Cardiology

KEYWORDS:
Polymorphism, Cytochrome P450 epoxygenase, Endothelial function and Acute coronary syndrome patients.

POLYMORPHISM OF CYTOCHROME P450 EPOXYGENASE AND ITS ASSOCIATION WITH ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH CORONARY ARTERY DISEASE

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Background: Cytochrome P450 (CYP) epoxygenase metabolise arachidonic acid (AA) into four epoxyeicosatrienoic acids (EETs) 5,6-EETs, 8,9 EETs, 11,12-EETs and 14,15-EETs. Since, EETs are unstable eicosanoids they rapidly get converted into dihydroxyeicosanoid trienoicacids (DHETs) by soluble hydrolase. These eicosanoids promote defence mechanism against inflammatory atherosclerosis process. However, 11,12-EETs are more potent eicosanoids in maintaining anti-atherosclerotic activity. Endothelial dysfunction is the key step in the pathogenesis of atherosclerosis. Polymorphism in CYP epoxygenase can alter individual's risk for events in coronary artery disease (CAD) patients. Therefore, we examined the impact of CYP epoxygenase polymorphism indirectly through evaluation of 11,12-DHET levels and its association with endothelial dysfunction.

Methods: It is a prospective case-control study consisting of 84 acute coronary syndrome (ACS) patients and 84 healthy controls of either gender aged above 18 years. Fasting serum lipid profile including total cholesterol (TC), high density lipid (HDL), triglycerides (TG) and homocysteine levels were measured in all subjects. We measured plasma 11,12-dihydroxyeicosatrienoic acid (11,12-DHET) as indicative of 11,12-EETs. Genotyping of CYP putative exons of CYP2C9, CYP2C19 and CYP2J2 epoxygenase were carried out by Polymerase Chain Reaction–Single Strand Conformation Polymorphism (PCR-SSCP) method. Sanger's sequencing chain termination method was carried out for SSCP positive samples. All the data obtained were analysed by using Ms-Excel, 2007 and SPSS, version 24.Software, IBM, USA.

Results: We observed significantly higher levels of homocysteine in CAD group (35.1 ± 13.8 µmol/L) indicating higher inflammatory condition in patients compared to control group (8.1 ± 2.9 µmol/L, $p < 0.001$). We also found higher 11,12-DHET levels in CAD group (628.6 ± 324.3 pg/mL) compared to healthy controls (332.1 pg/mL ± 203.2 pg/mL, $p = 0.0001$). In this connection, we observed positive correlation between homocysteine levels and 11,12- DHETs in CAD group ($p = 0.01$). Genotyping of CYP exons revealed 11 patients (13%) reporting 12 single nucleotide polymorphisms (SNPs). We found significant difference in the levels of 11,12- DHETs between the patients reporting CYP polymorphism and patients without CYP polymorphism compared with the control ($p<0.001$). Further, we observed negative correlation between homocysteine levels and 11,12-DHETs in CAD patients reporting CYP polymorphisms indicating decline of DHET mediated anti-atherosclerotic activity

Conclusions: Presence of lower levels of 11,12- DHETs is a reflection of poor reserve defence mechanism in CAD patients that might cause endothelial dysfunction and risk of cardiac events. Therefore, genotyping of CYP2C9, CYP2C19 and CYP2J2 genes can be recommended to be used as prognostic marker for risk stratification in CAD patients.

Introduction:

Despite the advancements in medical therapies for the past one decade, coronary artery disease (CAD) is still the leading cause of cardiovascular morbidity and mortality worldwide.1 CAD is a heterogenic, multi-factorial disease and varies with different ethnic populations. There is an exponential increase in the incidence of CAD in all age groups which may be attributed to genetic predisposition besides common risk factors.2 It is noteworthy that CAD patients are prone for cardiac events depending upon patient's individual risk. Most of the risk factors such as diabetes mellitus, hypertension, smoking and genetic defects for CAD are reported to act through atherosclerosis process.3 In human cardiovascular system, nitric oxide (NO) is a powerful vasodilator that prevents the vascular damage. However, when coronary endothelium is exposed to risk factors, NO production gets impaired.4,5 Endothelial dysfunction is the key process that precedes the development of CAD through atherosclerosis process. It can be characterised as an imbalance between the humoral and cellular factors that distract the structure and function of coronary wall.6 Therefore in order to maintain vascular homeostasis, coronary endothelium expresses Cytochrome P450 (CYP) that metabolises arachidonic acid (AA) to produce potent epoxyeicosatrienoic acids (EETs).These eicosanoids exert anti-inflammatory activity on vascular system and promote artery dilation, angiogenesis and protect ischemic myocardium.7 Most of the risk factors for cardiovascular disease such as hypertension, dyslipedemia, hyperglycemia, smoking and obesity can be controlled and treated through amelioration of endothelial function. A detailed review of Raja B S et al reveals the importance of novel risk factors and genetic predisposition that has further broadened our understanding of the pathogenesis of atherosclerosis.8 In this context, genetic factors have also been increasingly recognised as important contributors for risk stratification and patient prognostication. Thus, it is necessary to

study molecular mechanisms involved in genetic predisposition that may pave way for selecting optimal therapies and prevention of complications in CAD patients.9 Therefore, we sought to evaluate the effect of CYP polymorphism and its association with endothelial dysfunction in patients with coronary artery disease.

2. Methods:

2.1 Study population

It is a single center, prospective case-control study. Eighty four patients diagnosed with acute coronary syndromes, ST - elevation myocardial infarction (STEMI), Non - ST elevation myocardial infarction (NSTEMI) and unstable angina (UA) were recruited in to study from department of cardiology, Sri Venkateswara Institute of Medical Sciences, Tirupati, Andhra Pradesh, India and consequently 84 healthy volunteers without any reported cardiovascular risk factors were also included in the study.

2.2 Ethical Clearance

This study followed the ethical guidelines and it was approved by the Institutional ethical committee (IEC), EC Regn. No. ECR/488/Inst/AP/2013 with IEC approval no. 407. Oral and written informed consent was obtained from all the study participants.

2.3 Sampling

All the study participants were recruited between November 2015 and June 2016. Sampling was done on 2nd day of myocardial infarction (MI) from all the study patients. Similarly, sampling was carried out from healthy volunteers at 12 hours fasting state. Six millilitres (mL) of peripheral venous blood was collected from all the subjects and aliquated into separate sterile labelled vials for biochemical and genetic analysis. All the separated serum, plasma and whole blood samples were stored at -40°C until analysis.

2.4 Biochemical analysis

Fasting total cholesterol (TC), high density lipid-cholesterol (HDL-C), triglycerides (TG) and homocysteine were evaluated in all the study participants using appropriate commercially available kits on DXC600 Beckmann auto analyser.

2.5 Measurement of plasma 11,12-DHET levels

We measured plasma 11,12-DHET forms as a representative of 11,12-EETs (unstable). Anti-11,12-DHET competitive ELISA kit from Detroit R&D,Inc.,USA10 was used to quantify 11,12-DHETs of both the groups. Plasma samples were processed as per the manufacturer's instruction manual. One mL of plasma and 1mL ethyl acetate were mixed thoroughly and centrifuged at 2000 rpm for 10 min. The resultant upper organic phase were collected and allowed to evaporate and dry up at room temperature. Dried sample was dissolved in 2 mL of 20% potassium hydroxide (KOH) and incubated for 1 hour at 50° C. The pH was adjusted to approximately 5.5 with the help of formic acid. An equal volume of ethyl acetate was added to the mixture and centrifugation was repeated. The resultant organic phase was collected and dried up at room temperature. The dried pellet was reconstituted with 20 µL of ethyl alcohol for competitive enzyme assay.

2.6 Evaluation of CYP Polymorphism

Total genomic DNA of both the study group participants was extracted from ethylenediamine tetraacetic acid (EDTA) treated whole blood sample by using a standard phenol-chloroform extraction method.11 Genotyping was performed by polymerase chain reaction - single strand confirmation polymorphism (PCR-SSCP) technique. PCR amplification of three CYP exons, exon 3 of CYP2C9, exon 5 of CYP2C19 and exon 4 of CYP2J2 genes were carried out with the help of suitable primers designed by using primer3 online software tool (Table 1). PCR reaction mixture contained a final volume of 50µl, and consisted of 100µmol concentration of each primer, 100µmol of dNTPs mix, 10mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, 1U of Taq DNA Polymerase and 0.50µg of genomic DNA. The parameters for amplification included an initial denaturation for approximately 10 minutes at 94°C, denaturation at 94°C for 60 seconds, 30 seconds of corresponding annealing at 64.4°C, 45.5°C and 61.4°C respectively and 50 seconds of amplification at 72°C and this was followed by a final extension step for 5 minutes at 72°C in a master cycler gradient thermo cycler. Amplified PCR products were subjected to SSCP analysis using 6% polyacrylamide vertical gel electrophoresis. Further, Sanger's di-deoxy sequencing method was carried out for the samples that showed mobility difference compared with control in SSCP analysis.

2.7 Statistical analysis

All the data obtained were tabulated in Microsoft (Ms) Excel spreadsheets. Descriptive statistics including mean ± standard deviation (SD) for continuous variables and percentages for categorical variables were calculated. Unpaired student's t-test and ANOVA followed by multiple comparison tests for continuous data were performed for the data following normal distribution. Pearson's correlation was performed to assess the correlation between the variables. For all the analyses, values of $p \leq 0.05$ were considered to be statistically significant. All statistical analysis was performed using Ms-Excel 2007 and SPSS 24.0 (IBM Corporation, Chicago, IL, USA).

Results:

Male gender was dominant in both the groups and accounted for 77% and 69% respectively. Mean age of the CAD group was 51.2 ± 9.3 years and 42.1 ± 8.1 years in control group. Hypertension co-morbidity was more prevalent in 38 (45%) followed by smoking 37 (44%) and diabetes mellitus 37 (44%) in our CAD group. Majority of the patients 70(83%) presented with STEMI and single vessel disease (SVD) was predominant type of lesion in 49(58%) patients. Fasting lipid profile including total cholesterol, very low density lipid-cholesterol (VLDL-C) and triglycerides levels were higher in CAD group compared to control group (Table 2). We observed significantly higher levels of homocysteine in CAD group 35.1 ± 13.8 µmol/L compared to control group 8.1 ± 2.9 µmol/L, ($p < 0.001$) (Figure 1). In our study group, 73 (87%) of 84 CAD patients and 2 (3%) of 84 control individuals were found to report hyperhomocysteinemia. We found significantly higher levels of CYP derived 11,12-DHETs in CAD group 628.6 ± 324.3 pg/mL compared to healthy controls 331.2 ± 203.2 pg/mL, (p=0.0001) (Table 3).We also observed positive correlation between inflammatory marker homocysteine and vasoactive 11,12- DHET levels in CAD group ($R2 = 0.087$, p=0.01), (Figure 2).

PCR amplification of exon 3 of CYP2C9, exon 5 of CYP2C19 and exon 4 of CYP2J2 genes resulted in amplicon sizes of 308 base pair (bp), 287 bp and 157 bp respectively in both the groups. SSCP analysis of patient amplicons showed mobility differences in 11 patients compared with that of control amplicons. Further, Sanger's sequencing analysis revealed 12 SNPs in 11(13%) patients constituting base substitutions and base insertions. One patient, Case 58 was found to report both CYP2C9 gene novel mutations and CYP2C19*2 allele.

CYP2C9 gene base substitutions were found in 3 patients of which one patient was found to report CYP2C9*2 allele, c.430C>T (Case 47, Figure 3-A) and novel base substitutions in other two patients (Case 58 and Case 73). CYP2C19 gene SNP base substitutions were found in 5 patients of which 3 patients were found to report CYP2C19*2, c.681G>A (Figure 3-B) and two patients reported novel base substitutions (Case 5 and Case 10). CYP2J2 gene polymorphism constituting novel base substitutions (Figure 3-C) were found in 4 patients and with Cytosine base insertions in Case 31. In this connection, we also noticed comparatively reduced levels of 11,12-DHETs in the patients reporting CYP gene polymorphisms (Table 4). All the identified mutations of exon 3 of CYP2C9, exon 5 of CYP2C19 and exon 5 of CYP2J2 genes were communicated to GenBank and their corresponding accession numbers were depicted in Table 4. Further, data analysis revealed significant differences in the levels of 11,12-DHETs between patients reporting CYP polymorphism and patients without CYP polymorphism compared with the control individuals ($p < 0.001$),(Figure 4). We also observed negative

correlation between homocysteine and 11,12-DHETs levels (R2 = 0.222, p = 0.143) with the patients reporting CYP polymorphism (Figure 5). However, we did not find any association between diabetic and hypertensive CAD patients and 11,12-DHETs levels.

Discussion:

There is an increasing appreciation of the importance of EET/DHET mediated vasodilation.12,13 CYP2C9, CYP2C19 and CYP2J2 are the main epoxygenases in human cardiovascular system particularly found in cardiomyocytes, endothelium and smooth muscle cells (SMCs).14 CYP derived DHETs promote protective defence activity against the inflammatory stimulus and maintain cardiovascular homeostasis. Inter-individual differential expression of regulatory enzymes can influence the function and risk of developing disease.15 Thus, differential gene expression of CYP epoxygenase can lead to functional alterations of epoxygenase activity that may increase the individual's risk. In our CAD group, we observed hypertension, smoking and diabetes mellitus co-morbidities were more prevalent that can increase the risk of developing events. Hyperhomocysteinemia is also well established independent novel risk factor associated with early onset of CAD and risk of venous thrombosis.16,17 Jatin D P et al reported age wise hyperhomocysteinemia in 78-82% of CAD patients and only 5% in controls.18 Consistently, we also found higher levels of homocysteine in 87% of CAD patients and only 3% in control individuals. Higher levels of homocysteine can cause endothelial damage and increase the risk of developing events in CAD patients. Akasaka T et al in 2016 reported that higher levels of EETs/DHETs in CAD patients represent the protective defence mechanism against the inflammatory endothelial damage.19 In this connection, we also observed significantly higher levels of 11,12-DHETs in response to the higher inflammatory stimuli in CAD group compared to control group. Interestingly, we noticed significant positive correlation between homocysteine levels and 11,12-DHETs in CAD patients depicting the protective anti-inflammatory activity of DHETs against higher homocysteine levels in CAD group. Similar observations were found with the study of Yang T et al in 2013 who reported higher levels of DHETs corresponding to higher levels of high sensitive - C reactive protein (hs-CRP) and blood lipoproteins in CAD patients.10 However, we did not find any correlation between 11,12-DHET levels and other risk factors like hypertension, diabetes and blood lipoproteins.

It is note worthy that about 20% of the CAD patients report with low or no prevalence of traditional risk factors. Genetic polymorphism in CYP epoxygenases can alter the epoxygenase activity and result in poor defence mechanism that may add on risk of developing events in CAD patients. A study by Arun kumar A S et al., (2015) also suggests that the individuals with any confounding risk factors for CVD along with CYP epoxygenase polymorphism may be predisposed to risk of CAD.[20]

CYP2C9 is an important epoxygenase in endothelial cells that contributes higher 11,12-EET/DHET mediated vascular homeostasis.21 A study by Crespi CL and Miller VP in 1997 also reported that presence of CYP2C9*2 altered the interaction of epoxygenase with substrate and reduced metabolism.22 Any gene variation in this epoxygenase results in reduced DHET levels and presence of CYP2C9*2 in exon 3 was reported to show 50% reduced activity compared to the wild type.23 Consistently, we observed reduced levels of 11,12 DHETs in the patients reporting CYP2C9 polymorphism depicted in table 4. Harison M et al in 2008 reported two allele variants of CYP2C9*2 and CYP2C9*3 as having clinical significance and presence of these alleles showed decreased enzyme activity.24 In our study CYP2C9*2 allele (c.430C>T, R144C) was found only in one patient, case 47 and showed reduced levels of 11,12-DHETs,331.2 pg/mL representing poor epoxygenase activity. Presence of CYP2C9*2 in one patient in our CAD group accounted for 1.2% which is less in frequency compared to study reporting 4% by Jose R et al in 2004.[25]

CYP2C19 is also an important epoxygenase that mainly contributes 11,12-EET/DHET forms. CYP2C19*2 allele is extensively studied mutation in CAD patients and has attained clinical importance especially in acute coronary syndrome patients.12,26 Hokimoto S et al in 2015 revealed that presence of CYP2C19*2 polymorphism is an independent risk factor for cardiovascular events irrespective of clopidogrel resistance.27 In CAD group, we found CYP2C19*2 allele, c.681G>A, p.G228R in 3 of 84 patients (case 8, Case 58 & case 68) showing reduced levels of 11,12- DHETs (Table 4). Akasava T et al. in 2016 also reported that patients with CYP2C19*2 allele showed reduced levels of plasma 11,12- DHETs compared to the patients without the mutant allele.19 In our study CYP2C19*2 allele accounted for 3.6% and it was comparatively low with the studies reporting 12% and 10% by Shuldiner AR et al 28 and Tantray JA et al 29 respectively in Indian population.

CYP2J2 is cardiac specific epoxygenase expressed predominately in vascular endothelial cells and presence of mutant CYP2J2*4 allele in exon 4 was reported to express reduced levels of 11,12 DHETs compared to the wild type. But, we found none to report CYP2J2*4 allele in our CAD group and it was consistent with Xu M et al study reporting that Indians are rare (0-2%) to CYP2J2*4 allele.30 However, we found 4 of 84 patients (case 23, case 40, case 31 and case 73) reporting novel mutations in exon 4 of CYP2J2 gene constituting base substitutions and cytosine base insertions respectively (Table 4). We found novel cytosine base insertions, c.664_665ins C and c.673_674ins C in 2 of 84 patients (case 31 and case 73) respectively that resulted in frame shift mutation. A study by Indrayan A in 2013 reported that any gene variation in this epoxygenases can influence their activity that can act as important modifiers of cardiovascular risk in CAD patients.31 Consistently, we found that due to presence of frame shift mutation and enzyme deformativity of CYP2J2 gene in case 31 and case 73, reduced levels of 11,12-DHETs were observed depicting poor enzyme activity (Table 4).

Further, our analysis revealed the relationship of CYP polymorphism and 11,12-DHETs in patients reporting polymorphic genes. Interestingly, we observed negative correlation between 11,12-DHETs and homocysteine levels in 11 patients reporting CY2C9, CYP2C19 and CYP2J2 polymorphisms depicting reduced enzyme activity. Thus, our study findings showed that presence of CYP polymorphisms result in reduced levels of 11,12-DHETs and decline of DHET mediated vasodilation that can cause endothelial dysfunction and risk of events.

Conclusions:

Polymorphism in CYP2C9, CYP2C19 and CYP2J2 genes seems to have considerable effect on epoxygenase activity that may result in decreased levels of 11,12- DHETs in patients compared to the patients without CYP polymorphism. Presence of lower levels of 11,12- DHETs is a reflection of poor reserve defence mechanism in CAD patients that might result in endothelial dysfunction and risk of cardiac events.

Therefore, in acute coronary syndrome patients genotyping of CYP2C9, CYP2C19 and CYP2J2 genes can be recommended to use as prognostic markers for future events and risk stratification.

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Tables and Figures

Table 1 CYP genes and their primer sequences

CYP2C9	FP: TGCCTGTTTCAGCATCTGTCT RP: TCTCAACTCCTCCACAAGGC
CYP2C19	FP: AGAGCTTGGCATATTGTATC RP:CGCAAGCAGTCACATAACTAAGC
CYP2J2	FP: GCCTTTTGACCCTCATTTCA RP: CTGGCATGTCTTTGAAGCCT

FP: Forward primer, RP: Reverse primer

Table 2 Demographic and biochemical parameters of two groups.

Characterstics	CAD group (n=84)	Healthy group (n=84)	p value
Age , Mean ± SD years	51.2 ± 9.3	42.1 ± 8.1	P ≤ 0.05
Gender (Male: Female)	65:19	58:26	NA
Obesity, n (%)	10(11.9%)	0	NA
Smokers, n(%)	37(44.0%)	0	NA
Hypertension, n (%)	38(45.2%)	0	NA
Diabetes Mellitus, n (%)	37(44.0%)	0	NA
Family history, n (%)	14(16.7%)	0	NA
Single vessel disease n(%)	49(58%)	0	NA
Double vessel disease n(%)	19(23%)	0	NA
Triple vessel disease n(%)	12(14%)	0	NA
Total Cholesterol (mg/dL)	182.3 ± 49.2	160.6 ± 31.7	0.003s
Triglycerides (mg/dL)	185.7 ± 163.6	117.4 ± 51.3	0.001s
High density lipid Cholesterol (mg/dL)	38.9 ± 8.3	40.1 ± 9.5	0.26
Low density lipid - Cholesterol (mg/dL)	110.4 ± 50.1	97.0 ± 27.6	0.10
Very low density lipid Cholesterol (mg/dL)	37.1 ± 32.8	23.4 ± 10.3	0.001s

CAD: Coronary artery disease SD: Standard deviation, NA: Not applicable. s: significant

Table 3 Homocysteine and 11,12-DHETs between the two groups.

Variables	Cases Mean ± SD	Control Mean ± SD	p value
Homocysteine µmol/L	35.1± 13.8	8.1± 2.9	<0.001s
11,12-DHETs pg/ml	628.5± 322.3	332.1± 203.2	0.0001s

DHETs: Dihydroxyeicosatrienoic acids, s: significant

Table 4. Details of SNPs in CYP2C9 , CYP2C19 and CYP2J2 genes.

S.No	Gene	Case Id	11,12- DHET pg/mL	Base position	Novelty	GenBank accession no.
1		Case 47	331.2	c.430C>T	Reported	KY933642
				c.347A>G	Novel	
				c.357A>G	Novel	
				c.369C>A	Novel	
2	CYP2C9	Case 58	459.2	c.385A>G	Novel	MF043186
				c.347A>G	Novel	
				c.362A>G	Novel	
				c.363G>A	Novel	
3		Case 71	436.4	c.366G>T	Novel	MF043187
4.		Case 8	229.2	c.681A>G	Reported	KY823013
5.		Case 58	459.2	c.681A>G	Reported	KY823015
6.	CYP2C19	Case 68	332.4	c.681A>G	Reported	KY823012
7.		Case 5	971.6	c. 650A>T	Novel	KY823014
8.		Case 10	393.6	c. 650A>T	Novel	KY946734
9		Case 23	530	c. 646G>A	Novel	KY933638
10		Case 40	778	c. 646G>A	Novel	KY933639
	CYP2J2		247.2	c.630G>C,		
11		Case 31		c.664_665ins C	Novel	KY933640
12		Case 73	446.8	c.673_674ins C	Novel	KY933641

c. - coding gene

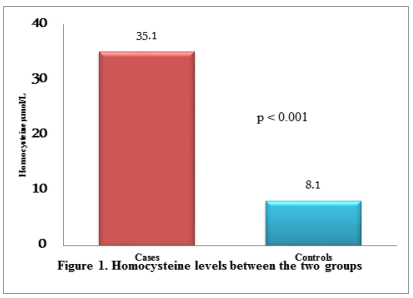


Figure 1. Homocysteine levels between the two groups

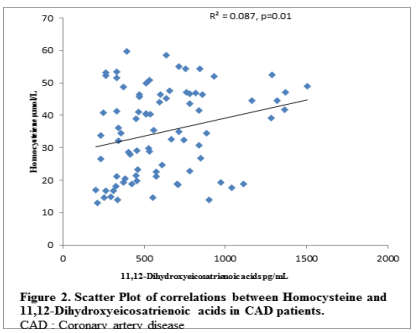
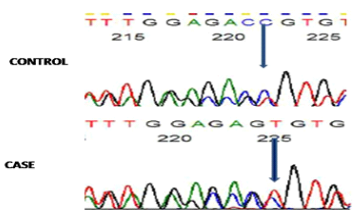


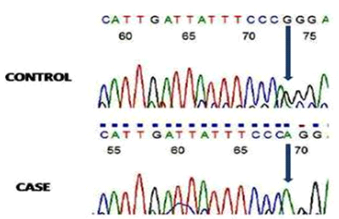
Figure 2. Scatter Plot of correlations between Homocysteine and 11,12-Dihydroxyeicosatrienoic acids in CAD patients.
CAD - Coronary artery disease

Figure: 3 Sequencing Chromatograms

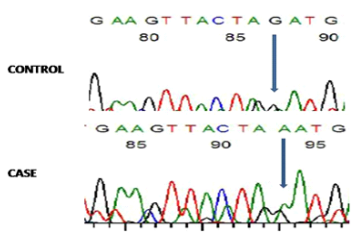
3-A. Arrow mark indicating CYP2C9*2, c.430C>T



3-B. Arrow indicating CYP2C19*2, c.681G>A



3-C. Arrow indicating c.646G>A novel mutation in CYP2J2



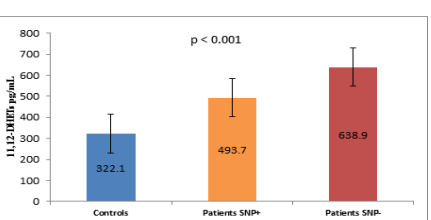


Figure 4. 11,12- DHETs levels between the groups.
DHETs Dihydroxyeicosatrienoic acids;
SNP+ : single nucleotide polymorphism present;
SNP- : single nucleotide polymorphism absent

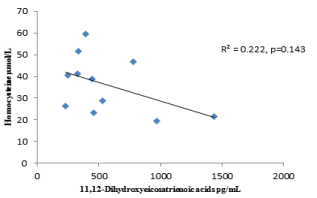


Figure 5. Scatter Plot of correlations between Homocysteiene and 11,12-Dihydroxyeicosatrienoic acids in CAD patients reporting CYP polymorphism.
CAD: Coronary artery disease ; CYP: Cytochrome P450

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