

## **Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes.**

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### Research Article

Paraoxonase was identified as a genetic risk factor for cardiovascular disease (CVD) in recent studies focusing on a polymorphism affecting position 191. A second polymorphism of the paraoxonase gene affects position 54 and involves a methionine (M allele) to leucine (L allele) change. It was investigated in diabetic patients (n = 408) with and without vascular disease. There were highly significant differences in plasma concentrations and activities of paraoxonase between genotypes defined by the 54 polymorphism: MMAA, MLAA, LLAA; protein, 65.3±18.0, 77.9±18.0, 93.5±26.0 microg/ml; P < 0.0001: activity (phenylacetate), 48.6±13.5, 64.1±14.5, 68.1±13.0 U/ml; P < 0.0001. The 191 variant had little impact on paraoxonase concentrations. Homozygosity for the L allele was an independent risk factor for CVD (odds ratio 1.98 (1.07-3.83); P = 0.031). A linkage disequilibrium (P < 0.0001) was apparent between the mutations giving rise to leucine and arginine at positions 54 and 191, respectively. The study underlines that susceptibility to CVD correlates with high activity paraoxonase alleles. The 54 polymorphism would appear to be of central importance to paraoxonase function by virtue of its association with modulated concentrations. The latter could explain the association between both the 54 and 191 polymorphisms and CVD.

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# Paraoxonase Polymorphism Met-Leu54 Is Associated with Modified Serum Concentrations of the Enzyme

## A Possible Link between the Paraoxonase Gene and Increased Risk of Cardiovascular Disease in Diabetes

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### Abstract

Paraoxonase was identified as a genetic risk factor for cardiovascular disease (CVD) in recent studies focusing on a polymorphism affecting position 191. A second polymorphism of the paraoxonase gene affects position 54 and involves a methionine (M allele) to leucine (L allele) change. It was investigated in diabetic patients ( $n = 408$ ) with and without vascular disease. There were highly significant differences in plasma concentrations and activities of paraoxonase between genotypes defined by the 54 polymorphism: MMAA, MLAA, LLAA; protein,  $65.3 \pm 18.0$ ,  $77.9 \pm 18.0$ ,  $93.5 \pm 26.0$   $\mu\text{g/ml}$ ;  $P < 0.0001$ ; activity (phenylacetate),  $48.6 \pm 13.5$ ,  $64.1 \pm 14.5$ ,  $68.1 \pm 13.0$  U/ml;  $P < 0.0001$ . The 191 variant had little impact on paraoxonase concentrations. Homozygosity for the L allele was an independent risk factor for CVD (odds ratio 1.98 (1.07–3.83);  $P = 0.031$ ). A linkage disequilibrium ( $P < 0.0001$ ) was apparent between the mutations giving rise to leucine and arginine at positions 54 and 191, respectively.

The study underlines that susceptibility to CVD correlates with high activity paraoxonase alleles. The 54 polymorphism would appear to be of central importance to paraoxonase function by virtue of its association with modulated concentrations. The latter could explain the association between both the 54 and 191 polymorphisms and CVD. (*J. Clin. Invest.* 1997; 99:62–66.) Key words: atherosclerosis • genetics • HDL • lipid peroxidation • diabetes

### Introduction

Cardiovascular disease (CVD)<sup>1</sup> is the principal cause of mortality and morbidity in developed countries. It is also the pre-

dominant source of early mortality in diabetes mellitus (1). Several features of this metabolic disorder increase susceptibility to CVD (2) including lipid anomalies, endothelial dysfunction, thrombosis, and protein glycation. Of particular interest is oxidative stress which is considered a major contributor to the atherogenic process (3). It may also be an important factor in the higher incidence of CVD in diabetic patients as hyperglycemia would appear to predispose to oxidative stress (4, 5).

Consistent with their designation as the major atherogenic lipoprotein particles, low density lipoproteins (LDL) appear to be the principal target for oxidative modifications (6). Attention has thus focused on factors that may protect LDL from oxidation and this has highlighted a potential role for high density lipoproteins (HDL). Several mechanisms involving HDL have been proposed (6), including enzymatic removal of lipid peroxides. Paraoxonase, a serum enzyme entirely bound to HDL (7), was recently hypothesized to fulfill such a role (8, 9). Although the natural substrate for the enzyme is unknown (10), it was shown to limit the accumulation of lipid oxidation products in LDL (8, 11) and prevent the transformation of LDL into biologically active, atherogenic particles (11). The hypothesis was recently bolstered by a report identifying an oxidized phospholipid as a potential substrate for paraoxonase (11).

The clinical relevance of paraoxonase was first demonstrated by observations from our group. In ongoing studies of susceptibility genes for vascular disease in diabetes, we identified a polymorphism of the paraoxonase gene (*HUMPONA*) provoking a glutamine (A allele) to arginine (B allele) interchange at amino acid 191, as a genetic risk factor for ischemic heart disease in non-insulin-dependent diabetic (NIDDM) patients (12). The association was subsequently confirmed in a study of non-diabetic patients with coronary artery disease (13). The mechanism by which the polymorphism could increase susceptibility to CVD is unknown. It gives rise to differences in enzyme activity but this is presently defined by exogenous substrates and is not valid for all substrates (10). It would nevertheless appear to implicate enzyme activity in the increased susceptibility to CVD, although the nature of the endogenous substrate of paraoxonase (14) and the precise location of the active site of the enzyme (15) are unknown.

There exists a second, frequent polymorphism of the human paraoxonase gene affecting amino acid 54; it involves a conservative interchange of leucine and methionine (16, 17). The polymorphism was reported to be without consequences for enzyme activity in early studies comprising small numbers of subjects (16, 17). Given the growing importance of paraoxonase as a CVD risk factor and its potential involvement in protection against oxidative stress, we examined the polymor-

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1. Abbreviations used in this paper: CHD, coronary heart disease; CVD, cardiovascular disease; NIDDM, non-insulin-dependent diabetes mellitus.

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phism at position 54 and its association with (a) the presence of CVD, (b) the polymorphism at position 191, (c) serum lipid concentrations, and (d) enzyme activity and concentration. The results indicate that the paraoxonase gene polymorphism influencing position 54 is of physiological and clinical importance independently of the 191 variant. The data have implications for the relationship between paraoxonase gene polymorphisms and susceptibility to CVD.

## Methods

**Study population.** The subjects were Caucasian, NIDDM patients recruited as described previously (12). Of the patients studied in the previous report, DNA samples from 408 and serum samples from 294 subjects were available for the present investigation. The subjects were classified (12) as having coronary heart disease (CHD+,  $n = 168$ ; confirmed transmural myocardial infarction or positive coronary angiogram) or free of heart disease (CHD-,  $n = 240$ ; no history of angina pectoris and a normal resting electrocardiogram). The study protocol was approved by the Ethics Committee of the Centre Hospitalo-Universitaire Lariboisière-St-Louis, Paris. Written, informed consent was obtained from participants.

**DNA extraction and analysis.** DNA was extracted using standard procedures (18) from cells obtained from a fasting blood sample (12). The gene polymorphism (A [glutamine] and B [arginine] alleles) corresponding to position 191 was analyzed by restriction isotyping (12) using the procedure of Humbert et al. (17). A similar procedure was used to analyze alleles M (methionine) and L (leucine) derived from the polymorphism affecting position 54 of the paraoxonase gene. Briefly, the primers described by Humbert et al. (17) were used to amplify this polymorphic region. DNA (500 ng) was denatured at 94°C for 5 min and then amplified for 35 cycles; each cycle comprised denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 30 s, with a final extension time of 6 min. The PCR product (170 bp) was digested with Hsp 92 II (Promega, Madison, WI) in the presence of BSA (37°C, 3 h). The digested products were separated by acrylamide gel (8%) electrophoresis and identified by ethidium bromide staining. Allele L corresponds to the presence of a non-digested fragment of 170 bp, while allele M corresponds to 2 digestion fragments of 126 and 44 bp, respectively.

**Paraoxonase enzyme activity and serum concentration.** Enzyme activities of serum samples were assayed in Geneva as described previously (12, 19) using a six-cell automated Uvikon 810 spectrophotometer (Kontron, Eching, Germany). A control of pooled human sera (stored at -70°C) was used to correct for interassay variations. The enzyme activities of the control pool were confirmed by measurements kindly performed by Dr. M. Mackness (Department of Medicine, University of Manchester, Manchester, United Kingdom). The paraoxonase protein concentration of the pool was determined as described (20). Paraoxonase concentrations in serum samples were measured using a competitive ELISA (20) based on a pan-monoclonal antibody against human paraoxonase (7).

**Statistical analyses.** Continuous clinical and biological variables were analyzed using either one-way analysis of variance or the Mann-Whitney U test, depending on the shapes of the distribution curves. Categorical variables were compared between groups using the  $\chi^2$  test and crude odds ratio. Allele frequencies were estimated by the gene counting method and Hardy-Weinberg's equilibrium was tested by the  $\chi^2$  test. For paraoxonase polymorphism, statistical analyses were based on the calculation of odds ratios (i.e., the ratio of CHD+ to CHD- patients in the test group compared to the control group) to provide an estimate of the relative risk of CVD associated with paraoxonase LL and ML+MM genotypes. Multivariate analyses were performed using a logistic regression model adjusted for all parameters. The relationships between polymorphism 54 of paraoxonase, lipids, paraoxonase activity and concentration were tested using Wilcoxon/Kruskal-Wallis tests.

**Table I. Clinical and Anthropometric Characteristics of NIDDM Patients with (CHD+) and without (CHD-) Cardiovascular Disease**

Parameter	CHD+	CHD-	P value
Age	63.0±9.6	59.3±10.0	0.0002
Sex (M/F)	132/36	160/80	0.01
Smoking (yes/no)	104/66	102/136	0.004
Duration diabetes (yr)	15.6±10.4	13.3±8.2	0.06
Waist-to-hip ratio (normal/high)	139/29	160/80	< 0.0001
Systolic blood pressure (mmHg)	146.3±20.0	142.2±19.0	0.04
Diastolic blood pressure (mmHg)	83.4±12.0	80.9±10.0	0.02
Cholesterol (mm/liter)	5.97±1.20	5.88±1.10	0.45
Triglycerides (mm/liter)	2.05±1.30	1.70±0.90	0.008
HDL-cholesterol (mm/liter)	1.14±0.30	1.28±0.40	0.0004
Apo A-I (g/liter)	1.46±0.30	1.60±0.30	< 0.0001
Apo B (g/liter)	1.40±0.30	1.33±0.30	0.10

## Results

**Association between polymorphism 54 and CVD.** The clinical characteristics of the two groups of diabetic patients are shown in Table I. As observed previously (12), risk indices were significantly more pronounced in the patients with CVD. When the association between the polymorphism and CVD was analyzed, patients homozygous for leucine at position 54 were more prevalent in the CHD+ group (46.4% vs. 35.8%; odds ratio (LL vs. LM + MM) 1.55 (1.04–2.32);  $P = 0.02$ ). The polymorphism was independently and significantly associated with the presence of CVD when subjected to logistic regression analysis (Table II).

**Association between polymorphisms 54 and 191.** The genotype frequencies at both positions were in Hardy-Weinberg equilibrium. The distributions of the two polymorphisms in the combined population ( $n = 408$ ) of patients are shown in Table III. The distribution of alleles defined by position 54 was concordant with frequencies noted in a much smaller group of subjects (16) with LL and ML genotypes present in similar frequencies (0.40 and 0.45) and a much lower frequency of MM homozygotes (0.15). The distribution of genotypes associated with the 191 polymorphism corresponded to that observed in other Caucasian populations (10). As clearly shown in Table III, methionine at position 54 (M allele) was rarely associated with arginine at position 191 (B allele). Analysis confirmed a

**Table II. Logistic Regression Analysis of Determinants of CVD**

	Odds ratio (95% CI)	P value
Waist-to-hip ratio	2.38 (1.59–3.56)	< 0.0001
Age	1.08 (1.04–1.13)	0.0001
Apo A-I	0.17 (0.03–0.46)	0.002
Diastolic blood pressure	1.04 (1.01–1.08)	0.023
Smoking	1.14 (1.01–1.96)	0.04
Polymorphism 54 (LL/LM + MM)	1.98 (1.07–3.38)	0.031

The following were also included in the analysis but were not significantly associated with the presence of CVD: sex, systolic blood pressure, cholesterol, triglycerides, HDL-cholesterol, apo B.

**Table III. Distribution of Genotypes Defined by Polymorphisms of the Paraoxonase Gene Affecting Amino Acids at Positions 54 and 191**

	AA	AB	BB	Total
LL	45	89	30	164
LM	87	96	0	183
MM	57	4	0	61
Total	189	189	30	408

Paraoxonase genotype distributions are shown for the combined NIDDM population ( $n = 408$ ). A refers to glutamine and B to arginine at position 191 of the peptide sequence; L refers to leucine and M to methionine at position 54 of the peptide sequence.

linkage disequilibrium between the polymorphisms giving rise to leucine at position 54 and arginine at position 191 ( $P < 0.0001$ ).

*Association between polymorphism 54 and plasma lipids.*

The relationships between the different genotypes and plasma lipid and apolipoprotein concentrations are shown in Table IV for the combined population of diabetic patients. There were no significant differences in concentrations between the three genotypes, with the exception of apo A-I which tended to be lower in MM homozygotes.

*Association between polymorphism 54 and paraoxonase activity/concentration.* Enzyme activities as a function of the 54 and 191 polymorphisms are illustrated in Table V for substrates that are discriminatory (paraoxon) and non-discriminatory (phenylacetate) with respect to position 191. With paraoxon as substrate and in the presence of 1M salt there was the expected, highly significant increase in activity (10) when passing from the A to the B allele (191 polymorphism). This was observed irrespective of the genotype defined by the polymorphism at position 54. With phenylacetate as substrate, increases in activity associated with polymorphism 191 were far less evident. In contrast, there were highly significant decreases in enzyme activities with both substrates when passing from the L to the M allele (54 polymorphism), independently of the polymorphism at position 191 (Table V).

Serum concentrations of paraoxonase as a function of the polymorphisms are shown in Table VI. There were highly significant ( $P < 0.0001$ ) differences in serum levels between genotypes defined by position 54; the L allele was associated with higher concentrations of the enzyme, irrespective of the presence of glutamine or arginine at position 191. Indeed, polymorphism at the latter position appeared to be without impact

**Table IV. Plasma Lipid and Apolipoprotein Concentrations as a Function of the Polymorphism at Position 54**

	LL	LM	MM
Cholesterol (mm/liter)	6.01±1.20	5.82±1.11	5.91±1.10
Triglycerides (mm/liter)	1.95±1.40	1.74±0.93	1.90±0.84
HDL-cholesterol (mm/liter)	1.21±0.40	1.25±0.37	1.19±0.38
Apo A-I (g/liter)	1.56±0.30	1.58±0.30	1.46±0.30*
Apo B (g/liter)	1.37±0.30	1.36±0.30	1.37±0.30

Analysis of variance was employed to examine differences between the groups. \*  $P < 0.05$ .

**Table V. Paraoxonase Activities as a Function of Polymorphisms at Positions 54 and 191**

	AA	AB	BB	P value
<b>Phenylacetate</b>				
LL	68.1±13.0	67.1±15.5	54.3±15.3	0.02
LM	64.1±14.5	57.1±15.0	—	< 0.01
MM	48.6±13.5	42.6±14.0	—	NS
P value	< 0.0001	< 0.0001	—	
<b>Paraoxon</b>				
LL	110.0±32.2	282.7±76.9	412.8±139.5	< 0.0001
LM	94.2±19.7	255.0±78.7	—	< 0.0001
MM	76.9±7.8	176.9±25.0	—	< 0.0001
P value	0.0003	0.03	—	

Activities (U/ml) are means±SD. Activity with paraoxon was measured in the presence of 1 M NaCl. Differences between genotypes were analyzed by ANOVA. NS, not significant.

on serum concentrations of paraoxonase. Some 19% of the variation in paraoxonase concentrations could be explained by the 54 polymorphism compared with 1–2% by the 191 polymorphism. Table VI also gives the specific activities (activity/mg protein) for the substrate phenylacetate. Corrected for protein, ANOVA revealed no significant activity differences between the three genotypes defined by polymorphism 54.

**Discussion**

The present study has furnished two important and novel observations concerning paraoxonase. First, it has demonstrated that the polymorphism affecting position 54 is associated with marked modulations of serum concentrations of the enzyme. As a consequence, the alleles exhibit substantial differences in enzyme activity not only towards substrates considered discriminatory (paraoxon) but also substrates considered non-discriminatory (phenylacetate) for position 191. Thus the polymorphism at position 54 is associated with a clear-cut physiological consequence. This contrasts with the polymorphism at position 191 whose physiological relevance is pres-

**Table VI. Plasma Concentrations and Specific Enzyme Activities of Paraoxonase as a Function of Polymorphisms 54 and 191**

	AA	AB	BB	P value
<b>Plasma concentration</b>				
LL	93.5±26.0	100.4±28.0	79.8±25.0	< 0.05
LM	77.9±18.0	78.9±18.0	—	NS
MM	65.3±18.0	57.1±13.0	—	NS
P value	< 0.0001	< 0.0001	—	
<b>Specific activity</b>				
LL	0.73±0.16	0.69±0.11	0.75±0.19	NS
LM	0.79±0.14	0.74±0.17	—	NS
MM	0.76±0.19	0.72±0.07	—	NS
P value	NS	NS	NS	

Paraoxonase concentrations are given as µg/ml (±SD). Specific activities were determined as U/µg. Analysis of variance was used to test for differences between the groups. NS, not significant.

ently unclear, defined as it is by non-natural substrates. The second observation is that the polymorphism at position 54 is associated with the occurrence of CVD. In addition to these observations, the study demonstrated that there is linkage disequilibrium between polymorphisms giving rise to leucine and arginine at positions 54 and 191 respectively.

Our previous report (20) that the B allele was associated with higher plasma concentrations of paraoxonase is clarified in this study. The polymorphism at position 191 does not influence serum concentrations as, for a given 54 allele, the 191 polymorphism has no consistent impact on paraoxonase concentrations. In contrast, for a given allele at position 191, there was a consistent decrease in concentrations when passing from the L to M allele. The earlier observation would appear to be related to the polymorphism at position 54 (L allele) and a linkage disequilibrium between the L and B alleles. Our results suggest that polymorphism at position 54 may be a major determinant of, or marker for, variable protein concentrations.

Whether the polymorphism at position 54 is causally implicated in modulated paraoxonase concentrations remains to be established. Interestingly, the mutation occurs in the NH<sub>2</sub>-terminal region of the peptide where a highly hydrophobic sequence may facilitate binding of paraoxonase to HDL (21). We have previously observed (20) a positive correlation between apo A-I, the structural peptide of HDL, and paraoxonase concentrations (confirmed in the present study, data not shown). It concurs with the greatly reduced paraoxonase activity reported in HDL deficient patients (22, 23). Thus HDL appears necessary to maintain an active form of paraoxonase. Accordingly, factors which influence the ability of paraoxonase to complex with HDL could modulate plasma concentrations of the enzyme. If the polymorphism at position 54 affects the conformation of the NH<sub>2</sub>-terminal region, this would provide a mechanism by which it may directly influence plasma paraoxonase concentrations.

Two independent studies have demonstrated that paraoxonase is a genetic risk factor for CVD (12, 13). The evident question is whether the enzyme directly influences susceptibility to disease and by what mechanism. Several groups (20, 24, 25) have observed weak associations between paraoxonase and plasma lipids/apolipoproteins. Hegele et al. (25) recently proposed that the 191 polymorphism (B allele) is associated with a comparatively more atherogenic lipid profile. We were unable to confirm this conclusion in our previous study of NIDDM patients (12). Neither did we observe an association of the 54 polymorphism with a less favorable lipid profile in the present study. Alternatively, evidence is accumulating for a role of paraoxonase in the hydrolysis of oxidized lipids and consequent protection of LDL against oxidative modifications (8, 9, 11). Herein lies a dilemma. The alleles (B and L) associated with greater susceptibility to CVD also display higher absolute enzyme activity, in large part due to modulated protein concentrations, as we demonstrate herein. One possible explanation therefore is that paraoxonase activity may, under certain conditions, be detrimental. It could give rise to potentially harmful lysolipids (26) or yield a more atherogenic profile (25). There exist precedents of factors implicated in normal lipid metabolism which can have a deleterious influence under certain conditions. A striking example is hepatic lipase. The enzyme functions to complete the transformation of very low density lipoproteins into LDL and remodel postprandially-modified HDL (27, 28). Under conditions where LDL and

HDL become pathologically enriched in triglycerides the action of hepatic lipase is accentuated producing smaller lipoprotein particles which are considered to be more atherogenic (LDL) or less protective against CVD (HDL) (27, 29).

An alternative point is that considerations of enzyme activity are influenced by the positive modulation exerted by the B allele on paraoxon hydrolysis and the implication that this has a physiological correlate. The latter has not been demonstrated. Indeed, as indicated by La Du (10), the non-discriminatory substrate phenylacetate is a more suitable substrate for paraoxonase. If one examines specific enzyme activity with phenylacetate as substrate, there are subtle but significant differences. Thus a comparison of patients with and without the susceptibility alleles (that is LL/AB + BB vs. ML + MM/AA) reveals significant differences in mean specific activities:  $0.681 \pm 0.015$  vs.  $0.776 \pm 0.019$  U/ $\mu$ g protein,  $P < 0.05$ . Could this be of physiological significance? In serum, paraoxonase appears to be restricted to and thus active within HDL particles. There is usually a defined stoichiometry concerning the apolipoproteins present in HDL complexes (30–32). If there is a defined number of paraoxonase molecules per HDL particle, activity of HDL containing B or L alleles will be less than that of HDL containing A or M alleles. While highly speculative, this could provide the basis for less efficient hydrolysis of lipid peroxides, concordant with the association of B and L alleles with an increased susceptibility to CVD.

The present study has several important implications for paraoxonase and its relationship with CVD. First, should the enzyme be considered a positive or negative risk factor for disease? The study underlines that high activity alleles are associated with CVD, and while a speculative case can be made for lower specific activity, the question remains whether it is increased or relatively reduced activity that determines susceptibility. Second, the 54 polymorphism may be in part responsible for the previously reported relationship between CVD and the 191 variant, due to the linkage disequilibrium between the L and B alleles. Finally, it is important to define the interaction between paraoxonase and HDL/apo A-I as this could explain modulated concentrations associated with the 54 variant and perhaps susceptibility to CVD. It would appear that the polymorphism affecting position 54 is of central importance to paraoxonase function by virtue of its association with serum concentrations and, consequently, enzyme activity. These effects are independent of the 191 polymorphism.

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