

Clinical Investigation and Reports

Low Paraoxonase Activity Predicts Coronary Events in the Caerphilly Prospective Study

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Background—The hypothesis that paraoxonase (PON1) has a role in preventing atherosclerosis is based on experimental, transgenic, and case-control studies but has not previously been tested prospectively.

Methods and Results—The Caerphilly Prospective Study is a cohort study of men aged 49 to 65 years observed for coronary heart disease (CHD) events (fatal and nonfatal myocardial infarction) over a mean period of 15 years. Serum PON1 activity toward paraoxon was measured in 1353 participants. PON1 activity was 20% lower in the 163 men who had a coronary event ($P=0.039$). Men in the highest quintile of PON1 activity had a decreased risk compared with those in the lowest quintile (OR 0.57 [95% CI, 0.33 to 0.96]). The inverse relationship between quintiles of serum PON1 activity and CHD risk was graded, the median change in OR across each quintile being 0.87 (0.77 to 0.98). After adjustment for all other CHD risk factors, including HDL cholesterol, this median value became 0.90 (0.78 to 1.02). PON1 was most predictive of a new CHD event in patients at highest risk by virtue of preexisting CHD (adjusted median OR for each quintile, 0.74 [0.59 to 0.93]; $n=313$) or the presence of other risk factors. For the highest tertile of CHD risk ($n=390$) calculated by the Framingham equation, adjusted median OR for each quintile was 0.84 (0.66 to 1.05); $n=390$.

Conclusions—Low serum PON1 activity toward paraoxon is an independent risk factor for coronary events in men at high risk because of preexisting disease or other CHD risk factors. (*Circulation*. 2003;107:2775-2779.)

Key Words: myocardial infarction ■ lipoproteins ■ coronary disease ■ antioxidants

Paraoxonase (EC.3.1.8.1, aryldialkylphosphatase) (PON1) has been extensively studied in the field of toxicology because it hydrolyses organophosphate compounds, used as insecticides and nerve gases.^{1,2} PON1 is synthesized in the liver and in serum is located on HDL. The serum HDL concentration is inversely correlated with atherosclerosis risk.³ The mechanism for this continues to be the subject of considerable debate. The initial focus of attention was on the role of HDL in reverse-cholesterol transport. However, recent studies have suggested more diverse mechanisms. HDL protects against oxidative modification of LDL,⁴⁻⁶ which is believed to be central to the initiation and progression of atherosclerosis.⁷ The antioxidant activity of HDL relates to its enzymes, primarily PON1, but also LCAT,⁸ and these can prevent lipid-peroxide accumulation on LDL both in vitro and in vivo.⁹⁻¹⁴

PON1 activity is partly genetically determined. An amino acid substitution at position 192 (Q→R) gives rise to 2 allozymes,^{15,16} the relative activities of which are substrate dependent. Substrates, such as paraoxon and fenitroxon, are hydrolyzed faster by the R alloenzyme, whereas other substrates, such as phenyl acetate, are hydrolyzed at the same rate

by both alloenzymes, and yet others, such as diazoxon and the nerve gases soman and sarin, are hydrolyzed more rapidly by the Q alloenzyme.¹⁷ The Q alloenzyme in vitro provides greater protection against the accumulation of lipid peroxides on LDL than the R alloenzyme.^{18,19} A second exonic polymorphism of the PON1 gene occurs at amino acid position 55 (L→M). This polymorphism also affects PON1 activity, although less than that of the 192 polymorphism.²⁰

There have been many case-control studies to test the hypothesis that the 192R allele of the PON1 gene is associated with coronary heart disease (CHD).²¹ A recent meta-analysis of these found that the PON1-192R allele was significantly related to the presence of CHD, but there was evidence of publication bias.²² Furthermore, genetic case-control studies fail to test the hypothesis that serum PON1 activity protects against CHD, because the 192 polymorphism accounts for only a small part of the 40-fold individual variation in serum PON1 activity.

Only 5 case-control studies have included measurements of PON1 activity as opposed to relying on genotype alone.²²⁻²⁶ In one of these, diminished protection of LDL against oxidation by HDL from patients with coronary atherosclero-

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sis was reported,²⁴ which, it was proposed, was attributable to low serum PON1 activity. In another study we found that serum PON1 activity was already decreased within 2 hours of the onset of symptoms of acute myocardial infarction²⁵ and remained low subsequently, suggesting that the decreased activity may have preceded the acute event.²⁵ Furthermore, the lower activity in the patients with myocardial infarction compared with controls was substantially greater than could be accounted for by PON1 polymorphisms. Two recently conducted large case-control studies in which PON1 activity and genotype were measured in people with angiographically proven CHD and controls also concluded that serum PON1 activity was more closely related to the presence of CHD than the PON1-192 genotype.^{22,26}

Case-control studies can give misleading results because of survivor effects, bias in case selection, and the effect of the disease and its treatment. It is thus important that the association of PON1 activity and CHD be tested prospectively. We therefore studied the relationship between new coronary events in the Caerphilly Prospective Study and serum PON1 activity with the specific a priori hypothesis that PON1 activity would be related to CHD. We also measured serum PON1 concentration and clusterin (apolipoprotein J [apoJ]), which has also been proposed as a component of HDL with a role in protecting tissues against oxidative damage.^{24,27}

Methods

Subjects

The general design and methods of the Caerphilly Prospective Study have been described elsewhere.²⁸ Men aged 49 to 65 years living in Caerphilly (South Wales), randomly selected from the electoral register, were invited to participate between 1984 and 1988. A detailed medical, lifestyle, and family history was obtained, the London School of Hygiene and Tropical Medicine chest pain questionnaire²⁹ was administered, a 12-lead ECG was recorded, and full anthropometric details and fasting blood samples were obtained.

Previously unfrozen serum samples stored at -80°C were available from 1353 men. In a pilot study, we analyzed 100 of them randomly chosen for PON1 activity toward paraoxon. Their median activity, 198.1 U/L (96.4 to 407.7), was not significantly different from fresh samples from a Manchester control population ($n=281$) in which it was 216.1 U/L (76.8 to 517.8). In 40 controls from Manchester, serum PON1 activity at the time samples was taken, and 6 years after storage at -80°C was also not significantly different (170.6 U/L [68.2 to 360.4] and 165.9 U/L [56.6 to 320.9], respectively). Similar comparisons of PON1 and apoJ concentration also revealed no significant differences (results not shown).

The occurrence of a coronary event was monitored over an average of 15 years as described²⁸ and was defined as fatal or nonfatal myocardial infarction or the appearance of major or moderate Q waves (Minnesota codes 1-1-any, 1-2-1 to 1-2-5, or 1-2-7) on any follow-up ECG where there were no Q waves (1-1-any, 1-2-any, 1-3-any) on the recruitment ECG. Preexisting CHD was defined as a history of severe chest pain, a positive history of angina on the chest pain questionnaire, or ischemic changes on the ECG as defined previously.²⁸ Family history of CHD was defined as having at least one first-degree relative with acute myocardial infarction before the age of 55 years.

Analysis of PON1

Serum PON1 activity toward paraoxon was analyzed spectrophotometrically as described previously.²⁰ PON 1 concentration was determined by using our in-house competitive ELISA using rabbit

anti-human PON1 monospecific antibodies as described previously.³⁰

Determination of Clusterin Concentration

Clusterin concentration was determined by ELISA using monoclonal antibodies to human clusterin and pure clusterin as standard (both purchased from Quidel, Aalkmar, the Netherlands) as described previously.²² Interassay and intra-assay coefficients of variation were 7.2% and 4.2%, respectively.

Other Laboratory Tests

Methods for serum cholesterol, HDL cholesterol (HDL-C), apoB, apoA1, and triglycerides have been previously described.²⁸

Statistical Analysis

Means were calculated for most variables in men who did and did not have a coronary event. Medians were used for serum triglycerides, total to HDL cholesterol ratio, PON1 activity, PON1 concentration, and clusterin, which were nongaussian. Differences were sought by Student's *t* test unless the frequency distribution was nongaussian, when the Mann-Whitney *U* test was used. Differences in the proportions of men with categorical variables were sought with the χ^2 test.

The independent contribution of quintiles of PON1 to CHD risk expressed as the OR (the median ratio of the proportion of men in 1 quintile who had a CHD event to that in the next) was tested by logistic regression analysis with age, smoking status, preexisting CHD, family history of CHD, body mass index, systolic blood pressure, diastolic blood pressure, diabetes, serum cholesterol, HDL-C, serum triglyceride, and total to HDL cholesterol ratio included as covariates.

Results

The risk factors of the men who did or did not have a new coronary event are given in Table 1. The former had significantly increased systolic and diastolic blood pressure, serum total cholesterol, triglycerides, and apoB, total serum to HDL cholesterol ratio, and likelihood of smoking, preexisting CHD, a family history of CHD, and lower HDL cholesterol, as previously reported.^{28,31} Serum PON1 activity was 20% lower in the men experiencing CHD events ($P<0.039$). PON1 (79.4 $\mu\text{g/mL}$ [2.3 to 275.9] versus 84.6 [3.3 to 447.8] $\mu\text{g/mL}$ median [range]), and clusterin (97.5 [12.6 to 192.7] versus 101.2 [1.0 to 320.5] $\mu\text{g/mL}$) concentrations in the men who did and did not have a coronary event were not significantly different.

There was a graded inverse relationship between quintiles of serum PON1 activity and the likelihood of a CHD event (Figure). Men in the highest quintile of PON1 activity had a decreased likelihood of having a coronary event, OR=0.57 (95% CI, 0.33 to 0.96) ($P=0.031$) compared with those in the lowest quintile, and the median decrease in risk from each quintile to the next highest was 0.87 (0.77 to 0.98) ($P<0.05$) (Table 1). The median OR for the likelihood of an event for each successive quintile of PON1 remained statistically significant after adjustment for history of CHD, family history of CHD, smoking, diabetes, body mass index, blood pressure, and total serum cholesterol (Table 2).

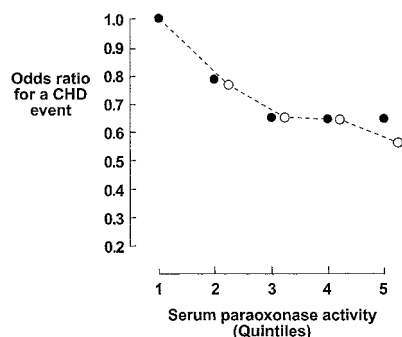
Adjustment for HDL-C had only a quantitatively small effect on the median OR between successive quintiles of PON1 activity and CHD risk, changing it from 0.87 to 0.89, although its confidence intervals just exceeded unity. PON1 and HDL-C were significantly correlated ($P=<0.001$), but the correlation coefficient (0.21) was too low to explain the

TABLE 1. Risk Factors in Men Who Did and Did Not Experience a New Coronary Event During the Study and the Median OR for Each Quintile Compared With the Next for Each Risk Factor

	Coronary Events		<i>P</i>	OR (95% CI)
	Yes	No		
Subjects, No.	163	1190		
Age, y	57.7 (57.0–58.3)	57.1 (56.8–57.3)	0.110	1.03 (0.99–1.07)
Preexisting CHD, %	39.3 (31.7–47.2)	20.9 (18.6–23.3)	<0.001	2.74 (1.96–3.81)
Family history of CHD, %	17.8 (12.3–24.5)	11.2 (9.4–13.1)	0.015	1.72 (1.11–2.67)
Body mass index, kg/m ²	27.1 (26.5–27.6)	26.5 (26.3–26.7)	0.054	1.11 (0.98–1.24)
Diabetes mellitus, %	4.9 (2.1–9.4)	2.6 (1.8–3.7)	0.99	1.93 (0.87–4.27)
Cigarette smokers, %	41.1 (33.5–49.1)	34.4 (31.7–37.1)	0.083	1.33 (0.95–1.86)
Systolic blood pressure, mm Hg	153.9 (149.8–157.9)	147.0 (145.7–148.3)	<0.001	1.20 (1.07–1.35)
Diastolic blood pressure, mm Hg	86.8 (84.7–88.9)	84.3 (83.6–85.0)	0.017	1.15 (1.02–1.30)
Serum cholesterol, mmol/L	5.93 (5.75–6.10)	5.64 (5.58–5.69)	<0.001	1.22 (1.08–1.38)
Serum triglycerides, mmol/L	1.96 (0.58, 11.27)	161 (0.34, 13.55)	<0.001	1.25 (1.11–1.41)
Serum apoB, mg/dL	99.9 (96.4–103.4)	95.2 (94.0–96.3)	0.005	1.18 (1.05–1.33)
HDL-C, mmol/L	0.96 (0.93–0.99)	1.03 (1.01–1.04)	0.002	0.83 (0.74–0.94)
ApoA1, mg/dL	122.5 (119.9–125.2)	125.2 (124.0–126.3)	0.111	0.92 (0.82–1.03)
Total serum cholesterol/HDL-C	6.24 (2.71, 15.73)	5.56 (1.39, 19.36)	<0.001	1.28 (1.14–1.45)
PON1 Activity, U/L	132 (28.6, 622.7)	158.2 (24, 834.4)	0.039	0.87 (0.77–0.98)

Values are means or percentages except for serum triglycerides, total to HDL-C ratio, PON1 activity, and ORs, which are medians. Figures in parentheses are 95% CI if separated by a hyphen and ranges if separated by a comma. *P* values are from a *t* test for means, Mann-Whitney *U* test for medians, and χ^2 test for proportions. ORs are unadjusted for any confounding variable and are statistically significant if their confidence interval does not cross unity.

whole of the effect of PON1 activity on CHD risk. Triglycerides correlated with HDL-C (Spearman's correlation coefficient -0.40 ; $P < 0.0001$), and addition of these after HDL-C made no difference to the model. When a similar analysis was performed for the predictive power of HDL-C, adjusting for all risk factors except PON1 activity, the OR for HDL was 0.83 (0.72 to 0.95). When PON1 activity was added to the model, HDL-C was relatively unaffected as a risk factor (OR 0.83 [0.76 to 0.96]) for the men overall, although in the men with preexisting CHD, PON1 adjusted for HDL-C was a stronger risk factor than HDL-C adjusted for PON1 (OR, 0.73 [0.58 to 0.92] versus 0.85 [0.69 to 1.05]). These results indicate that both HDL-C and PON1 activity contribute independently to variation in CHD risk.



The relative likelihood (OR) of a new CHD event occurring in the quintiles of serum paraoxonase activity in 1353 men, when quintile 1 has the lowest and quintile 5 has the highest activity. ●, ORs adjusted for age, family history, smoking, body mass index, history of CHD, diabetes, blood pressure, cholesterol, triglycerides, and HDL-C. ○, Unadjusted OR.

PON1 was a stronger prognostic factor in the presence of CHD. The gradient between quintiles in the 313 subjects with a definite history of CHD on entry to the study gave an OR of 0.74 (0.59 to 0.93) ($P = 0.011$) after adjustment for all other coronary risk factors including HDL-C, whereas the OR for the 1040 subjects with no history of CHD on entry to the study was 0.98 (0.83 to 1.15) ($P = 0.769$) after adjustment (Table 2). Men with CHD are at greater risk of new CHD events than men without such a history. To examine whether the greater predictive power of PON1 activity was related to the presence of CHD or to their greater risk, we studied those men who had no history of CHD but who were in the highest tertile of CHD risk predicted by the Framingham equation.³² The OR for this group after adjustment for all other risk factors was 0.84 (0.66 to 1.05), indicating a stronger predictive power of PON1 activity than in those without CHD at entry.

Discussion

This is the first prospective epidemiological study of PON1 and CHD. Its results indicate that low serum PON1 activity toward paraoxon is a predictive risk factor for subsequent coronary events independent of all other established risk factors, with the exception of HDL, of which PON1 is a component. However, the erosion of the independent effect of PON1 by HDL-C was small. The correlation of HDL-C with PON1 was weak and certainly not strong enough to explain the whole relationship between PON1 and CHD.

This, together with the results of earlier case-control studies of the PON1-192 polymorphisms, which influence PON1 activity toward paraoxon,²¹ support the view that

TABLE 2. Median OR (95% CIs) for the Likelihood of a CHD Event Between Each Quintile of PON1 Activity and the Next

	Total Population (n=1353)	No Preexisting CHD		
		All (n=1040)	Highest Tercile of CHD Risk (n=390)	Preexisting CHD (n=313)
Unadjusted	0.87 (0.77 to 0.98)	0.93 (0.86 to 1.08)	0.86 (0.70 to 1.05)	0.77 (0.62 to 0.94)
Adjusted for all risk factors except HDL-C and triglycerides	0.89 (0.78 to 1.00)	0.97 (0.82 to 1.15)	0.84 (0.67 to 1.05)	0.73 (0.58 to 0.92)
Adjusted for HDL-C alone	0.89 (0.78 to 1.02)	0.98 (0.83 to 1.16)	0.84 (0.67 to 1.06)	0.73 (0.58 to 0.92)
Adjusted for all risk factors including HDL-C and triglycerides	0.90 (0.78 to 1.02)	0.98 (0.83 to 1.15)	0.84 (0.66 to 1.05)	0.74 (0.59 to 0.93)

Values are (1) unadjusted for any other risk factor, (2) adjusted for history of CHD, family history, body mass index, diabetes, blood pressure, and serum cholesterol but not HDL-C, and triglycerides, (3) adjusted for HDL-C alone, and (4) adjusted for all risk factors listed for (2) and HDL-C and triglycerides. The result is the same if serum cholesterol and HDL-C are used as a ratio.

PON1 has a direct protective effect against CHD. Its capacity to predict coronary events, like its location and activity, is likely to be intimately linked with HDL. This would explain the suggestion from our present findings that PON1 activity and HDL-C are only partially independent CHD risk factors. We and others have shown that PON1 can prevent the oxidation of LDL both in vitro and in vivo. The present findings thus provide evidence in support of the hypothesis that the oxidative modification of LDL is important in the initiation and progression of atherosclerosis.⁷ The protection that the PON1 of HDL affords LDL against lipid peroxidation is substantially more prolonged than that of antioxidant vitamins,⁶ which have been notably unsuccessful in CHD prevention.^{33–36}

Our finding that PON1 activity was a stronger prognostic factor in the men at highest risk of a new CHD event, either because they already had clinical evidence of CHD or because other risk factors clustered in them as indicated by the Framingham risk equation, is consistent with several case-control studies in which low PON1 activity or PON1 polymorphisms associated with low activity have been reported to be more prevalent in patients at increased risk by virtue of other risk factors such as age, cigarette smoking, hypertension, hyperlipidemia, and diabetes mellitus.^{21,37} It suggests that, were it possible to increase PON1 activity by a nutritional or pharmacological intervention,³⁷ the adverse effect of some of other risk factors might be ameliorated.

The lack of any significant association between CHD and PON1 concentration requires explanation. We cannot exclude the possibility that PON1 concentration would have been predictive of coronary events in a larger study than the present one. However, our finding that the higher PON1 concentration was not statistically related to CHD risk, whereas hydrolytic activity toward paraoxon was, would suggest that the differential substrate activity of PON1 may be critical for its protective effect against atherosclerosis, at least within the concentration range encountered in our population. The concentration of the enzymatic catalyst is probably more critical to the reaction rate for hydrolytic activities of PON1, which are rapid, for example, with phenylacetate. The reaction rate with paraoxon (and lipid peroxides) is relatively low,^{1,9} and therefore the enzyme concentration may be less critical. Genetic and acquired factors might also influence the active site configuration on

PON1 so that PON1 activity may be more critical than concentration in atherogenesis.

A lipid environment is essential for the activity of PON1, because most of its substrates are highly hydrophobic.¹ The lipid composition of HDL, which is open to nutritional or pharmacological modification, may provide a means of modifying PON1 activity favorably. Furthermore, there may be opportunities to increase the expression of PON1 activity by nutritional or pharmacological effects on its genetic regulation³⁷ now that promoter polymorphisms of PON1 have been reported to be associated with early onset CHD.³⁸

Serum clusterin, which is present in the same HDL subspecies as PON1, did not predict new CHD events in this study, nor was it related to the presence of CHD in our previous case-control study.²² It is possible that the results of Navab et al,²⁴ obtained in fewer than 30 patients, were a reflection of an acute rather than chronic elevation, and additional studies should be conducted, specifically designed to test this possibility.

In conclusion, we have shown that low PON1 activity explains a significant proportion of the variation in CHD risk in middle-aged men. These results are likely to be explained by a derangement of PON1 activity toward lipid peroxides, which is reflected in its hydrolytic activity toward paraoxon.

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