, Ortho-Clinical Diagnostics). Serum levels of iron, glucose, urea, creatinine, uric acid, TG and total cholesterol (TC) and the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were quantified by standardized methods (Roche Diagnostics) in a COBAS C501 autoanalyser (Roche

Diagnostics). LDL-cholesterol (LDL-C) and HDL-C concentrations were determined by selective precipitation methods. Very-low-density lipoprotein-cholesterol (VLDL-C) was calculated. Insulin concentration was measured by radioimmunoassay (DPC). HsCRP concentration was measured by a high-sensitivity immunoassay (Roche Diagnostics). CETP activity was measured by an endogenous assay as previously described [14].

Impaired fasting glucose, altered glucose tolerance and Type 2 diabetes in IO patients were diagnosed in accordance to the American Diabetes Association (ADA) guidelines [25].

All subjects were tested for the three most common mutations in the HFE gene, encoding H63D, S65C and C282Y, by direct PCR-RFLP (restriction fragment length polymorphism) in whole blood without previous DNA extraction [26].

Metrics of insulin sensitivity, insulin secretion and β-cell function

The Matsuda index was calculated as previously described, using 0, 30, 60, 90 and 120 min measurements of glycaemia and insulinaemia [27]. Insulin secretion was estimated by the $\Delta Insulin_{0–120}/\Delta Glucose_{0–120}$ relationship [28]. The hyperbolic relationship between insulin secretion and sensitivity was tested by means of the slope of the double logarithmic plot. When the hyperbolic relationship was confirmed, β-cell function was estimated by the disposition index calculated as the product between metrics of insulin sensitivity and insulin secretion.

Lipoprotein isolation

Plasma lipoproteins were isolated from serum by a single step, isopycnic non-denaturing density gradient ultracentrifugation in a Beckman SW41 Ti rotor at 40 000 rpm for 44 h in a Beckman XL70 ultracentrifuge at 15 °C by the method of Chapman et al. [29] modified as previously described [30]. After centrifugation, each gradient was fractionated into 11 fractions corresponding to VLDL + intermediate density lipoprotein (IDL) (d < 1.019 g/ml), five LDL subfractions (LDL1 to LDL5, d 1.019–1.063 g/ml) and five HDL subfractions (HDL2b to HDL3c, d 1.063–1.179 g/ml). The validity and reproducibility of this density gradient procedure, which facilitates fractionation of lipoprotein particle subspecies in a non-oxidized native state, have been extensively documented [29,30]. Lipoproteins were dialysed against phosphate-buffered saline (PBS; pH 7.4) at 4 °C in the dark, stored at 4 °C and used within 10 days.

Lipoprotein chemical composition [total proteins (TP), TC, free cholesterol (FC), phospholipids (PL) and TG] was determined using commercially available assays. Cholesteryl ester (CE) concentration was calculated [29,30]. Total lipoprotein mass was also calculated as the sum of TP, CE, FC, PL and TG. For functionality assays, total HDL from each donor was prepared by mixing all five HDL subfractions at their equivalent serum concentrations.

HDL antioxidative activity

Antioxidative activity of total HDL (final concentration, 0.4 g total mass/l) was assessed towards reference LDL (0.1 g of TC/l) isolated from one healthy normolipidaemic control subject [31]. LDL oxidation was performed at 37 °C in PBS (pH 7.4) in the presence of a water-soluble azo-initiator 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH; 1 mM). HDL was added to LDL directly before oxidation. The HDL used for this assay was isolated from serum to ensure PON1 activity, which is inhibited by EDTA [32]. Accumulation of conjugated dienes was measured in the samples as the increase in absorbance at 234 nm [31,33]. The absorbance kinetics were corrected for the absorbance of AAPH itself assayed in parallel as a blank. The kinetics of diene accumulation revealed two characteristic phases, the lag and the propagation phases. For each curve, the duration of each phase, average oxidation rates within each phase and amount of dienes formed at the end of the propagation phase (maximal amount of dienes) were calculated.

HDL capacity to promote cellular cholesterol efflux

The capacity of total HDL to promote cellular cholesterol efflux was characterized using a human THP-1 monocytic cell system (ATCC, Manassas, VA, U.S.A.) at 15 ng of HDL-PL/l. Total HDL from each donor was prepared as described above. Assays of cellular cholesterol efflux were performed as previously described, with minor modifications [34]. Briefly, THP-1 monocytes were cultured on 24-well tissue culture plates and grown in RPMI 1640 medium with 10 % heat-inactivated foetal bovine serum and differentiated into macrophage-like cells with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 h. The cells were washed and loaded for 24 h with [^{3}H]cholesterol-labelled acetylated LDL (1 μCi/ml) in serum-free RPMI 1640 supplemented with 50 mM glucose, 2 mM glutamine, 0.2 % (w/v) bovine serum albumin (RGGB), 100 μg/ml penicillin and 100 μg/ml streptomycin to allow cell cholesterol pools to equilibrate. The labelling medium was removed and human macrophages were then equilibrated in RGGB for an additional 16–24 h period. Cellular cholesterol efflux to HDL was assayed in serum-free medium for a 4-h chase period; culture media were collected and cleared of cellular debris by a brief centrifugation. Cell radioactivity was determined by extraction in hexane/propan-2-ol (3:2), evaporation of the solvent and liquid scintillation counting (Wallac Trilux 1450 Microbeta, PerkinElmer). The percentage of cholesterol efflux was calculated as (medium cpm)/(medium cpm + cell cpm) × 100. Specific cholesterol efflux was determined by subtracting non-specific cholesterol efflux occurring in the absence of cholesterol acceptors.

Statistical analyses

Counts of categorical variables were compared by the χ^2 test applying Bonferroni's method to correct for multiple comparisons. The χ^2 test was also used to evaluate associations between allele presence and atherogenic lipoprotein alterations in IO patients. For these analyses, increased proportion of LDL4 and LDL5 particles was defined as above the third quartile. The Shapiro–Wilks method was used to assess data distribution. Normally distributed variables were expressed as means ± S.D. and skewed-distributed variables as median (interquartile range [IQR]). Differences were analysed by ANOVA using Tukey's *post-hoc* test or by Kruskal–Wallis and paired comparison *post-hoc* test according to normal or skewed data distribution, respectively. Different letters over the mean or the median indicate significantly dissimilar groups. Correlations in the IO group were analysed by the

Table 1 Clinical and biochemical characteristics of IO patients and control subjects
Results are expressed as means ± S.D. or medians (Q1–Q3) for normal or skewed data, respectively. Shown *P* values were obtained using parametric or non-parametric ANOVA tests as appropriate. Different letters indicate significantly dissimilar groups.

	C282Y patients ($n = 7$)	Non-C282Y patients ($n = 11$)	Control subjects ($n = 16$)	*P*
Age (years)	48 ± 18	47 ± 13	47 ± 15	0.991
BMI (kg/m^2)	28 ± 2	28 ± 2	26 ± 3	0.150
Overweight (*n*)	4	7	9	0.862
Obese (*n*)	2	2	2	0.730
Hypertension (*n*)	1	2	0	0.891
Hb (g/l)	148 ± 20	149 ± 8	146 ± 10	0.845
White blood cell count (10^9 cells/l)	5.8 ± 1.9	7.3 ± 1.4	6.3 ± 1.6	0.121
Platelet count (10^9 cells/l)	168 ± 78^a	220 ± 53^{ab}	237 ± 36^b	0.036
Transferrin saturation (%)	93 (72–98)^a	47 (34–54)^b	25 (23–29)^c	<0.001
Ferritin (μg/l)	2120 (456–2440)^a	444 (246–616)^a	124 (102–162)^b	<0.001
Total bilirubin (μmol/l)	17.1 (15.4–27.4)^a	13.7 (12.0–17.1)^a	8.6 (5.1–10.3)^b	<0.001
AST (IU/l)	41 (26–75)^a	29 (23–32)^a	20 (18–22)^b	<0.001
ALT (IU/l)	47 (27–57)^a	44 (28–48)^a	13 (9–21)^b	<0.001
ALP (IU/l)	102 (75–172)	74 (59–101)	56 (53–61)	0.213
hsCRP (mg/l)	1.4 (1.0–1.4)	1.9 (0.7–2.5)	0.9 (0.6–1.3)	0.052
Glucose (mmol/l)	5.4 ± 0.5^{ab}	5.7 ± 0.7^b	5.0 ± 0.4^a	0.016
Insulin (mU/l)	12 (11–18)^a	14 (10–18)^a	7 (4–8)^b	0.008
HOMA-IR	2.8 (2.6–4.6)^a	3.4 (2.0–4.8)^a	1.5 (0.9–1.7)^b	0.006

Pearson or Spearman test in agreement with data distribution. Significance was defined as $P < 0.05$ in the two-tail tests. For all statistical analyses, the software INFOSTAT (Grupo Infostat, Universidad Nacional de Córdoba, Argentina) was used.

RESULTS

Genotypic and phenotypic characterization

Seven out of the 18 IO patients were homozygous for the HFE C282Y mutation and were therefore diagnosed with HH. Of the remaining 11 patients (non-C282Y homozygous), two were compound C282Y/H63D heterozygous, one was H63D homozygous, four were H63D heterozygous and four presented none of the HFE mutations screened. Four of the 18 patients (22 %) presented mild hepatic steatosis. Eleven of the 18 patients were overweight and three were obese. The clinical and biochemical characteristics of the IO patients, according to C282Y homozygosis, and healthy controls are shown in Table 1. There were no differences in age, BMI and the prevalence of overweight/obesity among all the groups studied. As expected, both groups of IO patients exhibited elevated transferrin saturation and increased levels of ferritin, total bilirubin, AST and ALT, reflecting IO and iron-induced liver injury. C282Y homozygous patients were characterized by the highest levels of transferrin saturation among the groups.

Glucose metabolism

Fasting glucose concentration was significantly higher in non-C282Y homozygous patients than in controls (Table 1). In addition, a 2-fold increase in fasting insulin and HOMA-IR (homoeostasis model assessment-estimated insulin resistance) was consistently observed in both groups of IO patients in comparison with controls (Table 1).

Half of the IO patients presented alterations in glucose metabolism: 7/18 exhibited impaired fasting glucose, one had impaired glucose tolerance and one was diagnosed with Type 2 diabetes. No significant differences were evidenced in the prevalence of such alterations between genotype categories ($P > 0.05$). Nonetheless, patients with impaired glucose tolerance and diabetes belonged to the C282Y homozygous group.

Metrics of insulin sensitivity and secretion and of β-cell functionality derived from the OGTT are shown in Figure 1. Insulin sensitivity was similar in both groups of IO patients, but a significant decrease in insulin secretion was detected in C282Y homozygotes. Such modified insulin response was the leading cause of a significant reduction in β-cell functionality in this group (Figure 1).

Lipoprotein metabolism

Data regarding lipoprotein metabolism from C282Y and non-C282Y homozygous patients and controls are shown in Table 2. Both groups of IO patients presented significantly increased TG levels and TG/HDL-C ratio (Table 2). Furthermore, C282Y homozygous patients showed significantly lower HDL-C concentration and higher CETP activity than controls.

Figure 2 shows total plasma lipoprotein mass of the five LDL subfractions from the large, buoyant LDL1 to the small, dense LDL5 in IO patients and controls. Different LDL distribution profiles were observed between the genotype categories of IO patients. C282Y homozygous patients presented significantly higher LDL2 and lower small, dense LDL4 mass than non-C282Y homozygotes. In addition, they showed lower LDL3 levels than healthy controls (Figure 2).

The sum of all five LDL subfractions, equivalent to total LDL mass in the circulation, was decreased in C282Y homozygous

Table 2 Lipid and lipoprotein profile of IO patients and control subjects
Results are expressed as means ± S.D. or medians (Q1–Q3) for normal or skewed data, respectively. Shown *P* values were obtained using parametric or non-parametric ANOVA tests. Different letters indicate significantly dissimilar groups.

	C282Y patients ($n = 7$)	Non-C282Y patients ($n = 11$)	Control subjects ($n = 16$)	*P*
TG (mmol/l)	1.15 (0.85–1.93)[a]	1.37 (0.71–1.65)[a]	0.85 (0.55–0.93)[b]	0.024
TC (mmol/l)	3.9 ± 0.9	4.5 ± 1.0	4.8 ± 0.6	0.230
VLDL-C (mmol/l)	0.45 ± 0.29	0.51 ± 0.21	0.39 ± 0.21	0.244
LDL-C (mmol/l)	2.6 ± 0.7	2.8 ± 0.9	3.1 ± 0.7	0.410
HDL-C (mmol/l)	1.10 (0.95–1.20)[a]	1.15 (0.97–1.38)[ab]	1.35 (1.17–1.66)[b]	0.031
TG/HDL-C	2.6 (1.5–3.7)[a]	2.3 (1.2–3.7)[a]	1.3 (0.8–1.8)[b]	0.011
ApoB (g/l)	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.733
ApoA-I (g/l)	1.2 ± 0.2	1.4 ± 0.2	1.5 ± 0.2	0.072
CETP activity (%·ml^{-1}·h^{-1})	160±40[a]	157±50[ab]	128±36[b]	0.034

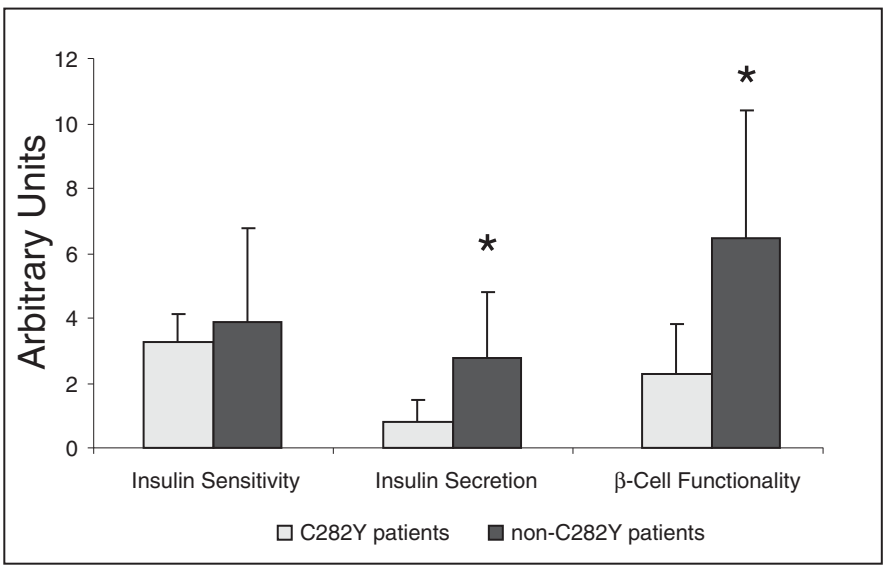

Figure 1 Metrics of insulin sensitivity, insulin secretion and β-cell function in IO patients
Surrogate markers of insulin sensitivity, insulin secretion and β-cell functionality in C282Y ($n = 7$) and non-C282Y homozygous patients ($n = 11$). Insulin sensitivity was estimated as the Matsuda index and insulin secretion as the ratio between $\Delta Insulin_{0–120}$ and $\Delta Glucose_{0–120}$. *$P < 0.05$.

patients (C282Y: 2.2 ± 0.2[a]; non-C282Y: 2.8 ± 0.6[ab]; controls: 3.0 ± 0.4[b] g/l, $P < 0.005$). Thus, proportions of the different LDL subfractions were calculated and compared among the groups. C282Y homozygous patients exhibited the highest proportion of large buoyant LDL1+2 particles (C282Y: 43 ± 5[a]; non-C282Y: 25 ± 8[b]; controls: 32 ± 7[b]%, $P < 0.001$), whereas non-C282Y homozygous patients were characterized by the predominance of small, dense LDL4+5 subfractions (C282Y: 23 ± 5[a]; non-C282Y homozygotes: 39 ± 10[b]; controls: 26 ± 4[ab]%, $P < 0.05$).

Moreover, LDL3, LDL4 and LDL5 particles from C282Y homozygous patients were significantly enriched in TG when compared with their counterparts from controls (Supplementary Table S1). Similarly, LDL subfractions from non-C282Y patients presented a mild increase in TG content which did not reach statistical significance (Supplementary Table S1).

Plasma levels of HDL subfractions in IO patients and controls are shown in Figure 3. C282Y homozygous patients presented a decrease in the concentration of small, dense HDL3b and HDL3c subfractions when compared with controls (Figure 3), whereas non-C282Y patients featured discrete reductions in plasma concentrations of all HDL subfractions, which did not reach statistical significance relative to controls. Chemical composition analysis revealed that HDL subfractions from IO patients were enriched in TG and depleted in CE (Supplementary Table S2). In particular, non-C282Y homozygous patients featured mild alterations in HDL chemical composition, whereas C282Y homozygotes exhibited more profound alterations in HDL chemical composition than controls (Supplementary Table S2).

HDL functionality

No difference was observed in the antioxidative activity of HDL between IO patients and controls (Supplementary Table S3). Similarly, no significant difference was detected in the capacity of HDL to promote cholesterol efflux from differentiated THP-1 cells between IO patients and controls (Supplementary Table S3).

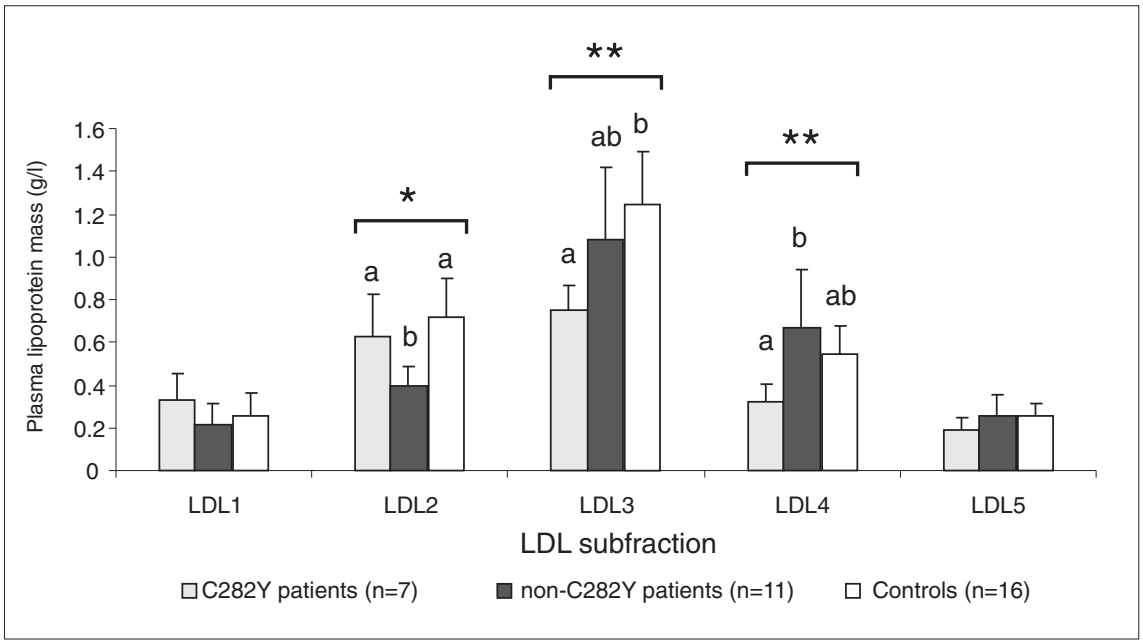

Figure 2 Plasma total lipoprotein mass of LDL subfractions in IO patients and controls
Distribution of total mass of plasma LDL subfractions from homozygous C282Y ($n = 7$) and non-C282Y patients ($n = 11$) and control subjects ($n = 16$). Total lipoprotein mass was calculated as the sum of its major chemical components (CE, FC, PL, TG and TP); $^{*}P < 0.005$; $^{**}P < 0.01$. Different letters indicate significantly dissimilar groups.

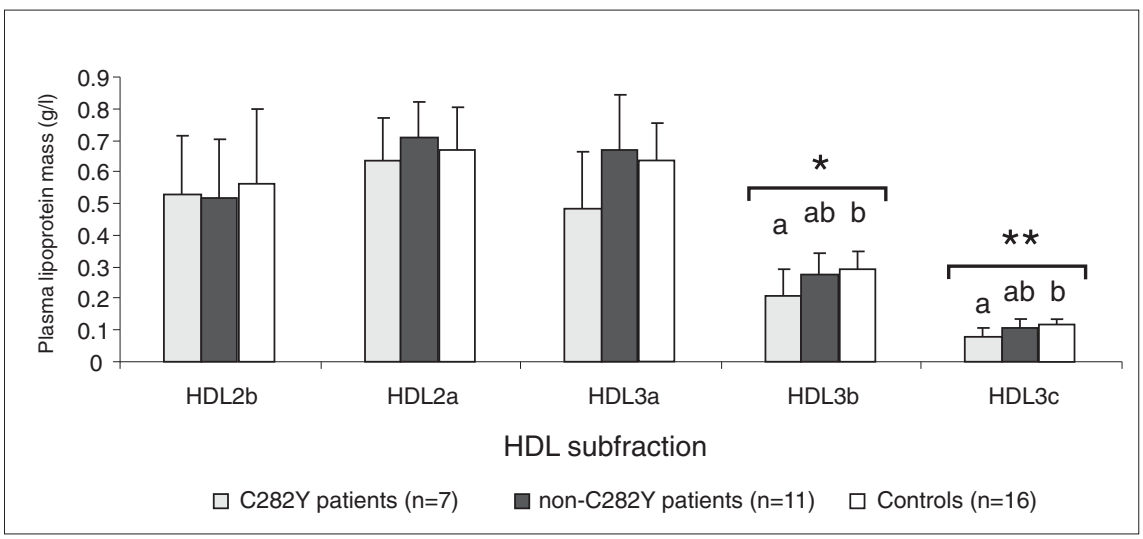

Figure 3 Plasma total lipoprotein mass of HDL subfractions in IO patients and controls
Distribution of total mass of plasma HDL subfractions from homozygous C282Y ($n = 7$) and non-C282Y patients ($n = 11$) and control subjects ($n = 16$). Total lipoprotein mass was calculated as the sum of its major chemical components (CE, FC, PL, TG and TP); $^{*}P < 0.005$; $^{**}P < 0.01$. Different letters indicate significantly dissimilar groups.

Interactions between iron levels, HFE mutations and metabolic characteristics

Regarding iron metabolism, ferritin concentration was poorly correlated with glucose metabolism markers and lipoprotein measurements within IO patients (Table 3). In contrast, transferrin saturation was correlated with the decline of insulin secretion and β-cell function, as well as with quantitative modifications in small, dense HDL levels (Table 3). Strikingly, insulin secretion and β-cell function were positively correlated with the proportion of small dense LDL4+5 subfractions ($r = 0.70$, $P < 0.01$ and $r = 0.77$, $P < 0.005$, respectively).

The presence of the H63D allele in IO patients was positively associated with an increased proportion of the small, dense LDL5 ($\chi^2 = 4.38$, $P < 0.05$). In contrast, the presence of the C282Y allele was negatively associated with increased proportions of LDL4 ($\chi^2 = 4.67$, $P < 0.05$) and LDL5 ($\chi^2 = 7.78$, $P < 0.005$).

Table 3 Correlations between iron metabolism markers and selected metabolic characteristics in IO patients ($n = 18$)
Insulin secretion was estimated as the ratio between $\Delta Insulin_{0–120}$ and $\Delta Glucose_{0–120}$. Total lipoprotein mass was calculated as the sum of its major chemical components (CE, FC, PL, TG and TP).

	Transferrin saturation	Ferritin
Insulin secretion [*r* (*P*)]	−0.67 (0.007)	−0.02 (0.867)
β-Cell function [*r* (*P*)]	−0.68 (0.007)	−0.20 (0.481)
HDL3a-mass [*r* (*P*)]	−0.64 (0.008)	−0.31 (0.229)
HDL3b-mass [*r* (*P*)]	−0.58 (0.019)	−0.30 (0.250)
HDL3c-mass [*r* (*P*)]	−0.63 (0.008)	−0.47 (0.066)

Table 4 Correlations between TG/HDL-C ratio and compositional lipoprotein characteristics in IO patients ($n = 18$)

	r	*P*
CETP activity	0.83	0.001
LDL3-TG	0.63	0.008
LDL5-TG	0.57	0.040
HDL3a-TG	0.68	0.007
HDL3a-CE	−0.71	0.006
HDL3b-TG	0.54	0.046
HDL3b-CE	−0.59	0.027
HDL3c-TG	0.54	0.046
HDL3c-CE	−0.64	0.008

Regarding the lipid profile, the TG/HDL-C ratio was correlated with CETP activity and with compositional changes in the LDL and HDL subfractions (Table 4). Finally, CETP activity was significantly correlated with all the changes observed in the chemical composition of HDL subfractions from IO patients (Supplementary Table S4).

DISCUSSION

The present study documents atherogenic lipoprotein alterations, which have been associated with high CVD risk [35–37], in male patients with primary IO. These alterations varied according to the presence of HFE mutations. Notably, C282Y homozygous patients exhibited diminished insulin secretion, reduced levels of small, dense HDL subfractions, and LDL and HDL particles with increased TG content. On the other hand, non-C282Y homozygous patients presented an atherogenic LDL subfraction profile characterized by an increased proportion of small, dense LDL, as well as mild alterations in their LDL and HDL chemical composition. IO patients also showed insulin resistance, dyslipidaemia and increased CETP activity, confirming previous results [14].

The impaired insulin secretion and β-cell function observed in C282Y homozygous patients is in agreement with the results of previous studies, in which the frequently sampled intravenous glucose tolerance test (IVGTT) was used [16]. Furthermore, impaired glucose tolerance and Type 2 diabetes, which are conditions primarily characterized by β-cell dysfunction, were exclusively present in C282Y homozygotes. In a mouse model analogous to human HFE C282Y homozygosis, such impaired insulin secretion was attributed to apoptosis due to iron-driven oxidative damage of β-cells [18]. In contrast, by using several surrogate markers calculated from the OGTT in a group of patients with IO, Hatunic et al. [17] found no defect in insulin secretion. This difference could be partly attributed to the fact that, in the latter study [17], the group of IO patients was heterogeneous in terms of the genotype (72% of C282Y homozygotes and 28% with other genotypes). Thus, C282Y homozygous patients could have been at higher risk of declined β-cell function compared with non-homozygous ones. To achieve iron accumulation in the pancreas, previous hepatic iron loading is needed [38]. Then, as an increased hepatic iron concentration was found in C282Y homozygotes [39], a more aggressive IO progression could be expected in these subjects. Together, these pieces of evidence suggest that C282Y homozygous patients present not only insulin resistance, which is common in primary IO, but also distinct pathophysiological characteristics leading to alterations in glucose metabolism.

Regarding lipoprotein metabolism, important differences were observed between the genotypes. Consistent with the results of the ARIC population study [40], C282Y homozygous patients presented the lowest LDL mass, even lower than normolipidaemic controls. Most interestingly, this low mass was attributed to the relatively small and dense LDL3 and LDL4 subfractions. Such LDL distribution profile can be considered as less atherogenic than that exhibited by non-C282Y patients. Indeed, the LDL distribution profile of non-C282Y patients, 64% of whom bore the H63D mutation, was characterized by the predominance of small, dense LDL4+5 subfractions. Importantly, we identified a significant association between a high proportion of the small, dense LDL5 and the H63D allele. Such association might contribute to the higher CVD risk estimates associated with the H63D allele [10,11,41,42]. Conversely, the C282Y allele was negatively associated with the proportion of small dense LDL4 and LDL5 subfractions and could partially explain the comparable CVD risk estimates observed between patients with C282Y homozygosis and wild-type HFE controls.

The positive correlation between the LDL4+5 proportion and β-cell function suggests that the differences observed between the groups of IO patients might be associated with the impact of low insulin secretion on LDL metabolism. Insulin is known to regulate LDL catabolism by hepatic lipase, which has been shown to contribute to small dense LDL formation [43,44]. Thus, in C282Y homozygotes, reduced hepatic lipase activity due to defects in insulin secretion could account for their particular LDL subfraction profile. Future studies of this association are needed. Regarding LDL chemical composition, IO patients with C282Y homozygosis presented a TG-enrichment in almost all LDL particle subpopulations. Importantly, some evidence has attributed a pro-atherogenic role to a TG-enrichment in LDL particles as it renders them more susceptible to oxidation and more injurious to the vascular endothelium [45,46]. It remains to be shown to what extent such lipoprotein modifications are relevant in the metabolic context of IO.

Noteworthily, alterations in the HDL subfraction profile observed in C282Y homozygous patients were independently

associated with CVD risk [37,47]. However, HDL functionality was not affected in C282Y homozygotes, in spite of the lower circulating levels of HDL3c, an HDL subfraction that displays potent anti-atherogenic properties [48]. Furthermore, although TG enrichment and CE depletion of HDL particles have been associated with altered HDL activities under other metabolic conditions [19,22], these alterations were not sufficient to impair HDL functionality. Thus, in spite of quantitative and compositional alterations, other factors might be contributing to the preservation of HDL functionality in IO. As previously suggested, the observed modifications in HDL would be preferentially associated with insulin resistance and increased CETP activity than with IO [14].

The present results have several points of clinical importance. High fasting glucose and insulin concentrations are common in primary IO patients and, therefore, the use of the OGTT seems to be adequate in this clinical setting. As the decrease in β-cell function represents the main pathway leading to impaired glucose tolerance and diabetes [49], the OGTT evaluation could be useful to understand the complex metabolic alterations that occur in IO. In this context, since insulin secretion could be already impaired in C282Y homozygous patients, it might be cost-effective to draw attention into factors that may compromise insulin sensitivity, such as obesity. On the other hand, lipid metabolism is not typically evaluated in IO patients and the presence of atherogenic lipoprotein modifications often remains unknown. Knowledge of the presence of atherogenic dyslipidaemia, including increased proportion of small, dense LDL, might be of importance to prevent future cardiovascular complications in IO patients. In this aspect, the TG/HDL-C ratio was the parameter that best reflected the multiple lipoprotein alterations observed. Thus, determining this parameter might be helpful to evaluate lipoprotein metabolism in IO patients.

In the present study, the use of surrogate metrics of insulin sensitivity and secretion, instead of the clamp technique, could be a source of bias. Also, the relatively small number of subjects could have limited the detection of subtle differences between the genotypes. Finally, our data do not allow inferral that carriers of HFE mutations, who do not display IO, will feature the metabolic alterations herein described. Nonetheless, the present results highlight the importance of early identification and treatment of patients with IO. Indeed, iron-depletion therapy has been proposed as an alternative to curb metabolic abnormalities associated with IO [15,50]. As a consequence, reductions in iron levels are now being considered as a therapeutic alternative not only for patients with overt IO but also for patients with other conditions [51].

In conclusion, our study reveals that metabolic alterations and HFE gene mutations are involved in the presence of atherogenic lipoprotein modifications in male patients with primary IO. It remains to be determined to what extent such alterations can account for increased CVD risk in this disorder.

CLINICAL PERSPECTIVES

- The associations between HFE gene mutations, iron overload (IO) and lipoprotein metabolism have been poorly studied. Insulin resistance and IO might promote the appearance of atherogenic lipoprotein alterations in patients with primary IO.
- We found that insulin resistance was common in IO patients and that HFE C282Y homozygous patients presented a significant reduction in β-cell function. In addition, C282Y patients presented a less atherogenic LDL subfraction distribution, whereas non-C282Y homozygotes displayed an increased proportion of circulating small, dense LDL. Finally, HDL particles from IO patients presented quantitative and qualitative alterations.
- Importantly, reduced β-cell function, TG-enrichment of LDL and HDL and an increased proportion of small, dense LDL were observed in IO patients as a function of HFE genotype. Thus, evaluation of glucose and lipoprotein metabolism should be included in the management of patients with primary IO. It remains to be determined whether iron depletion therapy can curb such atherogenic alterations and to which extent they contribute to increased cardiovascular risk.

AUTHOR CONTRIBUTION

Tomás Meroño, Carolane Dauteuille, Marie Lhomme, Martín Menafra, María Soledad Saez and Patricia Sorroche performed biochemical determinations and lipoprotein analyses. Tomás Meroño, Fernando Brites, Jorge Rey, Juan Andrés Sordá, Anatol Kontush and Jorge Daruich contributed to the protocol design and data analysis. Alejandra Arteaga, Esteban González Ballerga and Jorge Daruich recruited patients and performed their clinical evaluation. Marcelo Castro and Jorge Rey performed genetic testing. Tomás Meroño, Fernando Brites, Jorge Rey, Anatol Kontush and Jorge Daruich contributed to writing the manuscript.

FUNDING

The work was supported by a collaborative grant between CONICET (Buenos Aires, Argentina) and the National Institute for Health and Medical Research (INSERM, Paris, France) International Cooperation Program 2011-2012. We gratefully acknowledge support from the CODDIM Ile-de-France, ANR (CARINA project), University of Pierre and Marie Curie (UPMC), Fondation pour la Recherche Médicale (Paris, France), ICAN, University of Buenos Aires (UBACyT CB23), "Alberto J. Roemmers Foundation" and CONICET (PIP 516). A.K. acknowledges the award of a Contrat d'Interface from Assistance Publique-Hôpitaux de Paris/INSERM (France).

REFERENCES

1 Pietrangelo, A. (2010) Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. Gastroenterology **139**, 393–408 CrossRef PubMed

2 Piperno, A. (2013) Molecular diagnosis of hemochromatosis. Expert Opin. Med. Diagn. **7**, 161–177 CrossRef PubMed

3 Hanson, E.H., Imperatore, G. and Burke, W. (2001) HFE gene and hereditary hemochromatosis: a HuGE review. Human genome epidemiology. Am. J. Epidemiol. **154**, 193–206 CrossRef PubMed

4 Adams, P., Barton, J.C., McLaren, G.D., Acton, R.T., Speechley, M., McLaren, C.E., Reboussin, D.M., Leiendecker-Foster, C., Harris, E.L., Snively, B.M. et al. (2009) Screening for iron overload: lessons from the hemochromatosis and iron overload screening (HEIRS) study. Can. J. Gastroenterol. **23**, 769–772 PubMed

5 Rossetti, M.V., Mendez, M., Afonso, S., Gerez, E., Batlle, A., Munoz, A. and Parera, V. (2009) HFE gene mutations in patients with altered iron metabolism in Argentina. Cell. Mol. Biol. **55**, 31–35 PubMed

6 Fracanzani, A.L., Piperno, A., Valenti, L., Fraquelli, M., Coletti, S., Maraschi, A., Consonni, D., Coviello, E., Conte, D. and Fargion, S. (2010) Hemochromatosis in Italy in the last 30 years: role of genetic and acquired factors. Hepatology **51**, 501–510 CrossRef PubMed

7 Lapenna, D., Pierdomenico, S.D., Ciofani, G., Ucchino, S., Neri, M., Giamberardino, M.A. and Cuccurullo, F. (2007) Association of body iron stores with low molecular weight iron and oxidant damage of human atherosclerotic plaques. Free Radic. Biol. Med. **42**, 492–498 CrossRef PubMed

8 Stadler, N., Lindner, R.A. and Davies, M.J. (2004) Direct detection and quantification of transition metal ions in human atherosclerotic plaques: evidence for the presence of elevated levels of iron and copper. Arterioscler. Thromb. Vasc. Biol. **24**, 949–954 CrossRef PubMed

9 Gaenzer, H., Marschang, P., Sturm, W., Neumayr, G., Vogel, W., Patsch, J. and Weiss, G. (2002) Association between increased iron stores and impaired endothelial function in patients with hereditary hemochromatosis. J. Am. Coll. Cardiol. **40**, 2189–2194 CrossRef PubMed

10 van der, A.D., Rovers, M.M., Grobbee, D.E., Marx, J.J., Waalen, J., Ellervik, C., Nordestgaard, B.G., Olynyk, J.K., Mills, P.R., Shepherd, J. et al. (2008) Mutations in the HFE gene and cardiovascular disease risk: an individual patient data meta-analysis of 53 880 subjects. Circ. Cardiovasc. Genet. **1**, 43–50 CrossRef PubMed

11 Lian, J., Xu, L., Huang, Y., Le, Y., Jiang, D., Yang, X., Xu, W., Huang, X., Dong, C., Ye, M. et al. (2013) Meta-analyses of HFE variants in coronary heart disease. Gene **527**, 167–173 CrossRef PubMed

12 Libby, P. (2012) Inflammation in atherosclerosis. Arterioscler. Thromb. Vasc. Biol. **32**, 2045–2051 CrossRef PubMed

13 Chapman, M.J., Ginsberg, H.N., Amarenco, P., Andreotti, F., Boren, J., Catapano, A.L., Descamps, O.S., Fisher, E., Kovanen, P.T., Kuivenhoven, J.A. et al. (2011) Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. Eur. Heart J. **32**, 1345–1361 CrossRef PubMed

14 Merono, T., Rosso, L.G., Sorroche, P., Boero, L., Arbelbide, J. and Brites, F. (2011) High risk of cardiovascular disease in iron overload patients. Eur. J. Clin. Invest. **41**, 479–486 CrossRef PubMed

15 Casanova-Esteban, P., Guiral, N., Andres, E., Gonzalvo, C., Mateo-Gallego, R., Giraldo, P., Paramo, J.A. and Civeira, F. (2011) Effect of phlebotomy on lipid metabolism in subjects with hereditary hemochromatosis. Metabolism **60**, 830–834 CrossRef PubMed

16 McClain, D.A., Abraham, D., Rogers, J., Brady, R., Gault, P., Ajioka, R. and Kushner, J.P. (2006) High prevalence of abnormal glucose homeostasis secondary to decreased insulin secretion in individuals with hereditary haemochromatosis. Diabetologia **49**, 1661–1669 CrossRef PubMed

17 Hatunic, M., Finucane, F.M., Brennan, A.M., Norris, S., Pacini, G. and Nolan, J.J. (2010) Effect of iron overload on glucose metabolism in patients with hereditary hemochromatosis. Metabolism **59**, 380–384 CrossRef PubMed

18 Cooksey, R.C., Jouihan, H.A., Ajioka, R.S., Hazel, M.W., Jones, D.L., Kushner, J.P. and McClain, D.A. (2004) Oxidative stress, beta-cell apoptosis, and decreased insulin secretory capacity in mouse models of hemochromatosis. Endocrinology **145**, 5305–5312 CrossRef PubMed

19 de Souza, J.A., Vindis, C., Hansel, B., Negre-Salvayre, A., Therond, P., Serrano, Jr, C.V., Chantepie, S., Salvayre, R., Bruckert, E. et al. (2008) Metabolic syndrome features small, apolipoprotein A-I-poor, triglyceride-rich HDL3 particles with defective anti-apoptotic activity. Atherosclerosis **197**, 84–94 CrossRef PubMed

20 Gerber, P.A., Thalhammer, C., Schmied, C., Spring, S., Amann-Vesti, B., Spinas, G.A. and Berneis, K. (2013) Small, dense LDL particles predict changes in intima media thickness and insulin resistance in men with type 2 diabetes and prediabetes—a prospective cohort study. PLoS One **8**, e72763 CrossRef PubMed

21 Gomez Rosso, L., Benitez, M.B., Fornari, M.C., Berardi, V., Lynch, S., Schreier, L., Wikinski, R., Cuniberti, L. and Brites, F. (2008) Alterations in cell adhesion molecules and other biomarkers of cardiovascular disease in patients with metabolic syndrome. Atherosclerosis **199**, 415–423 CrossRef PubMed

22 Nobecourt, E., Jacqueminet, S., Hansel, B., Chantepie, S., Grimaldi, A., Chapman, M.J. and Kontush, A. (2005) Defective antioxidative activity of small dense HDL3 particles in type 2 diabetes: relationship to elevated oxidative stress and hyperglycaemia. Diabetologia **48**, 529–538 CrossRef PubMed

23 World Health Organization (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ. Tech. Rep. Ser. **894**, i–xii, 1–253 PubMed

24 Chobanian, A.V., Bakris, G.L., Black, H.R., Cushman, W.C., Green, L.A., Izzo, Jr, J.L., Jones, D.W., Materson, B.J., Oparil, S. et al. (2003) The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. JAMA **289**, 2560–2572 CrossRef PubMed

25 American Diabetes Association (2014) Executive summary: standards of medical care in diabetes—2014. Diabetes Care **37**, S5–S13 CrossRef PubMed

26 Mercier, B., Gaucher, C., Feugeas, O. and Mazurier, C. (1990) Direct PCR from whole blood, without DNA extraction. Nucleic Acids Res. **18**, 5908 CrossRef PubMed

27 Matsuda, M. and DeFronzo, R.A. (1999) Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care **22**, 1462–1470 CrossRef PubMed

28 Muniyappa, R., Lee, S., Chen, H. and Quon, M.J. (2008) Current approaches for assessing insulin sensitivity and resistance *in vivo*: advantages, limitations, and appropriate usage. Am. J. Physiol. Endocrinol. Metab. **294**, E15–E26 CrossRef PubMed

29 Chapman, M.J., Goldstein, S., Lagrange, D. and Laplaud, P.M. (1981) A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. J. Lipid Res. **22**, 339–358 PubMed

30 Guerin, M., Bruckert, E., Dolphin, P.J., Turpin, G. and Chapman, M.J. (1996) Fenofibrate reduces plasma cholesteryl ester transfer from HDL to VLDL and normalizes the atherogenic, dense LDL profile in combined hyperlipidemia. Arterioscler. Thromb. Vasc. Biol. **16**, 763–772 CrossRef PubMed

31 Hansel, B., Giral, P., Nobecourt, E., Chantepie, S., Bruckert, E., Chapman, M.J. and Kontush, A. (2004) Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity. J. Clin. Endocrinol. Metab. **89**, 4963–4971 CrossRef PubMed

32 Mackness, M.I. (1998) Human serum paraoxonase is inhibited in EDTA plasma. Biochem. Biophys. Res. Commun. **242**, 249 CrossRef PubMed

33 Kontush, A., Chantepie, S. and Chapman, M.J. (2003) Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress. Arterioscler. Thromb. Vasc. Biol. **23**, 1881–1888 CrossRef PubMed

34 Larrede, S., Quinn, C.M., Jessup, W., Frisdal, E., Olivier, M., Hsieh, V., Kim, M.J., Van Eck, M., Couvert, P., Carrie, A. et al. (2009) Stimulation of cholesterol efflux by LXR agonists in cholesterol-loaded human macrophages is ABCA1-dependent but ABCG1-independent. Arterioscler. Thromb. Vasc. Biol. **29**, 1930–1936 CrossRef PubMed

35 Jug, B., Papazian, J., Lee, R. and Budoff, M.J. (2013) Association of lipoprotein subfractions and coronary artery calcium in patient at intermediate cardiovascular risk. Am. J. Cardiol. **111**, 213–218 CrossRef PubMed

36 Hoogeveen, R.C., Gaubatz, J.W., Sun, W., Dodge, R.C., Crosby, J.R., Jiang, J., Couper, D., Virani, S.S., Kathiresan, S., Boerwinkle, E. and Ballantyne, C.M. (2014) Small dense low-density lipoprotein-cholesterol concentrations predict risk for coronary heart disease: the Atherosclerosis Risk In Communities (ARIC) study. Arterioscler. Thromb. Vasc. Biol. **34**, 1069–1077 CrossRef PubMed

37 Williams, P.T. and Feldman, D.E. (2011) Prospective study of coronary heart disease vs. HDL2, HDL3, and other lipoproteins in Gofman's Livermore Cohort. Atherosclerosis **214**, 196–202 CrossRef PubMed

38 Subramaniam, V.N., McDonald, C.J., Ostini, L., Lusby, P. E., Wockner, L.F., Ramm, G.A. and Wallace, D.F. (2012) Hepatic iron deposition does not predict extrahepatic iron loading in mouse models of hereditary hemochromatosis. Am. J. Pathol. **181**, 1173–1179 CrossRef PubMed

39 Cheng, R., Barton, J.C., Morrison, E.D., Phatak, P. D., Krawitt, E.L., Gordon, S.C. and Kowdley, K.V. (2009) Differences in hepatic phenotype between hemochromatosis patients with HFE C282Y homozygosity and other HFE genotypes. J. Clin. Gastroenterol. **43**, 569–573 CrossRef PubMed

40 Pankow, J.S., Boerwinkle, E., Adams, P. C., Guallar, E., Leiendecker-Foster, C., Rogowski, J. and Eckfeldt, J.H. (2008) HFE C282Y homozygotes have reduced low-density lipoprotein cholesterol: the Atherosclerosis Risk in Communities (ARIC) study. Transl. Res. **152**, 3–10 CrossRef PubMed

41 Ellervik, C., Tybjaerg-Hansen, A., Grande, P., Appleyard, M. and Nordestgaard, B.G. (2005) Hereditary hemochromatosis and risk of ischemic heart disease: a prospective study and a case–control study. Circulation **112**, 185–193 CrossRef PubMed

42 Ellervik, C., Tybjaerg-Hansen, A., Appleyard, M., Sillesen, H., Boysen, G. and Nordestgaard, B.G. (2007) Hereditary hemochromatosis genotypes and risk of ischemic stroke. Neurology **68**, 1025–1031 CrossRef PubMed

43 Lucero, D., Zago, V., Lopez, G.I., Graffigna, M., Lopez, G.H., Fainboim, H., Miksztowicz, V., Gomez Rosso, L., Belli, S., Levalle, O. et al. (2011) Does non-alcoholic fatty liver impair alterations of plasma lipoproteins and associated factors in metabolic syndrome? Clin. Chim. Acta **412**, 587–592 CrossRef PubMed

44 Carr, M.C., Ayyobi, A.F., Murdoch, S.J., Deeb, S.S. and Brunzell, J.D. (2002) Contribution of hepatic lipase, lipoprotein lipase, and cholesteryl ester transfer protein to LDL and HDL heterogeneity in healthy women. Arterioscler. Thromb. Vasc. Biol. **22**, 667–673 CrossRef PubMed

45 Schreier, L.E., Sanguinetti, S., Mosso, H., Lopez, G.I., Siri, L. and Wikinski, R.L. (1996) Low-density lipoprotein composition and oxidability in atherosclerotic cardiovascular disease. Clin. Biochem. **29**, 479–487 CrossRef PubMed

46 Tornvall, P., Bavenholm, P., Landou, C., de Faire, U. and Hamsten, A. (1993) Relation of plasma levels and composition of apolipoprotein B-containing lipoproteins to angiographically defined coronary artery disease in young patients with myocardial infarction. Circulation **88**, 2180–2189 CrossRef PubMed

47 Di Angelantonio, E., Sarwar, N., Perry, P., Kaptoge, S., Ray, K.K., Thompson, A., Wood, A.M., Lewington, S., Sattar, N., Packard, C.J. et al. (2009) Major lipids, apolipoproteins, and risk of vascular disease. JAMA **302**, 1993–2000 CrossRef PubMed

48 Kontush, A., Therond, P., Zerrad, A., Couturier, M., Negre-Salvayre, A., de Souza, J.A., Chantepie, S. and Chapman, M.J. (2007) Preferential sphingosine-1-phosphate enrichment and sphingomyelin depletion are key features of small dense HDL3 particles: relevance to antiapoptotic and antioxidative activities. Arterioscler. Thromb. Vasc. Biol. **27**, 1843–1849 CrossRef PubMed

49 DeFronzo, R.A. and Abdul-Ghani, M. (2011) Type 2 diabetes can be prevented with early pharmacological intervention. Diabetes Care **34**, S202–S209 CrossRef PubMed

50 Equitani, F., Fernandez-Real, J.M., Menichella, G., Koch, M., Calvani, M., Nobili, V., Mingrone, G. and Manco, M. (2008) Bloodletting ameliorates insulin sensitivity and secretion in parallel to reducing liver iron in carriers of HFE gene mutations. Diabetes Care **31**, 3–8 CrossRef PubMed

51 Zacharski, L.R., DePalma, R.G., Shamayeva, G. and Chow, B.K. (2013) The statin-iron nexus: anti-inflammatory intervention for arterial disease prevention. Am. J. Public Health **103**, e105–e112 CrossRef PubMed

Received 12 May 2014/26 November 2014; accepted 2 December 2014

Published as Immediate Publication 2 December 2014, doi: 10.1042/CS20140300

