Genetic Association of pro-inflammatory cytokine gene polymorphisms with coronary artery disease (CAD) in a North Indian population

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Key words: TNF-α -308 (G/A) (rs1800797), TNF-β +252 (A/G) (rs909253), IL-6 -174 (G/C) (rs1800795), IL6 -597 (G/A) (rs1800797), and IFN-ɣ +874 (T/A) (rs2430561), CAD, North India.

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Abstract:

Background: Cytokines regulate the expression of inflammatory molecules which destabilize the atheromatic plaques. This study focuses on studying the association of inflammatory cytokine polymorphisms like TNF-α -308 (G/A), TNF-β +252 (A/G), IL-6 -174 (G/C) and IL-6 -597 (G/A), and IFN-ɣ +874 (T/A) with coronary artery disease (CAD) among north Indian patients.

Materials and methods: 143 CAD and 137 normal healthy controls were recruited in this study. DNA extraction was carried out by high salting out method. TNF-α -308 (G/A) (rs1800797), TNF-β +252 (A/G) (rs909253), IL-6 -174 (G/C) (rs1800795), IL6 -597 (G/A) (rs1800797), and IFN-ɣ +874 (T/A) (rs2430561) SNPs were genotyped by TaqMan®SNP genotyping assays. Different statistical analyses were performed using SPSS v 22.0 and SNPStats. $p \leq 0.05$ was considered significant.

Results: Significant risk association with CAD was found for TNF-α -308 (G/A) “A” allele (OR =5.6, CI 1.8-17.4, p=0.001) and TNF-β +252 (A/G) “G” allele (OR=3.4, CI=1.9-6.0, $p<0.001$). However, no statistical significance was found for IL-6 -174 (G/C) or IL6 -597 (G/A), with CAD. TNF-α -308 (G/A), and TNF-β +252 (A/G) haplotype “GG” “AG” increased CAD risk significantly (GG haplotype, adjusted OR = 2.6, CI 1.4-5.0, p=0.003 and AG haplotype OR =8.5, CI 2.2-33.35, p=0.002) after adjustments for age, sex, TC, TG, HDL, APOB, smoking and diet.

Discussion: The present study found significant risk association for TNF-α -308 (G/A), and TNF-β +252 (A/G) genotypes, alleles and haplotypes, with CAD in a North Indian Population.

Introduction

Coronary artery disease (CAD) is a major cause of morbidity and mortality globally and has a complex developmental process with a number of contributing factors; gender, age, inflammation, hypertension, hypercholesterolemia, diabetes, obesity, tobacco smoking, alcohol drinking, total cholesterol and LDL cholesterol (Hansson, 2005;Yiannakouris et al., 2014). However, not all individuals who are exposed to the same risk factors of CAD necessarily develop disease, which suggests that inherited genetic factors contribute to the underlying pathogenesis of CAD. Indians are among the populations with the highest susceptibility and have an alarmingly high incidence of CAD (Enas & Senthilkumar, 2001). In addition to traditional CAD risk factors, strict endogamous marital patterns, rapid urbanisation and decreases in physical activity are some of the additional factors driving rampant increases in CAD among Indian populations. However, even all the risk factors taken together do not fully explain the complexity or the magnitude of disease burden (Anand et al., 2000). Thus genetic factors should be comprehensively and systematically studied in different populations of the Indian Subcontinent.

Inflammation plays a central role in atherosclerosis development and other manifestations of CAD (Hansson, 2005; Zakynthinos and Pappa, 2009). The underlying pathological mechanism of CAD is atheroma plaque instability, which is characterized by chronic inflammation caused by oxidized lipids adherent to the inner layer of the arterial wall. While the molecular mechanisms involved in the pathogenesis of CAD are still being de-convoluted (Libby at al., 2011) there is a consensus that the main events of unstable angina and myocardial infarction are due to the involvement of major inflammatory cytokines.

Cytokines are produced in a variety of tissues and regulate the expression of a number of other inflammatory molecules which can lead to destabilization and finally rupture of vulnerable atheromatic plaques. Several lines of evidence indicate that increased inflammatory cytokine levels and inflammatory cytokine activity detectable in peripheral blood have a prognostic role in predicting future cardiac events and clinical course (Tousoulis et al., 2006; De Gennaro et al., 2012). Recent studies have also shown that inflammation-related genes might be correlated with CAD risk (Zakynthinos and Pappa, 2009; Shankar and Kakkar 2010; Li et al., 2012; Xie et al., 2012; Singh et al., 2014), thus aligning a number of inflammatory cytokines, their expression and their activity, as key mediators of CAD. Inflammatory pathways are mediated by proximal cytokines, such as Tumor Necrosis Factor-Alpha (TNF-α) and Tumour Necrosis Factor Beta (TNF-β)/Lymphotoxin Alpha) (LT-α), Interferon gamma (IFN-ɣ) and interleukin-6 (IL-6) which suggest they may influence CAD risk. However, multiple pro-inflammatory and anti-inflammatory cytokines may be involved in the pathogenesis of CAD having overlapping, antagonistic and synergistic effects.

Tumor Necrosis Factor-α (TNF- α) and Tumour Necrosis Factor-β (TNF-β)/Lymphotoxin Alpha) (LT-α)

TNF-α, is a pleiotropic pro-inflammatory cytokine of 233 amino-acids, which is encoded by TNF-α gene on chromosome 6p21 falling within the major histocompatibility complex (MHC)

cluster on chromosome 6. It is generated by activated macrophages and facilitates the recruitment and infiltration of macrophages/monocytes into the sub-endothelium of arteries and contributes to the reduction of lipoprotein lipase activity. Elevated TNF-α levels have consistently been reported in patients with heart failure and increasing concentrations of the cytokine are related to the severity of CAD and mortality rate in patients. Different polymorphisms of the TNF-α gene may also lead to variation in the plasma level of TNF-α as there are several single nucleotide polymorphisms (SNPs) both within the gene and its promoter region. There have been significant susceptible/positive associations reported between TNF-α -308 (G/A) locus and CAD however other studies failed to find associations (Banerjee et al 2009; Bhanushali and Das 2013; Elahi et al., 2009; Garg et al., 2013; Wang et al., 2015).

Lymphotoxin-alpha (LTA) also known as TNF beta (TNF-β), is another pro-inflammatory cytokine which plays an important role in the immunologic response. The LTA gene, like the TNF-α gene, is also located within the tumour necrosis factor (TNF) gene cluster in the MHC region on chromosome 6 at position 6p21.3. TNF-β has been linked in a number of studies to the intensity and duration of local inflammation with TNF-β +252 (A/G) correlating with the altered production of TNF-β (Messer et al., 1991). In contrast to TNF-α there are only a few studies on the role of TNF-β in CAD and the paucity of data is especially limiting on Indian populations (Garg et al., 2013).

Interleukin-6

Interleukin-6 (IL-6) is another pro inflammatory and immunoregulatory cytokine which has a significant role in the development of CAD. IL-6 up-regulates the synthesis of the acute phase proteins involved in the process of inflammation (Gleeson et al., 2011). IL-6 consists of 184 amino acid residues and is located on chromosome 7 at 7p21–p24. A common G/C polymorphism in the promoter region (−174 base pairs) affects promoter function and is associated with differences in plasma IL-6 levels (Fishman et al., 1998). While total IL-6 expression may regulate the magnitude of inflammation and its correlation with CAD (Martins et al., 2006; Woo and Humphries, 2013) the IL-6 -174 (G/C) polymorphism has been associated with CAD (meta-analysis, GG vs. CC OR =0.80, CI 0.65-98, p=0.03, Yang et al., 2013). In addition some other polymorphisms in this gene promoter region (-572G/C and -597G/A) are also predictors of the cytokine concentrations in plasma following inflammatory stimulus. These polymorphisms may influence CAD susceptibility by altering gene regulation and protein expression (Elahi et al., 2009; Woo and Humphries, 2013). Some studies show that IL6 polymorphisms are significantly increase the risk for CAD in different populations, but results are inconsistent and conflicting (Banerjee et al., 2009; Bhanushali and Das, 2013; Cui et al., 2013; Elahi et al., 2009; Mishra et al., 2013; Satti et al., 2013; Hou et al., 2015).

Interferon gamma (IFN-γ)

Interferon gamma (IFN-γ) is a pleiotropic soluble cytokine with antiviral and anti-tumour properties and its levels are elevated in unstable angina and myocardial infarction (Pasqui et

al., 2006). The IFN- γ gene is located on chromosome 12q24.1, within this region, a SNP at position +874 (in the 1st intron) of the IFN-γ gene (rs2430561) maps to a putative nuclear factor-κB (NF-κB) binding site. This polymorphism (T allele) correlates with a higher production of IFN-γ and is associated with disease severity in different autoimmune and inflammatory diseases (Pacheco et al., 2008, Kohr at al., 2011) an example being the positive association of the T allele with CAD in an Indian population (OR =1.53, CI 1.10-2.13, p=0.01) (Garg et al., 2013).

As there are few studies focusing on the role of inflammatory cytokines in CAD specifically in Indian populations (Banerjee et al., 2009; Bhanushali and Das, 2013; Garg et al., 2013; Mishra et al., 2013) this study was designed to explore the roles of TNF-α -308 (G/A), TNF-β +252 (A/G), IL-6 -174 (G/C) and IL6 -597 (G/A), and IFN-ɣ +874 (T/A) polymorphisms in a North Indian CAD population. This study was designed to contribute to the knowledge of population specific genetic risk factors which might improve prediction of CAD risk in Indian populations.

Study population and Methods

The study population consisted of 137 healthy controls and 143 patients with clinically diagnosed CAD, from Uttar Pradesh, North India (achieving a Quanto predicted sample size of 130 (http://biostats.usc.edu/Quanto.html) to achieve an odds ratio of 1.75 and statistical power above 80% using previously determined allele frequencies and odds ratios where available (Banerjee et al., 2009; Bhanushali and Das, 2013; Garg et al., 2013)).

Patients were classified on the basis of at least 50% or more stenosis in one or more coronary arteries verified through coronary angiography. The healthy controls had no known history of ischemic heart disease, hypertension, diabetes, endocrine or metabolic disorders and were selected after administration of a treadmill exercise test to exclude the possibility of the patients having an underlying CAD (Rai et al., 2012). The DNA samples were collected with full written consent and the study protocol was approved by ethics committees of the SGPGIMS, Lucknow and Loughborough University. All recruitment and analyses followed the principles of Helsinki declaration.

Clinical data was gathered on age, gender, diet (vegetarian vs. non-vegetarian), smoking-status and lipid profiles (plasma total cholesterol (TC), triglycerides (TG), high density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and apolipoprotein B (ApoB). DNA extraction was performed using the salting out method (Sambrook and Russell, 2006) and genotyping of the TNF-α -308 (G/A) (rs1800629), TNF-β +252 (A/G) (rs909253), IL-6 -174 (G/C) (rs1800795) and IL6 -597 (G/A) (rs1800797) and IFN-ɣ +874 (T/A) (rs2430561) polymorphisms were performed using TaqMan®SNP Genotyping Assays. Genotypes were scored directly by viewing the presence or absence of fluorescence peaks using the StepOne TM Software v.2.3 (Applied Biosystems). Overall genotyping success rates varied from 94% for IL6 -597 (G/A) (rs1800797) to 99% for TNF-α -308 (G/A), (rs1800629). Failures were primarily due to DNA storage degradation and

quality issues with 10% of the sample randomly repeated to test the reliability of genotyping the results of which indicate 100% concordance at all SNPs.

Statistical analysis was carried out using the software packages EXCEL (Microsoft®) and SPSS v 22.0 (SPSS Inc., Chicago, IL, US).). The patient and control databases were both assessed for Hardy-Weinberg Equilibrium (HWE) by calculating a chi-square value. Association statistics like odds ratios (OR) associated confidence intervals and p values were calculated using online programme SNPSTATs (http://bioinfo.iconcologia.net/SNPstats) (Solé et al., 2006). Clinical and life style parameters were analysed using t-tests and where required Kruskal-Wallis test. A p-value ≤ 0.05 was considered statistically significant. SNPstats programme was also used for adjustments for various clinical and demographic parameters (age, TC, TG, HDL, LDL, VLDL, APOB) and calculation of linkage disequilibrium and construction of haplotypes.

Results

Clinical parameters

In the present sample, a statistically significant difference in Age, TC, TG, LDL, VLDL, and APOB was observed between CAD patients and controls. The patient cohort were older by nearly 4.5 years and as expected patients had higher levels of lipids (Table 1).

Genotype and allele frequency distribution

The genotype (number, percentage), and allele frequencies (with Standard errors (SE)) of the studied samples were in Hardy Weinberg Equilibrium (HWE) (Table 2). HWE was maintained for all the loci both in patients and controls except IFN-ɣ +874 (T/A) in controls (p=0.001) where there was an excess of homozygote genotypes and deficiency of heterozygotes. The crude association analysis showed that at TNF-α -308 (G/A) locus, AG genotype (OR =5.3, CI 2.0-14.4, p=0.0004), and A allele (OR =5.4, CI 2.0-14.5, p=0.0001) were significant susceptible contributors to CAD in this population (Table 2). These positive associations became stronger (AG genotype OR =5.5 CI 1.7-17.3, p =0.005 and A allele OR =5.6, CI 1.8-17.4, p<0.001) when adjusted for age, sex, TC, TG, HDL, APOB, smoking and diet. At this locus, all p values remained significant even after Bonferroni correction.

At TNF-β +252 (A/G) locus, GG genotype (OR = 4.7, CI 1.5-15.2, p <0.0001) and G allele (OR = 3.1, CI 2.0-4.9, p <0.0001) were positively associated with the CAD in this population. After adjusting for age, sex, TC, TG, HDL, APOB, smoking and diet, these odds ratios remained significant and improved in all models (e.g. GG OR = 6.3, CI 1.5-25.9, p<0.0001 and G allele OR = 3.4, CI 1.9-6.0, p<0.0001). All genotypes containing G allele remained significant following Bonferroni corrections except for the recessive model.

Both IL-6 (IL6 -174 (G/C) and IL6-597 (G/A)) loci showed no statistically significant association with CAD in any model or combination. The IFN-ɣ +874 (T/A) locus showed risk association (OR=1.8, CI=1.0-3.1) at co-dominant (AT vs AA) and over-dominant (AT vs AA+TT; OR=1.7, CI=1.1-2.8) models which became insignificant when adjusted for age, sex, TC, TG, HDL, APOB, smoking and diet. Other genotype combinations and models were

statistically non-significant. A caution is warranted in interpretation of the results at IFN-ɣ +874 (T/A) locus as controls showed departure from HWE.

Haplotype frequency

Haplotype analysis was carried out at TNF-α -308 (G/A), TNF-β +252 (A/G), and IL-6 -174 (G/C) and IL6 -597 (G/A) loci and results are presented in Table 3. TNF-α -308 (G/A), TNF-β +252 (A/G) haplotypes GG (crude OR =2.5, CI 1.5-4.1, p =0.0005) and AG (crude OR = 7.8, CI 2.6-24.0, p =0.0003) increased the disease risk significantly even after the adjustments for age, sex, TC, TG, HDL, APOB, smoking and diet (GG haplotype OR =2.6, CI 1.4-5.0, p =0.003 and AG haplotype OR = 8.5, CI 2.2-33.4, p =0.002). At the IL6 gene (IL-6 -174 (G/C) and IL6 -597 (G/A)), no haplotype were significantly susceptible or protective.

Interaction of SNPs with conventional CAD risk factors

One way ANOVAs were carried out for genotypes and clinical parameters in both patients and controls to evaluate the contribution of genotypes to these intermediate phenotypes (Table 4). At TNF-α -308 (G/A) and TNF-β +252 (A/G) loci, there were no significant interactions between genotypes and age or lipid parameters. At other polymorphisms, there were some p values < 0.05 before the Bonferroni correction but these become non-significant after correction. The IL-6 -174 (G/C) polymorphism is the only polymorphism showing highest number (3) interactions with TC, HDL, and LDL in control population before correction.

Cross tabulations were carried out for genotypes and categorical variables (gender, diet and smoking habit) to explore interactions. In these analyses none of the results achieved statistical significance either in patients or controls. Contrary to our expectations, none of the inflammatory polymorphisms studied here interacted with smoking habits (results not presented but available).

We also carried out binary logistic regression analysis (results not shown, but available) which confirmed that TNF-β +252 (A/G), age, two lipids; VLDL and APOB were independent contributors/predictors of CAD in this population.

Discussion

Genetic polymorphisms have an important role in the regulation of gene expression, and thus gene polymorphisms may contribute to the inter-individual differences in susceptibility, inception, progression and severity of disease. In this study we analysed five single nucleotide polymorphisms (SNPs) in four pro inflammatory genes (TNF-α -308 (G/A), TNF-β +252 (A/G), IL-6 -174 (G/C) and IL6 -597 (G/A), and IFN-ɣ +874 (T/A)) using sensitive genotyping assays. The allele frequencies observed in controls were comparable to many populations of the Indian subcontinent and online databases (1000 genomes (IGSR); Garg et al., 2013; Mishra et al., 2013).

In our study, TNF-α -308 (G/A), promoter region polymorphism was significantly associated with CAD, and this association remained significant in multivariate analysis, even after the

adjustment with confounding variables (A allele OR= 5.6, CI 1.8-17.4, p=0.001). Minor allele A was more frequent in patients (9%) compared to controls (2%). Previous studies have produced inconsistent results for this locus among Indian populations (Banerjee et al., 2009; Bhanushali and Das 2013; Garg et al., 2013). Bhanushali and Das (2013) did observe a positive association of TNF-α -308 (G/A) polymorphism with CAD (OR =3.37, CI 1.04-11.54, p= 0.03), in crude analysis, but it was not significant when confounders were taken into consideration. Garg et al. (2013) analysed an endogamous group from Delhi (India) and showed no significant association of CAD (adjusted OR =1.08, CI 0.94-1.22, NS) with this polymorphism but found it to be associated with BMI. Banerjee and colleagues (2009) also did not find a significant association with CAD and this locus (OR =1.07, CI 0.64-1.81, p =0.79). Wang et al. (2015) using a meta-analysis approach showed TNF-α -308 (G/A), polymorphism is not associated with CAD in Indian populations (OR =1.24, CI 0.86-1.78, p=0.26) though there were only a limited number of Indian studies (6) included in this analysis with limited total sample size of 1698 cases and 1459 controls. Further investigations are therefore required to explore the role of this locus in CAD among Indian populations.

TNF-β +252 (A/G), locus has not been extensively studied with reference to heart disease or CAD in global or Indian populations (Li et al., 2010; Gao et al., 2010; Garg et al., 2013). TNF-β +252 (A/G) odds ratios in the current CAD samples were highly significant in all models and remain significant after controlling for confounders (age, sex, TC, TG, HDL, APOB, smoking and diet) except in the recessive model. Garg et al. (2013) is the only study available on Indian populations for this locus and our results are consistent with their results though magnitude of effect is nearly 4 times higher in our study (e.g. AG vs. AA ORs 1.14 vs. 4.6). This could be due to sampling of different population groups and geographical position of samples, though patient groups are of similar size. This polymorphism was not associated with CAD in Chinese population or within a large meta-analysis (Gao et al., 2010, Li et al., 2010). As there are not many studies from India, it would be interesting to explore further if this is a regional/population specific disease effect.

Haplotype analysis of TNF-α -308 (G/A) and TNF-β +252 (A/G) (LTA) (Table 3) confirmed that carrying the risk haplotype 'AG' increases the CAD by nearly 8 fold and this remains significant even controlling for all other clinical variables. As control frequency for this haplotype is relatively low (0.014 compared to patients 0.093) caution is warranted in interpretations.

Both IL-6 polymorphisms, (IL-6 -174 (G/C) (rs1800795) and IL-6 -597 (G/A), (rs1800797)) showed no significant associations with CAD in this sample. Overall allele frequencies are comparable with previous studies from the region. Some previous studies looking at different ethnic/regional groups of India have also failed to document association between IL-6 -174 (G/C) (rs1800795) and CAD (Banerjee et al., 2009; Bhanushali and Das 2013). Hou et al. (2015) conducted a meta-analysis with 42 studies including 15,145 cases and 21,496 controls, and they reported that C allele of IL-6 -174 (G/C) was correlated with an increased risk of CAD in Caucasians (OR= 1.12, CI 1.03-1.22, p =0.007) but a similar effect was not observed in Indian populations (OR = 1.21, CI 0.74-1.99, p=0.45). There are some studies showing the opposite effect, with Phulukdaree et al. (2013) reporting that the presence of the IL-6 -174

(G/C) C allele influences levels of IL6 and increases the risk of CAD in South African Indians. Elsaid et al. (2014) reported that the G allele of IL-6 -174 (G/C) is significantly associated with CAD (OR = 3.44, CI 2.26-5.23, $p<0.0001$) while a recent meta-analysis showed that the association between CAD and IL-6 -174 (G/C) polymorphism is only a mild one (OR =0.80, CI 0.65-0.98, p =0.034) (Yang et al., 2013).

In case of IL-6 -597 (G/A) (rs1800797), no statistically significant genotypic or allelic association was observed in the present study. There are only limited studies on this locus with reference to CAD in literature and none on Indian populations. Sun et al (2014) also finding no significant association of this locus with CAD in a Chinese population. Overall control allele and genotype frequencies observed here are comparable to a large study on this locus (albeit different disease: leprosy) (Aggarwal et al 2011) but our results are significantly different from a diabetes study which reported a very high frequency of A allele (87%) in controls (Saxena et al 2014). Haplotype analysis of the two loci also did not reach statistical significance in any combination. Further large scale investigations are required to clarify the possible role of IL-6 polymorphisms in CAD among Indian populations.

IFN-ɣ +874 (T/A) locus is also not studied extensively within Indian populations (Garg et al 2013). Overall our results are comparable with the Garg et al. (2013) study however we only observed Codominant and Overdominant models to be statistically significant and this significance was lost when adjustments were made for clinical parameters. This polymorphism significantly interacted with Age in patients, TG and VLDL in controls in one way ANOVA (Table 4).

There are some limitations to our study which should be considered. Firstly CAD patients and control subjects were recruited from only one hospital leading to some selection bias. However, the genotype distributions for controls of all loci except IFN-ɣ +874 (T/A) are in HWE, which gives confidence that it could be considered as being representative of the general population. The relatively small sample size of our study may have limited the statistical power to find the association of these five polymorphisms in CAD risk. However, prior sample size calculations suggested a sample size of 130 patients and 130 controls was sufficient to achieve a large odds ratio of 1.75 and above with 80% power.

Overall the results provided here show that polymorphisms in TNF-α -308 (G/A) and TNF-β +252 (A/G) loci enhance the risk of heart disease in a North Indian population. These polymorphisms along with IFN-ɣ +874 (T/A) should be analysed in other Indian populations to confirm their role in CAD and other cardio metabolic diseases.

Acknowledgements

Authors acknowledge the financial support from Loughborough University and the Society for the Study of Human Biology (SSHB) (Undergraduate bursary award to M. Kirby). We also thank all our participants, without their participation this research would not have been possible.

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Table 1: Baseline characteristics of CAD patients and controls

Variable	CONTROL (n=137), Mean ± SD Or (n, and % for categorical variables)	PATIENTS (n=144) Mean±SD Or (n and % for categorical variables)	T-test/ Chi-Square Test	P value
Age	45.02 ±14.46	49.51±11.06	-2.910	0.004*
Sex, Male: Female	 111(81%) 26 (19%)	 125(87%) 19(13%)	1.75 (1df)	0.186
Diet Veg: Non Veg	 65 (47%): 72 (53%)	 60 (42%) 84 (58%)	0.95 (1df)	0.330
Smoking Non-smoker Smoker	 61 (44%) 76 (56%)	 49 (34%) 95 (66%)	3.25 (1df)	0.072
TC	132.76±34.38	182.32±106.03	-5.322	<0.001*
TG	145.64±66.03	208.01±109.45	-5.816	<0.001*
HDL	23.40±7.29	29.74±12.37	-5.261	<0.001*
LDL	80.71±27.25	106.37±42.94	-5.934	<0.001*
VLDL	30.08±13.46	42.79±18.04	-6.61	<0.001*
APOB	108.76±45.784	153.50±43.46	-8.394	<0.001*

* =Statistically significant at 5% level.

Table 2: Genotype, allele frequencies, Odds ratios and adjustments for various models for the studied SNPs among CAD patients

Model	Genotype/ Allele	Control No, (%) N=136	Patients No, (%) N=143	Crude OR	P value	Age, Sex, TC, TG, HDL, VLDL, APOB, Smoking and Diet Adjusted OR	P value
TNFα -308 (G/A), rs1800629							
	GG	131 (96.3)	118 (82.5)				
	AG	5 (3.7)	24 (16.7)				
	AA	0 (0.0)	1 (0.8)				
HWE P value		0.82	0.79				
Allele frequency	*G	0.98 (±0.001)	0.91 (±0.02)				
	*A	0.02 (±0.001)	0.09 (±0.02)				
Co-dominant (AG vs. GG)				5.3 (2.0-14.4)	<0.001	5.5 (1.7-17.3)	0.005
Co-dominant (AA vs. GG)				NA		NA	
Dominant (AA+AG vs.GG)				5.5(2.1-15.0)	<0.001	5.7 (1.8-17.8)	0.001
Recessive (A/A vs. AG+GG)				NA		NA	
Over-dominant (AG vs. GG+AA)				5.3(1.9-14.3)	<0.001	5.5 (1.7-17.3)	0.002
Log-additive				5.4 (2.0-14.5)	<0.001	5.6 (1.8-17.4)	0.001
TNFβ +252 (A/G), rs909253							
	AA	107 (79.3)	68 (48.3)				
	AG	24 (17.8)	61 (43.2)				
	GG	4 (2.9)	12 (8.5)				
HWE-P value		0.08	0.68				
Allele frequency	*A	0.88 (±0.02)	0.70 (±0.03)				
	*G	0.12 (±0.02)	0.30 (±0.03)				
Co-dominant (AG vs. AA)				4.0 (2.3-7.0)	<0.001	4.6 (2.1-9.8)	<0.001
Co-dominant (GG vs. AA)				4.7 (1.5-15.2)	<0.001	6.3 (1.5-25.9)	<0.001
Dominant (GG+AG vs. AA)				4.1 (2.4-7.1)	<0.001	4.9 (2.4-9.9)	<0.001
Recessive (GG vs. AG+AA)				3.0 (1.0-9.6)	0.05	4.3 (1.1-17.6)	0.03
Overdominant (AG vs. AA+GG)				3.5 (2.0-6.1)	<0.001	4.0 (1.9-8.4)	0.002
Log-additive				3.1 (2.0-4.9)	<0.001	3.4 (1.9-6.0)	<0.001
IL6 -174 (G/C), rs1800795							
	GG	91 (69.5)	105 (76.1)				
	GC	39 (29.8)	32 (23.2)				
	CC	1 (0.7)	1 (0.7)				
HWE-P value		0.14	0.35				

Allele frequency	*G	0.84 (±0.02)	0.88 (±0.02)				
	*C	0.16 (±0.02)	0.12 (±0.02)				
Co-dominant (CG vs. GG)				0.7(0.4-1.2)	0.5	0.5 (0.2-1.1)	0.2
Co-dominant (CC vs. GG)				0.9 (0.1-14.1)	0.5	0.8 (0.1-1	0.2
Dominant (CC+CG vs. GG)				0.7 (0.4-1.2)	0.2	0.5(0.3-1.1)	0.1
Recessive (CC vs. CG+GG)				0.9(0.1-15.3)	1.0	1.0(0.1-17.2)	0.1
Overdominant (CG vs. GG+CC)				0.7(0.4-1.2)	0.2	0.5 (0.3-1.1)	0.1
Log-additive				0.7(0.5-1.3)	0.2	0.6(0.3-1.1)	0.1
IL6 -597 (G/A), rs1800797							
	GG	98 (76.6)	104 (76.4)				
	AG	29 (22.6)	30 (22.1)				
	AA	1 (0.8)	2 (1.5)				
HWE-P value		0.47	0.85				
Allele frequency	*G	0.88 (±0.02)	0.88 (±0.02)				
	*A	0.12 (±0.02)	0.12 (±0.02)				
Co-dominant (AG vs. AA)				0.5 (0.1-6.0)	0.9	1.0 (0.1-16.7)	1.0
Co-dominant (AA vs. GG)				0.5 (0.1-5.9)	0.9	1.0 (0.1-16.5)	0.9
Dominant (GG+AG vs. AA)				0.5 (0.1-5.9)	0.6	1.02 (0.1-16.4)	0.9
Recessive (GG vs. AG+AA)				0.9 (0.6-1.8)	0.9	1.0 (0.6-1.8)	0.9
Overdominant (AG vs. AA+GG)				1.0 (0.5-1.7)	0.9	1.0 (0.6-1.8)	0.9
Log-additive				1.0 (0.6-1.6)	0.9	1.0 (0.6-1.7)	0.9
IFNγ +874 (T/A), rs2430561							
	AA	60 (45.8)	49 (35.2)				
	AT	44 (33.6)	65 (46.8)				
	TT	27 (20.6)	25 (18.0)				
HWE-P value		0.001*	0.63				
Allele frequency	*A	0.63 (±0.03)	0.59 (±0.03)				
	*T	0.37 (±0.03)	0.41 (±0.03)				
Co-dominant (AT vs. AA)				1.8(1.0-3.1)	0.1	1.5(0.7-2.9)	0.6
Co-dominant (TT vs. AA)				1.1(0.6-2.2)	0.1	1.06(0.5-2.5)	0.6
Dominant (TT+AT vs. AA)				1.5 (0.9-2.5)	0.1	1.30 (0.7-2.5)	0.4
Recessive (TT vs. AT+TT)				0.8 (0.5-1.5)	0.6	0.9 (0.4-1.9)	0.8
Over-dominant (AT vs. AA+TT)				1.7 (1.1-2.8)	0.03	1.4 (0.7-2.7)	0.3
Log-additive				1.1(0.8-1.6)	0.4	1.1(0.7-1.6)	0.7

Table 3: Haplotype analysis of TNF-α (-308 A/G), TNF-β (+252 A/G) and IL-6 (-174 G/C; -597 G/A) polymorphisms

Haplotype	Overall Frequency	Control Frequency	Patients Frequency	Crude OR	P value	Age, Sex, TC, TG, HDL, VLDL, APOB, Smoking and Diet Adjusted OR	P value
TNF-α (-308 A/G) –TNF-β (+252 A/G)							
GA	0.782	0.878	0.697	1.0 (ref)		1.0 (ref)	
GG	0.159	0.104	0.212	2.5(1.5-4.1)	<0.001	2.6 (1.4-5.0)	0.003
AG	0.053	0.014	0.093	7.8 (2.6-24.0)	<0.001	8.5(2.2-33.35)	0.002
AA	0.002	0.004	0.000	NA		NA	
Global Haplotype association P value					<0.001		<0.001
D´	0.95						
r	0.45						
P value	<0.001						
IL-6 -174 (G/C) – IL-6 -597 (G/A)							
GG	0.849	0.847	0.851	1.0 (ref)		1.0 (ref)	
CA	0.113	0.121	0.105	0.8(0.5-1.4)	0.4	0.7(0.3-1.3)	0.3
CG	0.026	0.032	0.020	0.6 (0.2-1.9)	0.4	0.7 (0.2-2.9)	0.6
GA	0.012	0.0	0.024	NA		NA	
Global Haplotype association P value					0.02		0.26
D’	0.89						
r	0.83						
P value	<0.001						

Table 4: One Way ANOVA tests for interaction of genotypes with conventional risk factors

	Mean+SD	Mean+SD	P-value									
			TNF-α (-308 G/A)		TNF-β (+252 A/G)		IFN-γ (+874 T/A)		IL-6 (-174 G/C)		IL-6 (-597G/A)	
	Patients	Controls	Patients	Controls	Patients	Controls	Patients	Controls	Patients	Controls	Patients	Controls
Age	49.51±11.06	45.02 ±14.46	0.68	0.37	0.65	0.17	0.01*	0.84	0.40	0.21	0.41	0.17
TC	182.32±106.03	132.76±34.38	0.65	0.25	0.86	0.08	0.56	0.48	0.21	0.02*	0.28	0.02*
TG	208.01±109.45	145.64±66.03	0.79	0.41	0.99	0.42	0.79	0.02*	0.46	0.78	0.14	0.74
HDL	29.74±12.37	23.40±7.29	0.71	0.33	0.14	0.16	0.74	0.49	0.18	0.05*	0.25	0.04
LDL	106.37±42.94	80.71±27.25	0.56	0.23	0.37	0.16	0.14	0.91	0.69	0.02*	0.95	0.02
VLDL	42.79±18.04	30.08±13.46	0.12	0.12	0.46	0.83	0.24	0.05*	0.10	0.94	0.01*	0.94
APOB	153.50±43.46	108.76±45.78	0.67	0.67	0.91	0.97	0.92	0.70	0.97	0.09	0.41	0.05
							Post Hoc tests Age: TT vs. AT =0.03 And AT vs AA =0.04	Post Hoc tests TG: TT vs. AT =0.047 And TT vs. AA =0.024. VLDL: TT vs. AA =0.04				

* = Statistically significant at 5% level