Lymphotoxin-α Gene and Risk of Myocardial Infarction in 6,928 Cases and 2,712 Controls in the ISIS Case-Control Study

Robert Clarke1*, Peng Xu2*, Derrick Bennett1, Sarah Lewington1, Krina Zondervan3, Sarah Parish1, Alison Palmer1, Sarah Clark1, Lon Cardon3, Richard Peto1, Mark Lathrop2, Rory Collins1 for the International Study of Infarct Survival (ISIS) Collaborators

1 Clinical Trial Service Unit, Old Road Campus, Oxford, United Kingdom, 2 Centre National de Genotypage, Paris, France, 3 Wellcome Trust Centre for Human Genetics, Old Road Campus, Oxford, United Kingdom

Lymphotoxin-α (LTA) is a pro-inflammatory cytokine that plays an important role in the immune system and local inflammatory response. LTA is expressed in atherosclerotic plaques and has been implicated in the pathogenesis of atherosclerosis and coronary heart disease (CHD). Polymorphisms in the gene encoding lymphotoxin-α (LTA) on Chromosome 6p21 have been associated with susceptibility to CHD, but results in different studies appear to be conflicting. We examined the association of seven single nucleotide polymorphisms (SNPs) across the LTA gene, and their related haplotypes, with risk of myocardial infarction (MI) in the International Study of Infarct Survival (ISIS) case-control study involving 6,928 non-fatal MI cases and 2,712 unrelated controls. The seven SNPs (including the rs909253 and rs1041981 SNPs previously implicated in the risk of CHD) were in strong linkage disequilibrium with each other and contributed to six common haplotypes. Some of the haplotypes for LTA were associated with higher plasma concentrations of C-reactive protein (p = 0.004) and lower concentrations of albumin (p = 0.023). However, none of the SNPs or related haplotypes were significantly associated with risk of MI. The results of the ISIS study were considered in the context of six previously published studies that had assessed this association, and this meta-analysis found no significant association with CHD risk using a recessive model and only a modest association using a dominant model (with narrow confidence intervals around these risk estimates). Overall, these studies provide reliable evidence that these common polymorphisms for the LTA gene are not strongly associated with susceptibility to coronary disease.

Introduction

Lymphotoxin-α (LTA) is a cytokine produced by all cells in the body in response to tissue injury, and it plays an important role in regulation of the immune system and local inflammatory response [1]. LTA and tumour necrosis factor share a common receptor that controls the expression of vascular endothelial cell adhesion molecules and nitric oxide production [2]. LTA is expressed in atherosclerotic plaques in animal models, and has been implicated in the pathogenesis of atherosclerosis and coronary heart disease (CHD) [3,4].

In an initial genome-wide case-control screen involving 65,671 single nucleotide polymorphisms (SNPs) from 13,738 genes in 94 myocardial infarction (MI) cases and 658 controls in Japan, susceptibility to MI appeared to be associated with the SNP 252A→G (rs909253) in the LTA gene, which encodes LTA on Chromosome 6p21 [5]. This possible association with rs909253, and with two further SNPs (rs1800683 and rs1041981) in near-perfect linkage disequilibrium (LD) with it, was subsequently replicated by the same investigators in a further 1,133 MI cases and 1,006 controls. Among these SNPs, the non-synonymous coding SNP rs1041981 seemed to be most strongly implicated because of its association with induction of vascular endothelial adhesion molecules [5]. An association of rs909253 or rs1041981 with the risk of CHD has subsequently been observed in some smaller studies [6-7], but not in others [8-10].

If genetic variants associated with the control of pro-inflammatory cytokines are associated even modestly with MI risk, this could have important implications for understanding the pathogenesis of atherosclerosis. However, reliable confirmation or refutation of such associations typically requires studies involving much larger numbers of cases than in the original hypothesis-generating studies [11,12]. We have examined the association between seven SNPs in the LTA gene, and their related haplotypes, with risk of MI in the large ISIS [13] case-control study, and then included it in a meta-analysis of all previously published reports on this association.

Citation: Clarke R, Xu P, Bennett D, Lewington S, Zondervan K, et al. (2006) Lymphotoxin-α gene and risk of myocardial infarction in 6,928 cases and 2,712 controls in the ISIS case-control study. PLoS Genet 2(7): e107. DOI: 10.1371/journal.pgen.0020107

Editor: Trudy Mackay, North Carolina State University, United States of America
Received January 27, 2006; Accepted May 26, 2006; Published July 7, 2006
DOI: 10.1371/journal.pgen.0020107
Copyright: © 2006 Clarke et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: BMI, body mass index; CHD, coronary heart disease; CI, confidence interval; CRP, C-reactive protein; HBE, Hardy-Weinberg Equilibrium; ISIS, International Study of Infarct Survival; LD, linkage disequilibrium; LTA, lymphotoxin-α; MI, myocardial infarction; SNP, single nucleotide polymorphism
* To whom correspondence should be addressed. E-mail: robert.clarke@ctsu.ox.ac.uk
† These authors contributed equally to this work.
Synopsis

Lymphotoxin-α gene (LTA) encodes a cytokine involved in the immune system that has been linked with risk of coronary heart disease (CHD). In one of the first genome-wide association studies, a Japanese group reported that polymorphisms for the LTA gene were associated with a 1.8-fold higher risk of CHD. The authors of this current paper examined associations of several polymorphisms for the LTA gene with risk of CHD in a case-control study of 6,928 heart attack survivors and 2,712 unrelated controls in the United Kingdom. None of the polymorphisms for LTA (including those examined in the original Japanese study) were associated with CHD risk. Moreover, a meta-analysis of all published studies found no significant association of LTA with CHD risk. These findings emphasize the need for large-scale studies to have sufficient power to detect associations of genetic variants with CHD risk and the need for replication of any such associations in independent studies of even larger size.

Results

Table 1 shows the location and relevant characteristics of the seven SNPs in the LTA gene on chromosome 6p21 that were examined in the International Study of Infarct Survival (ISIS) study. Data for six of these SNPs were available in 6,928 cases and 2,712 controls, and for one SNP (rs1041981) in only 6,858 cases and 2,690 controls who had sufficient DNA. The mean age was 54.8 y (standard deviation ±7.3 y) in cases and 46.2 y (standard deviation ±9.6 y) in controls (Table 2). The MI cases had a higher mean body mass index (BMI) and higher prevalence of current smokers, hypertension, and diabetes mellitus compared with controls. Table 2 also shows that, compared with controls, MI cases had higher plasma concentrations of apolipoprotein B, fibrinogen, and C-reactive protein (CRP), and lower mean concentrations of apolipoprotein A1 and albumin.

LTA SNPs and Haplotypes

All seven SNPs studied in the LTA gene were in Hardy-Weinberg Equilibrium (HWE) among both cases and controls (unpublished data). Reproducibility of the genotyping was high, with a weighted kappa score of 0.99–1.00 for each of these seven SNPs in the LTA gene (unpublished data). The region of LTA investigated in our study, which spanned about 1 kb and was bounded by rs2071590 and rs1041981, and included all three SNPs examined in the study that had generated the hypothesis of association with CHD [5]. It also included the rs1041981 SNP examined in the PROCARDIS and other published studies of the LTA gene and CHD [9,10]. Table 3 shows that all seven SNPs examined were in high LD, with pairwise LD measured in terms of D² ≥ 0.85 between each SNP. Although the pairwise r² values between some of the SNPs were low (perhaps due to differences in allele frequency), the r² values between the rs909253, rs1800683, and rs1041981 SNPs implicated in the hypothesis-generating study [5] were ≥0.98. Hence, all of the SNPs studied were incorporated in the haplotype analysis.

Associations with CRP, Fibrinogen, and Albumin

Table 4 shows the mean (standard error) values of cardiovascular risk factors by individual LTA haplotypes in the controls. Using the haplotype-based General Linear Model (GLM), statistically significant differences were observed between these haplotypes and plasma concentrations of CRP and albumin (although the absolute differences were generally small), which is consistent with some of these SNPs being functional. There were no significant differences between the haplotypes in BMI or plasma concentrations of apolipoprotein A1, apolipoprotein B, or fibrinogen.

Association with MI Risk

Table 5 displays the associations with MI risk in the ISIS case-control study for individual SNPs of the LTA gene, after adjustment for age and sex, when analyzed using co-dominant, recessive, and dominant genetic models, respectively. Neither the rs909253 nor the rs1041981 SNP for the LTA gene that was studied by Ozaki et al. [5] was significantly associated with MI risk using any of the genetic models. Although the most frequent genotype has been used as the reference category, any group can be compared with any other since each is displayed with its appropriate confidence interval (CI) using “ floated” absolute risks (see Materials and Methods). None of the individual haplotypes for the LTA gene was associated with MI risk (χ² test of heterogeneity: χ² = 7.97, p = 0.24) (unpublished data).

Comparison with Other Studies

Figure 1 shows the associations of CHD risk with rs909253 or rs1041981 in all previously published studies [5–10] and in the present ISIS study. Among the six previous studies, three

Table 1. Identity and Base-Pair Position of SNPs for the LTA Gene on Chromosome 6, and Minor Allele Frequencies in Controls in the ISIS Study

| dbSNP* | rsID    | Base-Pair Position | Inter-SNP Distanceb (bp) | Gene Location | Alternative Name | Minor Allele (Frequency) |
|--------|---------|--------------------|--------------------------|---------------|-----------------|--------------------------|
| rs2071590 | 31647747 | 0                  | Upstream                 | —             | A (0.35)        |
| rs1800683 | 31648050 | 303                | Upstream                 | 10G/A         | A (0.36)        |
| rs909253 | 31648292 | 242                | Intron-1                 | 252A/G        | G (0.36)        |
| rs746868  | 31648408 | 116                | Intron-1                 | C (0.38)      |
| rs2857713 | 31648535 | 127                | Exon-2                   | 319C/A        | C (0.26)        |
| rs3093543 | 31648736 | 201                | Exon-3                   | —             | C (0.06)        |
| rs1041981 | 31648763 | 27                 | Exon-3                   | 804C/A        | A (0.36)        |

*dbSNP, the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP).

bDistance from the preceding SNP listed above.

bp, base pairs.

DOI: 10.1371/journal.pgen.0020107.t001
of this meta-analysis materially, with a combined odds ratio for rs909253 instead of rs1041981 did not change the results (Table 2). For the dominant model, there was significant heterogeneity between the results of the individual studies (\( \chi^2 = 18.0, p = 0.007 \)), although there was a highly significant trend towards the null with increasing study size (\( \chi^2 \_1 = 7.0, p = 0.008 \)). For the dominant model, there was significant heterogeneity between the results of the individual studies (\( \chi^2 \_6 = 18.0, p = 0.007 \)), but this chiefly reflected a trend with study size (\( \chi^2 \_1 = 9.9, p = 0.002 \)) and no significant residual heterogeneity (\( \chi^2 \_5 = 8.1, p = 0.1 \)). Inclusion of the ISIS results for rs909253 instead of rs1041981 did not change the results of this meta-analysis materially, with a combined odds ratio of 1.06 (95% CI: 0.97–1.16) in the recessive model and 1.08 (95% CI: 1.02–1.15) in the dominant model.

### Discussion

The ISIS study is the largest genetic association study of MI risk and polymorphisms in LTA, involving 6,928 MI cases and 2,712 unrelated controls. The recruitment procedure used for controls in the ISIS case-control study (involving spouses of siblings and their children: see Materials and Methods) should have minimised the risk of population admixture between samples and cases. The seven SNPs studied included three that had been previously been reported [5], as well as others identified by resequencing to be in high LD with each other (see Materials and Methods), and covered a genomic region of about 1 kb in the LTA gene. The ISIS case-control study found no significant association of MI risk with any of these individual SNPs or with any of the six common haplotypes studied (including those associated with significantly higher plasma concentrations of CRP and albumin).

The present findings differ from those of the hypothesis-generating study [5] which reported that, in a recessive model, the SNPs rs909253 and rs1800683 in the LTA gene were associated with odds ratios for MI of 0.99 (95% CI: 1.24–2.36) and 1.78 (95% CI: 1.39–2.27), respectively. That study used a two-stage design in which 65,671 genetic markers in 13,738 genes were initially screened in 94 MI cases and 658 controls, and then the 1% of markers that appeared most strongly associated with MI were re-examined in a further 1,133 MI cases and 1,006 controls. In that study, a third SNP in LTA (rs1041981) also appeared to be associated with risk of MI with an odds ratio of 1.78 (95% CI: 1.39–2.27) [5]. It is possible that those results represented false-positive results identified among the relatively small number of cases. Alternatively, it may reflect an imbalance in the control population which was selected from several different population sources, since significant departures from Hardy-Weinberg equilibrium were observed in the genotype distributions in case and control samples in the Ozaki et al. study [5] (possibly indicating population stratification) [14]. Two further case-control studies carried out in Japanese populations have reported conflicting results, with one appearing to confirm an association of the rs909253 SNP with risk of MI [6] and the other not doing so [8].

In a multicentre European study of 400 trio families, the examined associations with rs909253 [5,6,8] and four examined associations with rs1041981 [5,7,9,10] (and one study reported data separately for males and females [8]). For the ISIS case-control study (without adjustment for age and sex), the association with rs1041981 is displayed in Figure 1, but that SNP was in near perfect LD with rs909253 (which had an \( r^2 = 1.00 \) [99% CI: 0.89–1.13] in a dominant model: see Figure 1). After excluding the hypothesis-generating study [5], the combined odds ratio in a recessive model was 1.07 with a 95% CI of 1.02–1.16 (Figure 1A), and in an autosomal model and 1.00 [99% CI: 0.89–1.13] in a dominant model: see Figure 1). After excluding the hypothesis-generating study [5], the combined odds ratio in a recessive model was 1.07 with a 95% CI of 1.02–1.16 (Figure 1A), and in an autosomal model and 1.00 [99% CI: 0.89–1.13] in a dominant model: see Figure 1).

### Table 2. Characteristics of Confirmed Myocardial Infarction Cases and Controls (Mean (SD) or %)

| Characteristic                  | Cases of MI (n = 6,928) | Controls (n = 2,712) |
|--------------------------------|-------------------------|----------------------|
| Age (y)                        | 54.8 (7.3)              | 46.2 (9.6)           |
| Percent Male                   | 82.3                    | 44.6                 |
| Body mass index (kg/m²)*       | 26.1 (6.6)              | 24.7 (4.3)           |
| Cigarette smoker (%)           | 21.6                    | 11.2                 |
| Hypertension (%)               | 56.7                    | 19.5                 |
| Diabetes mellitus (%)*         | 5.5                     | 1.4                  |
| Apolipoprotein A1 (g/l)*       | 1.14 (0.22)             | 1.24 (0.20)          |
| Apolipoprotein B (g/l)*        | 1.16 (0.29)             | 0.99 (0.25)          |
| Fibrinogen (g/l)*              | 3.71 (0.94)             | 3.40 (0.84)          |
| CRP (log mg/dl)*               | 1.02 (1.95)             | 0.03 (1.21)          |
| Albumin (g/l)*                 | 37.8 (3.84)             | 40.7 (3.50)          |

*Values were adjusted for age and sex. Data for LTA were available for 6,928 cases and 2,712 controls, whereas plasma biochemistry results were available for between 4,979 and 5,242 cases and for between 2,348 and 2,712 controls.

### Table 3. Pair-Wise Standardised Disequilibrium (\( D^2 \)) and Correlation (\( r^2 \)) Coefficients, between Individual SNPs for the LTA Gene

| dbSNP* rsID | Polymorphism | \( D^2 \) Pair-wise LD (and \( r^2 \) Correlation Coefficients) |
|------------|--------------|---------------------------------------------------------------|
| rs2071590  | A/G          | 0.99 (0.30) 1.00 (0.30) 1.00 (0.86) 0.85 (0.13) 1.00 (0.04) 1.00 (0.30) |
| rs1800683  | A/G          | 1.00 (0.98) 1.00 (0.34) 0.86 (0.15) 1.00 (0.04) 1.00 (0.99) |
| rs909253   | A/G          | 1.00 (0.34) 0.87 (0.15) 1.00 (0.04) 1.00 (0.99) |
| rs746688   | C/G          | 0.88 (0.16) 1.00 (0.04) 1.00 (0.34) |
| rs2857713  | C/T          | 0.89 (0.15) 0.87 (0.14) 1.00 (0.04) |
| rs3093543  | A/C          | 1.00 (0.04) 1.00 (0.04) 1.00 (0.04) |
| rs1041981  | C/A          | 1.00 (0.04) 1.00 (0.04) 1.00 (0.04) |

*dbSNP*, the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP).

DOI: 10.1371/journal.pgen.0020107.t002

PLoS Genetics | www.plosgenetics.org July 2006 | Volume 2 | Issue 7 | e1070992
PROCARDIS Consortium reported that rs1041981 was associated in a recessive model with a 1.96 (95% CI: 1.25–3.13) higher risk of CHD before age 65 y [7]. In principle, a study design involving the use of trios should eliminate the potential risk of population admixture that may occur in unrelated case-control studies. However, the apparent association in PROCARDIS could still reflect the play of chance in a study involving relatively small numbers of diseased cases. A subsequent larger, trio family study of 671 UK families reported no significant association of rs1041981 with risk of MI [10].

An alternative explanation for these apparently discrepant findings might be that none of the SNPs in the \textit{LTA} gene studied by Ozaki et al. is causally associated with MI, but are instead in high LD with some other SNP which is present in higher frequency in Japanese than in populations of European descent [12]. In such circumstances, detection of this association via the common \textit{LTA} SNPs would be facilitated in Japanese populations. There would also be a better chance of observing it in trio families of European descent [7], since a sampling strategy based on at least one family member suffering an MI would inflate the frequency of a causal allele that is otherwise rare in the general population. Lack of SNPs in the \textit{LTA} gene that were genotyped in both the European-descent and Japanese populations in HapMap Phase II [15] did not allow us to investigate the possibility of differential LD patterns between the two populations. However, the absence of an association in a large Japanese population by Yamada et al. [8] and in another large UK trio family study [10] does not support this alternative hypothesis. The ISIS study had more than 90% power to detect a 10% higher risk of MI associated with rs909253 and rs1041981 SNPs for the \textit{LTA} gene (assuming an allele frequency of about 0.35) using co-dominant or recessive genetic models.

### Table 4. Mean (SE) Values for Selected Characteristics by Haplotypes for the LTA Gene in Controls

| LTA Haplotypes | Frequency | BMI (kg/m²) | ApoA (g/l) | ApoB (g/l) | Fibrinogen (g/l) | CRP (log units) | Albumin (g/l) |
|----------------|-----------|-------------|------------|------------|-----------------|----------------|---------------|
| GAGGTAA        | 0.35      | 25.0 (0.2)  | 1.24 (0.01)| 1.00 (0.01)| 3.47 (0.03)     | 0.18 (0.05)    | 40.0 (0.1)    |
| AGACTAC        | 0.33      | 24.8 (0.1)  | 1.24 (0.01)| 0.99 (0.01)| 3.41 (0.02)     | 0.08 (0.04)    | 40.0 (0.1)    |
| GGGAGCAC       | 0.18      | 24.9 (0.2)  | 1.24 (0.01)| 0.99 (0.01)| 3.44 (0.03)     | 0.06 (0.05)    | 38.9 (0.1)    |
| GGAGCC         | 0.06      | 24.5 (0.2)  | 1.24 (0.01)| 1.00 (0.02)| 3.41 (0.04)     | 0.02 (0.07)    | 39.8 (0.2)    |
| GGACTAC        | 0.04      | 24.3 (0.3)  | 1.24 (0.02)| 0.98 (0.02)| 3.37 (0.06)     | 0.11 (0.09)    | 40.2 (0.3)    |
| GGAGTAC        | 0.02      | 24.8 (0.4)  | 1.23 (0.02)| 1.02 (0.03)| 3.34 (0.07)     | 0.16 (0.12)    | 39.7 (0.4)    |
| All others     | 0.02      | 24.5 (0.3)  | 1.23 (0.02)| 1.00 (0.02)| 3.48 (0.07)     | 0.07 (0.12)    | 40.2 (0.3)    |
| p-Value* for heterogeneity | 0.153     | 0.129       | 0.652      | 0.051      | 0.004           | 0.023          |

*Based on heterogeneity between the six common haplotypes.

ApoA, apolipoprotein A; ApoB, apolipoprotein B.

DOI: 10.1371/journal.pgen.0020107.t004

### Table 5. Odds Ratio (95% CI) of MI Associated with SNPs for LTA in ISIS Study (Adjusted for Age and Sex)

| dbSNP* | Genotype | Frequency | Model for Individual SNPs | Recessive Model | Dominant Model |
|--------|----------|-----------|---------------------------|----------------|---------------|
|        |          | Cases (%) | Controls (%) | OR (95% CI) | OR (95% CI) |
| rs2071590 | GG | 44.2 | 42.7 | 1.00 (0.92–1.09) |
|         | AG | 43.9 | 45.1 | 0.95 (0.88–1.03) |
|         | AA | 11.9 | 12.2 | 0.87 (0.75–1.02) |
| rs1800683 | AA | 41.0 | 40.8 | 1.00 (0.92–1.09) |
|         | AG | 45.8 | 45.6 | 1.01 (0.93–1.09) |
|         | GG | 13.2 | 13.6 | 0.91 (0.79–1.06) |
| rs909253 | AA | 41.3 | 41.1 | 1.00 (0.92–1.09) |
|         | AG | 45.6 | 45.6 | 1.00 (0.92–1.08) |
|         | GG | 13.1 | 13.3 | 0.92 (0.79–1.06) |
| rs746688 | GG | 39.7 | 38.5 | 1.00 (0.92–1.09) |
|         | CG | 45.9 | 47.4 | 0.93 (0.86–1.01) |
|         | CC | 14.4 | 14.2 | 0.96 (0.83–1.10) |
| rs2857713 | TT | 54.5 | 54.8 | 1.00 (0.93–1.08) |
|         | CT | 38.5 | 38.7 | 1.07 (0.98–1.17) |
|         | CC | 7.0  | 6.5  | 1.06 (0.86–1.31) |
| rs3093543 | AA | 87.4 | 87.7 | 1.00 (0.94–1.06) |
|         | AC | 12.2 | 12.0 | 1.14 (0.98–1.33) |
|         | CC | 0.4  | 0.3  | 1.83 (0.68–4.97) |
| rs1041981 | CC | 41.4 | 41.4 | 1.00 (0.92–1.09) |
|         | AC | 45.6 | 45.5 | 1.01 (0.93–1.09) |
|         | AA | 13.0 | 13.1 | 0.95 (0.82–1.09) |

*dbSNP, the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP).

DOI: 10.1371/journal.pgen.0020107.t005
controls in the ISIS study from spouses of siblings or spouses of children, together with the very small proportion of participants of non-European descent in this study would indicate that the risk of population stratification is small.

A major problem in genetic association studies has been the high proportion of false-positive results suggested by studies involving relatively small numbers of diseased cases [11,12]. Attenuation in the strength of the observed association in larger studies is consistent with publication bias, whereby more extreme results are more likely to be published earlier [16]. The apparent discrepancy between the results of studies of LTA and CHD risk reinforces the need for studies involving larger numbers of disease cases, unrelated controls selected from the same population as the cases, and genotyping of markers that provide good coverage of the gene of interest. The ISIS case-control study of nearly 7,000 cases with MI before age 65 y and of family-based controls, considered in the context of previously reported studies of this association, provides reliable evidence that these common polymorphisms for the LTA gene are not strongly associated with susceptibility to coronary disease. The present study cannot exclude the possibility of involvement of other variants near the LTA gene (e.g., in more distal regulatory regions), which were not considered.

Figure 1. Odds ratio (CI) for Coronary Heart Disease Associated with Genotypes of the LTA Gene in ISIS and Other Studies Using Recessive and Dominant Models

(A) shows the recessive model and (B) shows the dominant model. Studies are of either the rs909253 SNP (A/G alleles) or the rs1041981 SNP (A/C alleles). The hypothesis-generating study [5] has been excluded from the combined estimates for all other studies that tested the hypothesised association. Size of the squares is proportional to the variance of the log odds ratio, and horizontal lines represent the 99% CIs, and the width of the diamonds represents the 95% CIs. The associations have not been adjusted for age or sex.

DOI: 10.1371/journal.pgen.0020107.g001
Materials and Methods

Study population. The design of the ISIS case-control study has been described in detail in previous publications [17,18]. Cases genotyped for this study were men aged 30–64 y and women aged 30–84 y, with non-fatal MI confirmed by cardiac enzyme or electrocardiographic criteria (or both). Blood was collected on admission to hospital with their index MI, and a few months later, information was sought from surviving cases about aspects of their previous lifestyle. A similar questionnaire was sent to their siblings and children older than 30 y, and to the spouses of those relatives, with blood sought from all those who completed a questionnaire. Controls genotyped in the present study were spouses of their siblings or children (i.e., unrelated to the MI cases except by marriage) aged 30–64 y, and with no history of MI, angina, or other definite heart disease. Ethnicity was not recorded, but the family-based recruitment strategy should have yielded similar distributions among cases and controls (and, based on other studies conducted by us in these UK hospitals, more than 95% would be expected to be of European descent [19]). Participants provided consent to participate in the study, which had research ethics committee approval.

Laboratory methods. DNA was extracted from frozen buffy coat samples using a Taqman and amplification technique, as previously described [17,18]. Seven SNPs for LTA (rs2071590, rs1800683, rs909253, rs746868, rs2857713, rs3093543, and rs1041981) were selected, in addition to the 22 SNPs from previous studies, from among all known SNPs for the LTA gene in the National Center for Biotechnology Information (NCBI) genome assembly 35. Genotyping using mass spectrometry was carried out at the Centre National de Genotypage (CNG) in Paris without knowledge of disease status, with sample blank absorbance readings subtracted from the final reaction absorbance to correct any interference due to haemolysis prior to sample separation [20]. Fibrinogen and high sensitivity CRP concentrations were measured using DadeBehring reagents on an automated Dade-Behringer Nephelometer II analyser (Dade-Behring GmbH, Marburg, Germany). Previous studies had indicated small changes in measured levels of plasma analytes due to delay in plasma separation [20], so the analyses were adjusted for time from collection to separation, as well as for date of assay to allow for any slight assay drift. The intra-assay coefficients of variation, based on repeat analyses of control material, were 3%–4% for all plasma analytes (apolipoprotein A1, apolipoprotein B, albumin, fibrinogen, and CRP).

Statistical analysis. The genotypic distributions of each SNP were assessed for HWE in cases and controls separately using an exact chi-square goodness of fit test. A threshold of p < 0.05 was used to indicate departure from HWE. Allelic association, or LD, between SNPs was assessed using the standardised disequilibrium (D') [21] and correlation (r) [22] coefficients. The maximum likelihood estimates of haplotype frequencies and posterior probabilities of pairs of haplotypes were estimated for each individual with an expectation-maximization (E-M) algorithm under the HWE assumption using the Haplostats suite of software in R [23]. Haplotypes with an estimated frequency >1% were analyzed separately, and the remaining haplotypes were combined. A weighted kappa score was used to assess reproducibility of each of the individual SNPs studied. Analysis of variance was used to assess the associations of haplotypes with levels of cardiovascular risk factors in controls. Logistic regression adjusted for age and sex was used to estimate odds ratios for MI of each SNP and haplotype. Floating absolute risks and CIs were used to share the variance of the log odds ratios appropriately between the different genotypes or haplotypes, which allows a CI to be computed for the reference category and any group to be compared directly with any other [24,25]. The power calculations for the ISIS case-control study to detect associations of MI risk with either the rs909253 or rs1041981 SNP for the LTA gene were estimated using standard methods [26,27].

Finally, a meta-analysis was conducted of the associations between rs909253 or rs1041981 (which are in perfect LD in Japanese and European-descent populations) and the incidence of CHD in the ISIS study and all other published studies. For the latter studies, odds ratios for CHD were calculated from the published number of cases and controls in each of the three categories, either assuming an autosomal dominant model (homozygous and heterozygous vs. wild type) or recessive model (homozygous vs. heterozygous and wild type). Since the Ozaki et al. study using a recessive model was the hypothesis-generating study [5], it was not included in the combined results for the meta-analysis. The results for all remaining studies were assessed for heterogeneity and for linear trend by study size [16,28]. Summary estimates of the odds ratios from all studies were obtained by combining the separate estimates of the inverse variance–weighted log odds ratios from each study [29]. To minimise the risk of false-positive results associated with multiple comparisons, 99% confidence intervals were used for individual studies and 95% confidence intervals for sub-totals. All analyses were carried out using SAS version 8.2 (Cary, North Carolina, United States) and R version 1.9.1 (http://www.r-project.org).

Acknowledgments

The chief acknowledgment for the ISIS study is to the patients and their relatives who collaborated, to their general practitioners, and to the medical and nursing staff from more than 100 hospitals in the UK. A list of the participating centres and collaborators is given in the ISIS-3 report [13]. We thank Peter Sleight (Chairman of the ISIS Steering Committee) and Paul Sherliker (for producing the figures) of the Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford, respectively, and Fiona Green of the Wellcome Trust Centre for Human Genetics, Oxford.

Author contributions. R. Clarke, P. Xu, D. Bennett, S. Parish, R. Peto, M. Lathrop, and R. Collins conceived and designed the experiments. R. Clarke, P. Xu, D. Bennett, S. Clark, M. Lathrop, and R. Collins performed the experiments. R. Clarke, P. Xu, D. Bennett, S. Parish, R. Peto, S. Parish, R. Lathrop, and R. Collins analyzed the data. R. Clarke, P. Xu, D. Bennett, S. Parish, R. Lathrop, and R. Collins contributed reagents/materials/analysis tools. R. Clarke, P. Xu, D. Bennett, S. Parish, R. Lathrop, and R. Collins wrote the paper.

Funding. The ISIS trials and epidemiological studies were supported by the manufacturers of the study drugs, and by the British Heart Foundation, Medical Research Council, Cancer Research United Kingdom, Tobacco Products Research Trust of the UK Department of Health Independent Scientific Committee on Smoking and Health, and Oxford National Health Service Genetic Knowledge Park.

Competing interests. The authors have declared that no competing interests exist.

References

1. Cope AP (1998) Regulation of autoimmunity by proinflammatory cytokines. Curr Opin Immunol 10: 669–676.
2. LeBoeuf RC, Schreyer SA (1998) The role of tumour necrosis factor-a receptors in atherosclerosis. Trends Cardiovasc Med 8: 131–138.
3. Barath P, Fishkin MC, Berenson J, Helfant RH, Forrester JS (1990) Detection and localization of human tumour necrosis factor in human atheroma. Am J Cardioil 65: 297–302.
4. Schreyer SA, Vick CM, LeBoeuf RC (1998) Loss of lymphotixin-a but not tumour necrosis factor-a reduces atherosclerosis in mice. J Biol Chem 273: 12364–12368.
5. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, et al. (2002) Functional SNP's in the lymphotixin-alpha gene that are associated with susceptibility to myocardial infarction. Nat Genet 32: 650–654.
6. Iwagaya Y, Ono K, Takagi S, Terashima M, Tsutsumi Y, et al. (2004) Association analysis between polymorphisms of the lymphotixin-alpha gene and myocardial infarction in a Japanese population. Atherosclerosis 172: 197–198.
7. PROCARDIS Consortium (2004) A trio family study showing association of the lymphotixin-a 126 (804A) allele with coronary artery disease. Eur J Cardioil 12: 770–774.
8. Yamada A, Ichicara S, Murase Y, Kato T, Izawa H, et al. (2004) Lack of association of polymorphisms of the lymphotixin-alpha gene with myocardial infarction in Japanese. J Mol Med 282: 477–483.
9. Tobin MD, Braund PS, Burton PR, Thompson JR, Steeds R, et al. (2004) Genotypes and haplotypes predisposing to myocardial infarction: A multilocus case-control study. Eur Heart J 25: 459–467.
10. Brown B, Lawrence R, Cheng S, Barrett J, Balmforth A, et al. (2006) Lack of association between premature myocardial infarction and the lymphotoxin-α C804A polymorphism in a UK discordant sibling population. Eur J Hum Genet In press.
11. Zondervan K, Cardon LR (2004) The complex interplay among factors that influence allelic association. Nat Rev Genet 5: 89–100.
12. Marchini J, Cardon LR, Phillips MS, Donnelly P (2004) The effects of human population structure on large genetic association studies. Nat Genet 36: 512–517.
13. ISIS-3 (Third International Study of Infarct Survival Collaborative Group) (1992) ISIS-3: A randomised comparison of streptokinase vs tissue plasminogen activator vs anistreplase and of aspirin plus heparin vs aspirin alone among 41,299 cases of suspected acute myocardial infarction. Lancet 339: 753–770.
14. Witte-Thompson JK, Pluzhnikov A, Cox NJ (2005) Rational inferences about departures from Hardy-Weinberg equilibrium. Am J Hum Genet 76: 967–986.
15. The International HapMap Consortium (2005) A haplotype map of the human genome. Nature 437: 1299–1320.
16. Sterne JA, Egger M, Smith GD (2001) Investigating and dealing with publication and other biases in meta-analysis. BMJ 323: 101–105.
17. Keavney B, McKenzie C, Parish S, Palmer A, Clark S, et al. (2000) Large-scale test of hypothesised associations between the angiotensin-converting enzyme insertion/deletion polymorphism and myocardial infarction in about 5000 cases and 6000 controls. International Studies of Infarct Survival (ISIS) Collaborators. Lancet 355: 434–442.
18. Keavney B, Palmer A, Parish S, Clark S, Youngman I, et al. for the International Studies of Infarct Survival (ISIS) Collaborators (2004) Lipid-related genes and myocardial infarction in 4685 cases and 3460 controls: Discrepancies between genotype, blood lipid concentrations, and coronary disease risk. Int J Epidemiol 35: 1092–1013.
19. MRC/BHF Heart Protection Study Collaborative Group (1999) MRC/BHF Heart Protection Study of cholesterol-lowering therapy and of antioxidant supplementation in a wide range of patients at increased risk of coronary heart disease death: early safety and efficacy experience. Eur Heart J 20: 725–741.
20. Clark S, Youngman LD, Palmer A, Parish S, Peto R, et al. (2003) Stability of plasma analytes after delayed separation of whole blood: Implications for epidemiological studies. Int J Epidemiol 32: 125–130.
21. Lewontin RC (1988) On measures of genic disequilibrium. Genetics 120: 849–852.
22. Hill WG, Robertson A (1968) Linkage disequilibrium in finite populations. Theor Appl Genet 38: 226–231.
23. Schaid DJ, Rowland CM, Tines DE, Jacobsen RM, Poland GA (2002) Score tests for association between traits and haplotypes when linkage phase is ambiguous. Am J Hum Genetics 70: 425–434.
24. Easton DF, Peto J, Babiker AG (1991) Floating absolute risk: An alternative to relative risk in survival in case-control analysis avoiding an arbitrary reference group. Stat Med 10: 1025–1035.
25. Plummer M (2004) Improved estimates of floating absolute risk. Stat Med 23: 93–104.
26. Slager SL, Schaid DJ (2001) Case-control studies of genetic markers: power and sample size approximations for Armitage’s test for trend. Hum Hered 52: 149–153.
27. Friedlin B, Zheng G, Zhaozhi L, Gastwirth JL (2002) Trend tests for case-control studies of genetic markers: Power, sample size and robustness. Hum Hered 55: 146–152.
28. Thompson SG (1994) Why sources of heterogeneity in meta-analysis should be investigated. BMJ 309: 1541–1555.
29. Yusuf S, Peto R, Lewis J, Collins R, Sleight P (1985) Beta blockade during and after myocardial infarction: An overview of the randomized trials. Prog Cardiovasc Dis 27: 335–371.