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## The association of the *PON1* Q192R polymorphism with coronary heart disease: findings from the British Women's Heart and Health cohort study and a meta-analysis

Debbie A Lawlor\*<sup>1</sup>, Ian NM Day<sup>2</sup>, Tom R Gaunt<sup>2</sup>, Lesley J Hinks<sup>2</sup>, Patricia J Briggs<sup>2</sup>, Matthew Kiessling<sup>2</sup>, Nick Timpson<sup>1</sup>, George Davey Smith<sup>1</sup> and Shah Ebrahim<sup>1</sup>

Address: <sup>1</sup>Department of Social Medicine, University of Bristol, Bristol, UK and <sup>2</sup>Human Genetics Division, School of Medicine, University of Southampton School of Medicine, Southampton, UK

Email: Debbie A Lawlor\* - d.a.lawlor@bristol.ac.uk; Ian NM Day - imnd@soton.ac.uk; Tom R Gaunt - trg1@soton.ac.uk; Lesley J Hinks - ijh3@soton.ac.uk; Patricia J Briggs - pjb1@soton.ac.uk; Matthew Kiessling - mgk@soton.ac.uk; Nick Timpson - n.timpson@bristol.ac.uk; George Davey Smith - zetkin@bristol.ac.uk; Shah Ebrahim - shah.ebrahim@bristol.ac.uk

\* Corresponding author

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### Abstract

**Background:** There have been inconsistent results from case-control studies assessing the association of the *PON1* Q192R polymorphism with coronary heart disease (CHD). Most studies have included predominantly men and the association in women is unclear. Since lipid levels vary between the sexes the antioxidant effect of *PON1* and any genes associated with it may also vary by sex. We have examined the association of the *PON1* Q192R polymorphism with CHD in a large cohort of British women and combined the results from our cohort study with those from all other published studies.

**Results:** The distribution of genotypes was the same among women with CHD and those without disease. The odds ratio (95% confidence interval) of having CHD comparing those with either the QR or RR genotype to those with QQ genotype (dominant model of association) was 1.03 (0.89, 1.21) and the per allele odds ratio was 0.98 (0.95, 1.01). In a meta-analysis of this and 38 other published studies (10,738 cases and 17,068 controls) the pooled odds ratio for the dominant effect was 1.14 (1.08, 1.20) and for the per allele effect was 1.10 (1.06, 1.13). There was evidence of small study bias in the meta-analyses and the dominant effect among those studies with 500 or more cases was 1.05 (0.96, 1.15). Ethnicity and reporting of whether the genotyping was done blind to the participants clinical status also contributed to heterogeneity between studies, but there was no difference in effect between studies with 50% or more women compared to those with fewer women and no difference between studies of healthy populations compared to those at high risk (with diabetes, renal disease or familial hypercholesterolaemia).

**Conclusion:** There is no robust evidence that the *PON1* Q192R polymorphism is associated with CHD risk in Caucasian women or men.

## Background

Coronary heart disease (CHD) has a multifactorial aetiology involving physiological, environmental and genetic factors, leading to increased susceptibility [1]. High-density lipoprotein cholesterol (HDLc) is protective against CHD but the mechanisms underlying this protective effect are not fully understood. One plausible mechanism is related to the antioxidant properties of HDLc [2]. HDLc appears to protect against oxidation of low-density lipoprotein cholesterol (LDLc) and oxidation of LDLc is important in the initiation and progression of atherosclerosis [3,4]. The antioxidant effect of HDLc is determined by its enzymes, in particular paraoxonase 1 (PON1), which catalyzes the hydrolysis of lipid peroxides, as well as being a potent hydrolyser of a number of other substrates, including the active metabolites of organophosphates [5-8]. These findings have led to the suggestion that PON1 activity has a role in susceptibility to atherosclerotic disease [5,9]. A strong association between a polymorphism in the *PON1* gene (in which an amino acid substitution (glutamine (Q) for arginine (R) at position 192 gives rise to 2 alloenzymes) and PON1 activity has been found [10,11]. The effect of the polymorphism varies depending upon the substrate, for example the R alloenzyme is associated with faster hydrolysis of paraoxon but slower hydrolysis of diazinonoxon [10,11]. It has been shown that the R alloenzyme confers least ability of HDLc to prevent oxidation of LDLc, mediated through the level of PON1 enzyme activity [12].

Several case-control studies have assessed the association of the *PON1* Q192R polymorphism with CHD, and a recent review and meta-analysis of these studies found a weak overall effect but with evidence of small study bias, such that there was no effect when results were pooled only for studies including 500 or more cases [13]. In the majority of studies to date 75% or more of the participants have been male (see Additional file 1) and the effect of the polymorphism in women is unclear. Since lipid levels vary by sex, with adult women having higher levels of HDLc than men, it is plausible that any effect of the polymorphism and of PON1 activity will vary by sex.

The aim of this study is to assess the association of the *PON1* Q192R polymorphism with CHD in a large cohort study of women only, and to update the previous meta-analysis in this area. We have identified an additional three studies that were not included in the previous meta-analysis which together with our own study add 865 cases and 5452 controls to that analysis.

## Results

### **The British Women's Heart and Health Study**

Of the 4,278 participants who gave consent for genetic testing 15 (5 Afro-Caribbean, 8 South Asian and 2 other)

were defined by the examining nurse as being non-Caucasian and have been excluded from further analysis. Of the remaining 4,263 women 3,545 (83%) had DNA available for genotyping and for 3,266 (92%) of these women genotypic data were available. There was no difference in mean age (68.8 (5.5) versus 69.0 (5.6) years,  $p = 0.23$ ) and no difference in the prevalence of CHD (14.7% [480 cases out of 3266] of those with genotypic data versus 15.4% [155 cases out of 1005] of those without genotypic data,  $p = 0.36$ ) between those with and without genotypic data. The allele frequencies were in Hardy Weinberg equilibrium among women with no evidence of CHD ( $p = 0.14$ ) and among those with CHD ( $p = 0.42$ ). Of the 3,266 women with genotypic data 480 (14.7%) had CHD.

### **The association of *PON1* Q192R polymorphism with coronary heart disease in the British Women's Heart and Health Study**

Table 1 shows the characteristics of the study population by genotype. Genotype was not associated with CHD, obesity, insulin resistance, diabetes, dyslipidaemia, socioeconomic position or smoking. Systolic blood pressure appeared higher among those with the RR genotype compared to either those with QQ or QR genotypes, but this may be a chance finding since, unlike lipid levels and CHD which would be hypothesised to be associated with this polymorphism, we had no strong *a priori* hypothesis of an association with blood pressure and would therefore only be convinced that the result was not due to chance by a very small  $p$  value. The genotype frequencies for those with CHD did not differ from those not experiencing such an event ( $p = 0.2$ , see final row of Additional file 1).

Table 2 shows the results of the logistic regression analyses of the association of the *PON1* Q192R genotype with myocardial infarction only, angina and CHD, with different genetic models (dominant, recessive and per allele effect). There was no evidence from any of these that *PON1* Q192R genotype was associated with CHD. The odds ratio (95% confidence interval) of having CHD comparing those with either the QR or RR genotype to those with QQ genotype (dominant model of association) was 1.03 (0.89, 1.21) and the per allele odds ratio was 0.98 (0.95, 1.01). Adjustment for potential mediating or confounding factors (as listed in the first column of table 1) did not alter any of the associations presented in Table 2.

### **Systematic review and meta-analysis of all previously published studies**

Table summarising all previously published studies (see Additional file 1)

A table summarising all previously published studies is located in word format in additional file 1. The file is

**Table 1: Characteristics of study participants by genotype for PON1 Q192R polymorphism (N = 3,266)**

| Variable                      | Means or [N] % (95% CI) by genotype |                          |                         | P heterogeneity <sup>a</sup> |
|-------------------------------|-------------------------------------|--------------------------|-------------------------|------------------------------|
|                               | QQ N = 1669                         | QR N = 1326              | RR N = 271              |                              |
| <b>Dichotomous variables</b>  |                                     |                          |                         |                              |
| Myocardial infarction (MI)    | [53] 3.2 (2.4, 4.3)                 | [46] 3.5 (2.6, 4.8)      | [12] 4.4 (2.3, 7.6)     | 0.51                         |
| Angina (but no history of MI) | [186] 11.1 (9.7, 12.8)              | [165] 12.4 (10.7, 14.3)  | [18] 6.6 (4.0, 10.3)    | 0.13                         |
| Any CHD (MI or angina)        | [239] 14.3 (12.7, 16.1)             | [211] 15.9 (14.1, 18.0)  | [30] 11.1 (7.6, 15.4)   | 0.14                         |
| Diabetes at baseline          | [75] 4.5 (3.6, 5.6)                 | [70] 5.3 (4.3, 6.7)      | [12] 4.4 (2.5, 7.6)     | 0.61                         |
| Current smoker                | [189] 11.3 (9.9, 12.9)              | [163] 12.3 (10.6, 14.1)  | [22] 8.1 (5.4, 11.9)    | 0.12                         |
| Ever smoker                   | [746] 44.6 (42.2, 47.0)             | [596] 44.9 (42.3, 47.6)  | [116] 42.9 (37.1, 48.8) | 0.82                         |
| Adult manual social class     | [964] 57.8 (55.4, 60.2)             | [725] 54.7 (52.0, 57.3)  | [157] 57.9 (51.9, 63.6) | 0.20                         |
| Childhood manual social class | [1334] 79.9 (77.9, 81.8)            | [1075] 81.1 (78.9, 83.1) | [219] 81.0 (75.9, 85.2) | 0.72                         |
| On statin                     | [126] 7.5 (6.4, 8.9)                | [95] 6.9 (5.6, 8.3)      | [25] 9.2 (6.3, 13.2)    | 0.41                         |
| <b>Continuous variables</b>   |                                     |                          |                         |                              |
| Age                           | 68.8 (68.6, 69.1)                   | 68.8 (68.5, 69.1)        | 68.9 (68.3, 68.3)       | 0.91                         |
| HOMA <sup>c</sup>             | 1.64 (1.59, 1.70)                   | 1.72 (1.66, 1.79)        | 1.69 (1.56, 1.82)       | 0.15                         |
| Total cholesterol             | 6.64 (6.58, 6.70)                   | 6.65 (6.58, 6.72)        | 6.61 (6.46, 6.75)       | 0.85                         |
| HDLc                          | 1.65 (1.63, 1.67)                   | 1.65 (1.63, 1.68)        | 1.70 (1.65, 1.76)       | 0.18                         |
| LDLc                          | 4.15 (4.09, 4.20)                   | 4.15 (4.09, 4.21)        | 4.02 (3.89, 4.16)       | 0.21                         |
| Triglycerides <sup>c</sup>    | 1.67 (1.64, 1.71)                   | 1.69 (1.65, 1.73)        | 1.71 (1.62, 1.81)       | 0.73                         |
| Systolic BP                   | 148.3 (147.1, 149.5)                | 146.0 (144.6, 147.3)     | 150.4 (147.4, 153.4)    | 0.006                        |
| Diastolic BP                  | 79.9 (79.3, 80.4)                   | 78.9 (78.3, 79.6)        | 80.3 (78.9, 81.8)       | 0.05                         |
| BMI                           | 27.6 (27.3, 27.8)                   | 27.6 (27.4, 27.9)        | 27.6 (27.0, 28.2)       | 0.96                         |
| WHR                           | 0.817 (0.814, 0.821)                | 0.821 (0.817, 0.824)     | 0.826 (0.818, 0.834)    | 0.11                         |

<sup>a</sup>  $\chi^2$  for dichotomous variables and ANOVA for continuous variables <sup>b</sup> Occurrence of a new CHD event (death from CHD, new diagnosis of MI / angina, new CABG or angioplasty). All other characteristics are from baseline measures <sup>c</sup> Geometric means

**Table 2: Results of logistic regression analysis for the association of PON1 Q192R genotype with coronary heart disease (CHD) in the British Women's Heart and Health study N = 3,266**

| Genotype               | OR (95% CI) for myocardial infarction [N = 111] | OR (95% CI) for angina [N = 369] | OR (95% CI) for any CHD [N = 480] |
|------------------------|---|----------------------------------|-----------------------------------|
| <i>Dominant model</i>  |   |                                  |                                   |
| QQ                     | 1   | 1                                | 1                                 |
| QR or RR               | 1.13 (0.76, 1.70) p = 0.49                      | 1.04 (0.84, 1.27) p = 0.64       | 1.03 (0.89, 1.21) p = 0.62        |
| <i>Recessive model</i> |   |                                  |                                   |
| QQ or QR               | 1   | 1                                | 1                                 |
| RR                     | 1.42 (0.76, 2.64) p = 0.26                      | 0.67 (0.44, 1.01) p = 0.06       | 0.79 (0.59, 1.05) p = 0.11        |
| <i>Per allele</i>      |   |                                  |                                   |
| QQ                     | 1   | 1                                | 1                                 |
| QR                     | 1.09 (0.72, 1.67)                               | 1.12 (0.91, 1.39)                | 1.09 (0.92, 1.28)                 |
| RR                     | 1.45 (0.76, 2.78)                               | 0.70 (0.46, 1.08)                | 0.82 (0.60, 1.11)                 |
| Per allele             | 1.04 (0.98, 1.10) p = 0.24                      | 0.98 (0.93, 1.02) p = 0.11       | 0.98 (0.95, 1.01) p = 0.16        |

OR: odds ratio; CI: confidence interval; CHD: coronary heart disease

named 'summaryT' and the title of the table is 'Summary of studies examining the association between PON1 Q192R polymorphism and coronary heart disease'. The table lists the study design, population details, numbers of cases and controls with each allele and information about study quality.

We identified 38 studies that assessed the association between PON1 Q192R genotype and CHD [14-51]. Together with our own, British Women's Heart and Health study, these studies provide data on 10,738 CHD cases and 17,068 controls. Compared to a recently published meta-analysis[13] we included three additional studies [39,40,50]. One of these studies [39] was identified by the previous reviewers but excluded because it was

**Table 3: Results of meta-analysis of 39 studies (N = 10,738 cases and 17,068 controls) of the association of PON1 Q192R genotype with coronary heart disease (CHD)**

|                        | OR (95% CI) for myocardial infarction | OR (95% CI) for angina | OR (95% CI) for any CHD |
|------------------------|---------------------------------------|------------------------|-------------------------|
| <i>Dominant model</i>  |                                       |                        |                         |
| Fixed effect           | 1.10 (1.03, 1.18)                     | 1.18 (1.09, 1.27)      | 1.14 (1.08, 1.20)       |
| Random effect          | 1.12 (1.01, 1.23)                     | 1.26 (1.08, 1.46)      | 1.19 (1.08, 1.30)       |
| p heterogeneity        | 0.08                                  | < 0.001                | < 0.001                 |
| <i>Recessive model</i> |                                       |                        |                         |
| Fixed effect           | 1.14 (1.01, 1.28)                     | 1.12 (1.00, 1.25)      | 1.13 (1.04, 1.22)       |
| Random effect          | 1.14 (1.01, 1.28)                     | 1.14 (0.99, 1.33)      | 1.13 (1.02, 1.24)       |
| p heterogeneity        | 0.72                                  | 0.01                   | 0.08                    |
| <i>Per allele</i>      |                                       |                        |                         |
| Fixed effect           | 1.06 (1.01, 1.12)                     | 1.13 (1.06, 1.20)      | 1.10 (1.06, 1.13)       |
| Random effect          | 1.07 (1.01, 1.13)                     | 1.15 (1.05, 1.24)      | 1.11 (1.06, 1.15)       |
| p heterogeneity        | 0.12                                  | < 0.001                | < 0.001                 |

OR: odds ratio; CI: confidence interval; CHD: coronary heart disease

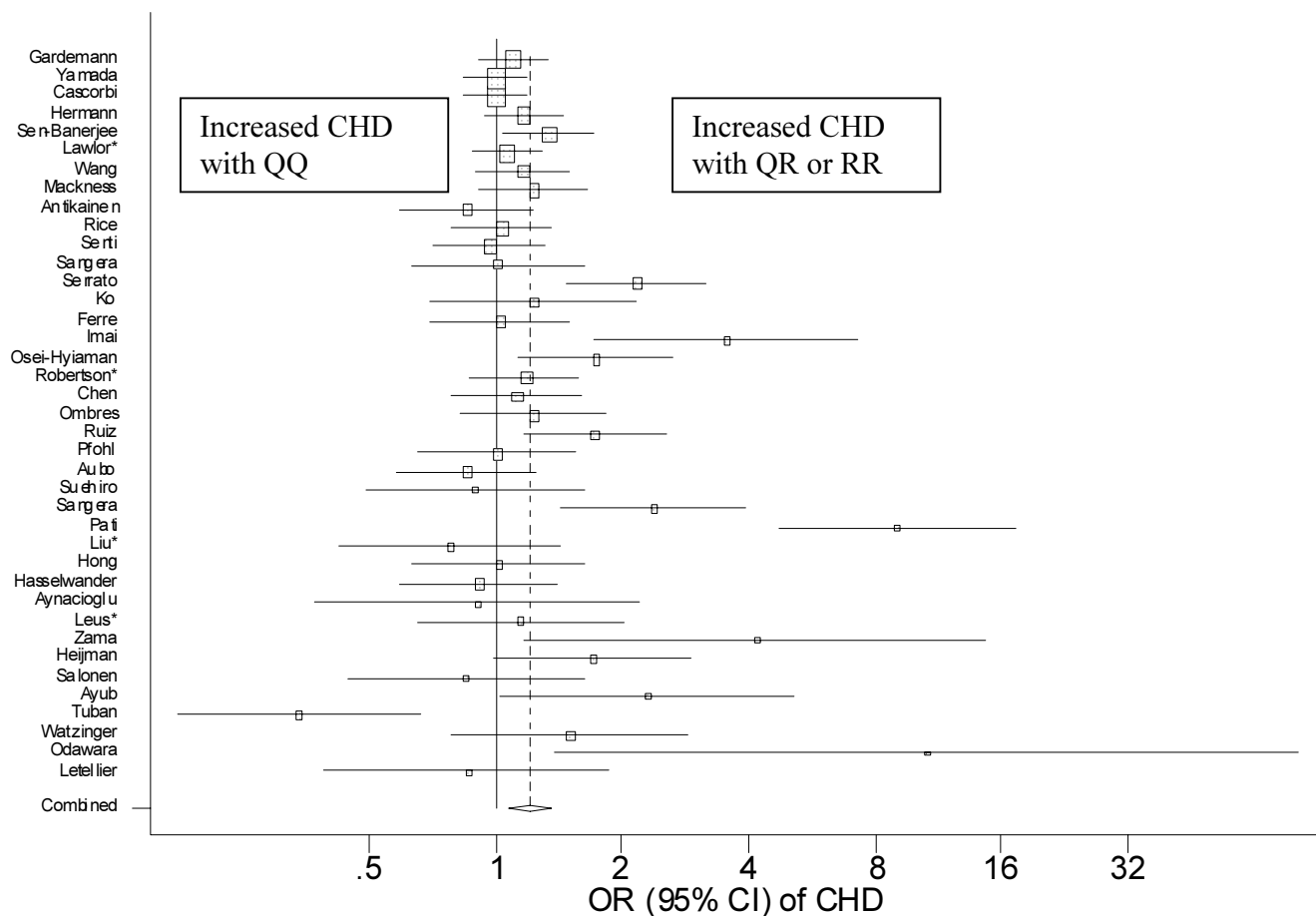
in a population with familial hypercholesterolaemia. It has been suggested that the *PON1* Q192R polymorphism has greater effects in populations at greater risk of CHD [5,9] and therefore we decided *a priori* not to exclude studies of high risk populations but to determine whether there was heterogeneity between these studies and those on healthy populations. The previous review did include studies in diabetic populations and one in patients who had undergone renal transplants [13]. A second study [40] was published within the time frame of the search of the previous review but was in an obscure Chinese journal which we had difficulty locating. The third study [50], a large prospective study, was published prior to publication of the recent review and meta-analysis but was later than the date at which those reviewers completed their search. Combining our study with these three studies provides an additional 865 cases and 5452 controls compared to the previous meta-analysis [13].

Table 3 presents the results of the meta-analyses and Figure 1 shows the meta-analysis of the odds ratio for any CHD comparing either QR or RR genotype to QQ genotype (dominant effect). These results suggest a weak association between the R allele and increased CHD risk. The pooled odds ratio for the dominant effect was 1.14 (1.08, 1.20) and for the per allele effect was 1.10 (1.06, 1.13). However, there was marked heterogeneity between studies in most of these meta-analyses and there was evidence of small study bias (both Egger and Begg's tests produced p-values between 0.003 and 0.04 for the different models).

Study size, ethnicity and whether the study reported blinding of the genotyping were all independently associated with heterogeneity between studies (all p-values <

0.04), though heterogeneity tended to remain even within these sub-groups. Figure 2 shows the differences in effect size according to study size, ethnicity, and blinding of the genotyping. The pooled effect from four studies with 500 or more cases suggested no association between genotype and CHD: the dominant effect (shown in Figure 2) was 1.05 (0.96, 1.15) and the per allele effect was 1.04 (0.98, 1.14). There appeared to be greater detrimental effect of the R allele among East-Asian and other non-Caucasian populations, but these pooled estimates were imprecise due to small numbers of small sized studies in these sub-groups. Studies that did not provide information on whether the genotyping was undertaken blind to the clinical status of study participants tended to show stronger effects. Lack of provision of this information may be a general marker of poor study quality, with meta-analyses in other areas suggesting that poor study quality tends to result in exaggerated effects [52].

There was no evidence of differences in the effect of the polymorphism between studies that included 50% or more women and those in which fewer than 50% of the participants were women: pooled odds ratio for the effect of QR or RR genotype versus QQ genotype among studies with 50% or more women was 1.13 (0.99, 1.24) and that among studies with fewer than 50% women was 1.15 (1.08, 1.22). There was no difference in effect between studies in healthy compared to diseased populations ( $p = 0.16$ ). Further, the method of diagnosis of CHD (angiographic diagnosis or diagnosis of MI based on at least two of three of classical chest pain symptoms, cardiac enzyme changes or ECG changes versus softer diagnoses of angina without angiography or self-report) did not explain any heterogeneity between studies ( $p = 0.87$ ).



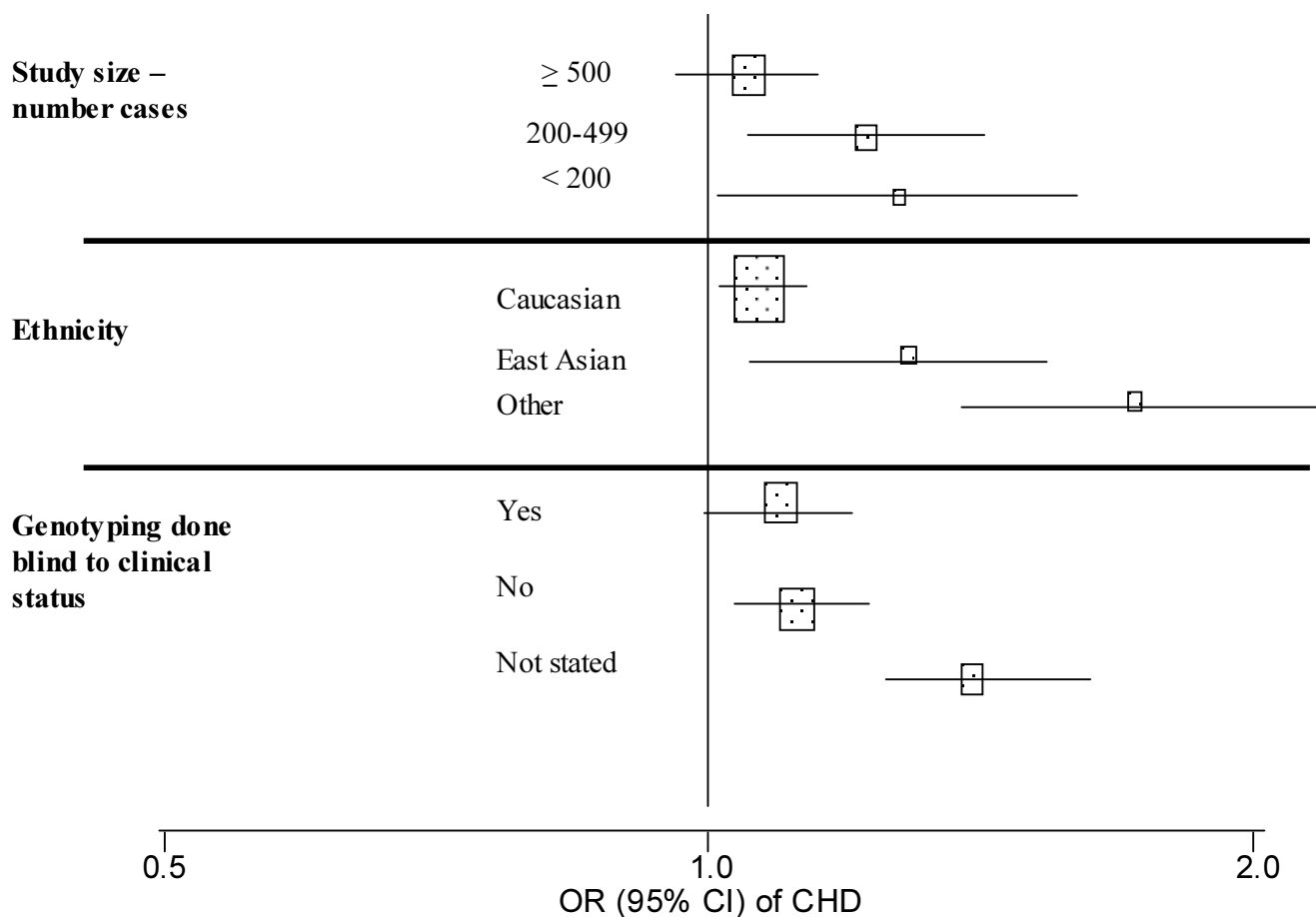
**Figure 1**  
 Meta-analysis of association of *PON1* Q192R polymorphism with coronary heart disease (dominant model: QR or RR genotype versus QQ genotype). Based on a total of 10738 cases and 17068 controls from 39 studies.

**Discussion**

In our cohort study of women we found no evidence of an association between *PON1* Q192R polymorphism and CHD. In a meta-analysis of this study together with 38 previously published studies there was a weak association between the R allele and increased CHD risk. However, there was evidence of small study bias and the pooled estimate from studies including 500 or more cases suggested no association between the polymorphism and CHD. The overall effect did not differ between studies containing predominantly men and those with a predominance of women. Overall we would conclude that there is no strong evidence that the *PON1* Q192R polymorphism is associated with CHD risk in either Caucasian men or women. Our results cannot rule out an important effect of this polymorphism in some non-Caucasian populations.

However, there is insufficient evidence in these subgroups to make precise estimates of the effect currently in these populations. Previous studies reporting an association demonstrate the particular problems of false positive genetic associations due to chance findings and small study bias [53].

It has been suggested that the polymorphism has a greater effect among individuals at high risk of heart disease, such as diabetics or those with familial hypercholesterolaemia [5,7], therefore heterogeneity between studies may be due to some consisting of high risk participants whereas others are in healthy populations. However, our meta-analysis suggested that effects were similar in those at high risk (individuals with diabetes, familial hypercholesterolaemia or renal disease) and healthy individuals.



**Figure 2**  
 Pooled estimates of the association of *PON1* Q192R polymorphism with coronary heart disease (dominant model: QR or RR genotype versus QQ genotype) by study characteristics

**Can the principles of Mendelian randomisation be used to deduce the effects of *PON1* activity on CHD from the *PON1* genotype-CHD association results?**

It has been proposed that the principles of Mendelian randomisation can be used to assess unconfounded associations between phenotypes and disease outcomes. [54,55] The basic idea uses the fact that the random assortment of genes from parents to offspring that occurs during gamete formation and conception results in genotype being independent of socio-environmental factors. Thus a genotype-disease association provides support for a causal association between a phenotype (that is functionally related to the genotype) and disease. Although it has been suggested that the Q192R polymorphism accounts for only a small part of the 40-fold individual variation in *PON1* activity [9], if, as is proposed the R allele is functionally related to *PON1* activity resulting in a reduced ability to prevent oxidation of LDLc, individuals with one or two R alleles

will, on average, have had lower exposure to *PON1* antioxidant effects during their lives than those who are homozygous for the Q allele, permitting an assessment of the effect of this lower exposure on risk of CHD. The many other environmental and genetic factors determining *PON1* activity, which would confound a comparison between *PON1* enzyme activity and CHD risk, should not confound the *PON1* genotype-CHD association owing to the random assortment of genes from parents to offspring that occurs during gamete formation and conception, the principle of Mendelian randomisation [54,55]. This is demonstrated in Table 1, where the major physiological and environmental risk factors for CHD are similarly distributed. In addition to avoidance of any confounding effect, the genotype-CHD association avoids the possibility that *PON1* antioxidant activity might be caused by the onset of CHD, that is reverse causality [55]. However, the use of Mendelian randomisation in this

instance may be complicated by the polymorphism being associated with a number of different intermediate phenotypes that could have opposing effects on CHD and by the fact that the principles of Mendelian randomisation depend upon a very clear understanding of the functional pathways from genotype, through intermediate phenotype, to disease outcome. Proving a negative using the principles of Mendelian randomisation is difficult since it requires the ability not simply to show a null genotype-outcome association (as we suggest is the case here) but the need to predict, from known effect sizes of the gene-intermediate phenotype association and the intermediate phenotype-outcome association, the size of effect that one would expect for the genotype-outcome association [55]. Since there is no clear epidemiological evidence of the effect sizes to make these predictions of the PON1 activity-CHD association (because of difficulties of reliably estimating PON1 activity and oxidation of LDL in large epidemiological studies) these calculations are not possible in this situation in the way that they have been shown to be useful in other examples, most notably with the fibrinogen-CHD association [55,56].

Knowledge of the link between PON1 activity and atherosclerosis comes largely from biological rather than epidemiological studies. There is good evidence that peroxidation of LDL lipids is an important risk factor for atherosclerosis and that HDL protects against atherosclerosis at least in part through its antioxidative properties [4,57]. Whilst a number of HDL enzymes have antioxidant properties purified PON1, in laboratory experiments, has been found to be highly effective in preventing lipid peroxidation of LDL and to be more effective than other HDL enzymes [6-8,58,59]. Avian HDL has no PON1 and in studies that have isolated avian HDL and tested its effects on human LDL it has been found to have no protective effect against lipid peroxidation [7]. Similarly in studies of PON1 knockout mice their HDL fails to protect against LDL oxidation [8]. Thus there is biological evidence that HDL PON1 activity has important LDL antioxidant effects. Since LDL oxidation is importantly linked to atherosclerosis a genetic factor that had a direct effect on PON1 activity should therefore be associated with CHD. However, without epidemiological studies which provide one with reliable estimates from which to clearly state the magnitude of association one would expect in this pathway we cannot rule out the possibility that an odds ratio of 1.15 (the upper limit of the 95% confidence interval for a dominant effect from the pooling of all studies with 500 or more cases) is consistent with the antioxidant activities of PON1.

If the Q192R polymorphism has pleiotropic effects (the potential for polymorphisms to have more than one specific phenotypic effect) with some, as yet unknown, phe-

notypic effect of the R allele that results in a decreased risk of CHD then the overall effect of this polymorphism could be null, but the antioxidant properties of PON1 activity could still be an important determinant of risk. However, our own findings do not suggest that the PON1 R allele is associated with phenotypes that would reduce CHD risk, and the unexpected association with increased systolic blood pressure would increase CHD risk. Other studies suggest that there are other effects of this phenotype that would actually increase rather than decrease CHD risk including a possible association with diabetes [14] and increased susceptibility to the effects of cigarette smoking [60]. Thus although it is clear that there are a number of different PON1 phenotypes and therefore possible that there is a phenotype which decreases CHD risk and leads to the null or weak findings we present here. To date the known PON1 phenotypes should in theory all increase CHD risk.

Finally, it is possible that the PON1 Q192R polymorphism is not the true functional polymorphism for PON1 activity but is linkage disequilibrium with the functional variant. Heterogeneity between studies could then result from different ethnic groups being included in different studies with different patterns of linkage disequilibrium in these groups. However, though we found that ethnicity was associated with heterogeneity it only explained a small proportion of the differences between studies.

In conclusion, whilst our results suggest that there is no robust evidence of an association between the PON1 Q192R polymorphism and CHD in Caucasian women and men. This does not mean that PON1 enzyme activity is not a determinant of atherosclerosis. Further, epidemiological studies of the association between the genotype and enzyme activity and between enzyme activity and CHD are required to be able to use the results from this meta-analysis and the principles of Mendelian randomisation to determine whether there is a causal link between the enzyme activity and CHD.

#### **Study strengths and limitations**

Ours is one of the few studies to examine this association in a predominantly female population. Although our results overall suggest that there is no difference in the association between women and men the differences in HDLc levels and CHD risk between the sexes warranted specific examination of the association in women. Our cohort study is moderately large with just under 500 clinically diagnosed cases and the null result in this study supports the conclusion of a recent meta-analysis that this polymorphism is not importantly associated with CHD risk [13]. Genotypic data were available on 76% of the total cohort but those without these data did not differ with respect to age or CHD status from those with these

data. Our cases were obtained from medical record. In the case of MI these were validated against WHO criteria. However, the MI cases accounted for only a small proportion of all CHD cases. Whilst medical diagnoses of angina are likely to be more accurate than self-reports it is possible that some of our cases are not truly CHD cases and as a result our results are an underestimate of the true effect. However, we found no heterogeneity between the pooled effects of studies with harder outcomes (angiographic assessments and well defined MI) and those with softer measurements of CHD. There are two known polymorphisms in the *PON1* gene and two in the *PON2* gene all of which could plausibly influence *PON1* activity and hence CHD risk [13]. We have genotypic data only on the *PON1* Q192R polymorphisms. However, this is the polymorphism which has been mostly studied in this area and a recent meta-analysis found no associations between the other three polymorphisms and CHD risk, with pooled point estimates of per allele odds ratios between 1.00 and 1.04.

We attempted to obtain all published studies of the association for our meta-analysis and as well as updating a recent meta-analysis with our own results we identified three studies that had not been included in that analysis [39,40,50]. Our analysis and the earlier publication found small study bias consistent with publication bias. This is demonstrated by one of the studies that we identified in our meta-analysis, but that was not included in the previous meta-analysis, which was a small null study that was difficult to obtain because of its publication in a difficult to obtain Chinese journal.[40] Publication is probably a greater problem for meta-analyses of genetic association studies than for randomised controlled trials as, for the latter, researchers and editors are much more aware of the importance of publishing important null findings. A solution to this problem is setting up central web-sites for the publication of all negative gene-disease association studies. We have initiated such a site (Archives of Genetic Epidemiological Analyses, <http://www.sgel.humgen.soton.ac.uk/agea/>) and other laboratories engaged also maintain web sites as a public domain data repository (e.g. GeneCanvas, <http://genecanvas.idf.inserm.fr/>). It seems likely that a synthesis of centralised and specific databases will evolve for complex trait genetics, mirroring the locus specific and centralised databases that currently exist for single gene disorders. There is heterogeneity of study approaches, data analyses and patterns of research, but the evolution of some common standards as these web-sites develop may help harmonisation in addition to dealing with the important issue of publication bias.

An important limitation of using Mendelian randomisation to provide trustworthy estimates of phenotype-dis-

ease associations is that as discussed above detailed information on genotype function is required. With advances in functional genomics, exciting new opportunities for using Mendelian randomisation to examine the effects of environmental exposures on common diseases that hitherto have proved difficult to study without confounding and bias are likely to arise [55].

## Conclusions

Our findings suggest that there is no strong evidence that the *PON1* Q192R polymorphism is associated with CHD risk in either Caucasian women or men. The role of the HDLc *PON1* enzyme in determining atherosclerotic risk is unclear and requires further epidemiological study. The results presented here may demonstrate the important pitfalls of false positive gene-disease associations that can occur due to failure to appreciate the play of chance and publication bias.

## Methods

### British Women's Heart and Health Study

Full details of this study have been previously reported [61,62]. Between 1999 and 2001 4,286 women aged 60 to 79 years, who were randomly selected from 23 British towns were interviewed, examined and completed medical questionnaires [61]. These women have been followed-up over a median of 3.5 years by flagging with the NHS central register for mortality data, two yearly review of their primary care medical records and a recently mailed 3-year follow up health questionnaire sent to all surviving participants between March and September 2003.

Methods used at baseline assessment have been previously described [61,62]. Briefly, blood samples were taken after a minimum 6 hour fast. These samples were used for assessment of insulin resistance (homeostasis model assessment calculated from fasting insulin and glucose levels) and lipids [62]. Blood pressure, height and weight (used for calculating body mass index) and waist and hip circumferences were measured [54]. In this study clinical diagnostic criteria for CHD have been applied: a case of myocardial infarction is defined as anyone with a diagnosis of infarction, based on world health organisation criteria [63], in their medical records at baseline or during the follow-up period and anyone who was assigned an underlying cause of death of CHD (ICD10 codes I20-I25, I51.6) during the follow-up period; a case of angina is defined as anyone with a clinical diagnosis of angina in their medical records at baseline or during follow-up and who was not included as an MI case; any CHD includes those defined as having either and MI or history of angina.



### Determination of PON1 genotype

DNA was extracted by salting out procedure [64] from K-EDTA whole bloods or red and white cell residues, which had been stored at  $-80^{\circ}\text{C}$  for 1 to 2 years. Quantitation was by picoGreen assay and DNA concentrations were equalised by dilutions with water. Long term stock DNA aliquots were laid down and working 96-well plates of DNA dilutions to 10 ng/ $\mu\text{l}$  prepared. Degenerate oligo primer amplifications ('DOP-DNA') were made from dilution plates in order to conserve stock DNA and 384-well PCRs were performed from DOP-DNA representing 0.1 ng of original genomic DNA. The DOP protocol was a modified version of the method used by Cheung and Nelson [65] designed to minimise loss of representation of %GC-rich genomic regions. The *PON1* Q192R genotype was determined using fluorescence-labelled oligonucleotide melting from matched or mismatched target, monitored in an Idaho Technology (Salt Lake City, Utah, USA) 384-well Odyssey. Detection utilised reduction of opposed G-base quenching of fluorescence during a thermal ramp. Asymmetric PCR was performed on 2  $\mu\text{l}$  of dried DOP amplified template in 384-well white PCR plates (Abgene<sup>®</sup>, Epsom, Surrey, UK) on a MJ Research PTC-225 DNA Engine Tetrad<sup>®</sup> (Genetic Research Instrumentation Ltd., Braintree, Essex, UK). Samples were amplified with primers 5'-CTGTGGGACCTGAGCACTTT-3' at 100 nM and 5'-GCCACCACTCGAACTTCACT-3' at 500 nM. A FITC-labelled probe (with 3' phosphate) 5'-FITC-CCCTACTTACAATCCTGGGAG-PHOS-3' matching the wild-type sequence (underlined) was included at 200 nM in the PCR for the Odyssey melting assay. 5  $\mu\text{l}$  PCR reaction mix also contained: 1x PCR buffer (Promega, Southampton, UK), 200  $\mu\text{M}$  dNTPs (Promega), 1.5 mM  $\text{MgCl}_2$  (Promega) and 0.1 units of Taq DNA polymerase (Promega). PCR cycling conditions were:  $94^{\circ}\text{C}$  for 2 minutes then 50 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $60^{\circ}\text{C}$  for 30 seconds and  $72^{\circ}\text{C}$  for 30 seconds, followed by  $72^{\circ}\text{C}$  for 2 minutes. Samples were overlaid with 5  $\mu\text{l}$  Chill-Out<sup>™</sup> wax (Genetic Research Instrumentation) to prevent evaporation during analysis. Following PCR amplification samples were melted from  $45^{\circ}\text{C}$  to  $75^{\circ}\text{C}$  in the 384-well Odyssey. LightTyper software (Roche Diagnostics Ltd.) was used to analyse the change in fluorescence during melting, and to group melting profiles into genotype groups. These were then manually verified using in-house software.

### Statistical analysis

Prevalences (for dichotomous variables) and means (for continuous variables) are presented by genotype. ANOVA was used for testing differences between genotypes for continuous variables and  $\chi^2$  tests were used for testing differences between genotypes for dichotomous variables. Logistic regression models were used for assessing the association between genotype and CHD. Robust standard

errors, which take into account possible non-independence between women from the same town, were used in all analyses to estimate confidence intervals and p-values. All analyses were conducted using Stata version 8.

### Study ethics

Local ethics committee approvals were obtained for the British Women's Heart and Health Study. Participants were asked for informed consent to review their medical records and for permission to perform anonymised genetic tests relating to cardiovascular disease on stored blood. Eight women declined to give consent and have not been included in this study.

### Systematic review and meta-analysis

Studies assessing the association of the *PON1* Q192R polymorphism with CHD were obtained through a search of Medline (1966-January 2004) and Embase (1980-January 2004) electronic databases (using the following search terms: paraoxonase, PON, PON1, Q192R, CHD, cardiovascular disease, atherosclerosis, myocardial infarction, angina) and review of the bibliographies of retrieved articles. For each study odds ratios for a dominant effect (comparing QR or RR genotype versus QQ genotype), a recessive effect (comparing RR genotype to QR or QQ genotype) and per (R) allele were abstracted from the publication or calculated from the data presented in the paper. Odds ratios for each study were pooled using both random and fixed effects methods [66]. Results from both models are presented because whilst random effects models are used when heterogeneity between studies is apparent (and this was likely among the studies we obtained), they do not "fix" the problem. The random effects method, compared to fixed effects, reduces the weight for each individual study proportional to the difference in effect size of an individual study from the pooled estimate of effect for all other studies. As heterogeneity may be the result of publication bias, a random effects model combining several small positive studies (and an absence of small negative studies due to publication bias) with a large null study will tend to underweight the latter, resulting in exaggeration of the true effect. Funnel plots and Egger and Begg's tests were used to assess whether smaller studies reported greater associations than larger studies, consistent with publication bias. [66] The effect of the proportion of women in the study population ( $> 50\%$  compared to  $\leq 50\%$ ), ethnicity of the study population (Caucasian, East-Asian or Other), disease/risk status of study participants (healthy versus high risk (diabetes, familial hypercholesterolaemia, renal disease)), blinding of the genotyping (blind to clinical status, not blind and not stated in the publication) and diagnosis of CHD (angiographic or clearly defined MI: two out of three of classical chest pain, cardiac enzyme change, ECG change versus

other) was assessed using meta-regression analysis [66]. All analyses were undertaken using Stata version 8 [66].

### Conflict of interest

None declared.

### Authors' contributions

All authors developed the study aim and design. INMD, together with SE and DAL, obtained funding for the DNA bank and genotyping set up. The DNA bank was set up by MK (supervised by PJB and LJH) and genotyping was set up by TRG. DAL undertook the initial analysis and coordinated writing of the paper. All authors contributed to the final version. DAL and INMD act as guarantors.

### Additional material

#### Additional File 1

Table showing a summary of all studies examining the association between PON1 Q192R polymorphism and coronary heart disease

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2156-5-17-S1.doc>]

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