SHORT COMMUNICATION

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The Impact on Coronary Artery Disease of Common Polymorphisms Known to Modulate Responses to Pathogens

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Summary

There are two distinct models to explain how genetic variants contributing to cardiovascular disease may have arisen. Firstly, variants may result from random, initially neutral, mutations whose effects are largely revealed in post-reproductive individuals in industrialized societies. Alternatively, the introduced variants may confer an adaptive advantage in certain circumstances. Resistance to pathogens is one of the strongest selection pressures on human proteins. To determine whether this evolutionary pressure has made a large contribution to heart disease we tested whether seventeen polymorphisms in fourteen innate-immunity genes, with documented evidence of modulating response to pathogens, had an impact on heart disease. Genotyping was performed in 1,598 CAD subjects (ACS or stable angina) and 332 controls. The *TLR4* 399Ile allele had the greatest impact on ACS risk (uncorrected $p = 0.006$); however there was no evidence overall that the resistance alleles cumulatively influenced the risk of ACS compared to controls or stable angina patients ($p = 0.12$, and $p = 0.40$, respectively). We did note a significant interaction between age at onset of disease and combined resistance allele carriership when the ACS and non-thrombotic, stable angina groups were compared ($p = 0.04$, 16 d.f.). This suggests that innate immunity factors could have a greater impact on thrombus formation among younger CAD patients

Keywords: Innate immunity, pathogen response, resistance alleles, cardiovascular genetics

Introduction

There are two population genetics models to explain how genetic variation in cardiovascular disease has arisen. The first "neutral" model states that the variation is largely a consequence of random mutation, which becomes important in post-reproductive individuals in industrialized societies. The second "adaptive" model states that there has been selection for variation (Stearns, 1999). An important group of variants that selection is likely to have raised to high frequencies are variants that alter resistance to pathogens.

From plants to insects to humans, the innate immune response system has evolved to rapidly alert the host to invasion of pathogenic microbes which have breached the integument of eukaryotic multicellular organisms (Hoffmann *et al*. 1999; O'Neill & Greene, 1998). Unlike the adaptive immune response, the innate immune response functions via non-clonal mechanisms (Akira *et al*. 2001; Hashimoto *et al*. 1988). There is clear evidence that genetic variation within the innate immunity genes plays an important role in susceptibility to a number of diseases (Hill & Motulsky, 1999); for example up to 80% of heritable variation in susceptibility to malaria is accounted for by variation outside the HLA system (Jepson *et al*. 1997). Thus, exposure to an

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array of microbial pathogens can drive human genetic diversity (Hill, 1998; Jeffery & Bangham, 2000). Segregating genetic polymorphisms that increase pathogen resistance may not have reached fixation for a number of reasons: firstly, the disease may be geographically limited, such as malaria, leading to different frequencies in different populations (Rascu *et al*. 1997); secondly, the resistance variant may be in a transition phase, having only recently arisen in the population; thirdly, the variant may be disadvantageous in some other respect, leading to "balancing" selection. Such negative effects may relate to increased susceptibility to other pathogens, for example by altering the T helper cell Th1/Th2 balance, or it may reflect other damaging effects of the variant (such as anaemia in homozygotes for variants conferring resistance to malaria, Fairhurst *et al*. 2003).

Several innate immunity genes have been shown to be both polymorphic and implicated in resistance/susceptibility to a wide variety of pathogenic infections. For example, variants within the tumour necrosis factor alpha (*TNF-α*) promoter have been associated with altered expression of the gene and susceptibility to malaria (Knight *et al*. 1999), while inactivating mutations of the interferon-gamma gene receptor (*IFNGR1*) lead to increased susceptibility to typical mycobacteria and disseminated BCG infection in homozygous children (Jouanguy *et al*. 1997).

Variants that modulate pathogen responses may impact on cardiovascular disease in a variety of ways. The first mechanism is that resistance variants may directly lower the pathogen burden that has been postulated to be a risk factor in cardiovascular disease (Epstein *et al*. 2000, Rothenbacher *et al*. 2003). The second mechanism is that the pathogen resistance factors may typically raise the general inflammatory status of the individual, which is itself postulated to be a risk factor for heart disease (Hansson, 2001; Ludewig *et al*. 2002). The third mechanism is that the resistance variants themselves have secondary roles in signalling among cellular and serum components of the vascular system, either increasing or decreasing the risk of atherosclerosis, thrombosis, plaque rupture, haemorrhage, or some other risk component.

Therefore, an accumulation of such alleles, while advantageous with respect to microbial infection, may also promote the secondary effect of cardiovascular dysfunction through a variety of mechanisms. Already some SNPs in immunity genes have been shown to modulate both susceptibility/resistance to infection and susceptibility to various categories of CAD. The Thr 280 Met polymorphism of the *CX3CR1* gene appears to confer susceptibility to HIV infection in Caucasians (Faure *et al*. 2000), but was also independently associated with a lower risk of cardiovascular disease (McDermott *et al*. 2003). This would seem to suggest that resistance/susceptibility alleles in host defense/pathogen response genes may indeed produce a secondary effect in the cardiovascular system.

In this study we assessed the impact on CAD of common polymorphisms, in a Caucasian population, for which there was documented evidence of their association with alterations in resistance to parasitic, bacterial and viral pathogens. Thus, the objective was to determine whether there is evidence consistent with the hypothesis that enrichment of resistance alleles in the genome, while conferring the advantage of resistance to a variety of pathogens, has the secondary consequence of compromising cardiovascular function leading to the manifestation of acute coronary syndromes (ACS).

Materials and Methods

Study Populations

The information and genetic samples provided by all the individuals in this study were obtained with written informed consent in accordance with the approval of the institutional Ethics Committees of the hospitals involved.

Coronary Artery Disease Population

These subjects were drawn from studies on the genetics of Acute Coronary Syndromes. There were 1,598 subjects in total, each having presented with a history of either stable angina (SA) or acute coronary syndromes (ACS), such as unstable angina (UA) and myocardial infarction (MI) (Table 1). Of the 1,598 a total of 437 individuals were recruited from a study of the genetics

Table 1 Breakdown of ACS case and control study groups by covariates and risk factors

	Coronary artery disease study population		Control population	
Coronary artery disease state				
ACS/SA	1,237 (77.4%)	361 (22.6%)	–	
Gender:				
Male/female	1,232 (77.1%)	366 (22.9%)	225 (58.3%)	161 (41.7%)
Age:				
<55yrs (male) or <60yrs (female)/ >=55yrs (male) or >=60yrs (female)	697 (43.9%)	890 (56.1%)	329 (85.2%)	57 (14.8%)
Smoking status:				
Current or previous/Never	1,205 (76.2%)	376 (23.8%)	116 (30.4%)	265 (69.6%)
Hypertension requiring medication:				
Yes/No	658 (41.3%)	935 (58.7%)	–	–
Hypercholesterolemia requiring medication:				
Yes/No	935 (58.7%)	657 (41.3%)	–	–
Diabetes mellitus requiring medication:				
Yes/No	148 (9.3%)	1,444 (90.7%)	–	–

of Early Onset Acute Coronary Syndromes (EO-ACS), and had suffered either unstable angina or myocardial infarction before the age of 55 (males) or 60 (females). Stable angina (n = 361) was diagnosed as chest pain occurring with exercise, typical of angina in a patient with known coronary artery disease (CAD) based on coronary angiogram or a positive treadmill test. Unstable angina (n = 336) was identified as chest pain typical of angina occurring at rest with duration of at least 20 minutes, and requiring hospitalisation in a patient with known CAD based on coronary angiogram or a positive stress test. Inclusion criteria for MI (n = 901) were defined by the occurrence of chest pain for at least 20 minutes duration, along with previous or current electrocardiogram and/or serum enzyme changes diagnostic of MI. Age, gender, smoking history, history of medication for hypertension, hypercholesterolemia and diabetes were also recorded (Table 1).

Control Population

This group was intended to provide estimates of gene frequencies in a representative non-diseased population. This comprised 386 individuals who were employees of an Irish financial institution (Table 1). Previous analysis of the ACS population indicated evidence for only very mild geographic stratification of genetic background in Ireland (Dolan *et al*. 2005).

Choice of SNPs

The references providing evidence that each variant studied confers susceptibility or resistance to a parasitic, bacterial or viral pathogen are shown in Table 2.

Genotyping Methodology

Genotyping was performed by Kbiosciences Ltd, UK, in 384-well microplates using a fluorescence resonance energy transfer (FRET)-based, competitive allele-specific PCR (CASP) genotyping method. Each patient in the ACS study group (n = 1,598) and control groups (n = 386) was genotyped for each pathogen response polymorphism. Three ng of genomic DNA provided the template for each genotyping assay. Amplification was initiated using allele-specific primers and a common downstream primer. The allele-specific primers were tailed with unique sequences that created corresponding complementary sequences in the two amplicons. In the second round of amplification, quenched Universal Amplifluor™ primers in a hairpin formation were used. These primers contained 3′ tails that specifically bound to the unique tailed sequences in the amplicons, and continued amplification.

Statistical Analysis

Error rates for each of the 17 genotyping assays were calculated based on duplicate genotyping of 87 samples

Table 2 Summary of pathogen response genotypes in control, ACS and SA populations (Part I; continued on next page)

Gene	Genotype	Control (%)	ACS (%)	MI (%)	SA (%)	Functional effect of variant	References
ABO P156L	CC	90.7	90.5	90.0	89.5	Inactivating.	(Barragan *et al.* 2000;
(Alpha glycosaminyltransferase)	CT	9.0	9.3	9.8	10.2	(156L exhibits lower	Schachter *et al.* 1973)
	TT	0.3	0.2	0.2	0.3	enzymatic activity)	
ABO G235S	GG	87.8	84.3	83.9	88.5	Undetermined.	(Hennessy *et al.* 2003)
(Alpha glycosaminyltransferase)	GA/AA*	12.2	15.7	16.1	11.5	(No direct functional evidence)	
CCR2 V64I	GG	89.9	88.8	88.5	88.8	Inactivating.	(Smith *et al.* 1997;
Chemokine (C-C Motif), Receptor 2	GA	10.1	11.0	11.3	10.7	(64I allele interferes with surface	Nakayama *et al.* 2004)
	AA	0.0	0.2	0.2	0.5	expression of chemokine receptors)	
CCR5 − 503 A/G	AA	27.6	31.5	31.8	36.4	Inactivating.	(McDermott *et al.* 1998)
Chemokine (C-C Motif) Receptor 5	AG	56.7	46.9	47.6	47.5	(− 503G allele lowers protein	
	GG	15.7	21.6	20.6	16.1	levels by 45%)	
CXCL12 3′ +880 G/A	GG	64.6	63.3	64.6	61.7	Undetermined.	(Winkler *et al.* 1998)
Chemokine (CXC Motif) Ligand 12	GA	32.2	32.9	30.9	33.2	(No direct functional evidence)	
	AA	3.2	3.8	4.5	5.1		
CX3CR1 T280M	CC	68.1	68.1	67.5	67.0	Inactivating.	(Faure *et al.* 2000;
Chemokine (CX3C Motif)	CT	29.9	29.6	30.3	29.3	(280M reduces fractalkine-mediated	McDermott *et al.* 2003)
Receptor 1	TT	2.0	2.3	2.2	3.7	immune signaling)	
FCGR2A R131H	AA	33.6	34.4	34.4	30.1	Activating.	(Yee *et al.* 2000;
(Fc fragment of IgG, Low Affinity	AG	51.2	50.0	49.1	50.5	(131H increases binding affinity to	Salmon *et al.* 1992)
RIIA Receptor)	GG	15.2	15.6	16.5	19.4	IgG2 complexes)	
FUT2 W156X	GG	33.4	35.5	37.2	34.4	Inactivating.	(Ali *et al.* 2000;
(Fucosyltransferase 2)	GA	49.3	45.7	45.0	48.4	(156X produces truncated protein)	Lindesmith *et al.* 2003;
	AA	17.3	18.8	17.8	17.2		Kelly *et al.* 1995)
HLA-A A269V	CC	91.0	86.6	86.3	88.7	Inactivating.	(Liu *et al.* 2003;
(Major Histocompatibility Complex,	CT	8.5	11.9	12.0	10.1	(269V does not bind to CD8	MacDonald *et al.* 2000;
Class 1A)	TT	0.5	1.5	1.7	1.2	presenting immune cells)	Salter *et al.* 1989)

Table 2 Continued.

Gene	Genotype	Control (%)	ACS (%)	MI (%)	SA (%)	Functional effect of variant	References
IL10 -1082 G/A	GG	35.3	30.2	29.1	32.3	Inactivating.	(Helminen *et al.* 2001;
(Interleukin 10)	GA	41.4	49.1	50.1	49.0	(−1082A reduces expression of IL10)	Mozzato-Chamay *et al.* 2000;
	AA	23.3	20.7	20.8	18.7		Rees *et al.* 2002)
IL12RB1 Q214R	AA	46.5	44.1	42.0	44.4	Inactivating.	(Akahoshi *et al.* 2003)
(Interleukin 12 receptor beta-1)	AG	42.4	42.7	44.3	46.2	(214R reduces IL12- mediated signaling)	
	GG	11.1	13.2	13.7	9.4		
IL13 R110Q	CC	67.8	68.3	68.1	72.1	Activating.	(Hoerauf *et al.* 2002;
(Interleukin 13)	CT	29.0	28.8	28.8	25.6	(110Q increases IL13- mediated	Vladich *et al.* 2005)
	TT	3.2	2.9	3.1	2.3	immune signaling	
SLC11A1 IN4 + 14G/C	GG	50.0	50.2	50.3	43.5	Undetermined.	(Bellamy *et al.* 1998;
(Solute carrier family 11, member 1.)	GC	42.4	40.3	39.7	47.5	(No direct functional evidence)	Cervino *et al.* 2000)
	CC	7.6	9.5	10.0	9.0		
TLR4 D299G	AA	88.9	84.7	85.2	85.0	Inactivating.	(Arbour *et al.* 2000;
(Toll-like Receptor 4)	AG	10.8	14.7	14.2	15.0	(299G blunts LPS-mediated	Agnese *et al.* 2002)
	GG	0.3	0.6	0.6	0.0	immune response)	
TLR4 T399I	CC	89.7	83.6	83.8	84.4	Undetermined.	(Akira *et al.* 2001;
(Toll-like Receptor 4)	CT	9.7	15.9	15.7	15.3	(No direct functional evidence)	Kiechl *et al.* 2002)
	TT	0.6	0.5	0.5	0.3		
VDR M1T	CC	33.9	38.2	39.0	39.4	Undetermined.	(Gelder *et al.* 2000)
(Vitamin D Receptor)	CT	53.7	47.3	47.0	47.3	(No direct functional evidence)	
	TT	12.4	14.5	14.0	13.3		
VDR INT8 + 1281G/T	TT	28.2	28.1	29.8	26.3	Undetermined.	(Gelder *et al.* 2000;
(Vitamin D Receptor)	TG	50.5	49.0	48.1	52.8	(No direct functional evidence)	Roy *et al.* 1999)
	GG	21.3	22.9	22.1	20.9		

*For the *ABO* G235S SNP, heterozygotes and minor allele homozygotes were pooled due to insensitivity of the assay when distinguishing these two genotypes

randomly chosen from the entire population. This revealed that 13 of the 17 assays had an error rate of 0.0%, while the *HLA-A*, *TLR4* T399I and *IL10* assays each had an error rate of 1.1%. The error rate for the *ABO* P156L variant was quite high, at 9.0%, but was included in the overall analysis since the error rates within the control and CAD populations were very similar (4.6 and 4.4 for controls and CAD groups, respectively), although any positive associations involving this variant were treated with due caution. This gave a combined error rate over all 17 SNPs of <0.85%.

All logistic regression and related analyses were performed using the STATA 8.0 Package. For each locus the genotypes were combined into two groups (as shown in Table 3). The choice of dominance/recessivity for each allele was primarily based on the similarity of biological effect observed in previous studies of pathogen responses, or secondarily on the basis of pooling the two rarer genotypes; this approach was adopted to reduce the number of degrees of freedom in order to increase statistical power. The primary hypothesis was to test whether the 16 variants within a single model (with 16 degrees of freedom) showed a difference in distribution between ACS and controls. A secondary hypothesis was to determine whether there was a significant difference between ACS and stable angina. In order to determine whether the effect of the polymorphisms with respect to gene function may be an important factor in CAD risk, the 17 variants were categorised as either activating or inactivating, i.e. the polymorphism reduces/increases the level or activity of the corresponding protein (Table 2). For these analyses we excluded the *TLR4* T399I variant, since it was in close disequilibrium with the *TLR4* D299G variant. To test these hypotheses the Log-Likelihood Ratio chi-squared ($LR\chi^2$) was estimated comparing a logistic regression including all 16 terms with a logistic regression that did not. Secondary analyses inspected whether individual loci had a significant effect, using logistic regression to estimate Odds Ratios (ORs), 95% confidence intervals (CI) and p-values.

Given the tendency for many complex disorders, including cardiovascular disease, to display stronger genetic effects in early onset disease, the impact on ACS

Table 3 Individual and combined carriership of Pathogen Response Alleles and Risk of CAD Disease State

Gene	Risk allele carriership	ACS vs. control			MI vs. control			ACS vs. SA		
		O.R.	95% CI	P Value	O.R.	95% CI	P Value	O.R.	95% CI	P Value
ABO P156L	TT/CT vs. CC	1.01	0.67–1.54	0.95	1.08	0.70–1.66	0.74	0.89	0.60–1.32	0.57
ABO G235S	AA/GA vs. GG	1.34	0.93–1.91	0.11	1.38	0.95–2.00	0.09	1.43	1.00–2.06	0.05
CCR2 V64I	AA/GA vs. GG	1.12	0.76–1.66	0.56	1.16	0.77–1.74	0.47	0.99	0.68–1.44	0.98
CCR5 −503 A/G	GG/AG vs. AA	0.83	0.64–1.07	0.15	0.82	0.63–1.07	0.14	1.24	0.97–1.59	0.08
CXCL12 3′ +880 G/A	AA/GA vs. GG	1.06	0.82–1.66	0.66	1.00	0.77–1.30	0.99	0.93	0.73–1.19	0.57
CX3CR1 T280M	TT/CT vs. CC	1.00	0.77–1.29	1.00	1.03	0.79–1.34	0.84	0.95	0.74–1.23	0.71
FCGR2A R131H	GG/AG vs. AA	0.96	0.75–1.23	0.76	0.96	0.75–1.24	0.78	0.82	0.64–1.06	0.13
FUT2 W156X	AA/GA vs. GG	0.91	0.72–1.16	0.47	0.85	0.66–1.09	0.20	0.95	0.74–1.22	0.71
HLA–A A269V	TT/CT vs. CC	1.56	1.06–2.30	0.03	1.60	1.07–2.39	0.02	1.22	0.84–1.77	0.30
IL10 −1082 G/A	AA/GA vs. GG	1.26	0.98–1.62	0.08	1.33	1.02–1.73	0.04	1.10	0.85–1.42	0.46
IL12RB1 Q214R	AA/AG vs. GG	0.82	0.56–1.19	0.29	1.38	0.95–2.00	0.09	0.68	0.46–1.01	0.06
IL13 R110Q	TT/CT vs. CC	0.98	0.76–1.27	0.88	1.16	0.77–1.74	0.47	1.20	0.92–1.56	0.17
SLC11A1 IN4 +14G/C	GG/GC vs. CC	0.78	0.51–1.19	0.26	0.82	0.63–1.07	0.14	0.94	0.63–1.42	0.79
TLR4 D299G	GG/AG vs. AA	1.45	1.00–2.10	0.05	1.00	0.77–1.30	0.99	1.03	0.74–1.43	0.88
TLR4 T399I	TT/CT vs. CC	1.71	1.17–2.50	0.006	1.03	0.79–1.34	0.84	1.06	0.77–1.47	0.72
VDR M1T	TT/CT vs. CC	0.83	0.65–1.06	0.13	0.96	0.75–1.24	0.78	1.05	0.82–1.34	0.70
VDR INT8 + 1281G/T	GG/TG vs. TT	1.00	0.77–1.29	1.00	0.85	0.66-1.09	0.20	0.91	0.69–1.19	0.49
		$LR\chi^2$	d.f.	P	$LR\chi^2$	d.f.	P	$LR\chi^2$	d.f.	P
Combined allele carriership		22.61	16	0.12	25.87	16	0.06	16.79	16	0.40
Combined inactivating allele carriership*		12.95	9	0.16	14.69	9	0.10	7.03	9	0.63

*Inactivating alleles (n = 9) are identified in Table 2.

of the interaction between the dichotomised age of onset (males under 55; females under 60) and multiple genetic variants was tested, by comparing the likelihood of a model with a term for each of the gene variants against a model which also had a term for the interaction with age for each variant (Shields *et al*. 2002). Haplotypic associations and tests of linkage disequilibrium were carried out using the "hapipf" module of STATA 8.0 (Mander, 2001).

Results

Linkage Disequilibrium Testing

Of the 17 pathogen response SNPs under study there were three pairs of SNPs located within three genes, i.e. the *ABO*, *TLR4* and *VDR* genes were each represented by two SNPs in the data set. We wished to include only variants in the overall test that were not in linkage disequilibrium. Neither the *ABO* nor the *VDR* pair of polymorphisms were in linkage disequilibrium in either the control ($p = 0.20$ and $p = 0.48$) or the CAD ($p = 0.85$ and $p = 0.66$) populations. However the *TLR4* polymorphisms were in virtually complete linkage disequilibrium (($p < 0.0001$), with less than 0.01% possessing either of the two rare haplotypes in both populations. Allele counts and frequencies for the three pairs of SNPs were also tabulated by STATA 8.0, Table 2. For this reason both the *TLR4* SNPs could not be included in the overall analysis, and so the *TLR4* 399I variant was omitted leaving 16 pathogen response variants. The *TLR4* T399I variant was investigated along with the other SNPs for individual gene effects.

Acute Coronary Syndrome versus Control Populations

Genotype frequencies for all seventeen pathogen response variants are shown in Table 2. When the combined contribution of the sixteen pathogen response variants to disease risk was analysed there was no significant risk of ACS compared to the control groups (Log ratios:LR $[\chi^2] = 22.61$, $p = 0.12$, 16 d.f.; Table 3).

The *TLR4* T399I polymorphism, in particular, was strongly associated with ACS compared to controls (Odds ratio [OR] = 1.68, 95% confidence interval [CI] = 1.16–2.45, $p = 0.006$). This remained significant after correction for 17 tests. Similarly, the *HLA-A* A269V variant was significantly associated with ACS (OR = 1.56, CI = 1.06–2.30, $p = 0.03$) compared to controls, but this was not significant after correction for multiple testing.

Since myocardial infarction (MI) represents the most severe ACS disease state, the combined contribution of the sixteen pathogen-response variants to MI risk was analysed. This revealed a risk of MI compared to the control group which approached the level of statistical significance (Log ratio LR $[\chi^2] = 25.87$, $p = 0.06$, 16 d.f.; Table 3). Of greatest impact were the *TLR4* T399I and *HLA-A* A269V variants again ($p = 0.01$ and 0.02 respectively), as well as the *IL10* −1082 G/A polymorphism ($p = 0.04$) (Table 3). Clearly these individual tests were no longer significant after correction for multiple testing.

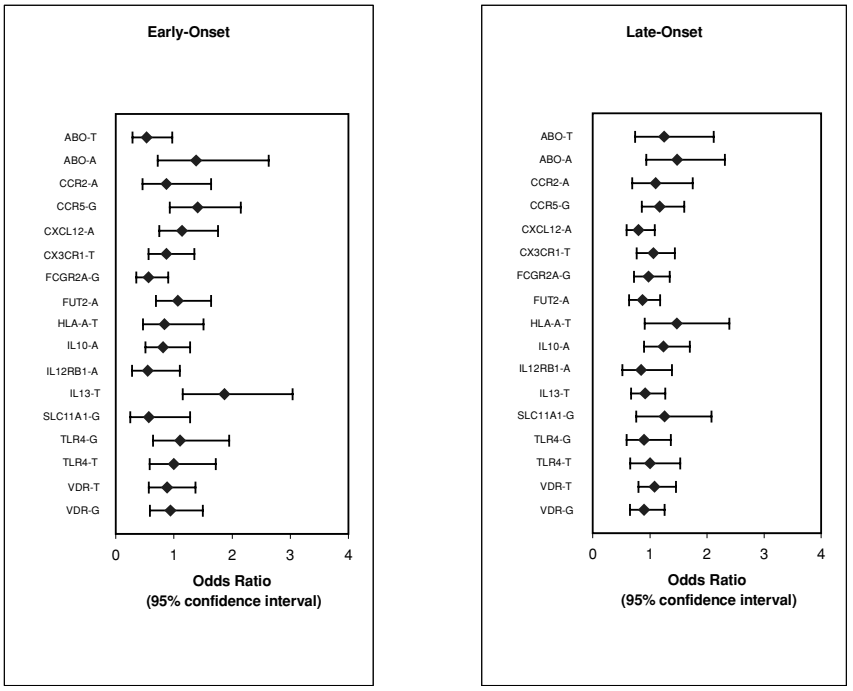
Acute Coronary Syndrome versus Stable Angina Population

There was no significant impact of combined carriership of the sixteen pathogen response variants on disease risk when individuals with a history of ACS were compared to those with stable angina (LR $[\chi^2] = 16.79$, $p = 0.40$, 16 d.f.)

Individual gene effects within this comparison group contrasted with those of the ACS versus the control comparison group. When individuals with ACS and stable disease were compared carriers of the *ABO* G235S variant were associated with the ACS group (OR = 1.43, CI = 1.00-2.06, $p = 0.05$). Two more polymorphisms, *IL12RB1* Q214R and *CCR5* −503 A/G, moderately influenced the risk of ACS compared to stable angina (OR = 0.68, CI = 0.46-1.01, $p = 0.06$ and OR = 1.24, CI = 0.97-1.59, $p = 0.08$ respectively; Table 3). Following Bonferroni correction for multiple testing these associations no longer remained significant.

Influence of Interaction between Age and Innate Immunity Variants on Disease

In order to uncover any interaction between age and genotype in relation to ACS outcome, the disease



Logistic regression of 16 Pathogen response alleles combined;
Early Onset ACS: LR χ^2 = 27.36, df = 16, P = 0.038

Logistic regression of 16 Pathogen response alleles combined;
Late Onset ACS: LR χ^2 = 16.15, df = 16, P = 0.4

Figure 1 Risk of ACS versus SA for allele carriers; Early and Late Onset Disease. Key: Diamond = Odds Ratio (OR) for each allele; Horizontal lines = 95% confidence intervals.

population was divided into those with earlier versus later onset of disease. Early-onset was defined as diagnosis of ACS or SA by the age of fifty-five years in men and by sixty years in women. There was no overall association between age and risk of ACS outcomes compared to controls for the sixteen pathogen response polymorphisms analysed (LR $\chi^2 = 7.95$, p = 0.95, 16 d.f.). However, within the CAD population itself a significant interaction was found between age and combined allele carriership leading to risk of ACS compared to stable disease (LR $\chi^2 = 26.99$, df = 16, P = 0.04).

Further analyses were performed to determine whether the combined set of pathogen response variants was responsible for risk of early-onset or late-onset ACS compared to SA. This revealed a significant association between combined allele carriership and ACS in those with early-onset disease (P < 0.038) but not late-onset disease (P = 0.44). This was mainly accounted for by a higher risk of ACS versus stable disease in the early onset group (Fig. 1) for the *IL13* 110Q polymorphism (OR = 1.87, CI = 1.15-3.04, p = 0.01), the *FCGR2A* 213H and *ABO* 156L variants (p = 0.02 and p = 0.04, respectively).

Impact of Inactivating Pathogen Response Alleles and Risk of CAD Disease State

We then investigated whether the effect of the polymorphisms with respect to gene function in innate immunity may be an important factor in CAD risk. The 17 variants were categorised as either activating or inactivating, i.e. the polymorphism reduces/increases the level or activity of the corresponding protein (Table 2). Only two variants were determined to be activating according to the literature, and six variants did not appear to have any functional evidence to support either an activating or inactivating impact on the corresponding protein. Therefore the remaining nine inactivating alleles were grouped together to determine whether their

effect on innate immune signalling influenced risk of CAD disease state.

No significant difference was observed when the distributions of the nine inactivating alleles in either the ACS or MI groups were compared to controls ($P = 0.16$ and 0.10 respectively). Nor was there an unequal distribution of the inactivating alleles for risk of thrombotic disease versus atherosclerotic disease when the ACS and SA populations were compared (Table 3).

Discussion

In our overall analysis there was no evidence that the set of pathogen response alleles cumulatively had a large significant impact on the risk of ACS when compared to controls. When we considered a group with the most acute cardiovascular outcome, namely MI, a modest impact was observed for this group of pathogen response alleles which approached levels of statistical significance. While the sample size was relatively modest it does suggest that the impact of selection on pathogen responses is not likely to have imposed a major burden on common cardiovascular disease. The suggestion of a higher degree of risk in patients presenting with MI may be consistent with such variants promoting myocardial injury, and it will be of interest to explore this in a larger population. This hypothesis will be worth addressing again as our knowledge increases concerning allelic variants with an impact on pathogen responses: this is likely to define a much larger group of variants, with more definitive evaluations of the scale of each variant's influence on pathogen responses. A broader epidemiological approach to this question could consider evaluating whether individuals whose genetic origin is a region of the world associated with a greater pathogen burden also suffer greater heart disease, or more serious myocardial damage, although the design of such a study is not straightforward.

Of the seventeen SNPs investigated the *TLR4* 399I SNP was most strongly associated with ACS, and was also elevated in the stable angina group compared with controls. This SNP is in strong disequilibrium with the 299G variant. TLR4 mediates the initiation of the innate immune response, primarily by recognition of lipopolysaccharides (LPS) on gram-negative bacteria. Activation of the TLR4 receptor stimulates transcription of several genes by NFkB, and this in turn leads to the synthesis and release of antimicrobial peptides, inflammatory cytokines and chemokines, and is a crucial link to mounting an adaptive immune response (Akira *et al*. 2001; Chow *et al*. 1999). These mechanisms have also been implicated in the chronic, low-grade, systemic inflammatory response involved in the development of atherosclerosis of the coronary arteries. Secondly, inactivating mutations of *TLR4* are known to reduce the cardiac depressant effect of LPS in mice (Thomas *et al*. 2002). It is not clear to what extent protection against myocardial damage is likely to have come under selection pressure in the past, for example in the context of sepsis-induced damage (Jenkins *et al*. 2005). One report associated the 299G allele with reduced carotid artery intima medial thickness (IMT) in healthy Italian individuals (Kiechl *et al*. 2002), while another found a significant reduction in acute coronary events associated with the 299G allele among a smaller group of French ACS patients ($n = 183$) compared to controls (Ameziane *et al*. 2003). Both these observations are contrary to our observation: it is possible that these differing results may have arisen through chance. It is also conceivable that they reflect differences in the pattern of genetic disequilibrium with the causative variants, or alternatively they could reflect strong differences in gene-specific mortality among case-control groups, which would best be resolved by a prospective study. One prospective study already supported the observation that the 299G allele is protective (Boekholdt *et al*. 2003). Finally, we cannot exclude the possibility that environmental covariates that are markedly different in the Irish population have reversed the direction of risk in other populations (such as statin therapy in the study by Boekholdt *et al*.). A large English study of coronary stenosis showed no effect of the 299G allele (Yang *et al*. 2003), while a Dutch study found no association of 299G with progression of atherosclerosis (Netea *et al*. 2004). This is the first study to show an effect in the opposite direction. It is conceivable that the haplotype increases risk in certain contexts, but decreases it in other contexts, and that some studies are a mixture of both types. However, in the absence of clear identification of any modulating risk factor it will be difficult to replicate this finding.

Comparison of ACS with stable disease is intended to highlight genetic factors that might contribute to

the thrombotic arterially occlusive phenotype of ACS, as distinct from genetic factors that contribute to an underlying atherosclerotic process. While in the overall group we found no evidence that the pathogen response variants distinguished these two groups, there was a suggestion that they may serve to distinguish them when analysis is restricted to early onset events (Fig. 1). The three variants that contributed most strongly to the pro-thrombotic phenotype in the early onset patients were the *IL13* 110Q allele, the *ABO* 156L allele, and the *FcGRIIA* 131H allele. FcγRIIA is expressed on platelets and is involved in mediating signalling via the GPIb-IX-V receptor (Canobbio *et al*. 2001; Sullam *et al*. 1998), and thus may have a role in thrombotic events. *ABO* genetic variation is implicated in the regulation of serum levels and activity of the platelet ligand vWF (Miller *et al*. 2003; Schleef *et al*. 2005), however the involvement of the *ABO* 156L allele must be treated with a degree of caution as a relatively increased error rate was recorded for this SNP. Lastly, whilst there is no obvious role for *IL13* in modulating thrombotic disease, it will be of great interest to determine if the IL13 association is replicated in other studies of thrombotic phenotypes.

In the logistic regression analysis shown in Fig. 1, the documented pathogen-resistance allele was treated as the "risk allele" for thrombosis. Hence it is clear from Figure 1 that of the three most interesting allelic effects, two of the resistance alleles were protective in relation to thrombosis while one allele increased thrombosis risk at an earlier age. This suggests that it is possible for different alleles to exert their effect by different mechanisms, e.g. carriership of a resistance allele may reduce the impact of pathogen burden on heart disease.

What are the implications of the findings of this study for attempts to reduce the burden of acute coronary events by down-regulating pathogen responses? Our study cannot distinguish with certainty whether any of the associations observed are direct effects of the protein variants on the vasculature (which was not supported by our limited analysis of the inactivating variants in this set of innate immunity genes), or are mediated through pathogens. If the associations of the genetic factors with acute versus stable events in early onset disease do indeed reflect alterations of the impact of pathogens on thrombosis, it is conceivable that antimicrobial therapies (Cercek *et al*. 2003; O'Connor *et al*. 2003; Stone *et al*. 2002) could show most promise in younger patients.

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