

# Promoter variants in interleukin-6 and tumor necrosis factor alpha and risk of coronary artery disease in a population from Western India

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**INTRODUCTION:** A central component of the atherosclerotic process is inflammation. Single nucleotide polymorphisms (SNPs) present in the promoter region of various cytokines can lead to altered levels of the transcript and a state of low-grade inflammation exacerbating the risk of coronary artery disease (CAD). The present work tries to understand the role of permissive promoter variants in the interleukin-6 gene (IL-6-174G/C) and the tumor necrosis factor alpha (TNF $\alpha$ -308G/A) in the causation of CAD and also dyslipidemia. **MATERIALS AND METHODS:** Genotyping was conducted on 100 cases of CAD and 150 controls by the allele termination assay SNaPshot. Biochemical parameters were determined by routine enzymatic endpoint methods. The results were analyzed by appropriate statistical methods.

**RESULTS:** No differences in the minor allele frequency IL-6-174G/C SNP were seen between cases and controls (0.13 vs. 0.12). The differences in the allele frequency of TNF $\alpha$ -308A between cases (6%) and controls (2%) have led to an odds ratio, 3.370; 95% confidence interval, 1.039-11.543;  $P=0.033$  in the univariate analysis. In the final logistic regression analysis, however none of the variants were associated with an increased risk of CAD.

**CONCLUSIONS:** In summary, no association of the permissive promoter variants in the IL-6 gene and the TNF $\alpha$  gene were seen with an increased CAD risk. These and other studies highlight the importance of doing population specific studies.

**Key words:** Association study, coronary artery disease, India, interleukin-6, SNaPshot, single nucleotide polymorphisms, tumor necrosis factor alpha

## Introduction

Over the last decade, there has been a consensus that inflammatory processes underlie atherosclerosis.<sup>[1,2]</sup> Inflammation acts at the initial stages of atherosclerosis, where the presence of modified low density lipoprotein (LDL) particles induces a number of specialized cytokines, which result in directed migration of the monocytes to the intimal layer.<sup>[3,4]</sup> In the later stages inflammation precipitates adverse clinical events.<sup>[5]</sup>

Since the levels of circulating cytokines vary considerably inter-individually, genetic variants have been implicated. Until date, many single nucleotide polymorphisms (SNPs) influencing the variance of lipid and proinflammatory molecules have been identified both by candidate-gene as well as genome wide association studies.<sup>[6-9]</sup> SNPs in the promoter region of cytokine genes have garnered attention since the presence of the distinct allele is often associated with the baseline cytokine levels and/or also with the response to insult.<sup>[10-12]</sup> Thus, these permissive promoter SNPs can lead to the state of low-grade chronic inflammation exacerbating the risk of coronary artery disease (CAD).<sup>[13]</sup>

The interleukin-6 (IL-6)-174G/C is one such well characterized polymorphism in the promoter<sup>[14]</sup> that affects the rate of IL-6 serum levels and IL-6 gene transcription.<sup>[15]</sup> IL-6 is the only cytokine that can stimulate the synthesis of all those involved in the inflammatory response: C-reactive protein, serum amyloid A, fibrinogen,  $\alpha$ 1-antichymotrypsin and haptoglobin.<sup>[16]</sup>

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Another promoter polymorphism in the tumor necrosis factor alpha (TNF $\alpha$ ) gene-308G/A polymorphism has been associated with an increased production of TNF $\alpha$ , with the uncommon A allele shown to be a significantly stronger activator of transcription *in vitro*.<sup>[17,18]</sup> TNF $\alpha$ , is a cytokine with a wide range of proinflammatory activities and it occupies a pivotal role in the initiation and amplification of the inflammatory cascade in clinical conditions such as rheumatoid arthritis and atherogenesis.<sup>[19,20]</sup>

With India gearing to develop the highest incidence of CAD, it is important to evaluate the new markers especially genetic variants in our population. It is necessary to determine their frequency, since it can translate into different risk related incidence for the population. In the present study, permissive promoter variants in (IL-6-174G > C) and (TNF $\alpha$ -308G > A) have been evaluated and their association with CAD risk and lipid levels determined in the select Indian population (Indo-Europeans).

## Materials and Methods

### Subjects

The study was performed on a total of 250 unrelated individuals which consisted of 100 patients with CAD confirmed by coronary angiography: >50% stenosis in one or more arteries and stable or unstable angina and 150 controls: Examined clinically and investigated by electrocardiography to exclude CAD. This cohort consisted of subjects of Indo European descent from Maharashtra in Western India. Informed consent was obtained from all the subjects. The study is in accordance with the Helsinki declaration and was approved by the local ethical committee. A detailed case record form pertaining to information on demographics, medical history and coronary risk factors such as the presence of diabetes, hypertension, smoking, life-style and current medication was completed for each participant through personal interviews and through perusal of their medical records.

Blood specimens were collected after an overnight fast of 12 h by venipuncture using the vacutainer system from Becton Dickinson (Franklin Lakes, NJ USA) in the anti-coagulant ethylenediaminetetraacetic acid (EDTA) as well as plain bulb for serum. Serum, EDTA plasma

samples were separated by centrifugation and aliquots were preserved at -20°C until analysis.

### Biochemical parameters

The laboratory parameters namely serum total cholesterol (TC) triglyceride (TG) and high density lipoprotein-cholesterol (HDL-C) levels were determined by routine enzymatic endpoint methods (X Imola; Randox Laboratories Ltd. UK). LDL-C and very low-density lipoprotein cholesterol (VLDL-C) were calculated according to Friedewald's formula.

### Deoxyribonucleic acid extraction

Genomic DNA was isolated from peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA yield and purity was determined by measuring absorbance at 260/280 nm.

### SNP genotyping

Polymorphisms IL-6-174G > C (rsid1800795), TNF-308G > A (rsid1800629) were determined by a variation of the allele termination assay (ABI PRISM<sup>®</sup> SNaPshot<sup>™</sup>). The chemistry of this assay is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer. The polymerase extends the specific SNaPshot primer by one nucleotide, adding a single ddNTP to its 3'-end.

Using the contig sequence specific to the SNP, SNaPshot primer was designed to end one base before the SNP. The length of the primer was modified by the addition of non-homologous tails, poly (dGACT) added at the 5'-end. The SNaPshot assay was performed in the following manner. Briefly, 25  $\mu$ l PCR reaction was carried out using the specific PCR primers and annealing temperature of 58°C for 30 cycles. The PCR products were then purified by a combined Exonuclease (ExoI) and shrimp alkaline phosphatase (SAP) treatment where 5  $\mu$ l of ExoSAP-IT was added to 15  $\mu$ l of post-PCR reaction product. The mixture was incubated at 37°C for 15 min and then inactivated by heating at 80°C for 15 min. The specific SNaPshot primer was reconstituted to give a final working dilution of 0.2  $\mu$ M, the SNaPshot reaction contained 5  $\mu$ l of 2  $\times$  multiplex ready reaction mix, 1  $\mu$ l of the SNaPshot primer, 1  $\mu$ l PCR product and 3  $\mu$ l of de-ionized water in a 10  $\mu$ l reaction. The reaction mixture was centrifuged briefly and subjected to

25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 30 s. Post SNaPshot PCR, the products were again purified, by the alcohol purification method. The purified product was reconstituted in 10 µl of de-ionized water. Subsequently, 0.5 µl of the SNaPshot product, 0.5 µl GeneScan-120 LIZ size standard along with 9 µl Hi-Di formamide was added to each tube, denatured at 95°C for 5 min, kept on ice, transferred into sequencing plate. Capillary electrophoresis was then undertaken on the automated ABI prism 3100 Avant genetic analyzer. The data was analyzed using GeneMapper™ 4.0 Software. The fragment length and color of the alleles obtained is indicated below. 10% of random samples were also sequenced using PCR primers to check concordance with the SNaPshot results.

**Statistical analysis**

Allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. Any deviation of the genotype frequencies from the Hardy-Weinberg equilibrium (HWE) was assessed by Fischer’s exact test. Chi-square tests were used for comparison of binary variables across groups. To determine the risk for CAD, odds ratio (OR) was determined by both univariate analysis, as well as multivariate analysis adjusting for other co-variables. Routine statistical analysis were carried out with the SPSS version 15 software (SPSS Inc., Chicago, IL).

**Results**

Table 1 displays the means and standard deviations (SDs) for the study subjects for relevant biochemical characteristics as well as risk factors. The mean age of CAD incidence was 48 years (SD ± 11). The mean age of controls was 50 years (SD ± 11). It was

seen that the cases showed a higher incidence of the traditional risk factors associated with CAD such as smoking, diabetes, hypertension and presence of family history. The genotype and allele frequencies have been enumerated in Table 2. The genotypes were identified by fragment analysis as indicated in Figure 1 along with the corroborating sequencing electropherogram. There was no significant departure from the HWE determined by the exact test ( $P < 0.05$ ). No discordance could be detected for samples analyzed twice and the success rate of genotyping was ~ 99%. In the present study, no significant differences were observed in the frequency of the IL-6-174G > C genotypes between cases: GG, 77%; GC, 20%; CC 3% and controls GG, 80%; GC, 17%; CC, 3%. On the other hand, the genotype frequency of TNF-308G > A in cases was: GG, 89%, GA, 10%; AA, 1% and in controls was: GG, 97%; GA, 3%; AA, 0%. The differences in the minor allele frequency-308A between cases and controls have led to an OR, 3.370;

**Table 1: General characteristics of cases and controls**

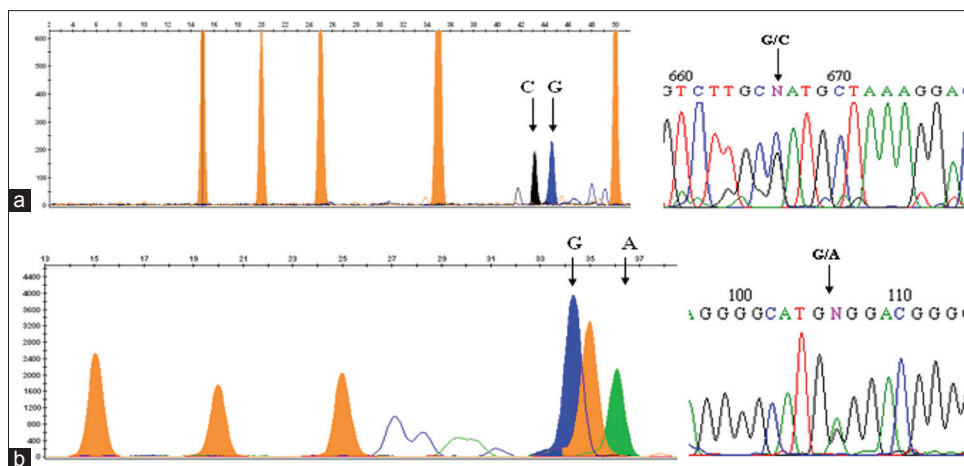
Characteristics	Cases n=100	Controls=150
Age	48±11	50±11
Sex (male) (%)	80	70
Smoking status (%)		
Smoker (up to 10 cigs/day)	23*	13
Ex-smoker	11*	6
Alcohol (%)	15	18
Hypertension (%)	38*	18
Diabetes (%)	23*	11
Family history (%)	23*	13
Plasma cholesterol (mg/dl)	179±55.1	193±36
HDL-cholesterol (mg/dl)	37±16.5	41±11
LDL-cholesterol (mg/dl)	110±51.2	125±30
VLDL-cholesterol (mg/dl)	36±23.9	30±18
Triglycerides (mg/dl)	178±124	152±88
Single vessel disease (%)	36	NA
Double vessel disease (%)	9	NA
Triple vessel disease (%)	55	NA
Myocardial infarction (%)	41	NA

Mean±SD for continuous variables. \*Cases significantly different from controls,  $P < 0.05$ . NA: Not applicable, SD: Standard deviation, HDL: High density lipoprotein, LDL: Low density lipoprotein, VLDL: Very low density lipoprotein

**Table 2: Genotype and allele frequencies of inflammation related variants**

	Genotype frequencies n (%)			Major G	Minor C	OR <sup>a</sup>	95% CI	P value
	GG	GC	CC					
IL-6-174G>C								
Cases (100)	77 (77)	20 (20)	3 (3)	0.87	0.13	1.186	0.624-2.251	0.645
Controls (150)	121 (80)	25 (17)	4 (3)	0.88	0.12			
	GG	GA	AA	G	A			
TNFα-308G>A								
Cases (100)	89 (89)	10 (10)	1 (1)	0.94	0.06	3.370	1.039-11.543	0.033*
Controls (150)	145 (97)	5 (3)	0	0.98	0.02			

<sup>a</sup>OR: Unadjusted odds ratio, 95% CI: 95% confidence interval, \*Statistically significant at  $P < 0.05$ , TNFα: Tumor necrosis factor alpha, IL: Interleukin



**Figure 1: Genotypes have been shown by SNaPshot assay. The genotypes are analysed based on the color of the peak and fragment length. The orange peaks indicate size standard LIZ120GS. (a) Interleukin-6-174G>C genotyping by SNaPshot indicates both G and C allele. The corroborating sequencing electropherogram is adjacent. (b) Tumor necrosis factor alpha-308G>A genotyping indicates both G and A allele. The sequencing electropherogram of the same is adjacent**

95% confidence interval (CI), 1.039-11.543;  $P$ , 0.033 in the univariate analysis. The effect of the variants on plasma lipid levels was assessed by comparing the mean concentration of the lipids based on genotypes and performing appropriate statistical analysis. The genotype-phenotype correlation was done only on controls as most of the cases were on lipid lowering medication. No association of the genotypes with any of the lipid levels were seen as indicated in Table 3. To determine if the variants are associated with an increased risk of CAD stepwise logistic regression analysis, which included nine variables was performed [Table 4]. In the final model hypertension (OR: 5.732, 95% CI: 2.194-14.970,  $P$  < 0.0001) and smoking (OR: 5.027, 95% CI: 1.908-13.243,  $P$ , 0.001) were associated with an increased risk of CAD, whereas alcohol consumption seemed to have a protective effect (OR: 0.272, 95% CI: 0.075-0.990,  $P$ , 0.048). The TNF-308G > A as a risk variable was removed on step 5 of the model (OR: 2.023, 95% CI: 0.358-11.426-14.970,  $P$ , 0.425) and IL-6-176G > C genotype on step 8 of the final model (OR: 1.598, 95% CI: 0.693-3.685,  $P$ , 0.271).

## Discussion

Inflammation is one of the hallmarks of atherosclerosis and hence several studies have evaluated the utility of inflammatory biomarkers in predicting cardiovascular

disease.<sup>[21]</sup> In the present study, two permissive promoter variants-174G/C in the IL-6 gene and-308G/A in the TNF $\alpha$  have been evaluated with respect to alterations in lipid metabolism and CAD risk in our population.

There is a considerable interethnic variation in the allelic distribution of IL-6 G > C polymorphism, with a C allele frequency of 0.41 in Caucasians,<sup>[14]</sup> 0.15 in Gujarati Indians in US and 0.05 in Afro-Caribbean's.<sup>[15]</sup> The minor allele frequency-174 (C) was relatively high in the Finnish population and varied between 0.53 and 0.56 in different studies.<sup>[22-24]</sup> In the current study, the minor allele frequency-174 (C) was 0.12 in controls which is almost similar to that of Gujarati Indians in US.

The frequency of the minor allele TNF $\alpha$ -308 (A) in the present cohort is low (0.02 in controls), which corroborates the findings of another study on North Indian population.<sup>[25]</sup> There is a wide variation in the incidence of the A allele in different populations across the world. The highest frequency (0.274) was recorded in a Caucasian population.<sup>[26]</sup> Data from the HapMap indicated frequency of the A allele in Europeans to be 0.217, whereas in the Japanese population it was 0.02.

The differences in the frequency of promoter polymorphisms of the cytokines are exciting, since they have functional significance and could partially explain interethnic variation found in other states such as autoimmune diseases.

**Table 3: IL-6 and TNF $\alpha$  genotypes and lipid levels**

SNP	TC	TG	LDL	HDL	VLDL	TC/HDL	LDL/HDL
IL-6-174G/C							
GG	188±38	143±93	123±30	41±8	29±19	4.76±1.1	3.13±0.93
GC	193±45	176±88	123±39	39±9	35±18	5.07±1.11	3.23±0.84
CC	178±25	167±40	114±26	33±6	33±8	5.45±0.91	3.49±0.94
P value	0.56	0.15	0.99	0.37	0.15	0.26	0.64
TNF $\alpha$ -308G/A							
GG	189±38	150±89	123±32	40±8	31±24	4.86±1.09	3.17±0.93
GA	175±46	156±40	109±46	35±5	31±8	5.04±1.43	3.15±1.47
P value	0.42	0.87	0.33	0.20	0.98	0.72	0.95

TC, TG, LDL-C, HDL-C and VLDL-C levels are in mg/dl, TC/HDL and LDL/HDL are ratios. The values indicate mean±SD of the lipid levels based on the genotypes. Assuming the dominant model, homozygotes for the minor allele and heterozygotes were pooled and lipid parameters compared with homozygotes for the major allele using the student t-test. SD: Standard deviation, TC: Total cholesterol, TG: Triglyceride, LDL: Low density lipoprotein, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, SNP: Single nucleotide polymorphisms, TNF $\alpha$ : Tumor necrosis factor alpha, IL: Interleukin

**Table 4: Stepwise backward logistic regression for inflammation related variants in IL-6, IL-10 and TNF $\alpha$  genes association with CAD risk**

Parameter	OR	95% CI	P value
Step 1 <sup>a</sup>			
Age	0.987	0.942-1.033	0.564
Gender	0.666	0.213-2.087	0.486
Alcohol	0.280	0.071-1.106	0.069
Diabetes	1.369	0.386-5.050	0.611
Hypertension	6.519	2.036-20.865	0.002
Family history	0.757	0.244-2.353	0.631
Smoking	3.598	1.292-10.019	0.014
IL-6-176G>C	1.679	0.696-4.048	0.248
TNF $\alpha$ -308G>A	1.920	0.338-10.893	0.462
Constant	0.126		0.223
Step 8 <sup>b</sup>			
Alcohol	0.272	0.075-0.990	0.048
Hypertension	5.732	2.194-14.970	<0.0001
Smoking	5.027	1.980-13.243	<0.001
Constant	0.032		0.001

<sup>a</sup>Variables entered on step 1 include: Age, gender, alcohol consumption, diabetes, hypertension, family history, smoking and IL-6-174G>C, and TNF $\alpha$ -308G>A genotypes. <sup>b</sup>In the final step only alcohol consumption, hypertension and smoking showed a significant association with CAD. CAD: Coronary artery disease, CI: Confidence interval, TNF $\alpha$ : Tumor necrosis factor alpha, OR: Odds ratio, IL: Interleukin

In recent times, the interplay between inflammation and lipid metabolism has also been the focus of research aimed at understanding the mechanisms of atherogenesis. In the present study, no association of IL-6-174G/C genotypes with lipid levels was seen, which is in agreement with a study on healthy Mexican subjects.<sup>[27]</sup> In contrast, the Genetics of Lipid Lowering Drugs and Diet Network study, the GG and CG subjects showed higher fasting plasma TG ( $P = 0.025$ ), VLDL ( $P = 0.04$ ) and large VLDL ( $P = 0.02$ ) concentrations than did CC subjects.<sup>[28]</sup> No association of the TNF $\alpha$ -308 genotypes with any of the lipid parameters was seen in the present

study, though in contrast the study on Mexican population indicated that high levels of TGs and TC, as well as low HDL-C levels were associated with GG genotype of the-308 TNF $\alpha$  polymorphism.<sup>[27]</sup> These contrasting results may be due to inherent difficulties in studying the interaction between inflammation and lipid metabolism *in vivo* due to anorexia triggered by the proinflammatory cytokines.<sup>[29]</sup>

No association with CAD risk was seen with the IL-6-174G > C SNP in our study, which is similar to the results of the Rotterdam Heart study, a meta-analysis<sup>[30]</sup> and the MONICA survey.<sup>[31]</sup> Our results are also in agreement with the Ludwigshafen Risk and Cardiovascular Health (LURIC) cohort study.<sup>[32]</sup> However, in contrast to our study and several others the ECTIM study implicated the-174C allele with over 1.34 times risk of MI.<sup>[33]</sup> Another study on Asian Indians observed the haplotype defined by this promoter SNPs to be associated with over 3.7 times risk of CAD (OR 3.676, 95% CI 1.68-8.05,  $P = 0.0017$ ),<sup>[34]</sup> which is in contrast to our findings and also to that in a study on North Indians.<sup>[35]</sup>

In the present study, the TNF $\alpha$ -308G > A did not associate with increased CAD risk after accounting for the confounders. The findings of the present study are similar to that of Koch *et al.* where no association was seen with both IL-10 and TNF $\alpha$  promoter polymorphisms -863C > A and -308G > A, either alone or acting co-operatively with the risk of CAD/MI.<sup>[36]</sup> A study on the North Indian population also has found no association of this variant with risk of CAD.<sup>[37]</sup> Recent meta-analysis<sup>[38]</sup> has also concluded that "It is probable that carrying the-308A variant is associated with CAD risk in Caucasians, but not in Asians, Indians or Africans. Further studies are merited to assess the association in greater details, especially in Asians, Indians and Africans".

In summary, no association of the permissive promoter variants in the IL-6 gene and the TNF $\alpha$  gene were seen with increased CAD risk. Though, one of the limitations of the study is the sample size and larger studies are ongoing, these and other studies highlight the importance of doing population specific studies taking into account the different ethnicities.

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