**J Cardiovasc Disease Res., 2018; 9(3):115-122**
A Multifaceted Peer Reviewed Journal in the field of Cardiology
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*Original Article*

# Association of *MTHFR, IL-6* and *ICAM-1* gene Polymorphisms with Coronary Artery Disease in South-Indian Ethnic Subset: A Case-Control Study

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## ABSTRACT

**Introduction:** Cardiovascular disease is the leading cause of mortality and morbidity all over the world. Among these cardiovascular deaths, half result from coronary artery disease (CAD). Increased prevalence of CAD is associated with increased levels of inflammatory markers. Phenotypic variations of these markers may depend on physiological stress or genetic variations. **Materials and Methods:** Single nucleotide polymorphism analysis of methylenetetrahydrofolate reductase (*MTHFR)*, interleukin-6 (*IL-6)* and intercellular adhesion molecule-1 (*ICAM-1)* genes was done by PCR-DNA sequencing method. **Results:** Statistically significant elevation of inflammatory markers- homocysteine, hsCRP and fibrinogen were found in CAD group ($p \leq 0.05$). Multiple sequence alignment showed a single nucleotide mutation i.e., c.677 C>T (p. A222V) in exon-4 of *MTHFR* in 10% of CAD group and was associated with an increased risk of CAD (OR: 12.21). Mutations observed in exon-4 of *IL-6* gene (in 26% of cases) was significantly associated with an increased risk of CAD (OR: 36.36). *ICAM-1* (exon-6) mutation i.e., c.1405 A>G (p. K469E) was observed in 18% of patients and an increased risk of CAD (OR: 23.12). **Conclusion:** Polymorphisms observed in *MTHFR, IL-6* and *ICAM-1* genes in South-Indian ethnic population which are associated with elevated levels of inflammatory markers – homocysteine, hsCRP and fibrinogen appear to be predisposing factors for atherosclerosis.

**Key words:** Coronary artery disease, Methylenetetrahydrofolate reductase, Interleukin-6, Intercellular adhesion molecule-1, Homocysteine, hsCRP, Polymorphism, South-India.

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**Submission Date:** 26-04-2018;
**Revision Date:** 08-08-2018;
**Accepted Date:** 19-09-2018.
**DOI :** 10.5530/jcdr.2018.3.27

## INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity all over the world. Among these cardiovascular deaths, half result from coronary artery disease (CAD). The prevalence of CAD in India is rising with time in both rural and urban areas.[1] CVDs are showing an escalation among the Indian population with a trend of affecting the younger age groups.

The most striking feature of premature CAD in Indians is low prevalence of traditional risk factors. Increased prevalence of CAD is associated with increased levels of inflammatory markers which damage the endothelium and increase the development of atheromatous plaques.[2-5] Phenotypic variations of these markers may depend on physiological stress or genetic variations.

Genetic susceptibility to CAD may depend on genes related to inflammatory process, hence in the present study we studied *Methylenetetrahydrofolate reductase (MTHFR), Interleukin-6 (IL-6)* and *Intercellular adhesion molecule-1 (ICAM-1)* genes in which genetic variations lead to hyperhomocysteinemia, release of inflammatory markers such as hsCRP, fibrinogen, LP[a] and elevated expression of soluble ICAM-1 levels respectively.[6-8]

Methylenetetrahydrofolate reductase (*MTHFR*) gene catalyzes the reaction that reduces 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which acts as a carbon (C1) donor in the remethylation of Homocysteine into Methionine. A variant of this enzyme called 'thermolabile MTHFR' that has reduced activity at higher temperatures is produced by a common single nucleotide mutation in the exon-4 of *MTHFR* gene and is incapable of reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate resulting in the elevated levels of homocysteine (hyperhomocysteinemia).[6] Homocysteine acts as a corrosive agent and causes oxidative damage to endothelium and results in atherosclerosis.[9]

IL-6 has been shown to contribute to both, atherosclerotic plaque development and plaque destabilization via a variety of mechanisms. IL-6 stimulates the expression of tissue factors, matrix degrading enzymes, LDL receptors in macrophages, as well as the aggregation of the platelets and the production of CRP, fibrinogen, LP[a] and serum amyloid A (SAA) by hepatocytes. These agents cause damage to the endothelium, increased occurrence of thrombosis, decreased the HDL-cholesterol and increased macrophage uptake of lipid which are events in CAD. This stimulation of IL-6 on acute phase proteins shows a link between IL-6 and CVD.[10,11]

*ICAM-1* is expressed on vascular endothelium and plays a key role in trans-endothelial migration of leukocytes. *ICAM-1* functions as a ligand for β2 integrin molecules (LFA-1 and Mac-1) present on leukocytes[12] and encodes a cell surface glycoprotein which is typically expressed on endothelial cells and cells of immune system and is involved in cell-cell adhesive interactions of the immune system. *ICAM-1* expression on endothelium is regulated by cytokine stimulation.[13] *ICAM-1* functions as a natural receptor for lymphocytes and is involved in the binding of leukocytes to the arterial endothelium which leads to the formation of strong plaques by the segregation of fat molecules and leukocytes on arterial walls.[14]

In view of lack of polymorphism studies on these genes in our ethnic population the present study was aimed to examine the polymorphisms

of *MTHFR, IL-6* and *ICAM-1* genes in young CAD patients of South-Indian ethnicity.

## MATERIALS AND METHODS

This was a prospective Case-Control study conducted in the department of Cardiology, SVIMS, Tirupati. This study was approved by the Institutional Ethics Committee of our institute with IEC approval no. 220.

50 CAD patients aged between 18 and 50 years, diagnosed with acute coronary syndromes [Unstable angina (USA), Myocardial infarction (ST-elevation myocardial infarction (STEMI) and Non-STEMI (NSTEMI)] were recruited in the CAD group. In addition, 50 healthy subjects aged between 18 and 50 years, without any known risk factors (diabetes mellitus, hypertension, smoking, obesity) and without family history of CAD (confirmed by history and physical examination) were recruited in control group. Patients with chronic stable angina, having other co-morbid conditions such as renal, lung, liver and other systemic illness, pregnant women were excluded from the study. A written informed consent was obtained from all the study participants following ethical guidelines of the 1975 declaration of Helsinki.

### Biochemical Analysis

Fasting lipid profile including total cholesterol, triglycerides, HDL cholesterol were estimated using commercial kits on DXC600 Beckman auto analyzer. LDL cholesterol and VLDL cholesterol were calculated using Friedwald formula.[15] Homocysteine levels were estimated using Dialab kit using enzymatic recycling method on DXC600 Beckman auto analyzer. High sensitivity C-reactive protein (hsCRP) levels were estimated on Beckman system pack by immunoturbidimetry assay method. Protein C,[16] Protein S[17] and anti-cardiolipin antibodies were estimated by ELISA method.

### Genetic Analysis

Genomic DNA was isolated from blood samples using QIAamp DNA Mini spin-column [Qiagen] DNA extraction kit.[18,19] Extracted DNA samples were analyzed on 1% agarose gel electrophoresis.[18] The following Oligonucleotide primers were designed using Oligo-6, NCBI blast and Primer-3 softwares and synthesized at Eurofins genomics India Pvt. Ltd. Bengaluru, India.[20]

Polymerase chain reaction (PCR) amplification was done in the Eppendorf Mastercycler nexus gradient-flexlid model, Hamburg, Germany, in a 50 µL reaction volume comprising of 1x assay buffer, 1.5 mmol $MgCl_2$, 500 ng template DNA, 50 pico mole forward primer, 50 pico mole reverse primer, 100 µmol dNTPs mix and 1U Taq DNA polymerase (Thermo Scientific, USA).

PCR amplification was performed with following conditions: Denaturation at 94°C; Annealing at 60°C, 55°C and 56°C for *MTHFR* (exon-4), *IL-6* (exon-4) and *ICAM-1* (exon-6) respectively; and Extension at 72°C. The amplified PCR products were analyzed on 2% agarose gel in 1X TAE [Tris-Acetate-EDTA, pH: 7.8] to confirm the targeted amplification.[18] The purification of PCR products resolved in 2% agarose gel was done by electro elution method with NucleoSpin® PCR (NP-PCR) Purification kit (Taurus Scientific, USA).[18] The amplified PCR products were sequenced by Sanger's dideoxy chain termination method at Eurofins Genomics India Pvt Ltd., Bengaluru, India.

The sequences were compared by performing multiple sequence alignment using *ClustalX tool* (Version 1.83, National Center for Biotechnology Information, Bethesda, MD) to identify the mutations in the sequences. Expert Protein Analysis System (ExPASy) analysis was used to translate nucleotide sequences into amino acid sequence and changes in amino acid sequences were noted for each sequence. All the mutated sequences were deposited at NCBI-GenBank database.[21]

### Statistical Analysis

Data was collected in a pre-designed proforma and entered in Microsoft Excel spread sheets. Normality of distribution was checked with *Kolmogorov-Smirnov test*. Descriptive statistics including mean and standard deviation (SD) for continuous variables and proportions for categorical data were calculated. Continuous data was tested for statistical significance with Independent Student's t-test. All the statistical analysis was performed with the help of Statistical Package for Social Sciences (SPSS) software for Microsoft Windows, version 20.0. (SPSS Inc., Chicago, IL, USA.).

## RESULTS

Mean age of the CAD group was 36.6 (±0.7) years and control group was 34.9 (±0.7) years ($p=0.11$), thus the two groups are comparable. By chance majority of the cases were males (86%) in both the groups. Smoking (60%) was the most prevalent risk factor in CAD group followed by diabetes mellitus (34%), hypertension (30%) and obesity (24%). Baseline and demographic details are summarized in Table 1.

Statistically significant higher levels of total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol and Lp[a] were observed in CAD group compared with control group ($p\leq0.05$). There was no statistically significant difference in HDL-cholesterol ($p=0.06$).

Risk factors related to inflammation (homocysteine and hsCRP) and coagulation (fibrinogen, protein-C and protein-S) were estimated and values are summarized in Table 2. A statistically significant elevation of inflammatory markers homocysteine, hsCRP and fibrinogen were found in CAD group ($p\leq0.05$). Protein-C deficiency was observed in 32% of the patients whereas protein-S deficiency was not observed in any case.

### Methylenetetrahydrofolate reductase (Exon-4) Mutation analysis

Multiple sequence alignment showed a single nucleotide mutation i.e., c.677 C>T in exon-4 of *MTHFR* in 10% of CAD group (Figure 1). This single nucleotide change (transition) causes a replacement of amino acid Alanine to Valine at position 222 (p.A222V) in the protein sequence (ExPASy analysis) of MTHFR which produces a thermolabile methylenetetrahydrofolate reductase enzyme which is incapable of conversion of homocysteine to methionine. The identified mutations were submitted to GenBank with accession numbers - KX234844, KX234845, KX234846, KX234847 and KX234848.

Statistically significant higher homocysteine levels were observed in patients with *MTHFR* mutation compared with patients without *MTHFR* mutation (35.8±4.7 µmol/L vs 17.5±7.7 µmol/L, $p<0.0001$).

| | Forward Primer [5′ to 3′] | Reverse Primer [5′ to 3′] | Amplicon Size |
|---|---|---|---|
| *MTHFR*, Exon-4 | *GGCAGGACAGTGTGGGAGTT* | AGGACGGTGCGGTGAGAGTG | 546 bp |
| *IL-6*, Exon-4 | TACATGGGGCCTCTGATTGTC | GGAAGTGGCATTGCATCCCT | 428 bp |
| *ICAM-1*, Exon-6 | CTTCGTGTCCTGTGTGAGTG | GGTGAGGATTGCATTAGGTC | 449 bp |

MTHFR: Methylenetetrahydrofolate reductase; IL-6: interleukin-6; ICAM-1: intercellular adhesion molecule 1.

**Table 1: Baseline and Demographic details of the Study group.**

| Variable | CAD group (n=50) |
|---|---|
| Age, in years | 36.6±0.7 |
| Male: Female, n | 43:07 |
| Obesity, n (%) | 12 (24%) |
| Smokers, n (%) | 30 (60%) |
| Alcoholics, n (%) | 19 (38%) |
| Hypertension, n (%) | 15 (30%) |
| Diabetes mellitus, n (%) | 17 (34%) |
| Family h/o CAD, n (%) | 12 (24%) |
| Presentation of Patients: | |
| STEMI | 38 (76%) |
| NSTEMI | 07 (14%) |
| USA | 05 (10%) |
| Type of disease: | |
| SVD | 35 (70%) |
| DVD | 09 (18%) |
| TVD | 02 (4%) |
| Normal Coronaries | 04 (8%) |
| Diseased Vessels: | |
| LAD | 35 (59.3%) |
| LCX | 07 (11.9%) |
| RCA | 17 (28.8%) |

CAD: Coronary artery disease; STEMI: ST-elevated myocardial infarction; NSTEMI: Non-ST-elevated myocardial infarction; USA: unstable angina; SVD: single vessel disease; DVD: double vessel disease; TVD: triple vessel disease; LAD: left anterior descending artery; LCX: left circumflex artery; RCA: right coronary artery; NS: not significant; NA: not applicable.

**Table 2: Comparison of Inflammatory and Coagulation Profile.**

| Variables | CAD group Mean± SD | Control group Mean± SD | p-val |
|---|---|---|---|
| Homocysteine (µmol/L) | 19.3±9.2 | 16.8±1.2 | p = 0.05* |
| hsCRP (mg/dl) | 2.3±2.2 | 0.4±0.1 | p<0.0001* |
| Fibrinogen (mg/dl) | 382.5±175.4 | 112.4±38.0 | p<0.0001* |
| Anti-Cardiolipin antibodies (MPL U/ml) | 7.96±1.09 | 6.80±4.51 | p = NS |
| Protein C deficiency | 16 (32%) | 00 | <0.0001* |
| Protein S deficiency | 00 | 00 | -- |

hsCRP: high sensitivity C-reactive protein; SD: standard deviation; NS: not significant. Independent sample t-test. *indicates statistical significance (p≤0.05).

Present study findings showed that the mutation c.677 C>T in exon-4 of *MTHFR* is associated with an increased risk of CAD (OR: 12.21, 95% CI: 0.66 to 226.98) in South-Indian ethnic population.

## Interleukin-6 (exon-4) Mutation analysis

Sequence analysis with multiple sequence alignment tool showed mutations (Figure 1) in exon-4 of *IL-6* in 13 CAD patients (C-5, C-9, C-10, C-12, C-14, C-15, C-21, C-24, C-28, C-29, C-32, C-45 and C-47). All the identified mutations were novel. There was no mutation in control group. Identified mutations were translated to amino acid sequences with ExPASy analysis and the changes in protein sequence was noted and deposited in GenBank. GenBank accession numbers are KX430119, KX430120, KX430121, KX430122, KX430123, KX430124, KX430125, KX430126, KX430127, KX430128, KX430129, KX430130 and KX430131 (Table 4).

**Table 3: Comparison of hsCRP and Fibrinogen– mutated vs normal genotype of *IL-6* gene.**

| *IL-6* SNP | hsCRP (mg/dl) | Fibrinogen (mg/dl) |
|---|---|---|
| Present | 5.1±1.3 | 480.6±140.3 |
| Absent | 1.3±0.2 | 347.9±175.0 |
| p-val | *p*<0.0001* | *p*=0.017* |

hsCRP: high sensitivity C-reactive protein; IL: interleukin; d.f: degrees of freedom. *indicates statistical significance (p≤0.05).

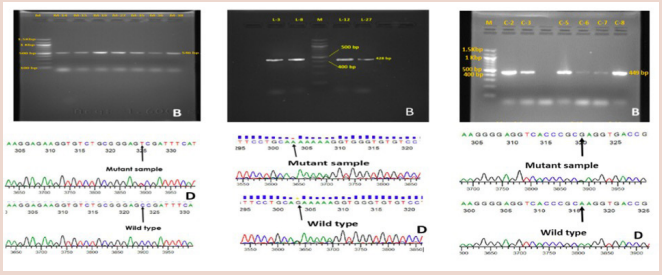


**Figure 1:** Agarose gels showing PCR amplicons. 2% agarose gels showing PCR amplicons and chromatograms showing polymorphisms in exon-4 of MTHFR, exon-4 of IL-6 and exon-6 of ICAM-1 genes respectively from left to right.

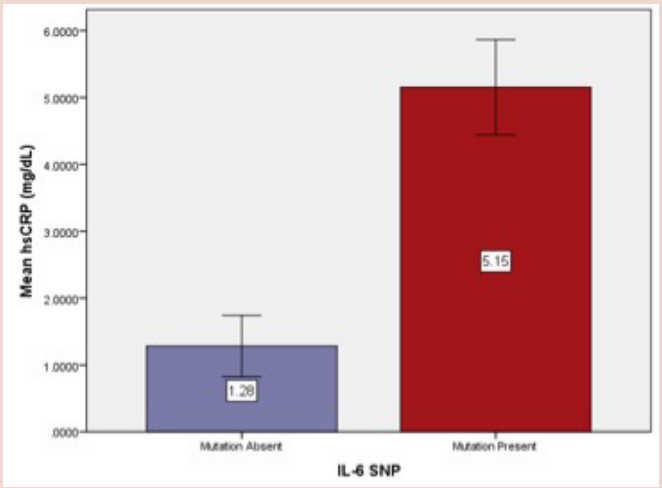


**Figure 2:** Comparison of mean hsCRP levels in patients with *IL-6* mutation and without *IL-6* mutation.

A higher hsCRP and fibrinogen levels were found in patients with mutations in *IL-6* gene which was statistically significant (*p<0.0001* and *p=0.017* respectively) (Table 3, Figures 2 and 3). Higher Lp[a] values were also found in patients with *IL-6* mutations but not statistically significant (*p=0.82*).

Present study findings showed that the mutations in exon-4 of *IL-6* gene is significantly associated with an increased risk of CAD (OR: 36.36, 95% CI: 2.09 to 631.21) in South-Indian ethnic subset.

**Table 4: Mutations identified in exon-4 of *IL-6* gene.**

| Patient ID | Nucleotide Sequence | Protein Sequence | hsCRP (mg/dl) | Fibrinogen (mg/dl) | GenBank Accession No. |
|---|---|---|---|---|---|
| C-9 | c.581 G>A | No change | 2.52 | 711 | KX430120 |
| C-24 | c.581 G>A | No change | 3.9 | 403 | KX430126 |
| C-28 | c.581 G>A | No change | 3.93 | 527 | KX430127 |
| C-29 | c.519 G>A, c.552 G>A | No change | 6.3 | 384 | KX430128 |
| C-32 | c.581 G>A | No change | 2.52 | 496 | KX430129 |
| C-45 | c.581 G>A | No change | 5.85 | 491 | KX430130 |
| C-47 | c.581 G>A | No change | 6.3 | 318 | KX430131 |
| C-12 | c.534 G>C, c.538 G>T, c.540 G>C, c.541 C>A, c.548 G>A, c.581 G>A | p.140 A>P, p.141 R>I, p.142 A>H | 5.42 | 436 | KX430122 |
| C-14 | c.506 G>A, c.509 C>T, c.538 G>T, c.548 G>A, c.581 G>A | p.141 R>I | 5.27 | 268 | KX430123 |
| C-10 | c.446 T>C, c.462 A>T, c.464 C>A, c.465 A>T, c.466 C>G, c.472 T>A, c.495 G>C, c.497 G>C, c.521 T>A, c.522 A>G, c.534 G>C, c.537 A>T, c.538 G>T, c.540 G>C, c.541 C>A, c.548 G>A, c.551 G>A, c.553 G>A | p.116 I>L, p.117 T>C, p.119 L>H, p.127 E>H, p.135 S>R, p.136 S>G, p.140 A>P, p.141 R>L, p.142 A>H, p.145 M>I, p.146S>N | 7.3 | 370 | KX430121 |
| C-21 | c.454 T>G, c.471 C>T, c.476 G>T, c.477 G>T, c.478 C ins, c.498 G>T, c.507 G>A, c.512 G>A, c.535 G>C, c.539 G>A, c.541 G>C, c.549 G>A, c.562 G>T, c.573 G>T, c.582 G>A | p.113 V>G, p.119 L>F, p.120 L>F, p.121 E>P, p.122 F>V, p.123 E> Stop codon. | 6.3 | 505 | KX430125 |
| C-5 | c.443 G>C, c.445 C>T, c.450 C>G, c.451 T>G, c.454 T>A, c.455 G>A, c.459 A>C, c.465 A del, c.466 C>T, c.467 T>G, c.471 C>T, c.478 A>G, c.482 T>G, c.484 A>G, c.487 G ins, c.490 T>C, c.491 A>C, c.496 G>A, c.497 A>T, c.498 G>T, c.499 T>C, c.500 A>C, c.503 T>C, c.507 G>A, c.512 G>A, c.513 A>T, c.516 T>G, c.518 A>G, c.520 A>G, c.522 T>G, c.523 A>G, c.525 T>G, c.527 A>G, c.529 G>A, c.531 A>C, c.533 A>C, c.534 A>C, c.535 G>C, c.539 G>A, c.541 G>T, c.546 G>C, c.549 G>A, c.550 A>T, c.551 T>A, c.552 G>A, c.554 G>A, c.555 T>C, c.557 C del, c.562 G>T, c.565 C>T, c.566 T>C, c.567 G>T, c.572 A>C, c.573 G>T, c.578 T>C, c.579 G>C, c.580 C>A, c.582 G>A, c.587 A>G | p.109 E>D, p.110 T>I, p.112 L>G, p.113 V>E, p.115 I>L, p.117 T>W, p.118 G>V, p.119 L>F, p.120 L>W, p.121 E>G, p.122 F>W, p.123 E>G, p.125 Y>P, p.127 E>I, p.128 Y>P, p.129 L>P, p.132 R>N, p.133 F>L, p.134 E>G, p.135 S>G, p.136 S>G, p.137 E>G, p.138 E>N, p.139 Q>P, p.140 A>P, p.141 R>K, p.142 A>S, p.145 Stop codon. | 6.3 | 609 | KX430119 |
| C-15 | c.451 T>G, c.454 T>G, c.462 A>T, c.465 A>T, c.467 T>G, c.495 G>C, c.497 G>C, c.505 A>C, c.506 G>C, c.511 G>T, c.521 T>A, c.522 A>G, c.533 A>C, c.534 G>C, c.540 G>C, c.541 C>A, c.543 G>C, c.548 G>C, c.551 G>A, c.561 G>T, c.571 A>C, c.572 G>C, c.581 G>C | p.112 L>R, p.113 V>G, p.116 I>F, p.127 T>S, p.130 Q>P, p.132 R>I, p.135 S>R, p.136 S>G, p.139 Q>H, p.140 A>P, p.141 R>T, p.142 A>H, p.143 V>L, p.144 Q>H, p.145 M>I, p.149 V>F, p.152 Q>P, p.155 Q>H | 5.69 | 730 | KX430124 |

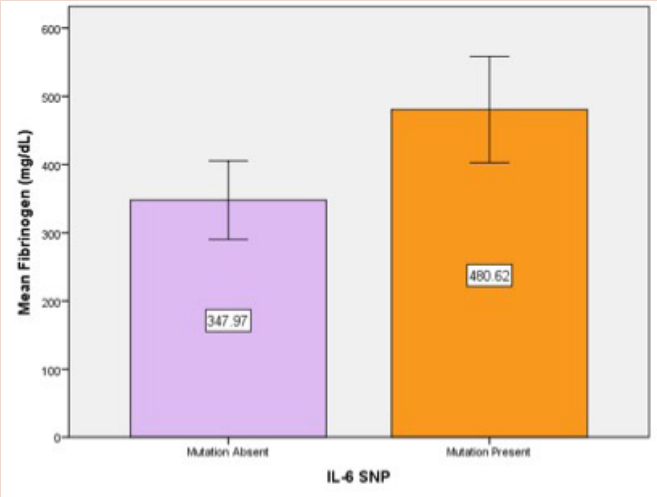


**Figure 3:** Comparison of mean Fibrinogen levels in patients with *IL-6* mutation and without *IL-6* mutation.

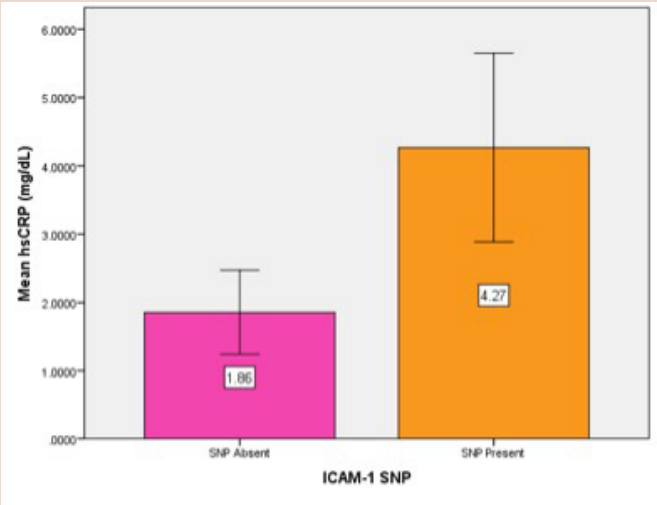


**Figure 4:** Comparison of mean hsCRP levels in patients with *ICAM-1* mutation and without *ICAM-1* mutation.

### *Intercellular adhesion molecule-1 (exon-6) gene Mutation analysis*

*ICAM-1* (exon-6) mutation i.e., c.1405 A>G was observed in 9 (18%) patients on Multiple sequence alignment (Figure 1). This mutation causes a change of amino acid Lysine to Glutamic acid (p. K 469 E) at position 469 (ExPASy analysis). Identified mutations were submitted to GenBank and the accession numbers are - KX239886, KX239887, KX239888, KX239889, KX239890, KX239891, KX239892, KX239893 and KX258233.

A statistically significant higher level of hsCRP was found in patients with *ICAM-1* polymorphism compared to patients without *ICAM-1* mutation (4.27±2.07 vs 1.86±1.98 mg/dl, *p= 0.002*) indicating that this mutation increases the inflammation (Figure 4).

Observations of the present study showed association of *ICAM-1* gene mutation with CAD risk (OR: 23.12, 95% CI: 1.31 to 409.15).

## DISCUSSION

Strong scientific evidence indicates that inflammatory pathway activation is important in the initiation, maintenance and progression of atherosclerosis. The inflammatory cascade has been implicated during the entire plaque formation, from the early stages of endothelial dysfunction to the development of acute coronary syndromes (ACS). This is in accordance with the presence of elevated circulating biomarkers of inflammation, which independently predicts the likelihood of adverse cardiovascular events.[22-24]

In the present study, a statistically significant elevation of inflammatory markers homocysteine, hsCRP and fibrinogen were found in CAD group which confirms the key role of inflammatory markers in CAD. Protein-C deficiency was observed in 32% of the patients. Protein-C and S are synthesized by the hepatocytes and endothelial cells. Protein-C upon activation by protein-S inhibits coagulation pathway by cleaving FVa and FVIIIa.[5] Therefore, Protein-C deficiency is suggestive of hypercoagulable state in CAD group.

In recent years, genomic susceptibility to diseases has attracted a growing attention to research the genetic polymorphisms involved in pathogenesis of diseases. Genetic polymorphisms can change the structure and quantity of the gene product, ultimately affecting the function of the product. The inflammatory status is an important step to start and promote the pathogenesis of atherosclerosis.

A single nucleotide mutation i.e., c.677 C>T in exon-4 of *MTHFR* gene was observed in 10% of CAD cases while it was absent in controls. This single nucleotide change (transition) causes production of thermolabile methylenetetrahydrofolate reductase enzyme which is incapable in conversion of homocysteine to methionine. The present study findings suggest that the T allele is associated with an increased risk of CAD in South-Indian ethnic population. This finding is consistent with previous reports[25-29] in which *MTHFR* C677T transition was found to be a risk factor for premature myocardial infarction (MI).

The current study showed that the T allele is significantly associated with CAD (OR: 12.21, 95% CI: 0.66 to 226.98). This finding is consistent with a previous report in which *MTHFR* C677T transition was found to be a risk factor for premature MI.[25, 27-30] On the other hand, other authors[30-32] reported insignificant differences for the T allele between MI subjects and the control group. Indian studies by SK Gupta *et al.*[33] and Ravi Kanth *et al.*[34] also showed no association of *MTHFR* polymorphism with CAD. In our study, the average homocysteine levels were significantly higher in the patient group than in controls. This is in agreement with observations by other investigators.[25,28,35]

The *MTHFR* C677T mutation is identified as a major determinant of homocysteine concentrations in Europeans, but is less prevalent and does not influence homocysteine concentrations in South Asians.[36] Each 5 µmol/L rise in homocysteine levels conferred ~9% increase in the risk of coronary heart disease (CHD) events, independent of other conventional CHD risk factors.[37] Various studies reported elevated levels of homocysteine with increased risk of CVD.[37-40] The level of plasma homocysteine depends on the combined effects of genetic and environmental factors.[41] *MTHFR* gene mutation (c.677 C>T; p.A222V) observed in our ethnic group is associated with Hyperhomocysteinemia which is a risk factor for CAD.

Cytokine-mediated inflammation accompanies atherosclerosis from its initiation to the occurrence of clinical endpoints.[42] In the present study, it has been found that the *IL-6* gene polymorphism is a risk factor of CAD susceptibility. Mutations in exon-4 of *IL-6* gene was observed in 13 (26%) patients. These polymorphisms may influence the expression and function of IL-6 protein and thus affect the susceptibility to cardiovascular diseases. This study showed a statistically significant higher levels of

hsCRP and fibrinogen levels in patients with *IL-6* gene mutations than in patients without mutations ($p<0.0001$ and $p=0.017$ respectively).

Elevated levels of serum hsCRP serve as a strong independent predictor of risk of MI, stroke, peripheral arterial diseases and cardiovascular mortality.[43] In 2002, Ridker *et al.*[44] reported the role of abnormal CR*P* values in the development of atherosclerotic CVD. Further in 2008, Ridker *et al.*[45] confirmed hsCRP as a strong, independent predictor of future heart disease. The Cardiovascular Health Study evaluated hsCRP levels in men and women aged 65 years or more without a history of vascular disease.[46] The study reported strong association between elevated levels of hsCRP with increased 10-year risk of CHD beyond traditional risk factors.

Guruprasad *et al.*[47] showed that elevated serum hs-CRP levels provide a useful marker for cardiovascular risk which, when combined with traditional risk factors, may help improve global risk prediction. There is evidence that hs-CRP, a leading inflammatory biomarker for clinical application, is independently associated with the risk of incidence or recurring cardiovascular events regardless of the lipid levels.[48]

Interleukin-6 induces the acute phase reactants such as fibrinogen, serum amyloid A, CRP and haptoglobin. Fibrinogen is produced by the liver on IL-6 induction. Fibrinogen is majorly involved in the blood coagulation cascade which results in thrombosis.[49] Plasma fibrinogen concentrations are recognized as an independent predictor of MI. It also binds to platelet glycoproteins, facilitating platelet aggregation.[50] In terms of atherogenesis, fibrinogen may act by binding to LDL and stimulating proliferation of vascular smooth muscle cells.[51-52] In addition to its role as a nonspecific marker of inflammation, fibrinogen may also have a direct role in atherogenesis and thrombogenesis by acting as a bridging molecule for many types of cell-cell adhesion events critical for atherogenesis.[53] It has been suggested that the association between fibrinogen haplotypes and MI is partly mediated through pleiotropic effects of the serum IL-6 concentration.[53]

Many studies assessed the relationship between *IL-6* gene polymorphisms and pathogenesis of CAD and reported conflicting results.[54] In present study, *IL-6* polymorphisms in exon-4 were studied which is not studied earlier by other researchers and found novel mutations which are significantly associated with elevated inflammatory markers in CAD patients.

ICAM-1 functions as a natural receptor for lymphocytes and is involved in the binding of leukocytes to the arterial endothelium which leads to the formation of strong plaques by the segregation of fat molecules and leukocytes on arterial walls.[14] Current study showed *ICAM-1* (exon-6) mutation i.e., c.1405 A>G in 18% (n=9) of the patients. *ICAM-1* 1405 A>G substitution determines change of Lysine to Glutamic acid at 469 position (K469E) in the fifth Ig-like domain. This domain is involved in binding to the LFA-1 ligand of *ICAM-1* which is expressed on leukocytes. This result suggests that the A>G genotype of the *ICAM-1* gene polymorphism in codon at position 469 (K>E) is a genetic risk factor that may determine an individual's susceptibility for CAD and MI.[55]

Genome wide association (GWA) investigation studied four novel loci and showed that *ICAM-1* (K469E) polymorphism determines the circulating concentration of sICAM-1[56] apart from G241R. In the current study, *ICAM-1* gene K469E polymorphism showed a significant association with CAD risk in our ethnic group (OR: 23.12, 95% CI: 1.31 to 409.15).

Nakashima *et al.* showed up-regulation of *ICAM-1* at atherosclerosis-prone sites and animal studies have shown a reduction in atherosclerosis in mice deficient in *ICAM-1*.[57] *ICAM-1* polymorphism (rs5498) has been suggested to have functional activity and affect mRNA splicing patterns that modify cell-cell interactions and influence inflammatory response.[58] In addition, this variant might have possible functional value in the etiology of atherosclerosis.[59] The present study showed that the mutation in exon-6 of *ICAM-1* gene is associated with an elevated levels of hsCRP in patients with CAD.

The present polymorphisms study of *MTHFR*, *IL-6* and *ICAM-1* genes helps us to better evaluate the prognosis of CAD in our ethnic patients. Thus future studies should focus on adopting reliable, cost effective and less time consuming methods to perform genotyping on large number of similar ethnic groups and thereby making it possible to establish the real effect of genotype for the benefit of the society.

### Limitations

1. Sample size is relatively small.
2. This is a single center study and the study population is from few districts of Andhra Pradesh state of India only. Hence multi-centric studies with diverse ethnic populations are needed to elucidate the impact of these genetic polymorphisms in the risk of CAD.

## CONCLUSION

Coronary artery disease a multifactorial arterial disease involves multiple genetic and environmental factors. Polymorphisms observed in *MTHFR*, *IL-6* and *ICAM-1* genes in South-Indian ethnic population which are associated with elevated levels of inflammatory markers – homocysteine, hsCRP and fibrinogen appear to be predisposing factors for atherosclerosis.

## ACKNOWLEDGEMENT

This research work was funded by Sri Balaji Aarogya Vara Prasadini (SBAVP) Scheme of Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati (*Grant No: SBAVP/Ph.D./05*).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**ACS:** Acute coronary syndromes; **bp:** base pairs; **CAD:** Coronary Artery Disease; **CHD:** Coronary heart disease; **CI:** Confidence intervals; **CVD:** Cardiovascular Disease; **DVD:** Double vessel disease; **ELISA:** Enzyme linked immunosorbent assay; **ExPASy:** Expert Protein Analysis System; **GWA:** Genome wide association; **hsCRP:** High-sensitivity C-reactive protein; **ICAM-1:** Intercellular Adhesion Molecule-1; **IEC:** Institutional ethics committee; **IL-6:** Interleukin-6; **LAD:** Left anterior descending artery; **LCX:** Left circumflex artery; **LP[a]:** Lipoprotein [a]; **MI:** Myocardial infarction; **MTHFR:** Methylenetetrahydrofolate reductase; **NCBI:** National Center for Biotechnology Information; **NSTEMI:** non ST-elevation myocardial infarction; **OR:** ODDs ratio; **PCR:** Polymerase chain reaction; **RCA:** Right coronary artery; **SAA:** Serum amyloid A; **SD:** Standard deviation; **SPSS:** Statistical Package for Social Sciences; **STEMI:** ST-elevation myocardial infarction; **SVD:** Single vessel disease; **TAE:** Tris-Acetate-EDTA; **TVD:** Triple vessel disease; **USA:** Unstable angina.

## SUMMARY

Genetic polymorphisms observed in MTHFR, IL-6 and ICAM-1 genes in South-Indian ethnic population which are associated with elevated levels of inflammatory markers – homocysteine, hsCRP and fibrinogen appear to be predisposing factors for atherosclerosis and further progression of CAD. Further studies with larger sample size including different ethnic populations are needed to elucidate the impact of these genetic polymorphisms in the risk of CAD.

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**Cite this article :** Latheef K, Rajasekhar D, Vanajakshamma V, Aparna BR, Chaudhury A, Sarma PVGK. Association of *MTHFR*, *IL-6* and *ICAM-1* gene Polymorphisms with Coronary Artery Disease in South-Indian Ethnic Subset: A Case-Control Study. J Cardiovasc Disease Res. 2018; 9(3):115-22.