

## ARTICLE

# The exon 1–8C/G SNP in the PSMA6 gene contributes only a small amount to the burden of myocardial infarction in 6946 cases and 2720 controls from a United Kingdom population

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The proteasome system is a proteolytic pathway that regulates the expression of genes involved in inflammation. Polymorphisms in the gene encoding subunit  $\alpha$  type 6 (PSMA6) – in particular the rs1048990 exon 1–8C/G SNP – have been implicated with susceptibility to myocardial infarction (MI) in a Japanese study. We examined whether several polymorphisms in the PSMA6 gene were related to MI risk in 6946 nonfatal MI cases and 2720 unrelated controls in a UK population. The homozygous GG genotype for rs1048990 was much less frequent in this UK population than in the Japanese population (2.1 vs 8.9%), and was associated with an odds ratio (OR) for MI of 1.09 (95% confidence interval (CI): 0.98–1.21) per G allele in a co-dominant genetic model and 1.32 (95% CI: 0.90–1.93) in a recessive genetic model. Although not statistically significant, these results for this variant are still consistent with the Japanese hypothesis-generating study. Our findings, when taken together with four other studies (including the hypothesis-generating one), yielded a combined OR for MI of 1.15 (95% CI: 1.08–1.21) per G allele in a co-dominant model and 1.38 (95% CI: 1.22–1.57) for the GG genotype in a recessive model. Larger studies involving more than 10 000 disease cases would be required to further elucidate the role of this variant for susceptibility to MI. However, given the rarity of this variant in Caucasians, the attributable risk of rs1048990 for MI is unlikely to be great in western populations.

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## Introduction

The ubiquitin–proteasome pathway plays an important role in the initiation and progression of atherosclerosis by controlling the activation of nuclear factor kappa B (NF $\kappa$ B) proteins, which control expression of genes encoding cytokines involved in inflammation.<sup>1,2</sup> Inflammation has been implicated in the initiation, progression, and complication stages of atherosclerosis. Drugs that inhibit the ubiquitin–proteasome pathway through their effect on NF $\kappa$ B have been shown to reduce the occurrence of stroke and atherosclerosis in animal models.<sup>3,4</sup>

Recently, a Japanese case–control study reported that susceptibility to myocardial infarction (MI) was associated with the exon 1–8C/G SNP at rs1048990 in the proteasome subunit  $\alpha$  type 6 (PSMA6) gene, which enhances transcription of PSMA6 on chromosome 14q13-q13.2.<sup>5</sup> The association of MI with rs1048990, and with a further SNP (intron 1 1233A/T) in near-perfect linkage disequilibrium (LD) with it, was originally found in a sample of 2592 MI cases and 2851 controls, and then replicated in a further 867 MI cases and 1104 controls. The rs1048990 SNP appeared to be functional because of its effects on the NF $\kappa$ B protein concentration in this population.<sup>5</sup> If genetic markers associated with the control of pro-inflammatory cytokines are implicated in susceptibility to MI risk, this could provide additional targets for the prevention of atherosclerosis. Consequently, we examined the association of six SNPs in the PSMA6 gene with biochemical markers of inflammation and with risk of MI in the large ISIS case–control study (6946 MI cases and 2720 controls) conducted in a UK population.

## Materials and methods

### DNA samples

The design of the ISIS case–control study has been described in detail in previous publications.<sup>6,7</sup> DNA was extracted from frozen buffy coat samples using a Taqman and amplifluor technique, as described previously.<sup>7,8</sup> Cases genotyped for this study were men and women aged 30–64 years, with nonfatal MI confirmed by cardiac enzyme or electrocardiographic criteria (or both). Controls genotyped in the present study were spouses of the siblings or children of cases (ie, unrelated to the MI cases except by marriage) aged 30–64 years, and with no history of MI, angina or other definite heart disease. Ethnicity was not recorded, but based on previous studies conducted by our research group in a similar setting, more than 95% would be expected to be Caucasian.<sup>9</sup> Participants provided consent to participate in the study, which had research ethics committee approval.

### SNP analysis

Six SNPs for PSMA6 were selected for analysis in the ISIS study. The rs1048990 and PSMA6\_1233 (intron 1 1233 A/T) SNPs were, respectively, the SNP originally associated with MI and a SNP in perfect LD with it in the Japanese study. The PSMA6\_7693 (intron 1 7693 G/A) SNP had been excluded from further analysis in the Japanese study, because it had a minor allele frequency (MAF) <5%, but it is more frequent in European populations (MAF = 18%) ([http://www.hapmap.org/Data/Release #21a](http://www.hapmap.org/Data/Release_#21a)). The three other SNPs (rs17458312, rs2277459, and rs2277461) studied had not been included in the Japanese study. Genotyping using mass-spectrometry was carried out at the Centre Nationale de Genotypage in Paris without the

knowledge of disease status, with samples from cases and controls distributed within each 384-well genotyping plate. Quality control procedures included assessment of consistency of genotyping between plates through internal controls and replication of measurements in a random sample of 250 controls, to assess the reproducibility of genotyping.

### Intermediate phenotypes

Plasma levels of high-sensitivity C-reactive protein (CRP), fibrinogen, albumin, apolipoprotein A<sub>1</sub> and apolipoprotein B were measured using standard methods.<sup>8</sup> Previous studies had indicated small changes in plasma levels of such analytes due to delay in plasma separation,<sup>10</sup> so levels were adjusted for time from collection to separation, as well as for date of assay, to compensate for extrinsic sources of variation.

### Statistical analysis

The distributions of genotypes for each SNP were assessed for departure from Hardy–Weinberg equilibrium (HWE) in cases and controls separately, using an exact  $\chi^2$  goodness of fit test.<sup>11</sup> A *P*-value <0.05 was used to indicate significant departure from HWE. LD between SNPs was assessed using both the standardised disequilibrium (*D'*)<sup>12</sup> and correlation (*r*<sup>2</sup>)<sup>13</sup> coefficients. A weighted kappa statistic was computed to assess reproducibility of each of the individual SNPs studied. Posterior probabilities of pairs of haplotypes and maximum likelihood estimates of haplotype frequencies were computed for each individual with an expectation-maximisation (E-M) algorithm under the assumption of HWE using the Haplo.stats suite of functions in R.<sup>14,15</sup> The associations of haplotypes that had an estimated frequency >1% with biochemical markers and with MI risk were analysed separately (while the remaining haplotypes with estimated frequencies <1% were ignored).

Standard one-way ANOVA and haplotype-based one-way ANOVA were used to assess the associations of SNPs and haplotypes with plasma levels of biochemical markers in controls. Standard logistic regression adjusted for age and sex, and haplotype-based logistic regression, were used to estimate odds ratios (ORs) for MI for each SNP and haplotype, respectively. Floated absolute risks and confidence intervals (CIs) were used to share the variance of the log ORs appropriately between the different genotypes, which allows a CI to be computed for the reference category and any group to be compared directly with any other.<sup>16,17</sup> Power calculations to detect the association of MI risk with the rs1048990 SNP for the PSMA6 gene in the ISIS case–control study were estimated using standard methods.<sup>18,19</sup> The results of the ISIS study of MI risk were combined with other similar studies using standard meta-analytic methods. We computed the population attributable risk<sup>20,21</sup> to compare the impact of these gene variants in a Western European population with that in a Japanese

population using the combined OR from all studies included in the meta-analysis. All analyses were carried out using SAS version 8.2<sup>22</sup> and R version 2.4.0.<sup>23</sup>

## Results

Data for all six SNPs were available in 6946 cases and 2720 controls who had sufficient DNA for analysis. Table 1 shows the characteristics of these cases and controls. The mean age was 55.1 years in cases and 46.3 years in controls, and a much higher proportion of cases were men. After adjustment for age and sex differences, cases had a higher mean body mass index and higher prevalence of current smokers, hypertension and diabetes mellitus compared with controls.

### PSMA6 SNPs and haplotypes

The reproducibility of the genotyping was high, with a weighted kappa score of 0.96–1.00 for each SNP in the PSMA6 gene, except for the rs2277459 SNP which was 0.75 (Supplementary Table A1). The rs2277459 and rs2277461 SNPs were not in HWE amongst controls (Supplementary Table A2), perhaps due to the very low frequencies of their minor alleles in Caucasians (both in ISIS and HapMap; [http://www.hapmap.org/Data Release #21a](http://www.hapmap.org/Data_Release_#21a)). We investigated a 9-kb region of PSMA6 in our study that was bounded by PSMA6\_1233 (intron 1 1233A/T) and

**Table 1** Characteristics of myocardial infarction cases and controls in the ISIS study (Mean (SD) or %)

Characteristic	Cases of MI (n = 6946)	Controls (n = 2720)
Age, years	55.1 (9.4)	46.3 (8.0)
Male, %	82.4	44.8
Body mass index (Kg/m <sup>2</sup> ) <sup>a</sup>	26.1 (4.7)	24.7 (4.3)
Cigarette smoker (%) <sup>a</sup>	56.9	19.7
Hypertension (%) <sup>a</sup>	21.6	11.2
Diabetes mellitus (%) <sup>a</sup>	5.4	1.4

<sup>a</sup>Values are adjusted for age and sex. The number of individuals with complete data may vary.

**Table 2** Pair-wise standardised disequilibrium ( $D^I$ ) and correlation ( $r^2$ ) coefficients, between individual SNPs for the PSMA6 gene in the ISIS study

DbSNP rsID	Polymorphism	Minor allele frequency	$D^I$ pair-wise linkage disequilibrium (and $r^2$ correlation coefficients)				
			PSMA 6_7693	rs1048990	rs17458312	rs2277459	rs2277461
PSMA6_1233	A/T	T (0.16)	1.00 (0.04)	1.00 (0.96)	1.00 (0.04)	1.00 (0.01)	1.00 (0.01)
PSMA6_7693	A/G	A (0.18)	—	1.00 (0.04)	1.00 (0.97)	1.00 (0.01)	1.00 (0.01)
rs1048990	C/G	G (0.16)	—	—	1.00 (0.04)	1.00 (0.01)	1.00 (0.01)
rs17458312	C/T	T (0.17)	—	—	—	1.00 (0.01)	1.00 (0.01)
rs2277459	A/G	A (0.04)	—	—	—	—	0.96 (0.88)
rs2277461	A/G	G (0.04)	—	—	—	—	—

rs2277461. Table 2 shows the minor allele frequencies and LD patterns of the six SNPs in the PSMA6 gene examined in the ISIS study. Ozaki *et al*<sup>5</sup> reported near complete LD in terms of  $r^2$  between rs1048990 and PSMA6\_1233, and the present study demonstrated similar findings for these two SNPs. Indeed, all six SNPs examined in the ISIS study were in high LD with respect to  $D^I$  (all >0.95), although most were in very low pair-wise LD with respect to  $r^2$  values (only the combinations of rs1048990 and PSMA6\_1233, rs17458312 and PSMA6\_7693, and rs2277459 and rs2277461 had  $r^2$  values >0.80). The four SNPs found to be in HWE were incorporated into a haplotype analysis, which yielded three common haplotypes.

### Associations with CRP, fibrinogen and albumin

Table 3 shows the mean (SE) values of cardiovascular risk factors for the rs1048990 SNP in the controls. The relatively few individuals with the GG genotype appeared to have higher levels of fibrinogen and CRP, accompanied by lower levels of albumin, compared with those without this genotype. But, these differences were marginally statistically significant ( $P=0.03$ ) only for fibrinogen and only in a recessive model. None of the other SNPs were associated with significant differences in mean plasma levels of these biochemical markers (Supplementary Table A3). In addition, there were no differences in the proportions of current smokers, or people with hypertension or diabetes mellitus by genotype for any of the SNPs investigated (Supplementary Table A4).

Considering the estimated haplotypes, slightly raised plasma levels of the inflammatory markers CRP and fibrinogen were observed for carriers of the TGGC haplotype, but these differences were not statistically significant (Supplementary Table A5). In addition, no statistically significant differences were observed between the three estimated haplotypes and plasma levels of albumin, apolipoprotein A<sub>1</sub> or apolipoprotein B.

### Association with MI risk

Table 4 shows the associations of rs1048990 with MI risk after adjustment for age and sex, when analysed using co-

**Table 3** Mean (SE) plasma levels of biochemical markers according to genotypes of rs1048990 in controls using co-dominant and recessive genetic models in the ISIS study

Marker and genotype	N	CRP <sup>a</sup> (mg/l)	N	Fibrinogen (g/l)	N	Albumin (g/l)	N	ApoB (g/l)	N	Apo A <sub>1</sub> (g/l)
<i>Co-dominant model</i>										
C C	1583	1.04 (0.027)	1702	3.39 (0.014)	1743	40.7 (0.074)	1746	0.99 (0.005)	1746	1.49 (0.005)
C G	630	1.04 (0.043)	670	3.41 (0.025)	683	40.7 (0.118)	684	1.00 (0.009)	684	1.50 (0.008)
G G	44	1.26 (0.161)	48	3.61 (0.094)	49	40.4 (0.442)	49	0.99 (0.033)	49	1.51 (0.031)
Per G allele	2257	1.03 (0.045)	2420	0.041(0.028)	2475	0.008 (0.123)	2479	0.006 (0.009)	2479	0.01 (0.009)
Trend <i>P</i> -value		0.48		0.14		0.95		0.53		0.11
<i>Recessive model</i>										
C C+C G	2213	1.04 (0.023)	2372	3.39 (0.014)	2426	40.7 (0.061)	2430	1.00 (0.005)	2430	1.49 (0.004)
G G	44	1.26 (0.161)	48	3.60 (0.094)	49	40.4 (0.422)	49	0.99 (0.033)	49	1.51 (0.026)
<i>P</i> -value		0.27		0.03		0.49		0.50		0.96

<sup>a</sup>Geometric mean.

ApoA<sub>1</sub>, apolipoprotein A<sub>1</sub>; ApoB, apolipoprotein B; CRP, C-reactive protein.

**Table 4** Odds ratio (95% CI) for MI associated with rs1048990 in the ISIS study (adjusted for age and sex)

SNPs	Frequency		OR (95% CI)	
	Cases (%)	Controls (%)	Co-dominant model	Recessive model
C C	4778 (69.1%)	1921 (70.8%)	1.00 (0.94–1.07)	GG vs CG+CC
C G	1980 (28.6%)	736 (27.1%)	1.07 (0.96–1.18)	1.32 (0.90–1.93)
G G	160 (2.3%)	56 (2.1%)	1.34 (0.92–1.96)	
Per G allele			1.09 (0.98–1.21)	
$\chi^2$ (df); <i>P</i> -value			2.57 (1); <i>P</i> =0.11	1.96 (1); <i>P</i> =0.16

dominant and recessive genetic models. In the co-dominant model, rs1048990 was associated with a nonsignificant OR for MI of 1.09 (95% CI: 0.98–1.21) per G allele (*P*=0.11). For a recessive model, the OR for MI was 1.32 (95% CI: 0.90–1.93) for the GG genotype, but again this association was not statistically significant (*P*=0.16). The other SNPs were less strongly associated with MI risk, with the exception of the rs2277459 SNP. However, the rs2277459 SNP had a poor kappa score, evidence of departure from HWE, and an MAF of <5%, so this marginally significant (*P*=0.01) result should be interpreted with caution (Supplementary Table A6). None of the three estimated individual haplotypes for the PSMA6 gene were significantly associated with MI risk (Global score-statistic  $\chi^2_3 = 5.65$ , *P*=0.13) (Supplementary Table A7).

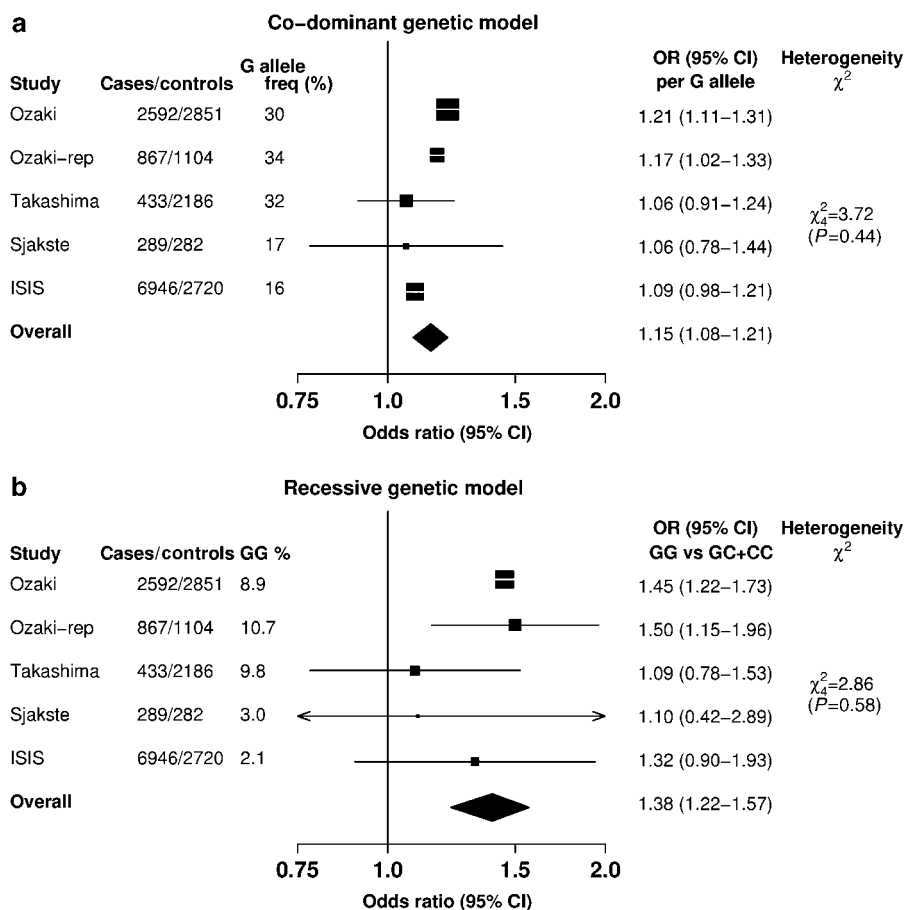
Figure 1 shows the results of a meta-analysis of the ISIS study and four other studies (three Japanese and one European). The overall pooled ORs for MI were 1.15 (95% CI: 1.08–1.21) when estimated using a co-dominant model and 1.38 (95% CI: 1.22–1.57) with a recessive model. There was no evidence of statistical heterogeneity between the results in the different studies for either genetic model (both *P*>0.10).

Using the combined OR of 1.38 for a recessive model, we estimated the population attributable risk to be 0.6% in a Western European population (as included in the ISIS

study) as compared to 3.5% in the Japanese population. For the co-dominant model, using the combined OR of 1.15, the estimated population attributable risk would be 2.4% in a western population compared to 8.1% in the Japanese population.

## Discussion

The ISIS study involving 6946 MI cases and 2720 unrelated controls is the largest genetic association study to date of MI risk in relation to polymorphisms of the PSMA6 gene, and one of the first studies to investigate the associations in a Caucasian population. In the ISIS study, the rs1048990 SNP was associated with an OR for MI of 1.09 (95% CI: 0.98–1.21) per G allele in a co-dominant model. This result is less extreme than the hypothesis-generating study by Ozaki *et al*,<sup>5</sup> which reported an OR for MI of 1.21 (95% CI: 1.11–1.31) per G allele in the Japanese population, but similar to the ORs for MI of 1.06 (95% CI: 0.91–1.24) and 1.06 (95% CI: 0.78–1.44) per G allele reported by Takashima *et al*<sup>24</sup> and Sjakste *et al*<sup>25</sup> in a Japanese and a Caucasian population, respectively (see Figure 1a). A meta-analysis of the results for the rs1048990 SNP in the ISIS study and the four other studies, involving a total of 11 127 cases and 9109 controls, yielded combined ORs of 1.15 (95% CI: 1.08–1.22) per G allele in a co-dominant model



**Figure 1** Odds ratio (& 95% CI) for MI associated with the rs1048990 polymorphism. The size of the squares are proportional to the variance of the log OR and horizontal lines represent the 95% CI. The diamond represents the pooled estimate with the width being the 95% CI. Panel (a) shows individual study estimates and the overall pooled odds ratios for myocardial infarction (MI) when estimated using a co-dominant genetic model. Panel (b) shows individual study estimates and the overall pooled odds ratios for MI when estimated using a recessive genetic model.

and 1.38 (95% CI: 1.22–1.57) in a recessive model, with no evidence of statistical heterogeneity between the results of these studies. The ISIS study had 98% power to detect a 39% increase in risk of MI associated with the rs1048990 SNP in a recessive genetic model, but only 78% power to detect a 15% increase in risk using a co-dominant model.<sup>18</sup> These associations are relatively modest, but similar in magnitude to those reported in previous studies involving other inflammatory-related genes.<sup>26</sup>

There was a substantial difference in the frequency of the homozygous GG genotype for rs1048990 in the two European and the three Japanese studies (2.1 and 3.0% vs 8.9, 10.7, and 9.8% respectively). The observed genotype frequencies of rs1048990 in these two populations are consistent with those reported by HapMap (<http://www.hapmap.org/Data Release #21a>). Despite the large number of cases in the ISIS study, the lower allele frequency results in a greater variability in the effect size and, hence, a proportionately smaller contribution to the meta-analysis (as depicted by a smaller square and wider CI

in Figure 1). Moreover, the low frequency of the GG genotype means that the estimated population attributable risk is only about 1–2% in this Western European population compared with 3–8% in the Japanese population (depending on whether a recessive or codominant genetic model is used).

The four other studies appear to be well conducted; controls were recruited from the same population as the cases, and the control genotype frequencies were in HWE. Even though selection of cases and controls of the same nationality and ethnicity does not rule out a false positive result due to population stratification,<sup>27</sup> the researchers in the Ozaki study also used genomic control methods<sup>28</sup> to account for this. The procedures used for recruitment of controls in the ISIS case–control study (involving spouses of siblings and their children) should also have minimised the impact of population admixture between controls and cases. There were departures from HWE for two rare SNPs in the ISIS study, but this was not the case for rs1048990, which was the main SNP of interest.

In the Ozaki study, the authors reported that the G allele was associated with 1.7–1.8 higher expression of PSMA6, which they suggested could have an effect on genes in the NF $\kappa$ B pathway. The Suita study conducted by Takashima *et al*<sup>24</sup> found the rs1048990 SNP to be significantly associated with intima-media thickness of the carotid artery and suggested that it may play a role in the pathogenesis of atherosclerosis. PSMA6 concentrations have not been measured in the ISIS study, but measurements were made of plasma concentrations of CRP and fibrinogen (which are established markers of inflammation). Both the p50 and p60 subunits of NF $\kappa$ B play a role in the transcription of CRP,<sup>29</sup> which appeared to have proatherogenic properties through upregulation of expression of AT<sub>1</sub> receptor<sup>30</sup> and activation of vascular smooth muscle cells in some studies.<sup>31,32</sup> Over 30 studies have reported that higher plasma levels of CRP are associated with higher risks of CHD,<sup>33</sup> but other studies have not been able to confirm these associations,<sup>34,35</sup> and there is uncertainty as to whether these associations are causal. Fibrinogen has been observed to regulate NF $\kappa$ B activation and the expression of chemokines in vascular endothelial cells,<sup>36</sup> but for it too there is uncertainty about the nature of the relationship with vascular disease.<sup>37,38</sup> In the ISIS study, CRP and fibrinogen levels were not significantly associated with the number of copies of the G allele for the rs1048990 SNP. In a recessive genetic model, carriers of the GG genotype appeared to have slightly higher levels of fibrinogen and CRP, but the low frequency of this genotype made these differences inconclusive.

Ozaki *et al*<sup>39</sup> investigated the PSMA6 gene, because they had previously observed that SNPs encoding lymphotoxin  $\alpha$  (LTA), a cytokine produced in the early stages of inflammation, was associated with an increased susceptibility to MI in a Japanese population. Binding of LTA to its receptor is known to activate NF $\kappa$ B, which has an effect on the ubiquitin–proteasome system. We have previously reported no significant association of the rs1041981 SNP in LTA gene with MI risk in the ISIS study.<sup>8</sup> The frequency of the minor A allele for rs1041981 is similar for European and Japanese populations from HapMap (0.36 and 0.43, respectively) ([http://www.hapmap.org/Data Release #21a](http://www.hapmap.org/Data/Release_#21a)). Thus, the lack of association of LTA with MI risk in the ISIS study was unlikely to be due to differences in the frequency of this allele, although it could be that this particular pathway is less important in Caucasians than in Japanese populations.

In conclusion, although the ISIS case–control study failed to identify statistically significant associations with MI for rs1048990 in the PSMA6 gene, the results remain entirely consistent with the findings of the previous studies. The results of a meta-analysis of these studies were homogenous, but further population-based studies (or meta-analyses of such studies) involving very large sample

sizes (in excess of 10 000 cases and 10 000 controls) would help to confirm the relevance of this gene for MI risk. Even if this variant is found to be causal, however, the low prevalence of the GG genotype means that the impact on overall MI risk is likely to be small, especially in western populations.

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