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Serum Paraoxonase Activity: A New Additional Test for the Improved Evaluation of Chronic Liver Damage

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Background: **Paraoxonase 1 (PON1) is an ester hydrolase present in serum and in the liver. The aims of the present study were to investigate the following: (*a*) the relationship between serum PON1 activity alterations and the degree of liver damage in patients with chronic liver disease; (*b*) the influence of genetic variability on serum PON1 activity; and (*c*) the efficacy of serum PON1 activity measurement, alone and in combination with standard liver function tests, in the assessment of liver damage.**

Methods: **We studied 68 patients with liver cirrhosis, 107 patients with chronic hepatitis, and 368 apparently healthy volunteers. Baseline and salt-stimulated PON1 activities were measured by the hydrolysis of paraoxon. PON1 genotyping at positions 55 and 192 was analyzed by PCR and restriction isotyping.**

Results: **Baseline and stimulated PON1 activities were decreased (P <0.001) in chronic hepatitis and in liver cirrhosis. PON1 activity was significantly correlated with serum total proteins, albumin, and bilirubin in patients but not in controls. There were no significant differences with respect to allele and genotype frequencies between patients and controls. The combination of baseline serum PON1 with five standard biochemical tests had a higher classification accuracy (94% of patients; 96% of controls) than the five standard tests alone (75% of patients; 96% of controls). ROC plots demonstrated a high diagnostic accuracy for baseline serum PON1 [area under the curve, 0.89 (95% confidence interval, 0.86–0.93) in chronic hepatitis and 0.96 (95% confidence interval, 0.94–0.99) in cirrhosis]. Baseline PON1 provided the highest ROC area for cirrhosis vs controls.**

Conclusions: **The significant decrease of PON1 activity in chronic liver diseases is related to the degree of hepatic dysfunction and not to allelic or genotypic differences. Addition of serum PON1 activity measurement to the current battery of tests may improve the evaluation of chronic liver diseases.**

Paraoxonase [(PON)[3]; aryldialkylphosphatase (EC 3.1.8.1)] is an ester hydrolase that catalyzes the hydrolysis of some xenobiotics, such as organophosphorates, unsaturated aliphatic esters, aromatic carboxylic esters, and possibly, carbamates *(1)*. The paraoxonase gene family contains at least three members, *PON1*, *PON2*, and *PON3*, which are located on chromosome 7q21.3-22.1 *(2, 3)*. The products of *PON2* and *PON3* gene expression are the subject of ongoing research *(3, 4)*. The enzyme PON1 is known to be tightly bound with HDL in serum, and several studies suggest that it is this association that contributes to the protection conferred by HDL against LDL oxidation *(5–11)*.

The liver plays a key role in the synthesis of serum PON1. To date, the gene expression has been observed only in the liver *(12, 13)*, and in vitro biochemical studies indicate properties shared by hepatic and serum PON1 [optimum pH, affinity for substrate (K_m), heat inactivation, and calcium requirement] *(14)*. These data support the concept of a common identity for both enzymes. Of considerable clinical interest is whether the measurement

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[3] Nonstandard abbreviations: PON, paraoxonase; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; ALP, alkaline phosphatase; AUC, area(s) under the curve(s); CI, confidence interval; and LCAT, lecithin: cholesterol acyl transferase.

of serum PON1 activity would be useful as an index of liver function status. Indeed, preliminary studies observed a significant decrease of serum arylesterase activity in patients with liver cirrhosis *(15–17)*, and furthermore, arylesterase and PON1 activities have been shown to be functions of a single enzyme *(18)*.

Any investigation of the variation in serum PON1 activity secondary to impairment of liver function needs to take into account the influence of genetic variability on this enzyme. Serum PON1 has two genetically determined polymorphic sites, one at position 192, which is a Gln→Arg substitution, and one at position 55, which is a Met→Leu substitution *(4)*. The Gln→Arg polymorphism leads to two alloenzymes that clearly differ in paraoxon-hydrolyzing ability: the Arg alloenzyme has a relatively higher activity and shows a greater degree of stimulation by 1 mol/L NaCl than does the Gln alloenzyme *(18)*. The Met→Leu substitution is associated with changes in the concentrations of the circulating enzyme, the Leu alloenzyme being associated with higher PON1 concentrations *(13, 19)*.

The key objectives of the present study were to investigate the following: (*a*) the relationship between serum PON1 activity and the degree of liver damage in patients with chronic liver disease; (*b*) the influence of genetic variability on serum PON1 activity in these types of patients; and (*c*) the efficacy of serum PON1 activity measurement, alone and in combination with the standard liver function tests, in the assessment of liver damage.

Patients and Methods

STUDY PARTICIPANTS

A prospective cohort of 175 patients with chronic liver disease was consecutively recruited from among those attending the outpatient clinics of the Hospital Universitari de Sant Joan between October 1999 and December 2000. The study included 68 patients with liver cirrhosis (42 men, 26 women; age, 60 ± 10 years) and 107 patients with chronic hepatitis (69 men, 38 women; age, 46 ± 14 years). Chronic hepatitis was diagnosed by liver biopsy. Cirrhosis was diagnosed on the basis of clinical evidence, including echography to evaluate splenomegaly or portal vein dilation and fibrogastroscopy to detect the presence of gastroesophageal varices. Hepatitis was mostly secondary to hepatitis C virus infection (73% of the cases).

The control group consisted of 368 healthy volunteers participating in an epidemiologic study currently being conducted in our region of Catalunya (Spain). A detailed description of this study sample will be published elsewhere [Homocysteine-folate (HOMFOL) study; Murphy MM, Vilella E, Santiago C, Figuera L, Sánchez M, et al., manuscript in preparation]. The controls (182 men, 186 women; age, 42 ± 15 years) were ostensibly healthy with no clinical or analytical evidence of diabetes, neoplasia, renal disease, hepatic damage, or cardiovascular disease. All procedures were in accordance with the ethical standards of the Hospital Universitari de Sant Joan, and the anonymity of data was guaranteed.

BIOCHEMICAL MEASUREMENTS

Serum PON1 activity was determined with an automated adaptation of an assay described previously *(20, 21)*. Operators were not aware of the diagnoses of patients and controls. Briefly, PON1 assays were performed without additional NaCl (baseline activity) and with 1 mol/L NaCl included in the assay buffer (salt-stimulated activity). We measured the rate of hydrolysis of paraoxon by monitoring the increase of absorbance at 410 nm and 37 °C. The assay reagent contained 1 mmol/L paraoxon of >96% purity (Sigma Chemical Co.) and 1 mmol/L $CaCl_2$ in 0.05 mol/L glycine buffer (pH 10.5). The two assays were performed in an ILab 900® automated analyzer (Instrumentation Laboratories) in which sample (8 μL) was incubated with reagent (250 μL) and the absorbance was determined after 17 and 51 s, respectively. The blank absorbance was never >0.700. Although this blank may be relatively high, the background was automatically subtracted by the analyzer in the course of the analyses. Because of its toxicity, paraoxon stock solutions were handled in an extraction cabinet, with the operator wearing protective gloves and mask. Paraoxonase activity was expressed as U/mL. The interassay CV was <8%. The intraassay CV was <6%. The traditional measures of liver dysfunction, such as serum alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), and alkaline phosphatase (ALP) activities, as well as total protein, albumin,

Table 1. Results of the standard liver function tests (values are expressed as the mean ± SD).

Marker	Control group (n = 368)	Chronic hepatitis (n = 107)	Cirrhosis (n = 68)
Total proteins, g/L	73 ± 5	73 ± 6	70 ± 10[a]
Albumin, g/L	42 ± 2	40 ± 5[b]	35 ± 8[b]
ALT, μkat/L	0.36 ± 0.15	1.68 ± 1.71[b]	0.78 ± 0.91[b]
GGT, μkat/L	0.30 ± 0.28	1.23 ± 1.76[b]	1.21 ± 1.40[b]
ALP, μkat/L	2.76 ± 0.80	3.50 ± 2.15[b]	4.21 ± 2.01[b]
Bilirubin, μmol/L	9.5 ± 3.4	16.5 ± 19.3[b]	34.8 ± 37.7[b]

[a] $P < 0.01$ with respect to the control group.
[b] $P < 0.001$ with respect to the control group.

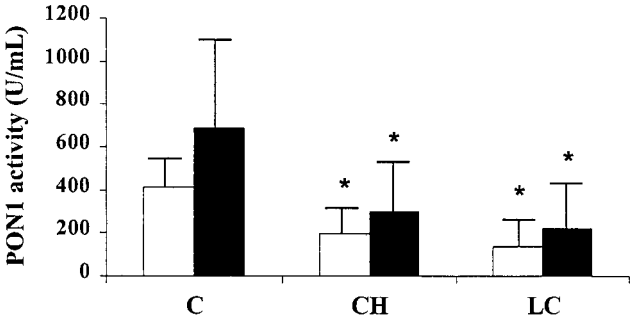

Fig. 1. Baseline (*open columns*) and stimulated (*filled columns*) serum PON1 activity in controls (*C*), patients with chronic hepatitis (*CH*), and patients with liver cirrhosis (*LC*).

*, $P < 0.001$ with respect to the control group.

and total bilirubin concentrations, were analyzed with standard techniques (ITC Diagnostics).

PON GENOTYPING

Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set; Gentra), and the $PON1_{55}$ and $PON1_{192}$ polymorphisms of the *PON1* gene were analyzed by PCR amplification and restriction isotyping. The primers used were 5′-GAAGAGTGATGTATAGCCCCAG-3′ and 5′-TTTAATCCAGAGCTAATGAAAGCC-3′ for the $PON1_{55}$ polymorphism and 5′-TATTGTTGCTGTGGGGACCT-GAG-3′ and 5′-CACGCTAAACCCAAATACATCTC-3′ for the $PON1_{192}$. The PCR conditions were as previously described *(22)*. PCR products of 171 bp for the $PON1_{55}$ and 99 bp for the $PON1_{192}$ polymorphisms were cleaved with the restriction enzymes *Nla*III and *Alw*I (New England BioLabs), respectively, separated by electrophoresis in 12% polyacrylamide gels, and the banding patterns were developed by silver staining. Characteristic bands were obtained at the following molecular weights: (*a*) M_r 171 for the $PON1_{55}$ LL polymorphism; M_r 127 and 44 for the MM polymorphism, and M_r 171, 127, and 44 for the LM polymorphism; and (*b*) M_r 99 for the $PON1_{192}$ QQ polymorphism, M_r 68 and 31 for the RR polymorphism, and M_r 99, 68, and 31 for the QR polymorphism.

STATISTICAL ANALYSIS

Differences between means were assessed with the Student *t* test. The Pearson correlation coefficient was used to evaluate the degree of association between two variables. When variables presented with skewed distributions, these statistical analyses were applied to log-transformed data. Multiple ANOVA was used to assess the effect of the different variables on the serum PON1 activity. Diagnostic accuracy for serum PON1 and other biochemical tests was calculated with ROC analysis *(23, 24)*. Multiple logistic regression was used to estimate the ability of groups of variables to predict the presence or absence of liver disease. χ^2 analysis was used to determine whether the genotype distribution was in Hardy–Weinberg equilibrium and to compare distributions of alleles and genotypes in the different groups of patients and controls. Statistical analyses performed were with the SPSS 10.0 statistical package and the Epi-Info program (CDC).

Results

RELATIONSHIP BETWEEN SERUM PON1 ACTIVITY AND LIVER DISEASE

The results of the standard liver function tests (Table 1) provide evidence of the spectrum of disease. Patients with chronic hepatitis had a moderate increase in serum ALT, as well as other analytes. For ALP activity and albumin and bilirubin concentrations, liver function values in cirrhotic patients were more altered than in patients with hepatitis.

Baseline and salt-stimulated serum PON1 activities are shown in Fig. 1. Baseline activity was 412 ± 153 U/mL in the control group. This was significantly decreased ($P < 0.001$) in patients with chronic hepatitis (196 ± 109 U/mL; mean decrease, 52%) and in patients with liver

Table 2. Correlation coefficients between serum PON1 activity and the standard liver function tests.[a]

	Baseline PON1 activity		
	Control group (n = 368)	**Chronic hepatitis (n = 107)**	**Cirrhosis (n = 68)**
Proteins	0.08 [−0.02 to 0.18]	0.20[b] [−0.01 to 0.38]	0.43[c] [0.21 to 0.61]
Albumin	0.01 [−0.10 to 0.10]	0.53[c] [0.38 to 0.66]	0.59[c] [0.40 to 0.72]
Bilirubin	−0.03 [−0.13 to 0.07]	−0.32[c] [−0.47 to −0.12]	−0.32[d] [−0.52 to −0.09]
	Stimulated PON1 activity		
Proteins	0.07 [−0.04 to 0.17]	0.12 [−0.08 to 0.30]	0.27[b] [0.03 to 0.48]
Albumin	0.01 [−0.10 to 0.10]	0.38[c] [0.20 to 0.53]	0.40[d] [0.18 to 0.59]
Bilirubin	−0.01 [−0.16 to 0.05]	−0.20[b] [−0.37 to 0.01]	−0.17 [−0.39 to 0.08]

[a] Values in brackets represent 95% CIs. There were no statistically significant relationships between PON1 activity and ALT, GGT, or ALP in any of the groups studied. Statistical analyses were applied on log-transformed values of bilirubin, baseline PON1, and stimulated PON1.

[b] $P < 0.05$.

[c] $P < 0.001$.

[d] $P < 0.01$.

Table 3. Distribution, in patients and controls, of PON1 genotypes defined by polymorphisms at positions 55 and 192 of the mature enzyme protein.[a]

Genotype	Control group (n = 368)	Chronic hepatitis (n = 107)	Cirrhosis (n = 68)
$PON1_{192}$ genotype frequency			
QQ	0.46	0.48	0.50
QR	0.46	0.39	0.41
RR	0.08	0.13	0.09
$PON1_{192}$ allele frequency (Q/R)	0.69/0.31	0.68/0.32	0.70/0.30
$PON1_{55}$ genotype frequency			
LL	0.40	0.36	0.44
LM	0.47	0.48	0.44
MM	0.13	0.16	0.12
$PON1_{55}$ allele frequency (L/M)	0.63/0.37	0.60/0.40	0.66/0.34

[a] No significant differences of genotype or allele distributions were observed between patients and controls.

cirrhosis (137 ± 89 U/mL; mean decrease, 67%). Stimulated PON1 activity was 688 ± 412 U/mL in the control group. This was also significantly decreased ($P < 0.001$) in chronic hepatitis (302 ± 242 U/mL; mean decrease, 56%) and liver cirrhosis (219 ± 223 U/mL; mean decrease, 68%).

The correlation coefficients of the regression lines between serum PON1 activity and the other biochemical analytes are depicted in Table 2. There were significant relationships between serum PON1 activity and total proteins, albumin, and bilirubin concentrations in patients with chronic liver disease, but not in the control group. These relationships were similar when the study participants were segregated with respect to PON1 genotypes; the correlation coefficients were slightly higher and reflected a lower variance of serum PON1 activity (data not shown).

RELATIONSHIP BETWEEN SERUM PON1 ACTIVITY AND GENOTYPES

The genotype frequencies at both restriction enzyme sites were in Hardy–Weinberg equilibrium. The distributions of the two polymorphisms in the control group and in the patient group with liver disease are shown in Table 3. There were no significant differences in genotype or allele frequencies between cases and controls for either the $PON1_{192}$ or $PON1_{55}$ polymorphisms. Baseline and stimulated serum PON1 activities as a function of the different polymorphisms are shown in Fig. 2. The mean decreases of serum PON1 activity in the patient groups relative to the control group were similar (~55–65%) and independent of the genotype.

Multiple ANOVA showed that baseline and stimulated serum PON1 activities were significantly influenced ($P < 0.001$) by both the diagnostic group and the polymorphisms $PON1_{55}$ and $PON1_{192}$, but not by age or gender.

PON1 MEASUREMENT AS A TEST OF LIVER FUNCTION

The results of the ROC analysis for baseline and stimulated PON1 activities, as well as for the standard biochemical tests of liver function, are shown in Table 4 and in Figs. 3 and 4. In patients with chronic hepatitis, the area under the curve (AUC) for baseline PON1 activity was similar to that of ALT, and both were significantly ($P < 0.05$) higher than those of the other tests. In the patients with liver cirrhosis, the AUC for basal PON1 was signif-

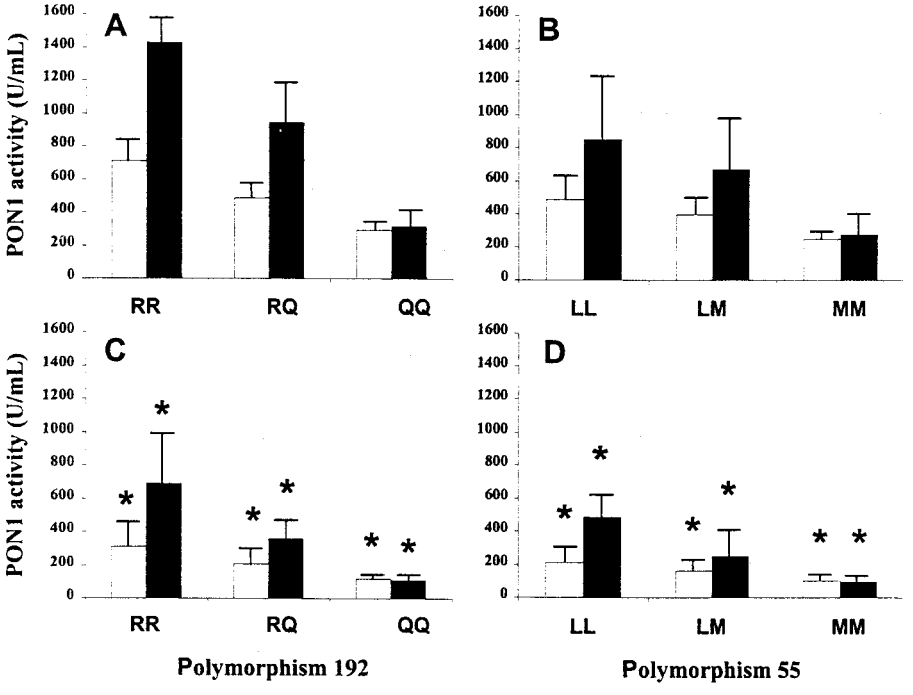

Fig. 2. Baseline (*open columns*) and stimulated (*filled columns*) serum PON1 activity in relation to the polymorphisms at positions 192 and 55 in controls (*A* and *B*) and in patients with liver disease (hepatitis and cirrhosis grouped together; *C* and *D*).

*, $P < 0.001$ with respect to the control group.

Table 4. AUC of ROC plots for serum PON1 activity and the standard liver function tests.

	AUC (95% CI)	
	Chronic hepatitis	**Cirrhosis**
Baseline PON1	0.89 (0.86–0.93)	0.96 (0.94–0.99)
Stimulated PON1	0.79 (0.74–0.84)	0.88 (0.85–0.91)
ALT	0.90 (0.86–0.95)	0.69 (0.61–0.77)
GGT	0.80 (0.76–0.85)	0.87 (0.83–0.92)
ALP	0.65 (0.59–0.71)	0.78 (0.71–0.85)
Bilirubin	0.69 (0.63–0.76)	0.85 (0.79–0.92)
Albumin	0.60 (0.53–0.67)	0.78 (0.70–0.86)
Total proteins	0.53 (0.46–0.59)	0.53 (0.43–0.62)

icantly higher than that of all the other tests. When ROC analyses were performed with data from patients with the same genotype, the AUC were similar to or better than with the combined groups (baseline PON1, 0.98–0.99; stimulated PON1, 0.96–0.99).

The usefulness of adding basal serum PON1 measurement to the standard panel of liver function tests was analyzed by multiple logistic regression analysis. We compared the ability of two different models to correctly differentiate between patients and controls. The coefficients of the equations of both models are shown in Table 5. The equation for model 1 (standard liver function tests) was: $x = 0.1433(\text{albumin}) - 4.1427(\text{ALT}) - 1.1656(\text{GGT}) - 0.2674(\text{ALP}) - 0.1048(\text{bilirubin}) - 0.2772$. To predict the status of any one individual with respect to chronic liver disease, the biochemical terms in the equation should be substituted with their corresponding measured values. If the result (x) calculated from the equation is <0, this classifies the individual as a patient. Similarly, the equation for model 2 (basal PON1 + standard liver function tests) was: $x = 0.0185(\text{albumin}) - 4.5597(\text{ALT}) - 1.0951(\text{GGT}) - 0.0305(\text{ALP}) - 0.0735(\text{bilirubin}) + 0.0172(\text{PON1}) - 0.8575$. Diagnostic sensitivity of model 2 was superior to that of model 1 without any impairment of specificity. Thus, the traditional tests correctly classified 75% [95% confidence interval (CI), 68–81%] of the patients (i.e., sensitivity) and 96% (95% CI, 93–98%) of the controls (i.e., specificity). The addition of serum PON1 activity measurement to the group of standard tests increased the sensitivity of patient classification to 94% (95% CI, 89–97%), whereas the specificity remained unchanged at 96% (95% CI, 93–98%).

Discussion

Recently, serum PON1 has been studied extensively in relation to cardiovascular diseases *(25–29)*, whereas in contrast, there is a paucity of data on the hepatic enzyme. PON1 activity has been observed in rat *(30–34)* and human *(14, 35)* hepatic microsomes. Some of this enzyme is secreted into the circulation, bound to HDL *(36)*, whereas another portion is stored in the liver *(14)*. The physiologic role played by PON1 in the liver is unknown, although preliminary observations suggest that this en-

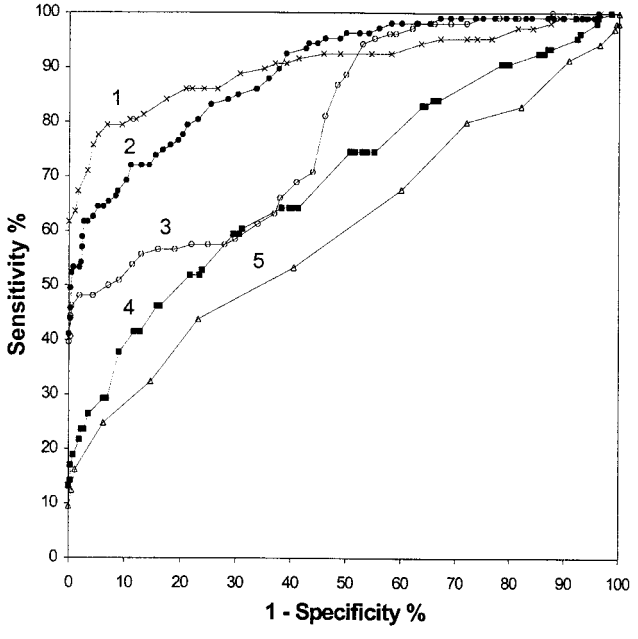

Fig. 3. ROC plots for serum PON1 activity compared with standard biochemical tests in controls (n = 368) vs patients with chronic hepatitis (n = 107).

1, ALT; *2*, baseline PON1; *3*, stimulated PON1; *4*, bilirubin; *5*, albumin. Baseline PON1 activity cutoff was 208 U/mL for 60% sensitivity and 220 U/mL for 95% specificity.

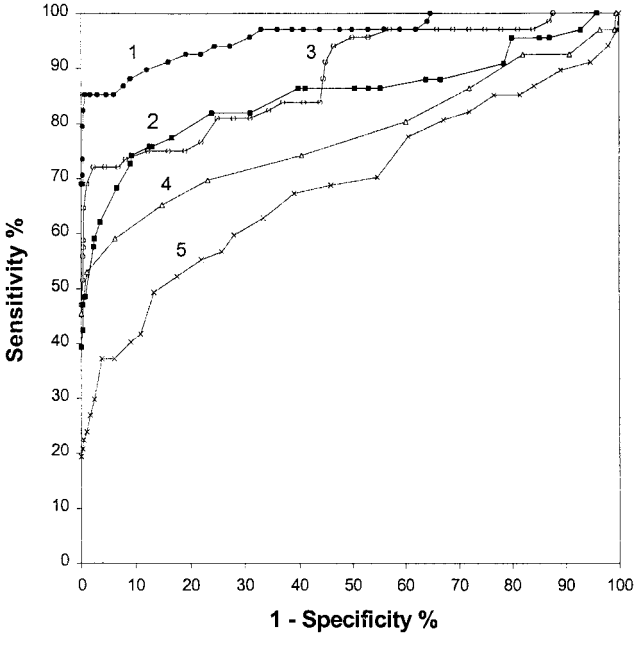

Fig. 4. ROC plots for serum PON1 activity compared with standard biochemical tests in controls (n = 368) vs patients with liver cirrhosis (n = 68).

1, baseline PON1; *2*, stimulated PON1; *3*, bilirubin; *4*, albumin; *5*, ALT. Baseline PON1 activity cutoff was 139 U/mL for 60% sensitivity and 220 U/mL for 95% specificity.

zyme provides hepatic protection against oxidative stress *(37)*.

In the present study, the decrease in PON1 activity in the serum of patients with chronic liver diseases was related to the degree of liver damage. PON1 activity was lower in patients with cirrhosis than in those with hepatitis and was correlated with serum albumin and bilirubin concentrations. Two mechanisms may explain this relationship. In one mechanism, a decrease in PON1 enzymatic activity or gene expression could be the consequence of the hepatic dysfunction. Supporting this hypothesis is the observation of an inhibition of microsomal PON1 activity in rats with chronically administered CCl_4 *(37)*. In the other mechanism, although hepatic PON1 concentrations may be normal, serum PON1 activity could be decreased as a consequence of an altered synthesis and/or secretion of HDL secondary, for example, to impaired lecithin:cholesterol acyl transferase (LCAT) activity. Alterations in HDL structure and concentration associated with decreases in hepatic LCAT activity are frequent in chronic liver diseases *(38)*, and a recent study described a decrease of serum PON1 activity in mice with LCAT deficiency resulting from *LCAT* gene targeted disruption *(39)*.

The main determinant of decreased serum PON1 activity in our study appears to be the severity of liver damage. However, because PON1 activity is also influenced by genetic polymorphisms, an alternative hypothesis would be that of differences in allele or genotype frequency distribution in the patients and the controls. It could be hypothesized, for example, that the associations could be between liver diseases and the Gln allele of the position 192 polymorphism or the Met allele of the position 55 polymorphism. This hypothesis can be discarded because our results indicated that genotype and allele frequencies were similar to those previously published for Caucasian populations in Europe and the US *(19, 25–27, 40–42)*. Also, the distributions were not statistically different between patients with chronic hepatitis and liver cirrhosis or between those with liver cirrhosis and controls. Furthermore, the mean decreases of serum PON1 activity in the patients vs the controls were similar irrespective of the genotype (Fig. 2).

Table 5. Coefficients of the two logistic regression analyses.

Variable	Coefficient	SE	*P*
Model 1: standard biochemical tests			
Albumin	0.1433	0.0396	<0.001
ALT	−4.1427	0.6226	<0.001
GGT	−1.1656	0.3233	<0.001
ALP	−0.2674	0.1719	NS[a]
Bilirubin	−0.1048	0.0306	<0.001
Constant	−0.2772	1.7502	NS
Model 2: basal PON1 and standard biochemical tests			
Albumin	0.0185	0.0501	NS
ALT	−4.5597	0.7883	<0.001
GGT	−1.0951	0.4601	NS
ALP	−0.0305	0.2212	<0.001
Bilirubin	−0.0735	0.0411	NS
PON1	0.0172	0.0024	<0.001
Constant	−0.8575	2.1806	NS

[a] NS, not significant.

The addition of serum PON1 activity measurement to the ensemble of standard liver function tests may be very useful when testing for liver dysfunction. Chronic liver diseases are slow, progressive diseases characterized by advancing hepatocellular necrosis, inflammation, and fibrosis. The progression is subtle and is not associated with dramatic changes in plasma liver function variables, which often remain within laboratory reference values until gross disease becomes evident. Currently, it is widely accepted that the sensitivities of standard biochemical tests for liver function are low and insufficient for a reliable determination of the presence or absence of liver disease *(43)*. Consequently, a "battery" of tests needs to be performed to increase the sensitivity and specificity of the evaluation. However, in many cases, especially when the status of the disease is not severe, these batteries of tests still fail to classify a high percentage of patients. Indeed, more reliable confirmation of suspected impairment is often achieved with only liver biopsy and histologic examination of the tissue. In the present investigation, the combination of five standard tests incorrectly classified 25% of the patients and misclassified 4% of the controls. On the other hand, the addition of baseline serum PON1 activity measurement to these tests increased the sensitivity without a decrease in the specificity (i.e., only 6% of the patients and 4% of the controls were misclassified). These results demonstrate that serum PON1 activity measurement may add a significant contribution to liver function tests. However, our findings need to be confirmed in other populations to preclude the slight possible bias of the present study, which could result from us having estimated sensitivity and specificity using the same volunteers that we used for describing the model parameters and for validating the model. ROC analysis also supports our proposition that the measurement of baseline serum PON1 activity is a good candidate test for inclusion among those used to evaluate liver damage: its diagnostic accuracy is equivalent to that of ALT in patients with chronic hepatitis and far superior to that of the other tests in patients with cirrhosis.

The measurement of serum PON1 activity is a simple, reliable, fast, inexpensive, readily automated method that is compatible with random access analysis in barcoded primary tubes and can be performed in most automated analyzers used for standard biochemical liver function tests. A drawback of the assay is the toxicity of paraoxon. However, with due care and attention, this should not be a major hazard. Further studies need to be conducted to

clarify the mechanisms leading to the decreased serum PON1 activity in liver diseases and the potential pathophysiologic implications. However, a practical result of the present study is the demonstration that the relatively simple PON1 activity measurement could significantly improve the current efficiency of a laboratory's evaluation of patients with suspected chronic liver disease.

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