

## Effects of 5' Regulatory-Region Polymorphisms on Paraoxonase-Gene (*PON1*) Expression

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Human HDL-associated paraoxonase (*PON1*) hydrolyzes a number of toxic organophosphorous compounds and reduces oxidation of LDLs and HDLs. These properties of *PON1* account for its ability to protect against pesticide poisonings and atherosclerosis. *PON1* also hydrolyzes a number of lactone and cyclic-carbonate drugs. Among individuals in a population, *PON1* levels vary widely. We previously identified three polymorphisms in the *PON1* regulatory region that affect expression levels in cultured human hepatocytes. In this study, we determined the genotypes of three regulatory-region polymorphisms for 376 white individuals and examined their effect on plasma-*PON1* levels, determined by rates of phenylacetate hydrolysis. The –108 polymorphism had a significant effect on *PON1*-activity level, whereas the –162 polymorphism had a lesser effect. The –909 polymorphism, which is in linkage disequilibrium with the other sites, appears to have little or no independent effect on *PON1*-activity level in vivo. Other studies have found that the L55M polymorphism in the *PON1*-coding region is associated with differences in both *PON1*-mRNA and *PON1*-activity levels. The results presented here indicate that the L55M effect of lowered activity is not due to the amino acid change but is, rather, largely due to linkage disequilibrium with the –108 regulatory-region polymorphism. The codon 55 polymorphism marginally appeared to account for 15.3% of the variance in *PON1* activity, but this dropped to 5% after adjustments for the effects of the –108 and Q192R polymorphisms were made. The –108C/T polymorphism accounted for 22.8% of the observed variability in *PON1*-expression levels, which was much greater than that attributable to the other *PON1* polymorphisms. We also identified four sequence differences in the 3' UTR of the *PON1* mRNA.

### Introduction

Human paraoxonase (*PON1* [MIM 168820]) is an HDL-associated enzyme (Kitchen et al. 1973; Don et al. 1975; Mackness et al. 1985). *PON1* was first investigated for its ability to hydrolyze organophosphorous (OP) compounds, such as the highly toxic oxon forms of the pesticides parathion (Aldridge 1953; Playfer et al. 1976; Geldmacher-v Mallinckrodt et al. 1979), chlorpyrifos (Furlong et al. 1988, 1989), and diazinon, as well as the nerve agents sarin and soman (Davies et al. 1996). In recent years, *PON1* has been found to protect LDL and HDL from oxidation (Watson et al. 1995; Aviram et al. 1998a, 1998b; Mackness et al. 1998b; Cao et al. 1999). The classes of substrates for *PON1* that most recently have been identified include a number of

drugs and a drug precursor, with important pharmacokinetic implications. Tongou et al. (1998) have shown that the prodrug NM441 is converted to its active form (NM394) by *PON1*, and Billecke et al. (2000) have shown that *PON1* metabolizes lactones and cyclic-carbonate esters, including several of the statin drugs used to control cholesterol levels. The hydrolysis of glucocorticoid  $\gamma$ -lactones and cyclic-carbonate esters by *PON1* provides a mechanism to confine the active forms of drugs to their target sites (e.g., lung) by rapidly metabolizing them as they enter the plasma (Biggadike et al. 2000).

Several types of evidence suggest that low levels of *PON1* protein raise the risk and severity of both OP poisoning and atherosclerosis. Species with low *PON1* activity are more sensitive to OP poisoning than are species with higher *PON1* activity (Costa et al. 1987). *PON1*-null mice on a high-fat diet had larger atherosclerotic lesions than were seen in either wild-type or heterozygous mice (Shih et al. 1998). Additionally, *PON1*-null mice are 5–10 times more sensitive to cholinesterase inhibition by diazoxon and chlorpyrifos oxon than are wild-type mice (Furlong et al. 1998; Shih et al. 1998; Li et al. 2000); however, intraperitoneal injection of purified *PON1* into these mice reconstitutes

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**Table 1****Allele Frequencies of the *PON1* Polymorphisms**

| POSITION<br>AND ALLELE | FREQUENCY REPORTED BY |                                 |                             |                         |      |
|------------------------|-----------------------|---------------------------------|-----------------------------|-------------------------|------|
|                        | Present<br>Study      | Levie<br>and<br>James<br>(2000) | Suehiro<br>et al.<br>(2000) | James et al.<br>(2000b) |      |
|                        |                       |                                 |                             | +CHD                    | −CHD |
| −909:                  |                       |                                 |                             |                         |      |
| G                      | .46                   | .41                             | ...                         | ...                     | ...  |
| C                      | .54                   | .59                             | ...                         | ...                     | ...  |
| −162:                  |                       |                                 |                             |                         |      |
| A                      | .23                   | ...                             | .10                         | ...                     | ...  |
| G                      | .77                   | ...                             | .90                         | ...                     | ...  |
| −108:                  |                       |                                 |                             |                         |      |
| C                      | .50                   | .46                             | .48                         | .44                     | .51  |
| T                      | .50                   | .54                             | .52                         | .56                     | .49  |
| 55:                    |                       |                                 |                             |                         |      |
| L                      | .64                   | .65                             | .94                         | .62                     | .60  |
| M                      | .36                   | .35                             | .06                         | .38                     | .40  |
| 192:                   |                       |                                 |                             |                         |      |
| Q                      | .73                   | .69                             | .40                         | .67                     | .73  |
| R                      | .27                   | .31                             | .60                         | .33                     | .27  |

plasma PON1 and resistance to diazoxon and chlorpyrifos oxon (Li et al. 2000). In humans, wide variation (i.e.,  $\geq 13$ -fold) in PON1-protein levels has been found among individuals (La Du et al. 1986; Furlong et al. 1989; Davies et al. 1996; Richter and Furlong 1999). Relative to that in controls, PON1 activity has been found to be lower in individuals with either non-insulin-dependent diabetes mellitus (Mackness et al. 1998c; Sakai et al. 1998), familial hypercholesterolemia (Tomas et al. 2000), coronary artery disease (James et al. 2000b), or carotid artery disease (Jarvik et al. 2000).

The *PON1*-coding region contains two common polymorphisms, a leucine (L) to methionine (M) substitution at codon 55 and a glutamine (Q) to arginine (R) substitution at codon 192 (Adkins et al. 1993; Humbert et al. 1993). The Q192R polymorphism causes substrate-dependent differences in the kinetics of hydrolysis by each PON1<sub>192</sub> isoform, such that paraoxon (Adkins et al. 1993; Humbert et al. 1993; Li et al. 2000) and chlorpyrifos oxon (Li et al. 2000) are hydrolyzed in vitro more efficiently by PON1<sub>R192</sub>, whereas soman and sarin are hydrolyzed more rapidly by PON1<sub>Q192</sub> (Davies et al. 1996). The Q192R polymorphism has been reported to affect the enzyme's in vivo ability to hydrolyze oxidized lipids, in both LDL and HDL (Aviram et al. 1998a; Mackness et al. 1998b; Cao et al. 1999). The PON1<sub>55</sub> polymorphism does not affect the catalytic efficiency of substrate hydrolysis by the enzyme, but the PON1<sub>M55</sub> allele is correlated with decreased mRNA and protein levels (Blatter Garin et al. 1997; Leviev et al. 1997; Mackness et al. 1998c; Brophy et al. 2000).

Although the substrate-dependent PON1 Q192R polymorphism has been examined in some detail, the

genetic basis for the high interindividual variability in serum PON1 levels has just recently begun to be understood. Our laboratory and two other groups recently identified five polymorphisms in the *PON1* regulatory region (Levie and James 2000; Suehiro et al. 2000; Brophy et al. 2001); these polymorphisms are at −107/−108, −126, −160/−162, −824/−832, and −907/−909, where the base immediately preceding the start codon is numbered as “−1.” The nomenclature differences for four of the polymorphisms are likely due to small variations in the sequences examined by the three different groups. In our laboratory, data generated from cell-culture experiments with a reporter gene indicated that three of the five polymorphisms had a functional effect on *PON1* expression (Brophy et al. 2001). In this report, we examine the three *PON1* regulatory-region polymorphisms—that is, −108, −162, and −909—to determine their effects on PON1 activity level in a white population sample.

**Material and Methods***Samples*

Genomic DNA and lithium-heparin–plasma samples were obtained from volunteers who were participants in a project at the Puget Sound Veterans Affairs Health Care System (PSVAHCS) Epidemiology Research and Information Center. The white sample population consisted of control subjects and patients (97.0% male) with varying degrees of carotid artery disease. Individuals with internal stenosis <15% (controls), 15%–80%, or >80% comprised 49.1%, 10.5%, and 40.4% of the population, respectively. No differences in *PON1*<sub>192</sub> gene frequencies between the patients and the controls were observed in this group; details of subject selection can be found in the report by Jarvik et al. (2000). Subject DNA was prepared from buffy-coat preparations, by a modification of the procedure of Miller et al. (1988), with Puregene reagents (Gentra). The study was approved by both the University of Washington and the PSVAHCS human-subject review processes. All subjects gave written, informed consent.

**Table 2** **$\chi^2$  P Values for Pairs of *PON1* Polymorphisms Demonstrating Linkage Disequilibrium**

| POSITION | P AT |       |       |       |       |
|----------|------|-------|-------|-------|-------|
|          | −909 | −162  | −108  | 55    | 192   |
| −909     | ...  | <.001 | <.001 | <.001 | .004  |
| −162     |      | ...   | <.001 | <.001 | .017  |
| −108     |      |       | ...   | <.001 | .002  |
| 55       |      |       |       | ...   | <.001 |
| 192      |      |       |       |       | ...   |

**Table 3****Mean  $\pm$  SD Arylesterase Activity, for Each *PON1* Genotype**

| Position and Genotype (No. of Cases) | Mean $\pm$ SD Arylesterase Activity (U/ml) | <i>P</i> <sup>a</sup> |
|--------------------------------------|--------------------------------------------|-----------------------|
| –909:                                |                                            | <.001                 |
| GG (79)                              | 128.8 $\pm$ 46.0                           |                       |
| CG (189)                             | 103.0 $\pm$ 37.5                           |                       |
| CC (108)                             | 73.9 $\pm$ 32.9                            |                       |
| –162:                                |                                            | <.001                 |
| AA (18)                              | 135.9 $\pm$ 50.4                           |                       |
| AG (140)                             | 117.1 $\pm$ 41.4                           |                       |
| GG (218)                             | 86.2 $\pm$ 37.2                            |                       |
| –108:                                |                                            | <.001                 |
| CC (94)                              | 125.8 $\pm$ 45.6                           |                       |
| CT (188)                             | 102.9 $\pm$ 37.6                           |                       |
| TT (94)                              | 68.6 $\pm$ 27.9                            |                       |
| 55:                                  |                                            | <.001                 |
| LL (147)                             | 115.0 $\pm$ 42.8                           |                       |
| LM (185)                             | 94.0 $\pm$ 39.7                            |                       |
| MM (44)                              | 75.9 $\pm$ 38.3                            |                       |
| 192:                                 |                                            | .015                  |
| QQ (195)                             | 104.1 $\pm$ 45.4                           |                       |
| QR (158)                             | 98.5 $\pm$ 40.3                            |                       |
| RR (23)                              | 77.4 $\pm$ 27.4                            |                       |

<sup>a</sup> Evaluated by ANOVA.

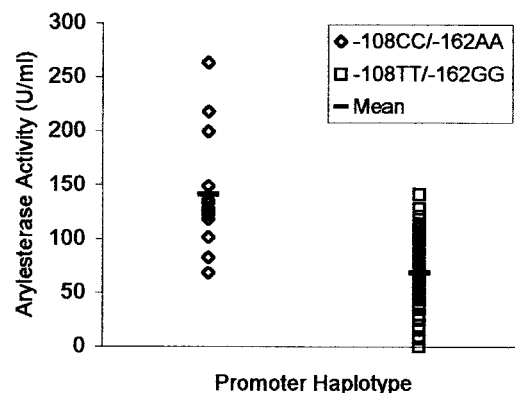
### Genotyping

All genotyping was conducted by PCR amplification, followed by polymorphism-specific restriction digestion and gel electrophoresis. The Q192R polymorphism was detected by *AluI* digestion, and the L55M polymorphism was detected by *NlaIII* digestion, as described elsewhere (Humbert et al. 1993). The genotype of the –108 polymorphism was determined by PCR amplification with primers GACCGCAAGCCACGCC-TTCTGTGCACC and TATATTTAATTGCAGCCGCA-GCCCTGCTGGGGCAGCGCCGATTGGCCCG-CCGC, with 5% dimethyl sulfoxide (DMSO) and *Taq* polymerase (Promega) and at an annealing temperature of 63°C, for 25 cycles. The latter primer creates a *Bst*UI site (New England Biolabs) when a C is present at –108. After digestion, the products were analyzed by 3% agarose gel (Sigma). Presence of a –108C allele results in digested bands of 52 bp and 67 bp (instead of an undigested band of 119 bp). The –162A/G polymorphism was PCR amplified with primers GCTATT-CTTCAGCAGAGGGT and TGAATCTGTAGCCAG-GGCAC, with 5% DMSO and *Taq* polymerase and at an annealing temperature of 56°C, for 30 cycles. The 1,210-bp PCR product was digested with *Bst*UI and was electrophoresed through 1% agarose. Digested bands (674 bp and 536 bp) indicated the presence of G at –162, whereas absence of digestion indicated the presence of an A allele. The –909G/C polymorphism

was PCR amplified with primers AACATGTCACTGT-GGCATATATAATGCTC and TATTATAATATATT-ATATCATTACAGTAACAGCAGACAGCAGAGA-AAAGA, with 5% DMSO and *Taq* polymerase and at an annealing temperature of 60°C, for 35 cycles. The latter primer removes a second *Bsm*AI site, leaving one *Bsm*AI site when a G is present at –909, resulting in digested bands of 50 bp and 206 bp.

### *PON1*-Activity Assays

Rates of phenylacetate hydrolysis (arylesterase activity) are neutral with respect to the Q192R polymorphism and are in a linear relationship with *PON1*-expression levels (Furlong et al. 1993; Blatter Garin et al. 1997). Arylesterase activities for individuals with sequence differences in the 3' UTR were determined as described elsewhere (Kitchen et al. 1973). Arylesterase activities of the population whose regulatory-region polymorphisms were studied were determined as above, except that a SpectraMax Plus (Molecular Devices) plate reader was used to measure rates of hydrolysis. Ten microliters of a 1:40 dilution of human plasma was mixed with 200  $\mu$ l of substrate (3.26 mM phenylacetate in 9 mM Tris-Cl pH 8 and 0.9 mM CaCl<sub>2</sub>). The rates of hydrolysis were determined by analysis in a plastic UV-transparent 96-well plate (Costar), at 270 nm for 4 min. The initial, linear rates of hydrolysis were used for rate calculation. Samples were assayed in triplicate. Because of the difference in assay format, a conversion factor was necessary for comparison of the data versus the results of the standard assay, in a 1-cm quartz cuvette. A subset of samples was assayed by both methods, and the arylesterase activity (in U/ml) was calculated; the results were plotted against each other, and a linear-regression



**Figure 1** Arylesterase activity in individuals homozygous for both polymorphisms associated with either high or low plasma-*PON1* activity. For –108CC/–162AA, *N* = 14 and mean = 140.9 U/ml; for –108TT/–162GG, *N* = 89, mean = 67.5 U/ml, and *P* < .001 (*t*-test). (Data available on request from the corresponding author.)

**Table 4****Mean  $\pm$  SD Arylesterase Activity, for *PON1* Regulatory-Region Genotypes**

| Position  | -909 | -162 | -108 | No. of Cases | Mean $\pm$ SD Arylesterase Activity (U/ml) | P     |
|-----------|------|------|------|--------------|--------------------------------------------|-------|
| -909      | CC   | GG   | CT   | 20           | 96.0 $\pm$ 41.5                            | .959  |
|           | CG   | GG   | CT   | 83           | 96.5 $\pm$ 35.8                            |       |
| -162      | CG   | AG   | CT   | 80           | 109.3 $\pm$ 36.9                           | .026  |
|           | CG   | GG   | CT   | 83           | 96.5 $\pm$ 35.8                            |       |
| -108      | CC   | GG   | CT   | 20           | 96.0 $\pm$ 41.5                            | .009  |
|           | CC   | GG   | TT   | 86           | 68.2 $\pm$ 28.1                            |       |
| All three | GG   | AA   | CC   | 14           | 140.9 $\pm$ 52.7                           | <.001 |
|           | CG   | AG   | CT   | 80           | 109.3 $\pm$ 36.9                           |       |
|           | CC   | GG   | TT   | 86           | 68.2 $\pm$ 28.1                            |       |

line was drawn ( $R^2 = .93$ ). To convert the plate-reader results for comparison with standard DU70 spectrophotometer-determined rates, the intercept (18,950 U/ml) was subtracted from the plate-reader assay arylesterase activity, and the result was divided by the slope (2,105.8 U/ml).

#### Identification of 3' UTR Sequence Differences

The regions encompassing the poly(A) signal sequences were PCR amplified from genomic DNA preparations from four individuals: one with low plasma *PON1* arylesterase activity, one with intermediate plasma *PON1* arylesterase activity, and two with high plasma *PON1* arylesterase activity. The PCR primers used were GGACATCATGAAGCATCAAAGC and CCTATGTGTCATTGCAACAGG. The PCR products were cloned into plasmid pCRII and then were sequenced by AmpliTaq FS dye terminator cycle sequencing (Perkin-Elmer) and primer M13 (forward).

#### Statistical Analyses

Statistical analysis was conducted with SPSS 8.0 (SPSS). The  $\chi^2$  test was used both to test if genotype frequencies deviated from Hardy-Weinberg-equilibrium expectations and to evaluate the significance of the linkage disequilibrium between each polymorphism pair. Arylesterase-activity differences between genotypes were evaluated by either analysis of variance (ANOVA) or *t*-test, and, of the total variance (vt) in arylesterase activity, the proportion (vg/vt) due to each of the five genetic polymorphisms was estimated two ways: first, the marginal effect that each polymorphism had on unadjusted arylesterase activity was estimated as the genetic variance divided by the total variance; second, the conditional effect of each polymorphism, given the effect of polymorphisms with a greater effect, was sequentially estimated. The *PON1*<sub>-108</sub> polymorphism had the greatest effect; thus, linear regression with two dummy variables was used to adjust arylesterase

for the *PON1*<sub>-108</sub> genotypes. The regression residuals were relocated to the arylesterase mean of 100.0742 U/ml, and, of the total variance of each relocated residual genetic variance, the proportion due to each additional polymorphism was computed. The polymorphism with the greatest effect was then adjusted for, and the new residuals were tested for the effects of the remaining polymorphisms. This process was repeated until all polymorphisms had been considered.

#### Results

A total of three *PON1* regulatory-region polymorphisms (-108C/T, -162A/G, and -909G/C) and two *PON1* coding-region polymorphisms (L55M and Q192R) were genotyped in a population of 376 white individuals. The allele frequencies determined, shown in table 1, are consistent with published reports on whites (Cascorbi et al. 1999; James et al. 2000b; Leviev and James 2000). The allele frequencies for both the coding-region and the -162 regulatory-region polymorphisms differed from those of a Japanese population, as expected (Imai et al. 2000; Suehiro et al. 2000), although the -108 allele frequencies did not differ. Genotypes at all five positions do not deviate from Hardy-Weinberg-equilibrium expectations. There is significant linkage disequilibrium across the entire regulatory region (table 2). As described elsewhere (Blatter Garin et al. 1997; Mackness et al. 1998c; Schmidt et al. 1998), the *PON1*<sub>55L</sub> allele also is in linkage disequilibrium with the *PON1*<sub>192R</sub> allele. In addition, we observed that *PON1*<sub>55L</sub> is in linkage disequilibrium with -108C, -162A, and -909G.

Arylesterase activities for each genotype were evaluated by ANOVA to test for evidence of genotype effect on activity level (table 3). Each polymorphism, whether coding or noncoding, is associated with statistically significant variability in arylesterase values. Comparison of individuals with two (fig. 1) or three (table 4) of the regulatory-region polymorphisms associated with high expression versus those with the regulatory-region polymorphisms associated with low expression reveals a twofold difference in activity levels.

Although each polymorphism genotyped in this study is associated with variability in arylesterase activity, the linkage disequilibrium among all of these polymorphisms makes it unclear whether activity differences were due to a specific polymorphism or to linkage disequilibrium with another position. We used two strategies to reduce this confounding effect: first, we subdivided the subjects by genotype at each of the three regulatory-region polymorphisms; second, we examined the residual genetic variance when adjustments were made for sites with greater effects. Each regulatory-region position was examined for *PON1*-activity differences while the genotype at the other two regu-

Table 5

Estimated Marginal and Serial Conditional Proportions  $vg/vt$  of the Total Variance  $vt$ , in Arylesterase Activity, Due to Each of the Five Genetic Polymorphisms

| PON1<br>SITE | $vg/vt$<br>MARGINAL | ARYLESTERASE<br>ADJUSTED FOR | $vt$     | MEAN <sup>a</sup> |        |        | $vg/vt$<br>CONDITIONAL |
|--------------|---------------------|------------------------------|----------|-------------------|--------|--------|------------------------|
|              |                     |                              |          | 1                 | 2      | 3      |                        |
| –108         | .228                |                              | 1,831.31 | 125.80            | 102.94 | 68.61  | .228                   |
| 192          | .022                | 108                          | 1,413.22 | 105.88            | 97.50  | 68.53  | .057                   |
| 55           | .153                | 108, 192                     | 1,330.32 | 109.09            | 95.40  | 89.60  | .041                   |
| –162         | .095                | 108, 192, 55                 | 1,250.83 | 112.17            | 102.71 | 97.38  | .011                   |
| –909         | .205                | 108, 192, 162, 55            | 1,231.25 | 101.16            | 99.09  | 101.01 | .001                   |

<sup>a</sup> Means 1, 2, and 3 are those of the three genotypes—pp, pq, and qq—for each site, for the arylesterase trait adjusted for the polymorphisms listed. The p allele for sites –108, 192, 55, 162, and –909, respectively, are C, Q, L, A, and C. The overall arylesterase mean was 100.0742 U/ml.

latory-region polymorphism positions was held constant (table 4). The –108 haplotype pair showed a significantly higher PON1-activity level when C was present compared to when T was present. Likewise, the –162A allele had a statistically significant effect, although the arylesterase-activity level differences were less than those for –108 and were not adjusted for linkage disequilibrium with 55 or 192. When the genotypes at 55 and 192 (LM and QQ, respectively) were also held constant, –162 remained significant ( $P = .025$ ). In contrast, the –909G/C polymorphism appears not to independently affect PON1-activity levels.

Estimated marginal proportions and conditional proportions,  $vg/vt$ , of the total variance,  $vt$ , in arylesterase activity that are due to each of the five genetic polymorphisms are given in table 5. The greatest effect appears to be that of the *PON1*<sub>–108</sub> site, accounting for 22.8% of the variation in arylesterase activity. The significant linkage disequilibrium between the polymorphisms results in dependence between the estimated marginal  $vg/vt$ . Given the effect of the *PON1*<sub>–108</sub> site, 5.7% of the remaining variance is associated with *PON1*<sub>192</sub>. Given the *PON1*<sub>–108</sub>- and *PON1*<sub>192</sub>-site effects, 4.1% of the remaining variance is associated with the *PON1*<sub>55</sub> polymorphism. The *PON1*<sub>–162</sub> site accounts for only 1.1% of the variance in *PON1*<sub>–108</sub>-, *PON1*<sub>192</sub>-, and *PON1*<sub>55</sub>-adjusted arylesterase activity. The effect of the *PON1*<sub>–909</sub> polymorphism that appeared, for marginal  $vg/vt$ , quite great (20.5 %; table 5) is trivial, once the other polymorphisms are accounted for.

As described elsewhere (Blatter Garin et al. 1997; Mackness et al. 1998c; Brophy et al. 2000), individuals with *PON1*<sub>55LL</sub> show, on average, a higher level of enzyme activity than do individuals with *PON1*<sub>55MM</sub> (table 3). The difference in expression level is statistically significant ( $P < .001$ ) and has been suggested to be due to linkage to other elements, such as regulatory-region polymorphisms (Levieu and James 2000). Our analysis showed that the M allele is indeed in linkage disequi-

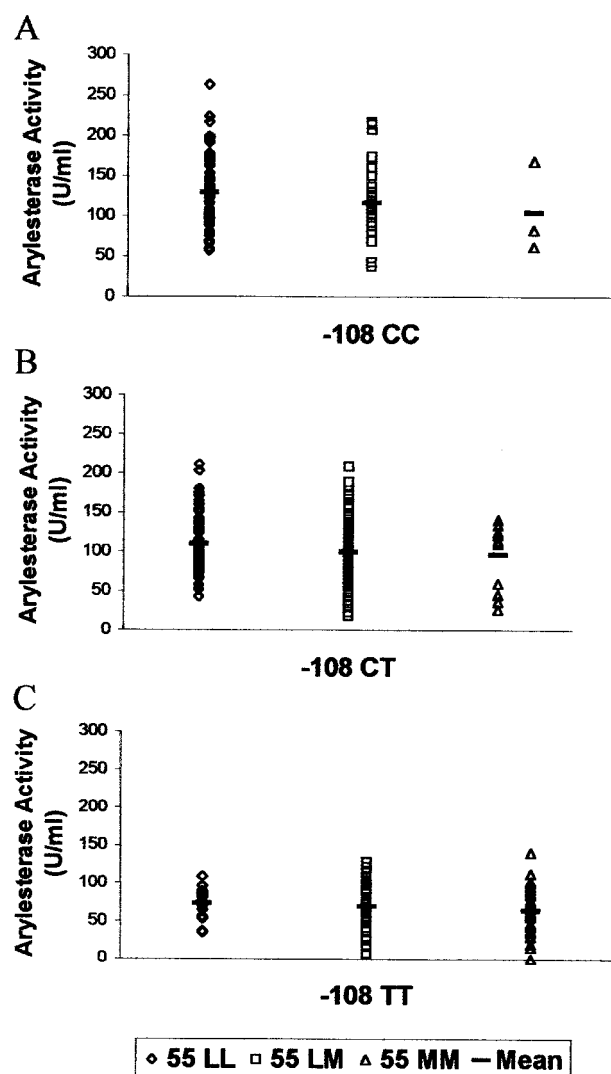
librium with the regulatory-region polymorphism (i.e., –108T) associated with lower PON1 expression. When the –108 genotype was held constant, arylesterase values shown were not significantly different between individuals with genotype LL and those with genotype MM (fig. 2 and table 6).

The polymorphisms identified in the regulatory region explain part of the variation in *PON1*-expression levels; however, a significant portion of that variation is still unexplained. We examined the 3' UTR of *PON1* mRNA from four individuals, by sequence analysis. The clones differed in the location of their poly(A) tails, and none of the sequences contained a canonical poly(A) signal of AATAAA; instead, sequences from the clones revealed the use of variants of this sequence (data not shown). Four sequence differences were identified in the 3' UTR (table 7), but none altered the poly(A) signal sequences. These 3' sequence differences have not yet been examined for their effects on expression level or for their frequencies in the population.

## Discussion

The allele frequencies for *PON1* polymorphisms differ among ethnic groups, as shown in table 1. Notably, the only position that does not differ, in allele frequency, between white and Japanese populations, in either the coding or the upstream regulatory region, is –108. This may be a coincidence, or selection pressure(s) may have acted on this polymorphism to maintain specific allele frequencies across different ethnic groups.

The three regulatory and the two coding polymorphisms all show significant linkage disequilibrium to each other, including the four alleles associated with high expression (i.e., –108C, –162A, 55L, and 192Q). The results summarized in table 5 suggest that the *PON1*<sub>–108</sub> polymorphism has the greatest effect on arylesterase activity, followed, in order, by *PON1*<sub>192</sub>, *PON1*<sub>55</sub>, *PON1*<sub>–162</sub>, and *PON1*<sub>–909</sub>. It is possible that



**Figure 2** Arylesterase activity in individuals with each 55/-108 haplotype. Means and *P* values are shown in table 6. The graphs indicate *PON1* regulatory-region genotypes of -108CC (A), -108CT (B), and -108TT (C). (Data available on request from the corresponding author.)

any of the effects listed are, at least in part, due to linkage disequilibrium with other, still unidentified polymorphisms. Our results indicate that the -909G/C polymorphism has little, if any, effect on *PON1*-activity level but is in linkage disequilibrium with the functional polymorphisms. Interestingly, -108C and -162A, the two regulatory-region alleles that may independently increase *PON1* expression (table 4) are in linkage disequilibrium with each other. The -108C allele is also in linkage disequilibrium with the *PON1*<sub>R192</sub> allele, which has been implicated in a lower level of protection against atherosclerosis, although the Q192R polymor-

phism is associated with activity variance that is independent of the -108C site. It is possible that the -108C allele that increases expression may partly compensate for the lowered protection afforded by the *PON1*<sub>R192</sub> isoform, complicating the apparent relationship between the *PON1*<sub>192</sub> genotype and disease. The L55M polymorphism has been associated with *PON1*-expression level (Blatter Garin et al. 1997; Leviev et al. 1997; Mackness et al. 1998c; Brophy et al. 2000). The results presented here (fig. 2 and tables 5 and 6) indicate that the *PON1*<sub>55</sub> effect is primarily due to linkage disequilibrium of *PON1*<sub>55M</sub> with the -108T lower-expression variant.

The lack of effect of the -909 polymorphism contrasts with the increased expression from -909G observed in our cell-culture experiments (Brophy et al. 2001). This discrepancy may be due to an inherent difference between a cultured hepatocarcinoma cell line and plasma from normal individuals. Alternatively, sequence-context effects may alter the significance of the -909 polymorphisms; for instance, the -909G allele may have an effect when -162A is present in cis (in vitro data) but not when other polymorphisms are present. We do not have plasma and genotypes from enough individuals of these specific haplotypes to investigate that possibility.

The -108 polymorphism lies within a probable binding site for Sp1, a ubiquitous transcription factor common in TATA-less genes such as *PON1*. James et al. (2000b) found that the -108T allele was associated with increased risk of coronary artery disease in patients with type 2 diabetes. The -162 polymorphism may be within a possible NF-1 transcription-factor-binding site (Brophy et al. 2001). Interestingly, a recent publication has identified an interleukin-6 (IL-6)-responsive element that shows homology to the sequence including and 3' of the -162 polymorphism (Ray 2000). IL-6, a

**Table 6**

Mean  $\pm$  SD Arylesterase Activity for Each 55/-108 Haplotype

| GENOTYPE |    | No. of<br>Cases | MEAN $\pm$ SD<br>ARYLESTERASE<br>ACTIVITY<br>(U/ml) |      | <i>P</i> |
|----------|----|-----------------|-----------------------------------------------------|------|----------|
| −108     | 55 |                 |                                                     |      |          |
| CC       | LL | 67              | 129.9 $\pm$ 43.2                                    | .349 |          |
| CC       | LM | 24              | 116.9 $\pm$ 50.8                                    |      |          |
| CC       | MM | 3               | 104.4 $\pm$ 56.7                                    |      |          |
| CT       | LL | 63              | 110.3 $\pm$ 38.6                                    | .154 |          |
| CT       | LM | 113             | 99.5 $\pm$ 36.4                                     |      |          |
| CT       | MM | 12              | 96.2 $\pm$ 41.3                                     |      |          |
| TT       | LL | 17              | 73.2 $\pm$ 20.4                                     | .572 |          |
| TT       | LM | 48              | 69.5 $\pm$ 28.5                                     |      |          |
| TT       | MM | 29              | 64.5 $\pm$ 30.9                                     |      |          |

**Table 7****Examination Results from 3' UTR of PON1 mRNA**

| INDIVIDUAL<br>(ALLELE AT<br>192) | ARYLESTERASE<br>ACTIVITY<br>(U/ml) | NO. OF<br>CLONES | SEQUENCE DIFFERENCE AT <sup>a</sup> |      |      |      |
|----------------------------------|------------------------------------|------------------|-------------------------------------|------|------|------|
|                                  |                                    |                  | 1290                                | 1314 | 1616 | 1647 |
| 1 (Q)                            | 47                                 | 6                | A                                   | A    | A    | C    |
| 2 (Q)                            | 142                                | 1                | A                                   | G    | A    | C    |
|                                  |                                    | 3                | A                                   | A    | A    | C    |
| 3 (R)                            | 103                                | 2                | A                                   | A    | G    | C    |
|                                  |                                    | 3                | A                                   | A    | A    | C    |
| 4 (R)                            | 169                                | 1                | G                                   | A    | A    | C    |
|                                  |                                    | 3                | A                                   | A    | A    | T    |

<sup>a</sup> Nucleotide position of mRNA, from initiator ATG.

proinflammatory cytokine, has been shown to decrease *PON1* expression in human HepG2 cells and *PON1* activity in the plasma of mice (Van Lenten et al. 2001).

The data shown in figure 1 indicate that possessing efficient *PON1* regulatory regions does not alone guarantee a high *PON1*-activity level. Environmental factors—such as smoking (Nishio and Watanabe 1997; James et al. 2000a; Jarvik et al. 2000) or a high-fat diet (Shih et al. 1996; Hedrick et al. 2000)—may decrease *PON1* expression and *PON1* activity and may also explain why individuals with efficient regulatory regions have low levels of plasma-*PON1* activity. Conversely, consumption of antioxidants—such as those found in pomegranate juice (Aviram et al. 2000), red wine (Hayek et al. 1997), and other alcoholic beverages (when consumed in moderation) (van der Gaag et al. 1999)—may allow an individual with inefficient *PON1* regulatory regions to express moderate levels of protein. However, individuals with inefficient regulatory regions appear to be unable to express very high levels of *PON1* (fig. 1).

Four sequence differences and variant poly(A) signal sequences were identified in the 3' UTR of *PON1* mRNA. Variant poly(A)-signal sequences are thought to be used inefficiently, resulting in low levels of polyadenylated (stable) mRNA (Sheets et al. 1990; Edwalds-Gilbert et al. 1997). Although the observed sequence differences are not within the variant signal sequences, they may affect the addition of the poly(A) tail or otherwise alter the stability of the *PON1* mRNA.

The –108 regulatory-region polymorphism has a significant effect on *PON1* expression in humans. Toxicological and epidemiological studies indicate that *PON1*-expression levels affect the degree of protection that an individual has against pesticide poisoning (Li et al. 1993, 1995, 2000) and heart disease (reviewed in Mackness et al. 1998a; Lusis 2000). Further understanding of *PON1* regulatory-region function may reveal approaches for increasing the *PON1* expression in at-risk individuals. Because the wide variation in *PON1*-

activity level among individuals cannot be predicted solely by genotype, future studies examining the relationship between *PON1* and disease should include determinations of plasma-*PON1*-activity levels. The determination of *PON1* status via the two-dimensional substrate-assay protocol provides an accurate inference of genotype at codon 192, as well as expression levels for individuals (Richter and Furlong 1999; Brophy et al. 2000).

*Note added in proof.*—It has recently been brought to our attention that the previously designed primers for genotyping the L55M polymorphism have a mismatch, in the reverse primer, that allows it to bind, but not completely. New primers were created that produce a larger PCR product but still use the same digestion. The new primers, AGAGGATTCTAGTCTTTGAGGAAA and CTGCCAGTCCTAGAAAACGTT, were used with *Taq* polymerase at an annealing temperature of 50°C, for 30 cycles. After digestion, the products were analyzed on a 2% agarose gel (Sigma). Digestion with *Nla*III generated either 296-bp and 90-bp fragments, when a 55M allele was present, or an undigested 386-bp fragment, when a 55L allele was present.

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## Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for *PON1* [MIM 168820])

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