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Phenotype selection for detecting variable genes: a survey of cardiovascular quantitative traits and *TNF* locus polymorphism

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The practice of using discrete clinical diagnoses in genetic association studies has seldom led to a replicable genetic model. If, as the literature suggests, weak genotype–phenotype relationships are detected when clinical diagnoses are used, power might be increased by exploring more fundamental biological traits. Emerging solutions to this include directly modeling levels of the protein product of a gene (usually in plasma) and sequence variation specifically in/around that gene, as well as exploring multiple quantitative traits related to a disease of interest. Here, we attempt a strategy based upon these premises examining sequence variants near the *TNF* locus, a region widely studied in cardiovascular disease. Multilocus genotype models were used to perform a systematic screen of 18 metabolic and anthropometric traits for genetic association. While there was no evidence for an effect of *TNF* polymorphism on plasma *TNF* levels, a relatively strong effect on plasma PAI-1 levels did emerge ($P=0.000019$), but this was only evident in post-myocardial infarction patients. Modeled jointly with the common 4G/5G insertion/deletion polymorphism of *SERPINE1* (formerly *PAI*), this effect appears large (10% of variance explained versus 2% for *SERPINE1* 4G/5G). We exhibit this finding cautiously, and use it to illustrate how transitioning the study of disease risk to quantitative traits might empower the identification of functionally variable genes. Further, a case is highlighted where association between sequence variation in a gene and its product is not readily apparent even in large samples, but where association with a down-stream pathway may be. *European Journal of Human Genetics* (2007) 15, 685–693. doi:10.1038/sj.ejhg.5201803; published online 14 March 2007

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Introduction

Non-replication of genetic association studies is extensive.^{1,2} The vast majority of such studies entail the examination of a single discretely defined disease state in

case-control and/or family-based models. The field is dominated by an approach where many genes (typically across an equal number of publications) are examined in relation to a single disease phenotype that is deemed important. At the extreme, whole genome association studies are now being planned and executed, typically with a single target phenotype.^{3,4} The focus on disease is natural, given the importance its resolution has to humanity, but directly tackling the questions of its genetic etiology may be overzealous until we understand more about the functional characteristics of gene

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polymorphism.⁵ Thus, it may be prudent to invert the question from 'is there a gene affecting my phenotype?' to 'what phenotype may allow me to find a genetic effect at all?'

In contrast to studies directly modeling disease risk, some of the strongest and most reliable associations that have emerged involve quantitative phenotypes that have been obtained without the inherent complexities of clinical diagnosis. One of the best examples is the effect of variation in the angiotensin converting enzyme gene (*ACE*) upon plasma levels of the ACE protein.^{6,7} The effect is overwhelming, with as much as 50% of the variance in plasma ACE levels explained by polymorphism at the *ACE* locus.⁶ This is illustrative of what can happen when one selects an appropriate phenotype to detect underlying genetic variability. The strength of the effect also allows for a more clear dissection of a genetic model that best fits the data. Consequently, the evidence is robust that there is allelic heterogeneity (multiple functional sites in a gene) that influence the ACE trait, something that can be seen clearly given the strong genotype-phenotype relationship.⁷ In contrast, if weak associations are evident for disease risk, then the reliability of the genetic model is going to be equally suspect. Other excellent examples include the strong effect of dopamine β -hydroxylase gene (*DBH*) polymorphism on plasma DBH levels⁸ an effect of histidine rich glycoprotein (*HRG*) polymorphism on plasma HRG levels⁹ and an association between *CRP* variation and plasma CRP levels.¹⁰

Despite evidence for weak effects on disease, some genes have enjoyed so much study that there is reasonable cumulative evidence that functional variation occurs in a region, even if the nature of that variation remains a mystery. One such gene encodes tumor necrosis factor- α (*TNF- α*), which has been the subject of perhaps more than 1000 genetic association studies (as returned from PubMed using the search string 'tnfa* or tnf-alpha, gene*, polymorph*, assoc*'). A cursory scan of those studies illustrates some of the primary problems of genetic association studies. Most have only examined a single common promoter variant (-308A/G), most are based upon small samples (a few hundred individuals), and the majority assesses a single disease or other clinical end point. There are, however, examples of well-powered studies implicating the *TNF* region, in particular in myocardial infarction,³ but this too has been followed by non-replication.¹¹ A previous study examining *TNF* variation in a large coronary heart disease (CHD) sample provided only weak evidence of an effect upon *TNF* plasma levels (in contrast to the above cited examples of *ACE* etc).¹² Against this background, we have performed a systematic evaluation of quantitative traits typically examined in cardiovascular disease, providing a broader search for phenotypes that may be able to detect the presence of functional variation within the *TNF* region.

Materials and methods

Clinical materials

Patient DNA samples employed in this study derive from individuals participating in the Stockholm Heart Epidemiology Program (SHEEP), a population-based case-control study aimed at investigating the effects of various risk factors for myocardial infarction in men and women.¹³

It consists of Swedish men and women aged 45–70 who were admitted to hospitals for treatment of first-time myocardial infarction (MI) events and population-based controls without a history of coronary disease. The present study is based on cases that survived 28 days after their first MI and had no further events before blood sampling, and their corresponding, frequency-matched controls who were randomly selected from the Stockholm County population registry after stratification for age (in 5-year intervals), sex and residential area. Five control candidates per case were sampled at the case incidence so that non-respondent controls could be replaced by another candidate who belonged to the same study base as the index case. Criteria for myocardial infarction were (i) certain symptoms according to case history information, (ii) specified changes in blood levels of the enzymes CK and LD and (iii) specified ECG changes, according to criteria accepted by the Swedish Association of Cardiologists in 1991. Participants were invited to a physical examination that included blood sampling after an overnight fast, approximately 3 months after the MI event to allow for a stable metabolic period. The ethics committee at the Karolinska Institute approved the study and all participants gave informed consent. A detailed description of these individuals and the examined traits can be found in Table 1.

Blood chemistry

Blood samples were collected approximately 3 months after the MI event. The samples were separated within 1 h and kept frozen until analysis. DNA was isolated using RapidPrep Macro Genomic DNA isolation kit (Pharmacia Biotech, Sweden). Blood lipid levels were determined using routine methods at the different hospitals using enzymatic colorimetric assays on fully automated equipment. Body mass index was calculated as kg/m² based on anthropometric data from each participant obtained at the health examination. The mean of two blood pressure measurements were used to calculate systolic and diastolic blood pressure. The assays used to measure fibrinogen, PAI-1, fasting glucose, IGFBP-1, CRP, IL-6, *TNF- α* have been described elsewhere.^{12,14–16}

SNP selection and verification

Five SNPs from the *TNF* promoter region were selected in this study (from 5' to 3' of the gene and from pter to qter along the chromosome) rs1799964 (-1031T/C), rs1800630 (-863C/A), rs1799724 (-857C/T), rs1800629 (-308G/A),

Table 1 Distribution of selected traits in MI cases and controls

Trait	Men				Women			
	Controls	N	Cases	N	Controls	N	Cases	N
Age (years)	58.8±0.22	1054	58.3±0.24	852	61.9±0.30	507	61.5±0.36	357
BMI (Body mass index kg/m ²)	25.6±0.13	702	26.5±0.16	508	25.7±0.20	394	26.7±0.33	236
DBP (Diastolic blood pressure mm Hg)	84.7±0.40	700	80.9±0.45	507	81.9±0.51	507	76.7±0.66	236
SBP (Systolic blood pressure mm Hg)	139.4±0.78	700	132.0±0.91	508	142.6±1.10	393	131.5±1.45	236
Ins (Insulin mU/ml)	10.6±0.29	754	14.4±0.61	602	9.9±0.38	414	14.4±0.85	297
HOMA* (Homoeostasis model assessment)	2.63±0.092	685	3.89±0.23	535	2.55±0.18	378	3.90±0.28	269
Gluc (Glucose mg/dl)	5.44±0.053	951	5.92±0.077	752	5.24±0.070	466	5.79±0.12	329
IGFBP1 (Insulin-like growth factor binding protein 1 mg/l)	26.1±0.74	755	26.6±0.88	602	33.3±1.02	414	37.5±1.67	299
FB (Fibrinogen g/l)	3.43±0.023	992	3.65±0.030	789	3.62±0.034	467	3.94±0.052	341
CRP (C-reactive protein mg/l)	2.49±0.22	735	3.77±0.28	590	2.58±0.22	424	4.33±0.38	295
IL6 (Interleukin-6 ng/l)	4.70±0.74	590	8.98±1.40	556	6.43±1.36	285	6.45±1.22	275
TNF- α (Tumor necrosis factor ng/l)	2.17±0.075	636	2.39±0.090	539	2.33±0.094	374	2.40±0.124	280
PAI-1 (Plasminogen activator inhibitor-1 mg/l)	15.3±0.42	1007	20.4±0.69	805	15.2±0.66	470	20.5±0.94	341
HDL-c (High density lipoprotein cholesterol mmol/l)	1.19±0.010	1046	1.02±0.009	842	1.49±0.016	503	1.23±0.018	353
LDL-c (Low density lipoprotein cholesterol mmol/l)	3.92±0.028	1041	4.17±0.033	822	4.05±0.047	503	4.36±0.059	351
TC (Total cholesterol mmol/l)	5.81±0.031	1053	6.08±0.038	849	6.15±0.050	505	6.47±0.066	358
TG (Triglyceride mmol/l)	1.55±0.029	1053	2.05±0.048	850	1.39±0.032	505	2.02±0.082	359
APOA1 (Apolipoprotein A1 g/l)	1.47±0.008	1051	1.35±0.008	848	1.66±0.013	505	1.50±0.015	357
APOB (Apolipoprotein B g/l)	1.47±0.011	1051	1.60±0.012	848	1.52±0.018	505	1.73±0.024	357

Anthropometric and metabolic traits (units) of clinical materials stratified by gender and disease. (values are mean±SEM). * Calculated as [fasting glucose (mg/dl) × fasting insulin (nU/l)]/22.5.

and rs3615525 (−238G/A), details on which may be found in the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) under their respective IDs. Surrounding 50 bp sequences in each direction were examined for repeats and duplicated sequences using Repeat-masker (<http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker/>) and Blast (<http://www.ncbi.nlm.nih.gov/blast>). To verify that SNPs were polymorphic in our study populations, each SNP was tested in a set of 16 Swedish control samples.

Genotyping

Genotyping of SNPs was performed using dynamic allele-specific hybridization.¹⁷ All PCR reactions were run in 10–20 μ l volumes with 1.5 mM MgCl₂ and using 5–20 ng genomic DNA.

Statistical analysis

Deviation from Hardy–Weinberg equilibrium for genotypes at individual loci as well as differences in haplotype and diplotype distributions between discrete groups were assessed using the χ^2 statistic. Correlations between traits were established using simple linear regression. Deviation from normality for trait distributions was assessed using a Kolgomorov–Smirnov test. Tests for association between genotypes and quantitative traits were performed using analysis of variance (ANOVA) with *post hoc* tests being

conducted with Fisher's protected least significant differences (PLSD) test. As the sample was large, all initial ANOVA analyses were performed on untransformed data. Effects with the greatest magnitude in the event of a non-normally distributed trait were also modeled using the Mann–Whitney *U*-test. The above statistical analyses were performed using StatView version 5.0 (Abacus Concepts). Haplotype frequencies and diplotype configurations were estimated using PHASE v2.1.¹⁸ LD between marker pairs was estimated using the r^2 metric.¹⁹

Results

We limited our initial investigation to the 18 phenotypes that are traditionally studied in relation to cardiovascular disease.^{20,21} While many of these exhibit strong correlations, we opted to investigate each independently recognizing that there would be some redundancy. The intention was to perform a systematic evaluation of traits without prior beliefs about which systems might be affected by *TNF* variation, other than that they might be CHD-related. For the genetic markers tested, there was no evidence of deviation from Hardy–Weinberg equilibrium.

An important consideration in our initial screen was how to compress genotypes into a single manageable genetic

Table 2 Common haplotypes extending across the TNF promoter

Name	Count (freq.)	rs1799964	rs1800630	rs1799724	rs1800629	rs3615525
H1	2997 (54.5%)	T	C	C	G	G
H2	911 (16.5%)	C	A	C	G	G
H3	881 (16.0%)	T	C	C	A	G
H4	429 (7.8%)	T	C	T	G	G
H5	172 (3.1%)	C	C	C	G	A
H6	79 (1.4%)	C	C	C	G	G
H7	30 (0.5%)	T	A	C	G	G

Detected haplotypes that have been estimated using PHASE v2.1. Four haplotypes with counts <10 are not shown. The two markers used for diplotype reconstruction are shaded.

factor. With five markers and 18 traits, the number of tests becomes relatively large. Towards this end, we identified two of the five single markers that delineate the most common (>10%) haplotypes (Table 2) and estimated diplotype configurations of those common haplotypes for each individual (see Gu *et al.*²² for details and an application of this strategy). We used PHASE v2.1 to arrive at the probable configurations using rs1800630 and rs1800629 and noted that the configurations based upon these two markers are essentially phase known (near 100% probability). This occurs since the 'A' allele at rs1800630 and the 'A' allele at rs1800629 never occur together (Table 2), indicating that only three of the four possible haplotypes exist at detectable frequencies for these two markers. For practical reference, we point out that this strategy is equivalent to what has been done for years in the case of *APOE*, using two markers to distinguish all diplotype configurations of e2, e3, and e4 alleles (eg Strittmatter *et al.*²³).

We tested diplotype-phenotype relationships initially in the whole population using ANOVA, adjusting for age, gender, and disease status in the model. We recognized that interactions with these factors might be present, but acknowledged that they would probably be easier to consider *post hoc* if any significant findings were made in the primary analyses. The results of this test are shown in Table 3. Three significant findings were made for CRP ($F_{5,1966}=2.7$, $P=0.019$), HOMA ($F_{5,1791}=2.6$, $P=0.023$), and PAI-1 ($F_{5,2522}=4.4$, $P=0.0005$). Of note, there was no evidence for an effect of *TNF* variation on plasma *TNF- α* protein levels. We focussed on the PAI-1 finding, as it withstands strict Bonferroni correction for multiple testing (considering 18 initial ANOVA omnibus tests). We then tested for possible interactions of diplotype with age, gender, or disease status in a second-order factorial ANOVA model. The disease by diplotype interaction term emerged as significant ($F_{5,2522}=3.2$, $P=0.0065$) and so stratified models were also tested to investigate this more closely. An effect was only evident in the disease group ($F_{5,1087}=6.0$, $P=0.000019$), in contrast to ($F_{5,1425}=0.2$, $P=0.93$) in controls (Figure 1). We estimated from this that variation at the *TNF* locus contributes to approximately 10% of the

variance in plasma PAI-1 levels in this group. For comparison, we also obtained *SERPINE1* locus genotypes (the common 4G/5G variant) and included it as a factor in ANOVA models. This factor was also significant in the disease group ($F_{2,1073}=4.5$, $P=0.012$) but not in the controls. We estimated that *SERPINE1* genotype accounted for approximately 2% of variation in PAI-1 plasma levels. Quantitatively, the greatest difference in PAI-1 levels from pair-wise diplotype comparisons was between the H2H2 and H2H3 categories (Figure 1). We note that this comparison is highly significant based upon non-parametric analysis (Mann-Whitney *U*-test) ($Z=3.7$, $P=0.0002$; compared with $P=0.0011$ for a simple *t*-test on un-transformed data). As the diplotype strategy used above compresses some of the rarer haplotypes, models were also tested using all the five markers for the PAI-1 phenotype. The results of this are shown in Figure 2. Finally, the 2-locus diplotype model was also tested for CRP and HOMA in cases and controls separately (since these traits showed borderline significance in the initial screen). Results for this analysis were negative (for CRP; $F_{5,1108}=1.3$, $P=0.24$ and $F_{5,830}=1.6$, $P=0.15$ in controls and cases respectively; for HOMA, $F_{5,1015}=1.7$, $P=0.15$ and $F_{5,748}=1.2$, $P=0.32$ in controls and cases, respectively).

Based upon the maximum effect being observed upon PAI-1 levels, and following from the broader search using diplotype models, we also explored for independent marker effects. To do this, we fitted first-order factorial ANOVA models with each of the markers in a step-wise forward selection algorithm. The value of doing this is to capture potential allelic heterogeneity that may be acting in the region (i.e. two or more sites that independently affect a phenotype) (e.g. Cox *et al.*⁷). The unusual model that emerged from the diplotype strategy indicated that addressing this might be important. The results of this analysis are shown in Table 4.

Both 2- and 5-locus diplotype models were also used to test for association with case-control status, the specific goal for which this sample was originally acquired.¹³ There was no evidence that diplotype group frequencies differed between cases and controls (not shown).

Table 3 Evaluation of TNF locus diplotypes with quantitative traits

Trait	H1H1	H1H2	H1H3	H2H2	H2H3	H3H3	Significance
APOA1	1.47±0.008 (1102)	1.48±0.011 (652)	1.46±0.011 (623)	1.49±0.035 (79)	1.46±0.023 (143)	1.51±0.037 (71)	F(5,2658)=0.9, <i>P</i> =0.49
APOB	1.55±0.012 (1102)	1.57±0.016 (652)	1.55±0.015 (623)	1.49±0.039 (79)	1.54±0.038 (143)	1.54±0.04 (71)	F(5,2648)=0.9, <i>P</i> =0.48
BMI	25.9±0.14 (741)	25.8±0.18 (436)	26.2±0.18 (413)	26.2±0.55 (48)	26.3±0.45 (102)	26.4±0.57 (38)	F(5,1766)=0.7, <i>P</i> =0.64
CRP	3.22±0.22 (820)	3.65±0.35 (490)	2.45±0.14 (459)	4.63±1.4 (58)	2.62±0.32 (108)	3.17±0.7 (43)	F(5,1966)=2.7, <i>P</i> = 0.019
DBP	81.7±0.39 (738)	82.3±0.53 (436)	82.2±0.51 (412)	80.2±1.26 (48)	82.6±1.13 (102)	83±1.44 (38)	F(5,1762)=0.6, <i>P</i> =0.72
FB	3.59±0.025 (1028)	3.62±0.035 (608)	3.59±0.033 (588)	3.59±0.085 (74)	3.62±0.067 (137)	3.56±0.102 (66)	F(5,2489)=0.2, <i>P</i> =0.97
Gluc	5.53±0.05 (994)	5.6±0.08 (584)	5.64±0.09 (568)	5.6±0.22 (70)	6.01±0.24 (133)	5.47±0.18 (65)	F(5,2402)=1.7, <i>P</i> =0.13
HDL-c	1.2±0.011 (1096)	1.22±0.014 (646)	1.17±0.014 (619)	1.23±0.043 (79)	1.2±0.033 (142)	1.21±0.046 (70)	F(5,2640)=1.7, <i>P</i> =0.14
HOMA	3±0.11 (740)	3.04±0.16 (448)	3.25±0.25 (418)	2.53±0.25 (51)	4.33±0.61 (107)	3.3±0.62 (39)	F(5,1791)=2.6, <i>P</i> = 0.023
IGFBP1	28.9±0.7 (823)	29.6±1 (501)	29.3±1.1 (461)	30.7±4.0 (56)	30.7±2.2 (116)	31±3.8 (44)	F(5,1989)=0.2, <i>P</i> =0.95
IL6	6.71±0.97 (686)	5.24±0.92 (418)	6.99±1.29 (375)	8.23±4.36 (46)	7.39±3.27 (90)	12.25±7.11 (34)	F(5,1637)=0.7, <i>P</i> =0.61
Ins	11.9±0.35 (822)	11.9±0.42 (501)	11.9±0.47 (460)	13.9±3.99 (55)	13.4±1.13 (116)	12.9±1.47 (44)	F(5,1986)=0.9, <i>P</i> =0.47
LDL-c	4.07±0.03 (1086)	4.11±0.041 (639)	4.07±0.038 (611)	4±0.094 (79)	3.94±0.093 (140)	4.2±0.12 (70)	F(5,2613)=1.3, <i>P</i> =0.26
PAI-1	17.8±0.5 (1042)	16.5±0.6 (615)	17.2±0.7 (595)	14.4±1.7 (75)	22.6±2 (140)	16.3±1.8 (67)	F(5,2522)=4.4, <i>P</i> = 0.0005
SBP	136.5±0.8 (739)	138.1±1.03 (436)	137±1.04 (412)	133.9±2.89 (48)	137.3±2.2 (102)	137.6±3.62 (38)	F(5,1763)=0.5, <i>P</i> =0.77
TC	1.72±0.032 (1103)	1.7±0.049 (652)	1.79±0.056 (625)	1.5±0.07 (79)	1.92±0.119 (144)	1.69±0.083 (72)	F(5,2663)=1.8, <i>P</i> =0.11
TC	6.03±0.033 (1103)	6.09±0.045 (652)	6.02±0.044 (624)	5.9±0.103 (79)	5.98±0.102 (144)	6.14±0.124 (71)	F(5,2661)=1.0, <i>P</i> =0.40
TNF	2.3±0.07 (744)	2.33±0.1 (433)	2.22±0.09 (405)	2.29±0.26 (49)	2.17±0.18 (97)	2.18±0.3 (37)	F(5,1753)=0.23, <i>P</i> =0.95

Trait means are shown ± s.e.m. (N) for untransformed data in relation to TNF locus diplotypes. Significance is shown from ANOVA omnibus tests. Units are as described in Table 1.

Figure 1 Diplotype means of plasma plasminogen activator inhibitor-1 levels in healthy controls (a) and in post myocardial infarction patients (b). The H2H3 diplotype class has significantly elevated levels compared with other groups. Significance from ANOVA omnibus tests is shown in the upper right of each diagram. The number of individuals in each diplotype class is shown at the base of each column. Of note, average PAI-1 levels are generally higher in the infarct group across diplotype classes. Models have been adjusted for gender and age. For these two cofactors, the only significant independent effect was seen for age in the case group ($F_{4,1087} = 10.9$, $P \leq 0.0001$).

In order to better understand the association with TNF diplotype only being present in cases, fundamental differences with controls were also considered. For this, we noted from our initial ANOVA of diplotype and PAI-1 levels that the significance of the age covariate was significant in the disease group (Figure 1). In itself, this could indicate that there is sampling error in the control group involving mislabeling or incorrect phenotype assignment. We thus performed simple linear regression of all 18 traits studied (Table 1) against age in the stratified MI and control groups. There were seven traits for which correlation existed in one group but not in the other, and these effects were not trivial (variance explained in excess of 1%). These consisted of insulin, triglyceride, glucose, CRP, total cholesterol, HOMA, and PAI-1 (see Table 1). We chose to

Figure 2 Diplotype means of plasma plasminogen activator inhibitor-1 levels in healthy controls (a) and in post-myocardial infarction patients (b) based upon all five TNF markers. Models have been adjusted for gender and age. Of note, the groups that drive the significance of the model are the same as is seen in Figure 1 (the H2H3 and H2H2 classes).

Table 4 Mixed linear ANOVA of five TNF markers and PAI-1 levels

	Marker	F Statistic	Significance
MI Cases	rs1799964	$F(2,934) = 4.2$	$P = 0.015$
	rs1800630	$F(2,934) = 2.8$	$P = 0.059$
	rs1799724	$F(2,934) = 0.2$	$P = 0.82$
	rs1800629	$F(2,934) = 0.03$	$P = 0.97$
	rs3615525	$F(2,934) = 8.6$	$P = 0.0002$
Controls	rs1799964	$F(2,1181) = 1.5$	$P = 0.22$
	rs1800630	$F(2,1181) = 1.4$	$P = 0.25$
	rs1799724	$F(2,1181) = 0.9$	$P = 0.41$
	rs1800629	$F(2,1181) = 0.4$	$P = 0.66$
	rs3615525	$F(2,1181) = 0.22$	$P = 0.80$

Age and gender have been included as covariates in all analyses. Significant results are highlighted that may indicate that there are 2 or more markers in the region acting independently.

highlight three that had the greatest effects (over 2% variance explained) in their respective groups. Coincidentally these were the traits that emerged as significantly influenced by *TNF* variation in our initial screen, CRP, HOMA, and PAI-1. We show these relationships in Figure 3.

Discussion

The *TNF* locus is one of the more widely studied in human populations, but there still is no unequivocal evidence that variation within it actually impacts any human trait. Our premise for this study has been that, if functional variation in this region actually occurs, and if cardiovascular phenotypes are a reasonable biological target of such variation, then it might be valuable to screen CHD traits for association in a comprehensive fashion. CHD samples are typically acquired under some of the more strict epidemiological standards for genetic association studies. In addition, CHD study protocols also commonly entail the accumulation of an impressive wealth of ancillary biological phenotypes. This provides an excellent test bed for the kind of experiments illustrated in the present study.

From a screen of 18 CHD-related traits, we provide evidence that *TNF* polymorphism may be influencing plasma PAI-1 levels. The indicated effect is large, exceeding that of the *SERPINE1* 4G/5G polymorphism, which is at present the only relatively well-replicated genetic factor affecting circulating levels of PAI-1.²⁴ We also note that although many hypotheses have been addressed, the main finding remains significant after a strict Bonferroni correction for multiple testing. As with any genetic association study, we are cautious about interpreting these results, especially as multiple independent samples are not available for replication purposes. We exhibit the result thus to encourage further independent replication in additional CHD samples that may be able to specifically focus on PAI-1. Fortunately, PAI-1 level measurements are fairly common in this field and it should be trivial for other groups to attempt to replicate the effect (or refute it). In addition many groups as judged from the scientific literature have already tested *TNF*-specific polymorphisms for association with primarily MI risk so no additional experimentation may be necessary.

We are also cautious in terms of speculation about the biological plausibility that PAI-1 would be specifically affected by *TNF*. This is important since linkage disequilibrium could cause association to be seen for loci other than *TNF*. Thus, variability of any of the genes in the region could underlie the observed genetic association, including *TNF*, lymphotoxin-alpha, lymphotoxin-beta, and nuclear factor of kappa light polypeptide all of which have been shown to contribute in some way to inflammatory processes. However, *TNF* has been demonstrated to be a potent activator of PAI-1,^{25,26} and there is some functional

Figure 3 Correlations between age and traits levels for CRP (a, b), HOMA (c, d) and PAI-1 (e, f) in MI cases (a, c, e) and healthy controls (b, d, f). Trait levels have been transformed for the presentation of this analysis ($\ln$ for CRP and HOMA and square of PAI-1). This highlights that there are probably fundamental differences in disease and healthy classes that make it important to consider each separately in QTL analyses.

evidence that *TNF* promoter polymorphisms actually do affect *TNF* transcription levels.^{27,28} We recognize that mRNA levels may be among the best phenotypes to use to detect functional genetic variability (e.g. Stranger *et al.*²⁹), but these typically are difficult to obtain in large samples, in contrast to the CHD traits reported here. No genetic effect was seen, however, on *TNF* plasma levels, but this could reflect the biological sample used (plasma) and

an effect might be seen in other tissues. Nonetheless, in contrast to, for example, ACE, regulation of *TNF* plasma levels may not be affected 'detectably' by variation at the *TNF* locus.

The two main caveats to the observed genetic association are that (a) the genetic model is unusual in that the main effect is mediated by a diplotype hybrid (composed of two unlike haplotypes) and (b) we only observe the effect

among MI cases. For the first point, similar models have emerged for other convincing genetic association publications (e.g. calpain-10)³⁰ One possibility here is that functional alleles occur on different genes in this LD region and that the combination of the two effects the greatest increase in PAI-1 levels. Regardless, this should serve as a warning for further work on the *TNF* region, that allelic heterogeneity may be present, and that it involves two (or more) different functional sites.

In terms of the association only being evident among cases, we speculate that this may have to do with either the disease process, changes in behavior (stabilization of diet or life-style) following the infarct event, or medication. We observed only the association of *SERPINE1* genotypes with PAI-1 levels in the disease population as well, indicating that this group is more in line with what has been observed in other association studies.²⁴ We also highlight (Figure 3) correlations that strongly differ between the healthy and the disease groups. This is included primarily to indicate that in retrospective studies, it is appropriate to consider study participants separately according to disease status, especially for CHD-related phenotypes. We acknowledge that this does not offer an explanation for the selective effect of *TNF* variation on PAI-1 levels. This does however, strengthen our conviction that in this particular sample group-specific effects are not negligible and can exist both when genetic factors and other exposures are considered. Also of note, PAI-1 distributions are very different between cases and controls (see Table 1), consistent with a heightened inflammatory process in MI sufferers. The degree of inflammatory response may be important to consider in future attempts to associate *TNF* polymorphism with disease end points.

In summary, we present a systematic screen of CHD phenotypes against polymorphism in the *TNF* locus. Although the predicted association of *TNF* polymorphism with plasma PAI-1 levels serves as the highlight of this report, we stress that it should primarily be a guide for replication efforts. It should be important to consider this phenotype (perhaps in addition to other metabolic traits) and the models that have emerged here towards the greater goal of proving that functional variation actually occurs in this genomic region and ultimately identifying the molecular character of that variation.

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