

## Paraoxonase-1 status in patients with hereditary hemochromatosis

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**Abstract** Hereditary hemochromatosis (HH) is characterized by accumulation of iron, oxidative stress, inflammation, and fibrogenesis in liver tissue. In this setting, research on the protection afforded by intracellular antioxidants is of clinical relevance. Paraoxonase-1 (PON1) is an enzyme that degrades lipid peroxides. This study investigates the alterations in serum PON1 status, *PON1* gene polymorphisms, and PON1 hepatic expression in patients with HH. We performed a case-control study in 77 patients with HH (80.5% men, 22–70 years of age) and 408 healthy individuals (43.1% men, 26–74 years of age). Serum PON1 activities against different substrates and *PON1*<sub>192</sub> and *PON1*<sub>55</sub> polymorphisms were analyzed. PON1 protein expression was investigated in 20 liver biopsies. HH patients had significantly lower serum PON1 activity, which was inversely correlated with ferritin (marker of iron stores) and serum 8-isoprostane concentrations (index of oxidative stress). PON1 protein expression in liver tissue was higher in patients and showed stronger staining in hepatocytes surrounding the areas of inflammation. **Our study provides preliminary evidence that PON1 may play a role in protecting against iron-induced oxidative stress in hereditary hemochromatosis.**—Martinelli, N., A. García-Heredia, H. Roca, N. Aranda, V. Arija, B. Mackness, M. Mackness, F. Busti, G. Aragonès, J. Pedro-Botet, F. Pedica, I. Cataldo, J. Marsillach, J. Joven, D. Girelli, and J. Camps. **Paraoxonase-1 status in patients with hereditary hemochromatosis.** *J. Lipid Res.* 2013. 54: 1484–1492.

**Supplementary key words** antioxidants • inflammation • iron metabolism • oxidative stress

Hereditary hemochromatosis (HH), one of the most common genetic disorders, is characterized by accumulation of iron in several tissues, but mainly in the liver (1). Intracellular iron is stored as ferritin to prevent the generation

of reactive oxygen species. The amount of ferritin within the cell is directly related to its iron content, and in hepatocytes of patients with HH, this can reach extreme levels (2). However, the protective capacity of ferritin is easily overwhelmed in HH, hepatic oxidative stress and fibrogenesis being characteristic of this disorder (3, 4). In this setting, research on liver protection by intracellular antioxidants is of clinical relevance. HH is frequently associated with mutations in the *HFE* gene, which cause insufficient production of hepcidin by hepatocytes. Hepcidin inhibits iron transport by binding to the iron channel ferroportin, which is located on the basolateral surface of gut enterocytes and on the plasma membrane of reticuloendothelial cells. Inhibiting ferroportin shuts off iron transport out of storage in these cells. By inhibiting ferroportin, hepcidin prevents enterocytes of the intestines from secreting iron into the hepatic portal system, thereby functionally reducing iron absorption (2, 5). Decreased hepatic hepcidin production then results in ineffective regulation of duodenal iron absorption, excessive delivery of iron to plasma, and iron accumulation in tissues.

Paraoxonase-1 (PON1), an enzyme with lactonase and esterase activities, degrades lipid peroxides (6–8) and plays an important role in the intracellular antioxidant system (9). In mammals, PON1 gene and protein expressions are observed in many cell types (10, 11), and the enzyme is found in the circulation bound to high-density lipoproteins (HDL) (12). Importantly, the liver plays a predominant role in the synthesis of PON1. We previously

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DEPCyMC, 7-*O*-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin; DTNB, dithio-bis-2-nitrobenzoic acid; HH, hereditary hemochromatosis; 4-HNE, 4-hydroxy-2-nonenal; MCP-1, monocyte chemoattractant protein-1; PON1, paraoxonase-1; TBBL, 5-thiobutyl butyrolactone.

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demonstrated that serum PON1 activity is decreased in patients with chronic hepatocellular liver diseases, despite serum PON1 concentration and hepatic PON1 protein expression being increased (13–15). More recent evidence indicates that PON1 overexpression provides protection against liver damage (16). Conversely, low PON1 levels are associated with an enhanced sensitivity to liver disease (17). PON1 levels are genetically determined; the polymorphisms Arg/Gln at position 192 (*PON1*<sub>192</sub>, with two alleles termed Q and R) and Leu/Met at position 55 (*PON1*<sub>55</sub>, with two alleles termed L and M), are strongly associated with the enzyme's activity (9). *PON1*<sub>55</sub> polymorphism indirectly influences serum PON1 activity and concentration; i.e., it is in linkage disequilibrium with the *PON1*<sub>108</sub> promoter polymorphism, the mutation really responsible for the observed changes (18).

Recently, novel assays to measure PON1 activity have been developed using 5-thiobutyl butyrolactone (TBBL) and 7-*O*-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin (DEPCyMC) as substrates (19). Both TBBLase and DEPCyMCase activities are highly specific to PON1. TBBL is a chromogenic lactone that resembles the most favorable PON1 lactone substrates, allowing a specific evaluation of PON1 lactonase activity.

To date, there have been no reports on PON1 activity in HH. Hence, the aim of this study was to investigate the possible alterations in serum PON1 activity, the influence of the main *PON1* gene polymorphisms, and PON1 hepatic expression in HH to ascertain whether this enzyme may be involved in the pathophysiology of this disease.

## MATERIALS AND METHODS

### Study participants

Patients with clinically diagnosed HH ( $n = 77$ , 80.5% men, 22–70 years of age) were recruited from the outpatient clinics of the Department of Medicine of the Policlinico G.B. Rossi, Verona, Italy. On entry into the study, relevant data were collected from clinical records, patient interviews, and physical examinations. The large majority of HH patients have HFE-related hemochromatosis (C282Y homozygotes,  $n = 30$ ; C282Y-H63D compound heterozygotes,  $n = 30$ ). Other patients have no HFE-related genetic variants but have other causal mutations (e.g., transferrin receptor 2, ferroportin, and hemojuvelin). Twenty-one patients had a history of previous phlebotomy therapy. PON1 hepatic protein expression was examined in nonelective liver biopsies of 20 patients who had had this specimen solicited by their physician for clinical diagnostic purposes. Of note is that all 20 liver biopsies confirmed the diagnosis of HH, with strong intensity of positivity by Perls staining.

The control group consisted of 408 healthy volunteers (43.1% men, 26–74 years of age) with no clinical or analytical evidence of renal insufficiency, liver disease, neoplasia, or neurological disorders. Healthy volunteers were 95.7% wild-type homozygotes and 4.3% heterozygotes for the C282Y polymorphism of the *HFE* gene, and 63.4% were wild-type homozygotes, 32.3% heterozygotes, and 4.3% mutant homozygotes for the H63D polymorphism (20, 21).

A fasting blood sample was obtained from all the participants, and serum and leukocytes were stored separately at  $-80^{\circ}\text{C}$ . Frozen aliquots were sent from Verona (Italy) to Reus (Spain) by

overnight courier in a dry-ice package. All the participants provided fully informed consent to participation in the study on the understanding that anonymity of all data was guaranteed. The study was approved by the ethics committees (institutional review boards) of the participating hospitals.

### Biochemical analyses

Serum PON1 paraoxonase and arylesterase activities were determined as the rate of hydrolysis of paraoxon (22) and phenyl acetate (23), respectively. Serum PON1 TBBLase and DEPCyMCase activities were analyzed as previously reported (19, 24–26). Serum markers of iron metabolism, as well as other common biochemical parameters, were measured by standard methods in a RXL Dimension automated analyzer (Dade International Inc., Newark, DE). As an index of oxidative stress, serum 8-isoprostane concentrations were measured by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI).

### PON1 genotyping

Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set, Gentra Systems Inc., Minneapolis, MN). Polymorphisms *PON1*<sub>192</sub> and *PON1*<sub>55</sub> were analyzed by the Iplex Gold MassArray method (Sequenom Inc., San Diego, CA).

### PON1 immunohistochemistry in liver tissue

Hepatic PON1 expression was assessed in sections (4  $\mu\text{m}$  thick) of paraffin-embedded tissue by immunohistochemistry using the anti-PON1 antibody, as previously described (11). As an index of lipid peroxidation, 4-hydroxy-2-nonenal (4-HNE) protein adducts were analyzed with a specific antibody purchased from the Japan Institute for the Control of Ageing (Shizuoka, Japan). Monocyte chemoattractant protein-1 (MCP-1) expression as a marker of inflammation (27) and  $\beta$ -actin as a hepatic housekeeping protein (28) were measured by immunohistochemistry using specific antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Five biopsies of commercial normal human liver tissue (ProteoGenex Inc., Culver City, CA) were analyzed as controls.

### Statistical analyses

All the calculations were performed with SPSS 18.0 statistical package (SPSS Inc., Chicago, IL). Normality of distributions was determined with the Kolmogorov-Smirnov test. Differences between two groups were assessed with the Student *t*-test (parametric) or the Mann-Whitney *U* test (nonparametric). Spearman correlation coefficients were used to evaluate the degree of association between variables. Qualitative data were analyzed with the  $\chi^2$  test. A multiple logistic regression model was fitted to evaluate the variables that were independently associated with HH. Results are shown as medians and 95%CI. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### Relationship between serum PON1-related variables and other biochemical indices

Demographic and biochemical variables are summarized in **Table 1**. HH patients were considered as a whole or segregated into two subgroups with respect to a history of previous phlebotomy therapy. As expected, patients with HH had significantly higher serum iron and ferritin concentrations, higher transferrin saturation, and lower serum transferrin concentrations than the control group.

TABLE 1. Selected demographic and biochemical variables in patients with HH and in the control group

Variable	Controls (n = 408)	Hemochromatosis (n = 77)	No Phlebotomy (n = 56)	Phlebotomy (n = 21)	<i>P</i> <sup>a</sup>	<i>P</i> <sup>b</sup>	<i>P</i> <sup>c</sup>
Age (years)	46 (26–74)	46 (22–70)	46 (26–72)	49 (14–69)	0.906	0.893	0.999
Male gender (%)	175 (43.1)	62 (80.5)	44 (78.6)	18 (85.7)	< 0.001	< 0.001	< 0.001
AST (μkat/l)	0.35 (0.25–0.57)	0.44 (0.27–1.17)	0.44 (0.27–0.97)	0.41 (0.29–2.74)	< 0.001	< 0.001	0.002
ALT (μkat/l)	0.32 (0.16–0.80)	0.56 (0.24–1.69)	0.54 (0.24–1.63)	0.65 (0.17–1.90)	< 0.001	< 0.001	< 0.001
Cholesterol (mmol/l)	5.20 (3.70–7.00)	5.17 (3.44–7.21)	5.30 (3.50–7.20)	5.02 (3.23–7.42)	0.316	0.238	0.664
HDL-cholesterol (mmol/l)	1.44 (0.92–2.19)	1.02 (0.53–1.69)	1.03 (0.48–2.02)	0.97 (0.57–1.45)	< 0.001	< 0.001	< 0.001
Apolipoprotein A-I (g/l)	1.67 (1.26–2.19)	1.58 (1.09–2.49)	1.58 (1.04–2.59)	1.57 (1.18–2.41)	0.009	0.110	0.327
Triglycerides (mmol/l)	1.10 (0.50–2.60)	1.24 (0.65–3.59)	1.21 (0.67–3.34)	1.37 (0.65–3.59)	0.013	0.089	0.046
Iron (μmol/l)	16.4 (6.2–31.0)	27.9 (13.5–54.4)	27.7 (13.1–50.1)	27.9 (12.4–54.9)	< 0.001	< 0.001	< 0.001
Transferrin (g/l)	2.49 (1.96–2.76)	1.98 (1.40–2.65)	1.95 (1.29–2.65)	1.99 (1.54–3.58)	< 0.001	< 0.001	< 0.001
Transferrin saturation (%)	34 (11–67)	53 (21–99)	55 (17–98)	48 (23–100)	< 0.001	< 0.001	< 0.001
Ferritin (μg/l)	84 (12–380)	609 (47–2611)	771 (219–2088)	199 (29–4678)	< 0.001	< 0.001	< 0.001
8-isoprostanes (μg/l)	8.6 (2.4–21.8)	53.2 (19.0–495.7)	43.3 (20.7–551.8)	85.8 (17.1–465.0)	< 0.001	< 0.001	< 0.001
Paraoxonase (U/ml)	279 (161–580)	213 (122–443)	222 (114–416)	205 (125–504)	< 0.001	< 0.001	0.012
Arylesterase (U/ml)	99.8 (64.5–141.3)	83.1 (40.4–126.6)	76.6 (37.1–123.0)	91.7 (68.2–132.0)	< 0.001	< 0.001	0.174
TBBLase (U/l)	5.9 (3.5–8.9)	5.7 (2.9–7.9)	5.7 (2.9–8.4)	5.6 (1.6–7.9)	0.182	0.306	0.332
DEPCyMCase (U/l)	24.5 (20.8–29.7)	19.9 (17.3–24.2)	19.9 (13.4–27.8)	22.1 (13.7–29.7)	< 0.001	< 0.001	< 0.001

Patients with HH were subgrouped with respect to history of previous phlebotomy therapy at time of enrolment

<sup>a</sup>*P* values comparing all hemochromatosis patients.

<sup>b</sup>*P* values comparing patients not treated with phlebotomy.

<sup>c</sup>*P* values comparing patients treated with phlebotomy versus the control group.

They also had slight, but significant, increases in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and a marked increase in serum 8-isoprostane concentrations. There were weak, but significant, inverse correlations between this parameter and arylesterase activity ( $r = -0.19$ ;  $P = 0.001$ ), DEPCyMCase activity ( $r = -0.17$ ;  $P = 0.005$ ), and paraoxonase activity ( $r = -0.012$ ;  $P = 0.025$ ) when all the participants were considered together ( $n = 485$ ). HDL-cholesterol concentrations were significantly lower in HH patients, as was apolipoprotein

A-I, albeit this latter parameter decreased only slightly. Serum PON1 paraoxonase, arylesterase, and DEPCyMCase activities were significantly decreased. HH patients with a previous history of phlebotomy were found to have lower ferritin concentrations than those without such a history. Moreover, in HH patients, there were highly significant associations between the four enzyme activities of PON1 (Fig. 1).

We observed, in all of the hemochromatosis patients, relatively weak associations between arylesterase activity and

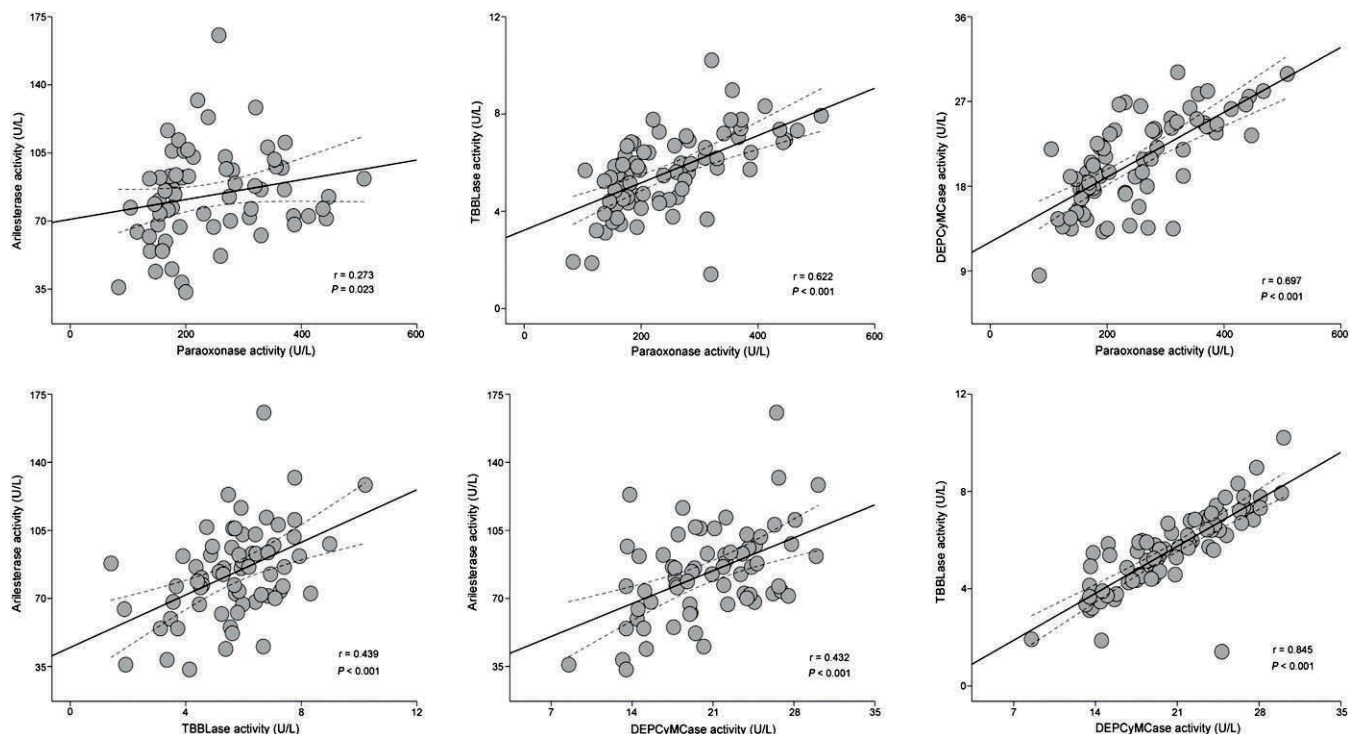


Fig. 1. Relationships between serum PON1 activities versus different substrates in hemochromatosis patients.

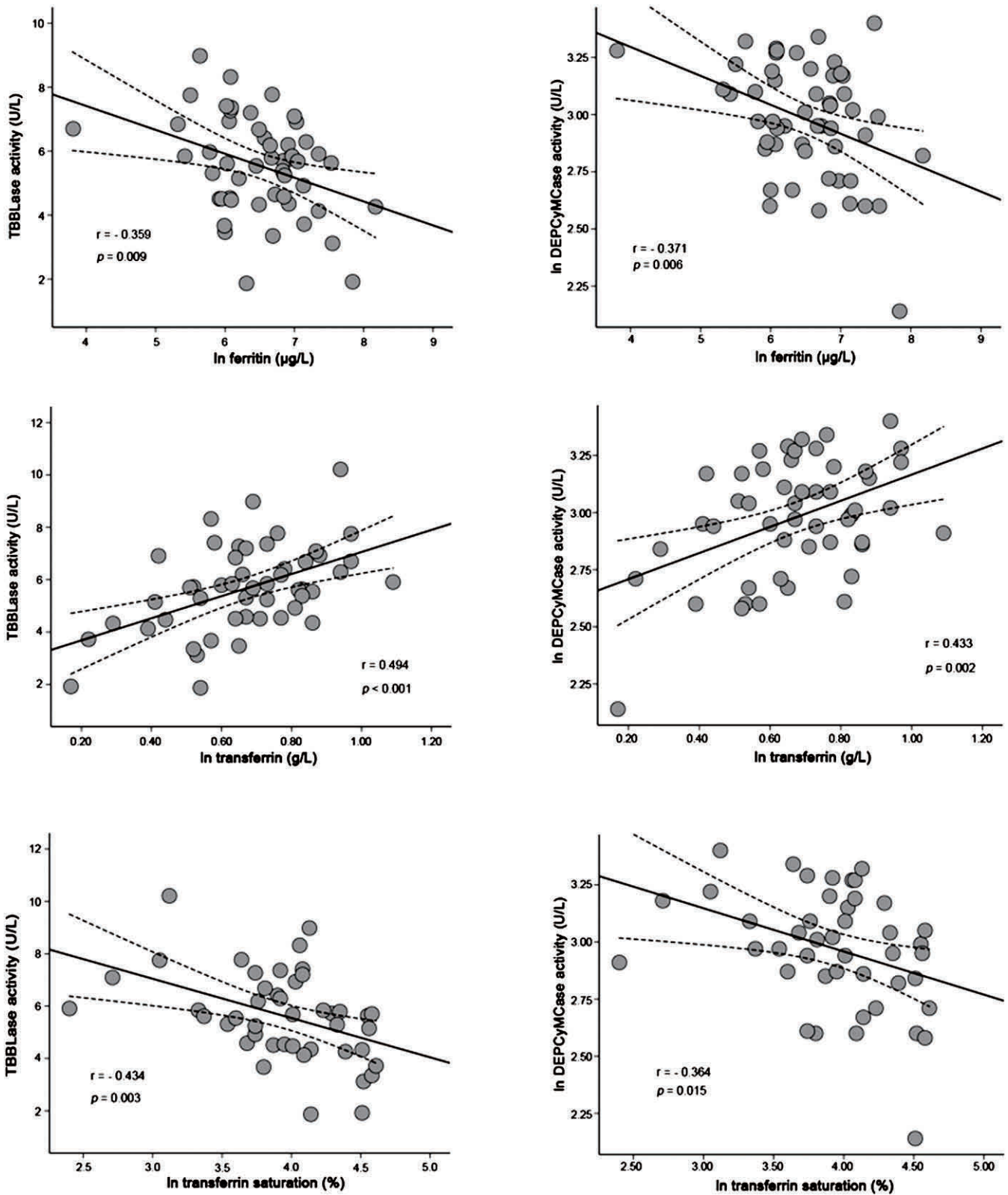


Fig. 2. Relationships between serum PON1 activities versus different substrates and iron-status markers in hemochromatosis patients without a history of previous phlebotomy.

transferrin ( $r = 0.357$ ;  $P = 0.004$ ), and between DEPCyMCCase activity and transferrin saturation ( $r = -0.263$ ;  $P = 0.042$ ). However, after exclusion of the 21 patients, who at the time of enrollment were receiving phlebotomy treatment,

stronger associations were found between serum iron status markers and PON1 TBBLase and DEPCyMCCase activities (Fig. 2). Serum paraoxonase activity was negatively associated with ferritin ( $r = -0.322$ ;  $P = 0.019$ ). On including all

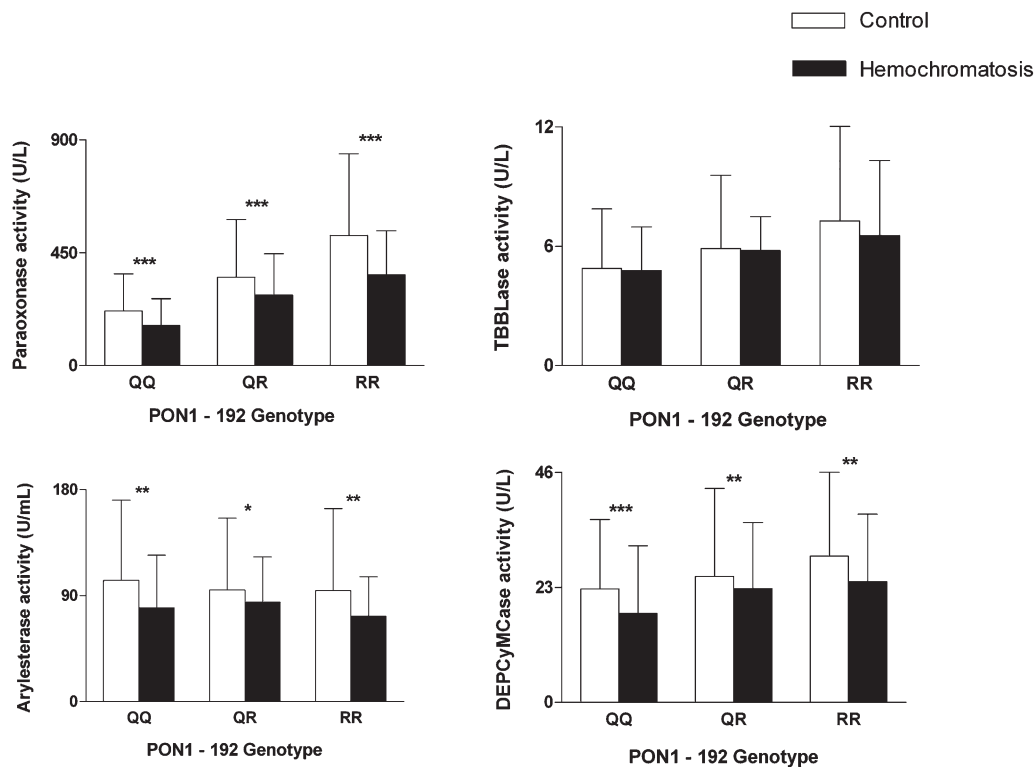


Fig. 3. Serum PON1 activities segregated according to *PON1*<sub>192</sub> polymorphism. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001, with respect to the control group.

the iron status markers in linear regression models with the different PON1 activities as dependent variable, only ferritin remained a significant predictor of PON1 activities (data not shown).

Interestingly, and consistent with the above-mentioned results, among patients without a history of phlebotomy therapy, the C282Y homozygous carriers (*n* = 17) compared with C282Y/H63D compound heterozygotes (*n* = 23) presented not only higher ferritin concentrations [986 (725–1342) versus 536 (432–664) μg/l, *P* = 0.001] but also lower PON1 activities [paraoxonase activity 194 (159–236) versus 257 (220–302) U/l, *P* = 0.022; TBBLase activity 4.8 (1.5–8.1) versus 5.97 (3.3–8.6), *P* = 0.012; DEPCyMCCase activity 17.7 (15.3–20.4) versus 21.2 (19.4–23.1) U/l, *P* = 0.023].

### PON1 genotyping

The allele distributions of the *PON1*<sub>192</sub> and *PON1*<sub>55</sub> polymorphisms followed Hardy-Weinberg equilibrium in cases as well as controls. The frequency of the *PON1*<sub>192</sub> RR genotype was significantly higher (*P* = 0.019) in the patient group [Controls (%): QQ, 49.9; QR, 41.4; RR, 8.8. Patients: QQ, 41.4; QR, 37.9; RR, 20.7], while no difference was found in the genotype distribution of the *PON1*<sub>55</sub> polymorphism [Controls (%): LL, 34.8; LM, 47.1; MM, 18.0. Patients: LL, 31.9; LM, 44.7; MM, 23.4]. When subjects were segregated with respect to *PON1*<sub>192</sub> genotype, we observed that the degrees of inhibition of serum PON1 activities were similar in HH patients compared with the control group, for all the different genotypes (Fig. 3).

### Multivariate analysis of PON1-related variables in hemochromatosis

We fitted three independent multiple logistic regression models to further investigate the potential link with HH of the three different PON1-related variables that had presented a significant association on univariate analysis. In the first series of analyses, paraoxonase (β coefficient −0.015; *P* < 0.001) and DEPCyMCCase activity (β coefficient −0.168; *P* = 0.003), remained significantly associated with HH in the models adjusted for gender, age, HDL-cholesterol concentration, and iron status markers. Arylesterase activity was not significantly related in this analysis (β coefficient −0.019; *P* = 0.107). However, when 8-isoprostanes were included in the regression models, the results showed that 8-isoprostanes, but not PON1 activities or *PON1*<sub>192</sub> genotype, were independently associated with HH (Table 2).

### Immunochemical analysis of hepatic PON1 protein expression

Representative examples of PON1 protein expression in normal livers and hepatic biopsies from patients with hemochromatosis are shown in Fig. 4. We observed a stronger anti-PON1 staining in hepatocytes of patient liver tissues than in the samples from normal subjects. We observed differences in PON1 expression among the hepatocytes, as shown by stronger staining in cells surrounding the inflammation areas. We did not find any positive staining either in endothelial cells or in inflammatory cells. These changes were accompanied by an enhanced expression of 4-HNE protein adducts and MCP-1, indicating increased

TABLE 2. Multiple logistic regression analysis for HH-associated variables

PON1-related Variable <sup>a</sup>	B	Exp(B)	P
Paraoxonase activity			
Gender <sup>a</sup>	-2.269	0.103	0.219
Age	-0.151	0.860	0.059
<i>PON1</i> <sub>192</sub> polymorphism <sup>a</sup>	4.786	119.797	0.095
Iron (μmol/l)	0.312	1.366	0.033
Transferrin (g/l)	-0.549	0.577	0.851
Ferritin (μg/l)	0.011	1.011	0.031
HDL-cholesterol (mmol/l)	-0.599	0.549	0.723
8-isoprostanes (μg/l)	0.280	1.323	0.015
Paraoxonase (U/ml)	-0.030	0.971	0.148
Arylesterase activity			
Gender <sup>a</sup>	-0.871	0.418	0.556
Age	-0.115	0.892	0.115
<i>PON1</i> <sub>192</sub> polymorphism <sup>a</sup>	0.405	1.499	0.691
Iron (μmol/l)	0.189	1.207	0.011
Transferrin (g/l)	-1.335	0.263	0.583
Ferritin (μg/l)	0.008	1.008	0.010
HDL-cholesterol (mmol/l)	0.363	1.438	0.793
8-isoprostanes (μg/l)	0.198	1.219	0.001
Arylesterase (U/ml)	-0.001	0.999	0.968
DEPCyMCase activity (U/l)			
Gender <sup>a</sup>	-1.632	0.196	0.348
Age	-0.191	0.826	0.069
<i>PON1</i> <sub>192</sub> polymorphism <sup>a</sup>	2.387	10.881	0.139
Iron (μmol/l)	0.237	1.267	0.014
Transferrin (g/l)	-0.094	0.910	0.969
Ferritin (μg/l)	0.009	1.009	0.010
HDL-cholesterol (mmol/l)	1.317	3.734	0.576
8-isoprostanes (μg/l)	0.273	1.313	0.020
DEPCyMCase (U/l)	-0.422	0.656	0.063

<sup>a</sup>References were male gender and *PON1*<sub>192</sub> QQ genotype.

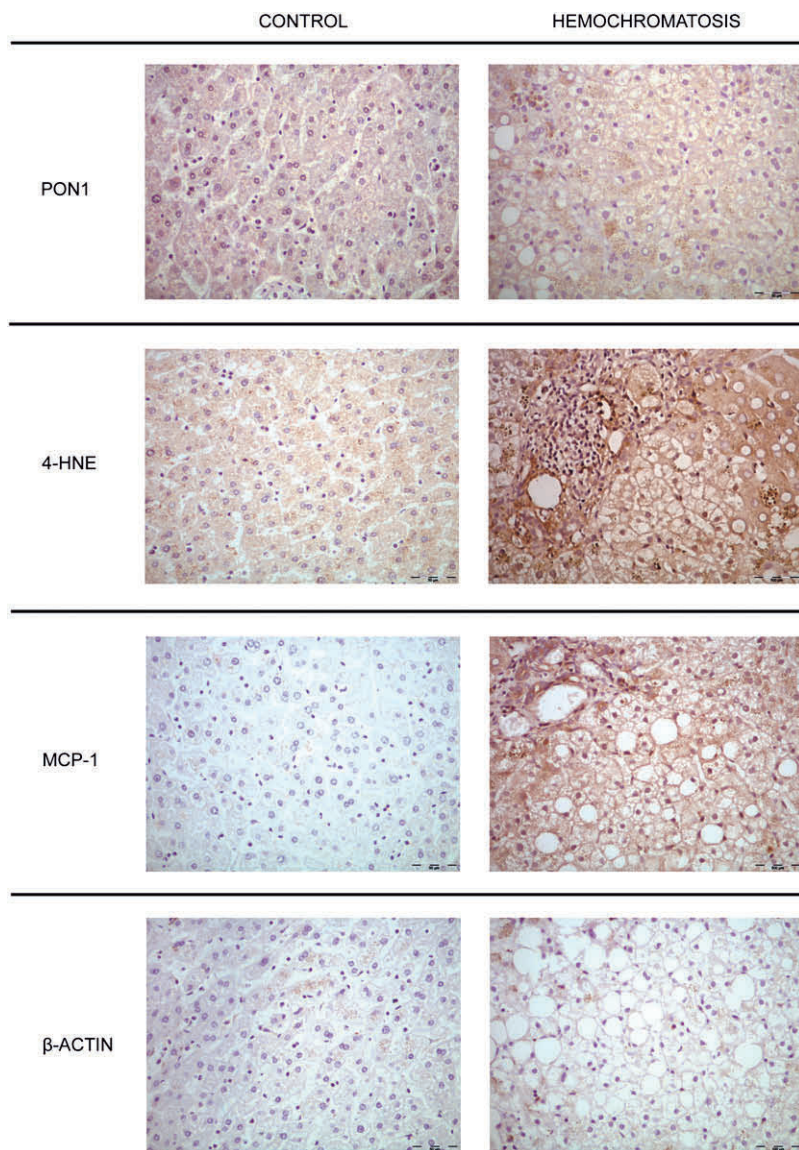
oxidative stress and inflammation. There were no noticeable changes in the expression of the housekeeping protein β-actin.

## DISCUSSION

The present study shows that hepatic PON1 protein expression is clearly increased in HH patients. Oxidative stress is a key feature of HH (3, 4, 29). PON1 is able to hydrolyze a great variety of substrates by means of its esterase and lactonase activities, and considerable evidence suggests that its physiological role is the intra- and extracellular hydrolysis of lipid peroxides, thereby playing an antioxidant role (6–9). Our results show a strong PON1 staining in the cytoplasm of hepatocytes while nuclei were not stained. Expression was stronger in areas surrounding inflammatory cell accumulation. The existence of a close link between oxidation and inflammation in the liver has been suggested previously (30). Immunochemical studies from our group showed that hepatic expressions of PON1 and MCP-1, a chemokine responsible for the recruitment of monocytes to the inflammatory areas, were increased in rats with CCl<sub>4</sub>-induced fibrosis, and the histological distribution was similar for both proteins (31). Moreover, hepatocytes were strongly stained for PON1 and MCP-1 when located in close proximity to inflammatory infiltrates and fibrosis septa. The present report adds more data in support of these conclusions.

In the current study, serum paraoxonase, arylesterase, and DEPCyMCase activities were significantly decreased in HH patients. Changes in HDL structure and composition are known to influence PON1 activity because this enzyme is profoundly dependent on the lipid and protein compositional environment of the HDL particles for its activity (32). Since our HH patients had a reduced HDL-cholesterol concentration but normal apolipoprotein A-I levels, such a compositional change could explain our findings. Indeed, low HDL-cholesterol concentration is a common feature of chronic liver diseases (14, 15) and has been associated with iron overload (33–35). Moreover, serum PON1 activity may be inhibited additionally by increased lipid peroxidation products in blood. Lipid peroxides are well documented as having the ability to inhibit PON1 activity by reacting with the free sulfhydryl group at cysteine 284 (36), and indeed, our patients had a marked increase in serum 8-isoprostane concentrations accompanying decreased PON1 activities. The consistent interrelationship between PON1 and oxidative stress was further emphasized in our study population by the observation that the statistical significant association between low PON1 activities and HH was lost following the inclusion of 8-isoprostanes in the regression model. This suggests an oxidative stress-related modulation of PON1 activities in HH.

Our results also show that the decrease in PON1 activity is related to serum ferritin concentration. High ferritin levels have been shown to be an important threshold that determines cirrhosis and the risk of iron overload-associated death (37). These results are in agreement with previous observations in patients with other forms of liver



**Fig. 4.** Representative examples of the immunohistochemical analyses of PON1, 4-HNE protein adducts, MCP-1, and  $\beta$ -actin expression in a normal liver biopsy and a liver biopsy from a HH patient (magnification  $\times 200$ .)

disease, in which a decreased serum PON1 activity was found to be associated with increased PON1 protein expression (13, 14). Similar results were recently obtained in patients with coronary artery disease (38). Previous studies showed that oxidized phospholipids and inflammatory cytokines decreased *PON1* gene expression in hepatocyte cultures (39–41). However, *in vivo* studies in rats with experimental cirrhosis showed that decreased serum PON1 activity was associated with increased PON1 protein expression despite decreased *PON1* gene expression. This apparently contradictory finding was explained by decreased PON1 proteolysis (31).

A question of considerable clinical relevance arising from the present study is whether these alterations in serum PON1-related variables play a role in the pathophysiology of the disease or whether they are merely an epiphenomenon. Our finding of an increased PON1 protein expression and its localization near the inflammation sites supports the hypothesis that PON1 may participate in the molecular mechanisms leading to the clinical manifestation of HH. Consistent with this view is the recent

evidence suggesting that protection against lipid peroxidation prevents clinical complications associated with increased body iron stores. An intriguing example within this hypothesis derives from recent observations relating to metabolic syndrome. For example, a cross-sectional study in more than 7,000 ostensibly-healthy volunteers showed that individuals with high serum ferritin concentrations had a higher predisposition to metabolic syndrome (42). Importantly, serum PON1 activity is decreased in metabolic syndrome (26, 43), and such impairment has been reported to increase the risk of developing cardiovascular disease (26).

Another finding of the present study is the higher frequency of patients homozygous for the R alloform of the *PON1*<sub>192</sub> polymorphism being associated with HH. This is of interest because *PON1*<sub>192</sub> polymorphism can greatly modify the ability of PON1 to protect lipids against oxidation. *In vitro* studies showed that RR homozygotes were less efficient than QQ and QR individuals in protecting low-density lipoproteins from copper-induced oxidation (44). In addition, several case-control studies have reported

increased frequencies of the RR genotype in diseases involving oxidative stress. These include hepatitis C infection (45), human immunodeficiency virus infection (46), atherosclerosis (47), and cancer (48). However, our results must be interpreted with caution because the numbers of HH patients were too low for a reliable genetic association study. Therefore, our conclusions need to be considered as preliminary and requiring confirmation in wider series of patients.

HH has a highly variable clinical and biochemical penetrance. Indeed, many patients do not develop severe iron overload or clinical symptoms, and hence, there is a growing interest in investigating the biological mechanisms and consequences of HH phenotypic heterogeneity (49, 50). In a population-based study (51), only 28.4% of men and 1.2% of women homozygous for C282Y mutation had clinical evidence of disease. Moreover, the number of subjects developing clinical disease (defined as hepatic cirrhosis) is even smaller than the number with iron overload (49). Such differences in HH clinical presentation may be related to environmental as well as genetic modifiers of iron overload mechanisms. Among the latter group, the hypothesis that genetic variants in antioxidant enzymes may have a phenotypic effect on HH is attractive, and previous studies have proposed a role for glutathione S transferase P1 and myeloperoxidase gene polymorphisms (52). Our preliminary results to date cannot demonstrate detrimental effects low PON1 activity or of PON1 genotype on HH prognosis. The higher prevalence of RR genotype in our HH patient series may reflect a selection bias, as we included patients with evident clinical/biochemical expression, not simply C282Y homozygotes and/or C282Y-H63D compound heterozygotes. Nonetheless, they provide evidence for a biologically plausible link between PON1 and HH, such that we are tempted to speculate that PON1 may be a modifying factor of the HH phenotype. Future studies that prospectively address such issues are awaited.

In conclusion, the current study showed a significant decrease in serum PON1 activity, together with an increased hepatic PON1 protein expression in HH patients. Our data suggest that these alterations may play a role in the protection against iron-induced oxidative stress and its clinical consequences. ■■

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