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Human paraoxonase gene cluster polymorphisms as predictors of coronary heart disease risk in the prospective Northwick Park Heart Study II

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Abstract

The anti-atherogenic effect of HDL has been suggested to be partly due to the action of HDL-associated paraoxonase (PON). Three distinct enzymes have been identified, encoded by *PON1*, *PON2* and *PON3*, clustered on chromosome 7q21–q22. Two cSNPs in *PON1* (L55M and Q192R) and one in *PON2* (S311C) have been implicated as independent risk factors for coronary heart disease (CHD) in some, but not all, studies. A *PON3* SNP (A99A) was identified and the effect of these four *PON* SNPs on HDL levels and CHD risk was examined in the prospective Northwick Park Heart Study II (NPHSII). Genotype frequencies did not differ between cases and controls but the CHD risk associated with smoking was significantly modified by *PON1* L55M genotype. Compared to LL non-smokers, LL smokers had a hazard ratio (HR) of 1.30 (95% CI 0.81–2.06) while M-allele carriers had a HR of 1.76 (1.17–2.67). When genotypes were analysed in combination, men with the genotype *PON1* 55 LM/MM + *PON2* 311 CC, had HR of 3.54 (1.81–6.93) compared to *PON1* LL + *PON2* SS/SC men (interaction $P = 0.004$). These effects were independent of classical risk factors. These data demonstrate the importance of stratifying by environmental factors and the use of multiple SNPs for genetic analysis.

Keywords: Paraoxonase; Polymorphism; *PON1*; *PON2*; *PON3*

1. Introduction

Human serum paraoxonase (PON1) is a 44-kDa glycoprotein [1] located almost exclusively on apolipoprotein (apo) AI and apoJ containing high-density lipoprotein (HDL) [2]. HDL levels are inversely proportional to the risk of coronary heart disease (CHD) (reviewed in Ref. [3]), and HDL has been shown to prevent the oxidative modification of low-density lipoprotein (LDL) in vivo and in vitro [4]. This anti-oxidative property has been attributed to serum PON [5,6], which also inhibits modification of HDL by lipid peroxidation [7]. These anti-oxidant properties have been confirmed in mouse models, with *pon1* knockout mice being more susceptible to developing atherosclerotic lesions than their littermates [8], in a dose dependent manner [9].

PON1 is a member of a multigene family, which includes *PON2* and *PON3* [10]. The gene cluster maps to chromosome 7q21–q22 [11] and covers approximately 136kb. Several *PON1* SNPs have been identified, among them L55M and Q192R. The Q192R 'activity' polymorphism [11] is so called because of the eightfold increase in hydrolysis activity toward paraoxon demonstrated by the R allele; this activity is substrate-dependent with the Q isoform showing greater activity against nerve gases such as soman and sarin [12]. Both the Q192R and L55M polymorphisms have been associated with CHD in some [13,14], but not all, studies [15,16]. PON1 levels are lower in patients suffering a myocardial infarction (MI) [17], in patients with familial hypercholesteraemia (FH) and insulin-dependent diabetes [18], suggesting an important role for serum PON in CHD. PON1 levels and activity vary markedly between individuals (10–30-fold) [12] as well as

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between different ethnic groups [19], which may be part of the reason for lack of clarity on this matter.

Several variants of *PON2* have also been reported, with almost complete allelic association between an A148G change and a C311S change [20]. The presence of the C allele of S311C variant has been associated with an independent protective effect in FH patients [21], and in an Asian–Indian study [22], but not in a Japanese study [23]. The *PON2* S311C variant has also been shown to interact with *PON1* Q192R in an additive manner in determining CHD risk [22]. The third *PON* gene (*PON3*) has, as yet, no confirmed coding region polymorphisms but a recent report [24] identified a −133C>A polymorphism in the promoter region which was not found to be associated with a significant effect on CHD risk.

The purpose of the present study was to examine in a large prospective study the previously reported associations of the *PON* gene cluster polymorphisms, specifically the combined effect of *PON1* and *PON2* genotypes on CHD risk and to identify and evaluate the effect of *PON3* variants. The fact that smoking reduces PON1 activity directly [25] suggests PON1 may play an important role in smoking-associated risk of CHD; thus, an additional aim of this study was to examine whether the *PON* cluster polymorphisms modified the smoking-associated risk of CHD.

2. Methods

2.1. Subjects

Three thousand and fifty two (3052) healthy men aged 50 to 61 years, registered with nine general medical practices, were recruited for prospective surveillance. The methods used have been described previously [26]. Briefly, all were free of a history of unstable angina, myocardial infarction or evidence of a silent infarction, coronary surgery, aspirin or anticoagulant therapy, cardiovascular disease, malignancy (except skin cancer other than melanoma), or any condition precluding informed consent. Each participant attended non-fasting, having been instructed to avoid heavy meals before examination and to refrain from smoking or vigorous exercise from the preceding midnight. Each answered a questionnaire for smoking habit [27]. A smoker was defined as any man who had smoked at least one cigarette/day on average for a year or more. Those smokers who had not smoked to this extent in the previous year were categorised as ex-smokers; all other men were classified as never-smokers. Smoking questionnaires were completed at baseline and annually to year 5. Alcohol consumption was assessed by questionnaire, and stratified according to WHO guidelines as those who consumed more or less than 21 units/week. A standard 12-lead electrocardiogram (ECG) was recorded and coded according to Minnesota criteria [28] (42 men with changes indicative of myocardial infarction (codes 1_1, $1_{2.1}$, to $1_{2.7}$ or $1_{2.8}$ plus 5_1 or 5_2) were excluded from the study). Height (m) was measured on a stadiometer and weight (kg) on a balance scale to calculate body mass index (BMI, kg/m^2). Survivors have been re-called annually for interview and repeat measurements. A routine ECG was repeated at the sixth examination. The study had ethical approval from the local MRC institutional review committee and patients gave written, informed consent. To date, there have been 205 documented CHD events, in the subjects with DNA available for analysis (fatal + non-fatal MI = 147, need for CABG = 39, ECG ischaemia = 19).

2.2. Plasma measures

A 5-ml sample of venous blood was taken by Vacutainer technique (Becton Dickenson, Cowley, Oxford) into a glass tube. Serum was transferred to plastic screw-cap vials (Nunc) and stored at −40 °C pending analysis. Cholesterol and triglyceride concentrations were determined by automated enzyme procedures with reagents from Sigma (Poole, Dorset, UK) and Wako Chemicals (Alpha Laboratories, Eastleigh, UK), respectively. Serum apolipoprotein AI (apoAI) concentrations were measured by immunoturbidometry with reagents from Incstar (Wokingham, UK). HDL-C was measured using polyethylene glycol 8000 and enzymatic colorimetry on the sample of plasma taken at year 6 [29].

2.3. DNA extraction and genotyping

DNA was extracted by the salting-out method [30]. All PCRs were performed using an MJ Research PTC-225 Peltier Thermal Cycler, in a 20-μl reaction volume using 1 × KCl buffer containing 50 mM KCl, 10 mM Tris (pH 8.3), 0.001% gelatin and 0.2 mM each dNTP. *PON1* L55M (sometimes referred to as L54M [31]) was determined using the conditions of Humbert et al. [11], with an annealing temperature of 58 °C and 1.5 mM $MgCl_2$. Digestion was performed using *Hsp92*II (Promega, Madison) resulting in a 170-bp undigested fragment for the L allele and 130- and 40-bp fragments for the M allele. For the *PON1* Q192R polymorphism, primers were designed as follows: 5′-GTGTGTATGTTTTAATTGCAGTTTGA-3′ and 5′-AGTAGACAACATACGACCACGCTA-3′. An annealing temperature of 58 °C was used and 2.0 mM $MgCl_2$. Digestion with *Alw*I (New England Biolabs, Cambridge) resulted in 76- and 70-bp fragments for the Q allele and 70-, 48- and 28-bp fragments for the R allele. *PON2* S311C polymorphism was determined according to Sanghera et al. [22]. Digestion of the PCR product by *Dde*I (New England Biolabs) resulted in fragments of 120, 75 and 65 bp for the S allele and fragments of 142 and 120 bp for the C allele. Digested products of the L55M and Q192R polymorphisms were separated on 7.5% MADGE [32], while the *PON2* S311C was separated on a 9% MADGE. All primers were obtained from Invitrogen.

For *PON3*, two unvalidated SNPs were identified from the database and primers were designed to span both polymorphisms, the forward sequence was 5′-TCCAGGCATGCCAAACTTT-3′ and the reverse sequence was 5′-TTTCCCTCATTTCCCCCTT-3′. Primers were obtained from Qiagen. Conditions used were 2.25 mM $MgCl_2$, with an annealing temperature of 54 °C for 30 cycles. Digestion recognition sequences were, for the A99A polymorphism GCGC (*Hha*I) or RGCGCY (*Hae*II), thus the G allele version would be digested by the enzyme. For the D107N polymorphism the recognition site was TTAA (*Mse*I), resulting in digestion in the presence of the A (Asp). Samples were amplified by PCR and 8 μl of PCR product was digested with 5 μl of digest mix containing 2 units of *Hha*I, or 2 units of *Mse*I, in separate reactions. In the case of the A99A digest, a GG homozygote resulted in 112-, 63- and 22-bp fragments, the AA homozygote in 175- and 22-bp fragments and the GA heterozygote in 175-, 112-, 63- and 22-bp fragments. These were detected on a 7.5% MADGE. For the D107N, two fragments of 120 and 77 bp were predicted for the GG homozygote, three fragments of 105, 77 and 15 bp for the AA homozygote, and for the GA heterozygote four fragments of 120, 105, 77 and 15 bp. All genotypes were read and cross-checked against the computer database entry by two observers blind to status. Discrepancies were resolved by re PCR and analysis; any samples lacking a genotype had failed to PCR on at least two occasions.

2.4. Statistical analysis

Statistical analysis was conducted in intercooled STATA version 7.0 (STATA Co-operation, Texas). The 71 individuals with diabetes at entry to the study were excluded from all analysis. Deviations from Hardy–Weinberg were considered using chi-squared tests. Linkage disequilibrium between variant sites was determined using Δ [33]. Log-transformations were conducted for data which were not normally distributed (body mass index, fibrinogen, apoB, systolic blood pressure, triglyceride and HDL). For variables that required log-transformations, geometric means and approximate S.D.'s are presented. One-way analysis of variance (or Kruscal–Wallis when appropriate) was used to assess differences in continuous baseline characteristics by CHD status, using either the raw values or log-transformed values as appropriate; there was no evidence of heteroscedacity between groups, considered via Bartlett's test. Differences in HDL, apoA1 and fibrinogen levels by genotype was considered using ANOVA. HDL was measured at year 6 and apoAI at baseline and there was strong correlation between these measures ($r=0.51$, P value <0.0005). Survival analysis with respect to genotypes was conducted using Cox's proportional hazards model, thus allowing for varying follow-up intervals and censoring due to competing events. For this modelling, 'failure time' was taken as the time to the first CHD event. The significance of the parameters in the Cox model was assessed using the Likelihood Ratio (LR) Test; 95% Confidence Intervals (CI) for the estimates were calculated from the standard errors assuming a normal distribution. All results were exponentiated and are presented as hazard ratios (HR) with their corresponding 95% CI. Survival analysis was adjusted for age by including the term in the model and differences in the baseline hazard by practice were permitted (using the strata option in STATA); adjustments for covariates were made by including them in the model. For all genotypes, individuals homozygous and carrying the rare allele were grouped for comparison of combined genotype effects; this was an a priori decision based on maintaining adequate sample size. Interactions were primarily considered as deviations from multiplicative effects in the survival model. The relative excess risk due to interaction (RERI) was used as a measure of deviation from additive effects and was calculated as: relative risk (combined genotype effect) − relative risk (effect of PON1 only) − relative risk (PON2 only) + 1. A value of 0 represents no deviation from additive effects, and 95% CI were calculated using bootstrapping [34]. Survival probability was examined graphically using a Kaplan–Meier plot. Haplotypes were estimated using PHASE [35]; individuals were only included in later analysis of haplotypes if their phase calls were made with greater than 0.9 probability. In all cases, a P value of <0.05 was considered statistically significant. Power calculations were performed, and based on previous genotype frequency estimates and the 205 CHD events, the study has 80% power to detect a risk of 1.52 in *PON1* 55 M carriers, 1.51 in *PON1* 192R carriers, and 1.61 in *PON2* 311 C carriers ($P=0.05$).

3. Results

3.1. Characteristics of the study group

The NPHSII (Northwick Park Heart Study II) study consists of healthy middle-aged Caucasian UK males, 2702 of whom were included in the analysis based on the fact that there was genotype data for at least one of the polymorphisms of interest. Baseline clinical and biochemical characteristics of the men are summarised in Table 1, divided on the basis of whether they had suffered a CHD event or were event-free. The classical risk factors for CHD show a significant difference between those individuals who have remained healthy and those who have had an event. In the CHD group, there was a higher percentage of current smokers ($P<0.0005$), and body mass index and systolic blood pressure were significantly higher. Cholesterol, triglycerides, apoB, and Lp(a) were higher in the event group with HDL and apoAI being significantly lower. Fibrinogen levels were also significantly different between the groups.

Table 1
Baseline characteristics (mean ± S.D.) by CHD event status and genotype frequency

Variable		No CHD event $n=2510$	CHD event $n=192$	P value	
Age		56.00 (3.43)	56.56 (3.55)	0.15	
Smoking (% current)		27.8%	40.6%	<0.0005	
BMI[a]		26.2 (3.3)	26.8 (3.4)	0.008	
Systolic BP[a]		136.6 (18.7)	141.6 (19.9)	0.0005	
Cholesterol		5.71 (1.00)	6.05 (0.99)	<0.00005	
Triglyceride[a]		1.76 (0.92)	2.07 (1.07)	<0.0001	
ApoB[a]		0.86 (0.24)	0.92 (0.22)	0.0001	
HDL[a]		0.80 (0.24)	0.74 (0.20)	0.02	
ApoA1		1.64 (0.32)	1.57 (0.27)	0.01	
Lp(a)[a]		8.7 (2.7, 25.5)	12.0 (4.0,28.8)	0.02	
Fibrinogen[a]		269.9 (51.5)	283.6 (50.4)	0.0005	
Genotype		Genotype distribution (11/12/22), Rare allele freq (95% CI)	Genotype distribution (11/12/22), Rare allele freq (95% CI)	P values for difference in	
				Genotype distribution	Allele frequency
PON1	L55M	980/982/249 0.34 (0.32, 0.35)	78/71/23 0.34 (0.29, 0.39)	0.60	0.98
	Q192R	1219/1000/205 0.29 (0.28, 0.30)	100/69/15 0.27 (0.22, 0.31)	0.56	0.96
PON2	S311C	1307/873/117 0.24 (0.23, 0.25)	109/53/13 0.23 (0.19, 0.28)	0.03	0.96
PON3	A99A	664/1201/588 0.47 (0.47, 0.50)	49/94/47 0.50 (0.44, 0.55)	0.92	0.93

[a] Distribution of data showed significant deviation from normality, so statistical analysis carried out on ln-transformed data, and geometric means and approximate S.D.'s are presented.

3.2. PON3 polymorphism identification

Two unvalidated, inferred polymorphisms in the coding region of *PON3* (both in exon 4) were identified from a search of the database (http://www.ncbi.nlm.nih.gov:80/LocusLink/). The first polymorphism at amino acid position 99 (rs1053275) is a G -to-A substitution resulting an synonymous change, Ala (GCG) to Ala (GCA). The second inferred polymorphism is present at amino acid position 107 (rs2345003) and is also a G-to-A change, predicting a non-synonymous change from an Asp (GAC) to an Asn (AAC). Primers were designed to amplify a 197-bp region spanning amino acid positions 99 and 107 using restriction enzyme digestion to distinguish alleles, *Hha*I for A99A and *Mse*I for D107N. One hundred and eighty-six samples from the NPHSII study were amplified by PCR and digested with *Hha*I. In this sample the allele frequencies of the G and A alleles were found to be 0.51 and 0.49, respectively; this did not differ significantly from the expected Hardy–Weinberg proportion. For the *Mse*I digestion, all 186 samples showed the same digestion pattern of two fragments, 120 and 77 bp in size, suggesting that no polymorphism is present at this site. The remaining samples from the NPHSII study were genotyped only for the A99A polymorphism.

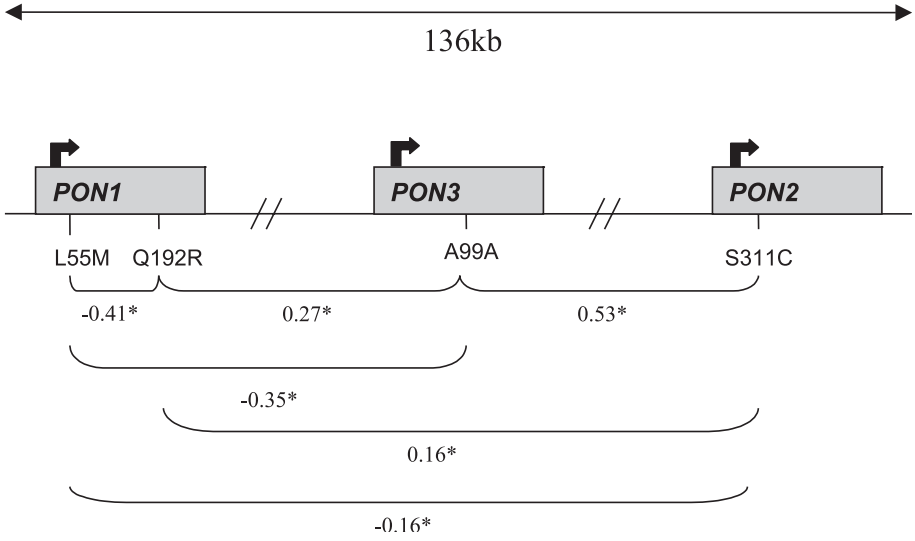

Fig. 1. *PON* gene cluster linkage disequilibrium analysis. Linkage disequilibrium, calculated as delta values, *$P<0.00005$.

Table 2
Mean (± S.D.) HDL and apoAI levels by genotypes

Genotype		HDL (mmol/l)[a]	*P* values	ApoA1 (mg/l)	*P* values
L55M	LL	0.81 (0.25)	0.35	1.63 (0.32)	0.77
		n = 716		*n* = 906	
	LM	0.80 (0.24)		1.63 (0.32)	
		n = 699		*n* = 909	
	MM	0.78 (0.23)		1.65 (0.30)	
		n = 176		*n* = 230	
Q192R	QQ	0.80 (0.24)	0.74	1.63 (0.30)	0.98
		n = 879		*n* = 1132	
	QR	0.81 (0.25)		1.63 (0.34)	
		n = 711		*n* = 894	
	RR	0.80 (0.25)		1.63 (0.32)	
		n = 153		*n* = 191	
S311C	SS	0.79 (0.25)	0.10	1.63 (0.33)	0.31
		n = 934		*n* = 1194	
	SC	0.82 (0.24)		1.64 (0.32)	
		n = 631		*n* = 800	
	CC	0.77 (0.24)		1.59 (0.32)	
		n = 86		*n* = 109	
$A_1 99 A_2$	A_1A_1	0.80 (0.24)	0.78	1.62 (0.32)	0.54
		n = 460		*n* = 592	
	A_1A_2	0.80 (0.24)		1.64 (0.32)	
		n = 885		*n* = 1120	
	A_2A_2	0.81 (0.25)		1.64 (0.33)	
		n = 426		*n* = 546	

[a] Distribution of data showed significant deviation from normality, so statistical analysis carried out on ln transformed data, and geometric means and approximate S.D.'s are presented.

3.3. Genotype frequency and distribution

The genotype distribution and frequencies are presented in Table 1; all were in Hardy–Weinberg equilibrium in both the CHD event and the event-free groups and allele frequencies did not differ between the two groups. The linkage disequilibrium (LD) across the cluster is shown in Fig. 1. Overall, there was significant LD across the cluster. *PON1* L55M showed negative LD with all the sites and this was strongest with the Q192R. LD between the *PON3* A99A and *PON1* and *PON2* polymorphisms was also significant in the whole sample. Using the PHASE haplotype assigning programme, it could be deduced that the frequency of the four halplotypes was 55L-192Q, 0.365; 55L-192R, 0.293; 55M-192Q,0.303; 55M-192R, 0.010; thus, the 55L allele occurs roughly 55% of the time with 192Q and 45% with 192R, while the 55M allele occurs with 192Q 97% of the time.

3.4. ApoAI and HDL level association with genotype

The relationship between *PON* genotypes and HDL and apoAI levels is presented in Table 2; overall, there was no significant association detected for any genotype. The correlations between HDL-cholesterol and ApoAI levels in individuals with different PON genotypes were high (between 0.41 and 0.61, all $P < 0.001$) but were not significantly different between groups (data not shown).

3.5. Genotype and smoking interaction

The possible modifying effect of *PON* genotype on the smoking-associated risk of CHD was examined. The reported number of cigarettes used per week was not different between genotype groups (all $P > 0.7$). Overall, in this sample smokers had 1.75-fold (1.31–2.34) higher risk of CHD compared to non-smokers. As shown in Table 3, compared to *PON1* LL non-smokers, in the smokers only carriers of the M allele, but not LL subjects, had a higher risk of an event, showing borderline interaction of smoking and genotype (LM + MM smokers HR = 1.76 (1.17–2.67); *P* for interaction = 0.09). This effect remained after adjusting for BMI, blood pressure, cholesterol, HDL-C and triglyceride level in addition to age and practice (HR = 1.92, 1.22–3.03). The higher rate of CHD events in this group is evident in the Kaplan–Meier survival plot (Fig. 2a). For the Q192R genotype, the smoking-associated

Table 3
Risk of CHD by genotype and smoking status[a]

Genotype		Non No/event	smokers HR (95% CI)	Current No/event	smokers HR (95% CI)	*P* value for interaction
L55M	LL	735/50	1	323/28	1.30 (0.81, 2.06)	0.22
	LM	760/40	0.76 (0.50, 1.15)	293/31	1.64 (1.05, 2.57)	
	MM	191/13	0.94 (0.51, 1.73)	68/10	2.29 (1.16, 4.53)	
Q192R	QQ	951/62	1	368/38	1.69 (1.13, 2.53)	0.31
	QR	777/38	0.75 (0.50, 1.13)	292/31	1.70 (1.10, 2.62)	
	RR	146/10	1.08 (0.55, 2.11)	74/5	1.04 (0.41, 2.59)	
S311C	SS	1012/65	1	404/44	1.71 (1.16, 2.51)	0.89
	SC	660/30	0.72 (0.47, 1.11)	266/23	1.44 (0.89, 2.33)	
	CC	93/9	1.56 (0.77, 3.14)	39/6	2.66 (1.14, 6.17)	
$A_1 99 A_2$	A_1A_1	514/31	1	199/18	1.60 (0.89, 2.87)	0.46
	A_1A_2	923/58	1.07 (0.69, 1.66)	372/36	1.69 (1.04, 2.74)	
	A_2A_2	450/24	0.93 (0.54, 1.58)	185/23	2.33 (1.30, 3.84)	

[a] Adjusted for age and practice.

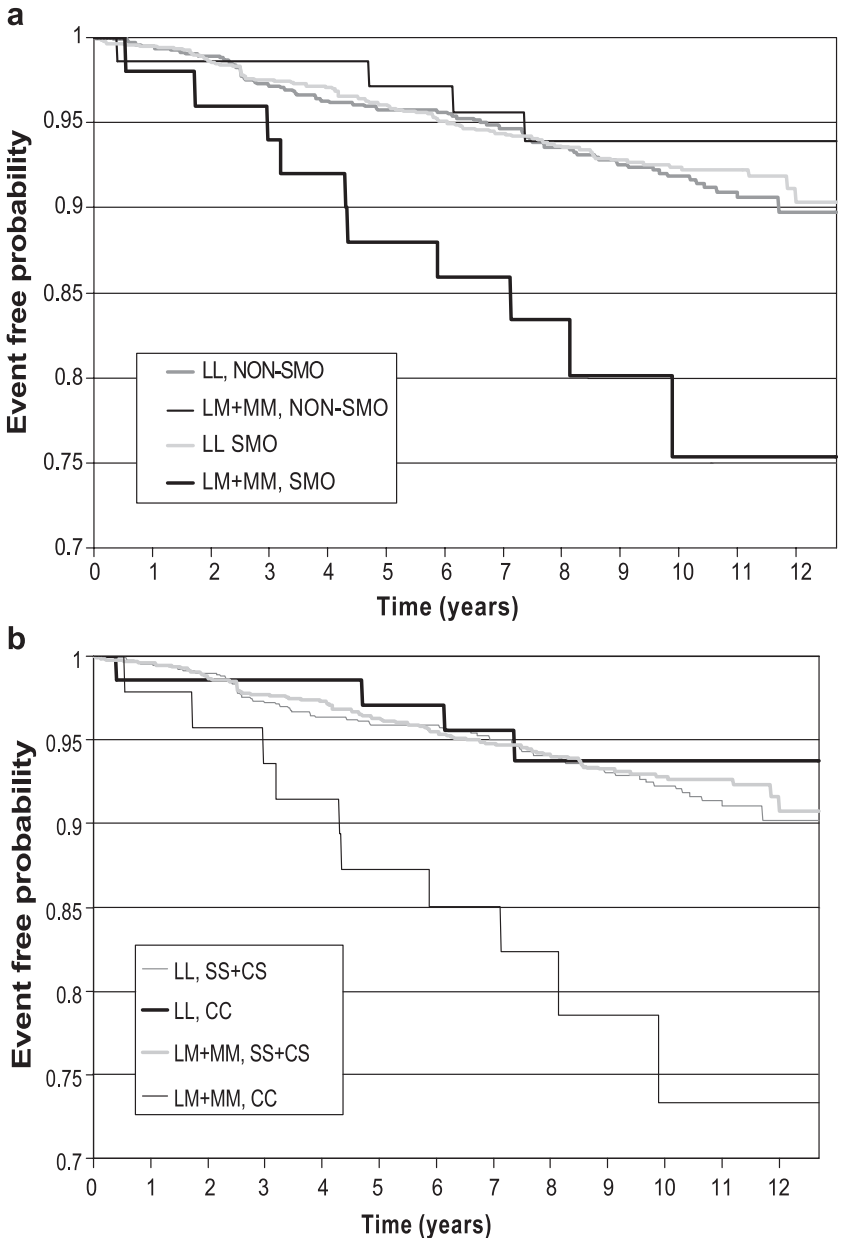

Fig. 2. Kaplan–Meier survival plots for: (a) smoking and *PON1* L55M, adjusted for age and practice, interaction $P = 0.003$; (b) *PON1* L55M and *PON2* S311C genotypes combined, adjusted for age and practice, interaction $P = 0.02$.

risk appeared to be less in the RR homozygotes, but this is the smallest group ($N = 79$) and no strong inference can be made.

3.6. Combined genotype effect on CHD risk

As shown in Fig. 3, when genotypes were analysed in pairwise combinations, a significant effect on CHD risk was found in men homozygous for the *PON2* C allele who also carried the *PON1* M allele (LM/MM), who showed higher CHD risk compared to SS/LL men (OR = 3.54; 95% CI = 1.81–6.93). Deviation from additive effects of the genotypes was analysed and greater than additive effects were confirmed [RERI: 2.67 (0.55, 6.18)]. The data are presented graphically in Fig. 2b as a survival plot, showing the higher event rate in this combined genotype group. The relatively small number of subjects with this genotype combination ($n = 47$) precluded an analysis of potential interaction with smoking, but after adjustment for classical risk factors, including smoking, the effect remained statistically significant (HR = 4.84(2.42–9.68). No significant effect on risk was observed for the *PON2* S311C and *PON1* Q192R genotype combination.

Haplotype analysis of all four genotypes from the three *PON* genes did not identify a risk-associated haplotype (data not shown). This is partly because of the low numbers of

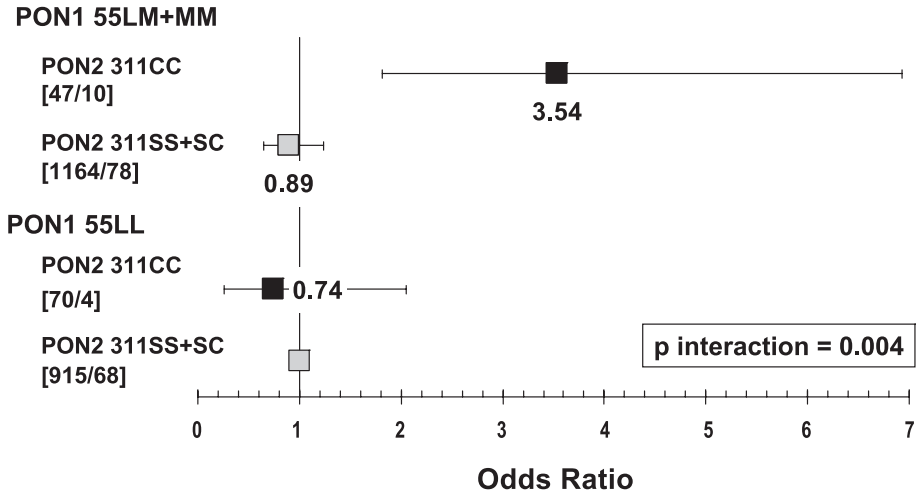

Fig. 3. Odds ratio for CHD by combined *PON1* L55M and *PON2* S311C genotypes.

individuals that could be considered for the analysis, since in only 61% of subjects could a 'phase call' be made with sufficient precision (probability>0.9). These low phase calls are most likely the result of the weaker LD between PON1 and PON3.

4. Discussion

4.1. PON gene variants and CHD risk

The present study of over 2700 healthy middle-aged men evaluated the association of four polymorphisms in the *PON* gene family with the risk of CHD. Although no single polymorphism was associated with a statistically significant independent effect on risk, the *PON1* L55M, when considered together with *PON2* S311C, did influence risk of CHD, and the *PON1* L55M modified the CHD risk associated with smoking. Two reported *PON3* variants were examined; one could not be verified and the A99A, although common, was not associated with any effect on CHD risk. Although some apparently silent sequence changes in exons have been reported to influence gene expression by altering the normal intron–exon splicing processes (reviewed in Ref. [36]), there is no direct evidence to suggest that the sequence change in codon 99 is functional, and further SNP detection is required to examine the potential role of *PON3* in detail.

4.2. PON1 and CHD risk

To date, studies that have examined the relationship between *PON1* and *PON2* and risk of CHD have in general been carried out in case-control cohorts. The NPHSII study comprises a prospective analysis of men healthy on entry which eliminates any risk of survival bias affecting the results. The 71 subjects who had diabetes at baseline were excluded from all follow-up analysis. This group of individuals was too small to allow any meaningful statistical comparisons of the impact of *PON* genotypes on development of CHD. We did not confirm the relationship between the L55M and Q192R polymorphisms and CHD risk seen by others [13]. Power calculations show that the smallest detectable odds ratio effect for L55 in NPHSII is 1.52, and since the previously reported risk estimates were in the range 1.7–1.9 [31,37], a similarly large risk effect in healthy men is unlikely, but we cannot rule out a more modest effect. A meta-analysis of studies performed up until 2001 on the Q192R genotype demonstrated that there was an overall relative risk of the R allele of 1.44 (1.17–1.77) [38]. There was, however, evidence of publication bias with the greatest effects being seen in the smaller studies. Our study found no increase in frequency of the 192R allele in CHD in cases, but power calculations show the smallest detectable odds ratio in NPHSII with this allele is 1.57, so the reported modest effects on risk cannot be ruled out. Thus, in healthy subjects the *PON1* polymorphisms may have little or no effect on risk but when other factors, such as FH or diabetes [18], are introduced, the impact of the polymorphisms on CHD risk may become clinically significant.

4.3. Smoking and PON1 genotype

It has been suggested that the mechanism by which smoking increases CHD risk may be directly on reducing PON activity and promoting lipid oxidation. The ability of PON1 to prevent lipid oxidation, and the fact that smoking reduces PON activity, implies that variants that cause low PON activity may play a role in modulating smoking-associated CHD risk (reviewed in Ref. [39]). When effects of smoking and genotype on CHD risk were examined, there was evidence for higher smoking-associated risk associated in men carrying the *PON1* 55 M allele, i.e. the LL subjects showed a degree of

protection from smoking. The 55 M risk-raising effect appeared to be co-dominant, but when LM + MM men were considered together, the interaction term was only of borderline statistical significance, and this suggestion of smoking risk-modification by the 55 M allele requires confirmation in other prospective studies. The lower risk in the RR individuals could also be attributable to the lack of MM individuals in this group. Although this is a possibility, the strong LD between these two sites means that it is not possible to tease this out with statistical certainty. It should be noted that if the proportion of smokers differs among studies, this interaction with smoking could account for why some of the previous studies failed to find an association with risk.

4.4. PON2 and CHD risk

Previous reports have identified the *PON2* 311 C allele as having a protective role in CHD risk [21,22]. We could not confirm this but, in contrast, men who carried the *PON1* 55 M allele and who were *PON2* 311 C homozygotes were significantly more likely to develop CHD (OR = 3.54, 95% CI 1.81–6.83) when compared to other genotype combinations. Since the 55 M allele has been independently associated with risk [32], it might have been assumed that the risk would be moderated if the *PON2* 311 C allele was indeed protective. However, the NPHSII data are consistent with the observation of an increased risk with this combined genotype in a small case-control study of Korean subjects [40]. Since there is almost complete allelic association (LD>0.99) between the C311S and the A148G change [20], only the C311S variant was genotyped here, but the effect on risk may be due to either change (or both acting together), and in vitro studies on functionality will be required to examine this. The 55 M isoform has been shown to be less stable than the 55 L isoform, which may indicate a mechanism of action [41], although since the 55 M allele is in linkage disequilibrium with the potentially functional −108T allele [42], the mechanism may alternatively be through effects on transcriptional control of PON1 synthesis. Another study identified an increased risk in individuals who were carriers of the 192R allele and the 311S allele [22], but no such effect was seen in the present study.

4.5. PON activity rather than genotype is a better risk marker

The unavailability of plasma measures of *PON1* activity is a limitation of this study. It has been reported that PON activity and mass is a more important marker for CHD risk than genotype [38] although the activity of PON is, at least in part, related to genotype [43]. There are, as yet, no standard assays to assess the levels of PON2 and PON3, and it is currently unclear as to the relative usefulness of measures of PON activity against different synthetic substrates, or of PON antigen levels, in determining CHD risk. It is likely that the levels of PON would be more important in risk determination than PON genotypes alone, or that the combination of phenotype and genotype may give a better risk estimate.

4.6. PON activity and anti-oxidant potential

Although PON1 (and PON3) are found in the plasma associated with HDL particles [44], the role of PON2 in vivo is unclear, and while the different substrates of PON1 are well studied, little is known for PON2. PON2 is ubiquitously expressed and is not detectable on either HDL or LDL. It is found in all of the cells associated with the artery wall, as well as in primary macrophages where neither PON1 nor PON3 are found, and has been described as a cellular antioxidant due to its ability to reduce the intracellular oxidative stress levels [45]. PON1 has a number of conserved cysteine residues, the one at position 284 being required for protection of LDL from oxidation [46]. The direct effect of the S311C variant on PON2 activity has not been reported but it may be that the introduction of a cysteine at position 311 in the *PON2* gene is having an important physical effect. Further studies into the action of PON2 need to be performed in order to explain this finding. PON2 and PON3 show no paraoxon hydrolytic activity and it has been suggested that it is lactonase activity, rather than arylesterase or organophosphate activity, that is the common feature of the PONs. This has lead to the mechanism of protection being postulated to be the hydrolysis of a potentially toxic endogenous lactone, which would otherwise produce vascular damage [44]. Whatever the actual physiological role of the PONs, their anti-oxidant ability in vitro has been proven beyond doubt.

4.7. PON gene cluster and CHD risk

The three *PON* genes lie within a 136-kb region of chromosome 7, and the frequency and linkage disequilibrium between the *PON1* and *PON2* polymorphisms was in agreement with the those previously published [23]. Linkage disequilibrium between the *PON3* polymorphism and the *PON1* and *PON2* alleles was significant. LD estimates in NPHSII were similar to those reported in previous Caucasian samples, although it has been suggested that there may be differences in linkage disequilibrium among populations [40]. Since *PON1*, *PON2* and *PON3* are closely linked, it could be that a variant in any of these genes may be in allelic association with a functional change elsewhere, and that they merely act as markers for this change, i.e. with an as yet unidentified polymorphism in *PON3* or, indeed, another closely linked gene. Several polymorphisms in the promoter region of the *PON1* gene have been identified [47,48] and some studies indicate a relationship between these polymorphisms and CHD [49,50]. One study suggested that the −108C>T promoter polymorphism accounted for much of the risk effect seen with the L55M

polymorphism [42]. Unfortunately, despite several attempts using different methods, accurate genotyping for this site could not be obtained on NPHSII DNA (unpublished). Haplotype analysis might be useful over short regions of single *PON1* genes such as the promoter region and the exons including the L55M and Q192R amino acid changes in the *PON1* gene.

Overall, the data suggest that, in combination, the PON1 protein found in HDL and PON2 found in arterial cells are playing an important role in protection against CHD. Polymorphisms within the *PON* genes may affect the ability of the PON proteins to exert their antioxidant effects. However, the NPHSII data suggest that this is not due to one polymorphism alone, and may be mediated by external factors such as smoking. This highlights the need for the study of multiple polymorphic sites, in combination with evaluation of environmental factors, to elucidate the genetic contribution of the paraoxonases and risk of CHD. Future studies should make use of protocols that would provide information on *PON* levels as well as further SNP characterisation. Taken together, these data may provide a more accurate prediction of *PON1* levels and allow an assessment of genetic polymorphism and activity levels on CHD risk.

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