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Effect of Coronary Artery Disease risk SNPs on serum cytokine levels and cytokine imbalance in Premature Coronary Artery Disease

Wafa M. Ansari[a,*], Steve E. Humphries[b], Abdul K. Naveed[c], Omer J. Khan[d], Dilshad A. Khan[e], Ejaz Hassan Khattak[f]

[a] Army Medical College, National University of Science and Technology, (NUST), Islamabad, Pakistan
[b] Cardiovascular Genetics Institute, University College London, UK
[c] Riphah International University, Rawalpindi, Pakistan
[d] Azad Jammu & Kashmir Medical College, Muzaffarabad, Pakistan
[e] National University of Medical Sciences (NUMS), Rawalpindi, Pakistan
[f] Khyber Medical College, Peshawar, Pakistan

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ABSTRACT

Background: Premature Coronary Artery Disease (PCAD) occurs almost a decade earlier in the South Asian population as compared to the West. Inclusion of genetic information can prove to be a robust measure to improve early risk prediction of PCAD. Aim was to estimate the genotypic distribution and risk allele frequencies of 13 Coronary Artery Disease (CAD) risk Single Nucleotide Polymorphisms in loci identified by the CARDIoGRAMplusC4D consortium namely *MIA3 rs17465637*; *9p21 rs10757274*; *CXCL12 rs1746048*; *APOA5 rs662799*; *APOB rs1042031*; *LPA rs3798220*; *LPA 10455872*; *MRAS rs9818870*; *LPL rs328*; *SORT1 rs646776*; *PCSK9 rs11591147*; *APOE rs429358*; *APOE rs7412* in Pakistani PCAD patients and controls. Moreover, the differential serum cytokine levels *(IL-18, IL-10, IL-6, TNF-alpha, IL-18:IL-10 & TNF-alpha:IL-10 ratios)* with respect to the genotypic distribution of these selected SNPs were determined.

Material and methods: The case-control study was carried out in National University of Sciences and Technology, Islamabad in collaboration with the Cardiovascular Genetics Institute, University College London, UK. Subjects (n = 340) with > 70% stenosis in at least a single major coronary artery on angiography were taken as PCAD cases along with 310 angiographically verified controls. ELISA was performed for measuring the concentrations of serum IL18, TNFA, IL6 and IL10. Genotyping was done using TAQMAN and KASPar assays.

Results: The risk allele frequencies (RAF) of *APOE rs7412, CXCL12 rs1746048, 9p21 rs10757274, MIA3 rs17465637* and *SORT1 rs646776* were significantly higher in the PCAD cases as compared to the controls. *APOE rs429358* had the greatest influence among the selected GWAS/CARDIoGRAMplusC4D consortium CAD risk SNPs by significantly altering the serum levels of TNF-alpha, IL-10 and TNF-alpha:IL-10 ratio. It was followed by *APOE rs7412* and *CXCL12 rs1746048* which significantly altered the serum levels of IL-18; TNF-alpha and IL-18; IL-18:IL-10 ratio respectively. The cytokine imbalance denoted by IL-18:IL-10 was significantly higher in the risk allele carriers *MIA3 rs17465637* and *CXCL12 rs1746048* while TNF-alpha:IL-10 ratio was significantly raised in the risk allele carriers of *APOE rs429358*; *MRAS rs9818870* and *LPL rs328*.

Conclusion: The association of the selected SNPs with differential serum cytokine levels especially the cytokine imbalance points towards their potential causal role in the immune inflammatory pathogenic pathway of PCAD.

1. Introduction

Coronary Artery disease (CAD) is the commonest cause of death world-wide [1]. This increase can be attributed to several dietary and environmental factors combined with a greater susceptibility to metabolic syndrome [2]. It has also been observed that it is the younger individuals who are developing this disease more frequently compared to the older ones [19]. Early detection and treatment of young individuals can lead to age specific, timely interventions. In order to achieve this milestone those individuals who are at the greatest risk should be promptly and accurately identified. Genetic testing is becoming increasingly popular because it is relatively inexpensive, needs to be performed only once and the genetic risk can be assessed well before the disease process has set in in the form of abnormalities in the

* Corresponding author at: Department of Chemical Pathology, Army Medical College, National University of Science and Technology (NUST), Islamabad, Pakistan.
E-mail address: wafamuniransari@gmail.com (W.M. Ansari).

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biochemical parameters [12]. Genome–wide association studies (GWAS) and CARDIoGRAMplusC4D consortium have completely revolutionized the concepts regarding the pathogenesis of CAD by highlighting the role of genomics in risk assessment of CAD. A number of genetic loci conferring the risk of CAD have been identified with 53 loci being validated [13]. Still however, these loci have only managed to demonstrate very little genetic variance involved in the pathogenesis of Coronary Heart Disease [6]. So 90% of the factors contributing towards CAD hereditability need to be unraveled. Moreover, due to various linkage disequilibrium patterns in different ethnic groups the need arises to see the allelic distribution of these genetic variants on CAD risk [21].

There is hardly any insight regarding the effect of the CAD risk SNPs identified in the Genome Wide Association Studies and the CARDIoGRAMplusC4D on the serum levels of inflammatory cytokines in Premature Coronary Artery Disease. This interplay between biological pathways (lipid metabolism, Immune regulation, cellular stress response) and human genetics can unravel novel biological knowledge in the field of integrative genomics giving an insight to the pathogenesis of PCAD [18].

Hence, the aim of this study was to compare the pro- and anti-inflammatory cytokine levels between PCAD patients and disease free controls. Further we aimed to investigate the effect of 13 selected CARDIoGRAMplusC4D consortium gene variants on these serum cytokine levels and the pro- & anti-inflammatory cytokine imbalance in Premature Coronary Artery Disease.

2. Material and methods

The case-control study was conducted in the Chemical Pathology Department, Army Medical College, National University of Sciences and Technology (NUST) Rawalpindi, Pakistan, in collaboration with Cardiovascular Genetics (CVG) Institute, University College London (UCL), London, United Kingdom (UK). Study was approved by the Institutional Ethical review committees of both the institutions. A total of 650 subjects aged ≤45 years who were stable and were due to undergo coronary angiography were consecutively recruited from Armed Forces Institute of Cardiology (AFIC) Rawalpindi. The participants had a recent history (< 2 months) of an episode of left sided chest pain and were advised angiography by consultant cardiologists after evaluation. All participants were citizens of Pakistan. Sampling technique employed was non-probability convenience sampling. 340 subjects who displayed > 70% stenosis in one coronary artery on angiography were categorized as Premature Coronary Artery Disease patients. Those patients who had a history of angina, previous MI, infectious, autoimmune diseases, hyperlipidemia (familial), heart disease by birth, arthritis, diabetes mellitus, expectance of life < 12 months along with those not giving informed or written consent were not included in the study. Moreover, 310 age and sex matched controls who were declared to be disease free on angiography were recruited. Any subject with acute or chronic ailment or taking anti-inflammatory drugs was not included in the control group. Expectant mothers or females taking contraceptives were excluded as well. General physical examination including detailed history and physical examination was done by a registered medical practitioner.

Ten ml blood sample was taken from the median cubital vein. Six ml blood was poured into a plain vacutainer tube to separate the serum. Serum total cholesterol levels by cholesterol oxidase method using Cholesterol-LQ kit (Pioneer Diagnostics) while GPO-POD colorimetric method was used to estimate serum triglyceride by TG kit by Linear Chemicals (Spain) on Selectra – E Chemistry Analyzer. Serum HDL was measured by direct enzymatic colorimeteric method for quantifying cholesterol in high density lipoprotein with HDL- Cholesterol kit (Linear Chemicals (Spain) on Selectra E Chemistry Analyzer. Serum LDL was measured by Friedewald formula. Four ml whole blood was poured into EDTA tube to extract genomic DNA using Gentra Puregene Blood kit (Qiagen, USA). Serum IL18, TNFA, IL6 and IL10 levels were measured using monoclonal antibodies on ELISA (Invitrogen). The CAD risk SNPs namely *MIA3 rs17465637*; *9p21 rs10757274*; *CXCL12 rs1746048*; *APOA5 rs662799*; *APOB rs1042031*; *LPA rs3798220*; *LPA 10455872*; *MRAS rs9818870*; *LPL rs328*; *SORT1 rs646776*; *PCSK9 rs11591147*; *APOE rs429358*; *APOE rs7412* were genotyped using TAQMAN and KASPar assays. Standard protocols and preformed primers were used and technique was selected depending on the efficacy and availability of respective assays. The reagent volumes for TAQMAN genotyping assays, per plate using TAQMAN buffer are shown in supplementary Table-1(a) while those for KASPar genotyping assay are shown in supplementary Table-1(b).

The PCR conditions for TAQMAN genotyping assays are summarized in supplementary Table-2(a) while those for KASPar genotyping assays are summarized in supplementary Table-2(b) and Table-2(c).

Sequencing was done to confirm the genotype of certain samples which were not tightly clustered on the allelic discrimination plot (TAQMAN/KASPar) to ascertain any rare variants in LPA rs3789220, LPA rs10455872 and PCSK9 rs11591147 which have low minor allele frequency. To do this, firstly the region surrounding the SNP was amplified by PCR. The reaction components are shown in supplementary Table-3(a) and the PCR conditions in supplementary Table-3(b). The sequences for the primers are shown in Supplementary Table-3(c). Following PCR the purified PCR products were sent for sequencing to either source Bioscience/Eurofins.

The CAD risk SNPs were selected after the most recent publication from the CARDIoGRAMplusC4D consortium [10] was studied in detail. 50 SNPs from 45 loci were identified by the Consortium to be associated with CAD. These SNPs were developed as a CAD risk panel in 2010 (Cardiac Risk Prediction Array-Randox Laboratories Ltd, Crumlin, Co Antrim, UK) as part of a 19-SNP coronary heart disease gene score profile [3]. This gene score profile has been validated as having clinical utility in UK subjects [2] and or African origin [17].

These SNPs were further searched for in CARDIoGRAMplusC4D data and finally 13 CAD SNPs in loci meeting the genome wide significance threshold ($p < 5 \times 10^{-8}$) were selected [2]. Details of SNPs selected for the study along with the respective references are shown in supplementary Table 4.

Data analysis was done using standard SPSS software version-21 (SPSS Inc, Chicago, Illinois, USA) and R v3.0.3. The allelic frequencies of all SNPs were checked for Hardy-Weinberg equilibrium (HWE) equation. Kolmogorov-Smirnov test of uniformity was applied on the data to assess its distribution. Mean ± SD was calculated for continuous normally distributed (Gaussian distribution) variables. Continuous variables were compared amongst PCAD cases and controls using Independent t-tests. Categorical variables between PCAD cases and controls were compared using chi-square (x2-tests). Association between the gene polymorphisms and the serum cytokine levels were assessed by ANOVA and Post-hoc Tukey's test. Odds ratios were calculated using multi-variate logistic regression analysis after adjusting for confounding variables. A two-tailed p value < 0.05 was taken as significant.

3. Results

The patients of PCAD had mean ± SD age of 42 ± 3.80 years comprising 329 males and 11 females. Demographic features are given in Table 1. Body Mass Index (BMI), body weight, systolic & diastolic blood pressure was significantly higher in PCAD patients compared to controls ($p < 0.05$). The patients were mostly smokers with a positive family history of PCAD and hypertension ($p < 0.01$). Serum pro-inflammatory cytokines were significantly higher in the cases as compared to the controls (Table 2). The genotypic distribution of the 13 CAD SNPs along with their odds ratios and risk allele frequencies are shown in Tables 3 & 4 respectively. The results of sequencing done to confirm the genotypes of samples with data points outside the clusters

Table 1
Baseline characteristics (Mean ± SD) of PCAD cases and controls (n = 650).

Parameters	PCAD patients n = 340	Controls n = 310	p-Value
Age (y)	42 ± 3.80	39 ± 7.8	0.12
Sex (m/f)	329/11	298/12	0.66
Weight (kg)	76.5 ± 12.7*	69.0 ± 11.8	0.303
Height (m)	1.7 ± 0.12	1.68 ± 0.06	0.301
Ethnicity			
Punjabis n (%)	250 (74%)	193 (62%)	0.22
Pathans n (%)	90 (26%)	117 (38%)	
BMI (kg/m^2)	26.6 ± 6.7*	24.1 ± 4.03	0.175
Diastolic BP (mm Hg)	83.0 ± 9.6**	73.1 ± 3.8	0.0001
Systolic BP (mm of Hg)	124.7 ± 11.0**	112.0 ± 5.1	0.0001
Smokers n (%)	197 (58%)**	81 (26%)	< 0.01
HTN self n (%)	163 (48%)**	34 (11%)	< 0.01
Family history HTN n (%)	136 (40%)*	37 (12%)	< 0.05
Family history PCAD n (%)	112 (33%)*	28(9%)	< 0.05
Family history DM n (%)	78 (23%)*	25 (8%)	< 0.05
Family history IHD n (%)	136 (40%)**	31(10%)	< 0.01
Total Cholesterol (mmol/l)	4.47 ± 0.87*	4.2 ± 0.77	0.019
Triglycerides (mmol/l)	2.4 ± 1.15**	1.9 ± 0.73	0.0001
LDL (mmol/l)	2.32 ± 0.77*	2.12 ± 0.75	0.043
HDL (mmol/l)	1.07 ± 0.23**	1.24 ± 0.25	0.0001
VLDL (mmol/l)	1.07 ± 0.52**	0.87 ± 0.33	0.001

PCAD: Premature Coronary Artery Disease; DM: Diabetes Mellitus; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; SD = Standard Deviation; CAD: Coronary Artery Disease; VLDL: Very Low Density Lipoprotein.
Categorical variables were compared using a χ^2 test while continuous variables were compared using independent t-tests.
** $p < 0.01$.
* $p < 0.05$.

especially in case of monomorphic genes *LPA rs3789220*, *LPA rs10455872 and PCSK9 rs11591147* are shown in supplementary Figs. 1-(a) (b) and (c) respectively.

APOE rs7412 had the highest odds ratio and 95% CI for PCAD among the 13 CAD SNPs.

The 13 CAD SNPs complied with Hardy-Weinberg Equilibrium. The risk allele frequencies of *MIA3 rs17465637*; *APOE rs7412*; *CXCL12 rs1746048*; *9p21 rs1042031* and *SORT1 rs646776* were significantly greater in the patients compared to the controls ($p < 0.05$).

Serum IL-18/IL10 ratio was significantly higher in the MIA3 ra17465637 CC genotype patients as compared to CA and AA genotypes ($p < 0.05$). Comparison of serum IL-18/IL-10 ratio with respect to the genotypic distribution of *MIA3 rs17465637* SNP is shown in Fig. 1a. Serum IL-18 and TNF-alpha levels were significantly higher in the CT heterozygote PCAD patients bearing *APOE rs7412* SNP as compared to TT and CC genotypes ($p < 0.01$). The comparison of serum IL-18 and TNF-alpha levels with respect to the genotypic distribution of *APOE rs7412* SNP is shown in Fig. 1b. There was no significant difference in the serum IL-6, IL-10, IL-18:IL-10 and TNF-alpha:IL-10 ratios with respect to the genotypic distribution of *APOE rs7412* ($p < 0.05$).

Serum TNF-alpha and TNF-alpha:IL-10 ratio was significantly raised in the homozygous C-allele carriers of the *APOE rs429358* SNP as compared to CT and TT genotypes ($p < 0.05$). On the contrary serum IL-10 was significantly lower in these patients ($p < 0.01$). Comparison of serum TNF-alpha, TNF-alpha:IL-10 ratio and IL-10 with respect to the genotypic distribution of *APOE rs429358* SNP is shown in Fig. 1c. Serum IL-18, IL-6, TNF-alpha, IL-10 and IL-18:IL-10 ratio did not differ in the risk allele carriers of *MRAS rs9818870* SNP ($p > 0.05$) however the TNF-alpha:IL-10 ratio was significantly higher in the TT genotype patients as compared to CT and CC genotypes ($p < 0.05$). Comparison of serum TNF-alpha:IL-10 ratio with respect to the genotypic distribution of *MRAS rs9818870* SNP is shown in Fig. 1d.

Serum IL-18 and IL-18:IL-10 ratio were significantly higher in the *CXCL12 rs1746048* CC genotype patients as compared to CT and CC genotypes ($p < 0.05$). The comparison of serum IL-18 and IL-18:IL-10 ratio with respect to the genotypic distribution of *CXCL12 rs1746048* SNP is shown in Fig. 1e. Serum IL-18 was significantly higher in the GA heterozygotes of *9p21 rs10757274* as compared to GG and AA genotypes ($p < 0.05$). However, no significant difference was seen in the serum levels of IL-6, IL-10, TNF-alpha or the pro-inflammatory/anti-inflammatory ratios with respect to the genotypic distribution of *9p21 rs10757274* SNP. The comparison of serum IL-18 level keeping in view the genotypic distribution of *9p21 rs10757274* SNP is shown in Fig. 1f.

Serum TNF-alpha was significantly higher in the GG genotype patients of *LPA rs10455872* SNP as compared to GT and TT genotypes ($p = 0.01$). However, no significant difference was seen in the serum levels of IL-6, IL-10, IL-18 or the pro-inflammatory/anti-inflammatory ratios with respect to the genotypic distribution of *LPA rs10455872* SNP. The comparison of serum TNF-alpha level keeping in view the genotypic distribution of *LPA rs10455872* SNP is shown in Fig. 1g The GG genotype patients of *PCSK9 rs11591147 SNP* had higher serum IL-18 levels as compared to GT and TT genotypes ($p < 0.05$) shown in Fig. 1h while the TNF-alpha:IL-10 ratio was significantly higher in the CC genotype patients with *LPL rs328* SNP as compared to CG and GG genotypes shown in Fig. 1i. *APOE SNP rs429358* had the greatest influence among the selected GWAS/CARDIoGRAMplusC4D consortium CAD risk SNPs by significantly altering the serum levels of TNF-alpha, IL-10 and TNF-alpha:IL-10 ratio followed by *APOE rs7412* and *CXCL12 rs1746048* which significantly altered the serum levels of IL-18; TNF-alpha and IL-18; IL-18:IL-10 ratio respectively. The serum cytokine levels did not vary significantly with respect to the genotypic distribution of the selected 13 CAD risk gene variants in disease free controls though the serum cytokine levels showed a trend towards rise in the risk allele carriers but did not reach statistical significance (see Table 5).

In order to further assess the role of cytokines in the prediction of premature atherosclerosis multivariate logistic regression analysis was performed after adjustment for age, sex, smoking, HTN, lipids and BMI. This revealed that IL-18, TNF-alpha, IL-6 and TNF-alpha/IL-10 still remained significant in the prediction of atherosclerosis as shown in Supplementary Table 5.

Table 2
Comparison of cytokine levels in PCAD patients and controls.

Variable	PCAD Patients (n = 340) Mean ± SD	Controls (n = 310) Mean ± SD	p-Value
IL-18 (pg/ml)	263.6 ± 42.5**	175.6 ± 21.8	0.0001
TNF-alpha (pg/ml)	6.9 ± 1.4**	3.24 ± 1.13	0.0001
Serum IL-6 (ng/dl)	3.8 ± 1.5*	2.9 ± 1.9	0.001
IL-10 (pg/ml)	0.83 ± 0.53*	0.87 ± 0.36	0.011
IL-18/IL-10	453.4 ± 97.6**	243.7 ± 81.4	0.001
TNF-alpha/IL-10	11.9 ± 8.7**	4.33 ± 2.06	0.0001

PCAD: Premature Coronary Artery Disease; IL-18: Interleukin-18; IL-10: Interleukin-10; TNF-alpha: Tumor Necrosis Factor-alpha; SD = Standard Deviation.
** $p < 0.01$.
* $p < 0.05$ applying the Independent *t*-test.

Table 3
Genotypic distribution of the 13SNPs in PCAD patients (n = 340) and controls (n = 310).

Gene	Genotypes	Patients n (%)	Alleles n (%)	Controls n (%)	Alleles n (%)	OR (95% CI)	p-value HWE
MIA3	*AA*	23 (7)	*C = 316 (93)	38 (12)	*C = 272(88)	1.16	0.56
rs17465367	*AC*	136 (40)	A = 159 (47)	129 (4)	A = 167(54)	(0.76-1.56)	
	CC	180 (53)		143 (47)			
APOE	*TT*	6 (2)	*T = 34 (10)	2 (1)	*T = 12 (4)	2.83	0.42
rs7412	*CT*	28 (8)	C = 334(98)	10 (3)	C = 307 (99)	(1.19-3.21)	
	CC	306 (90)		297 (96)			
APOE	*CC*	4 (1)	*C = 65 (19)	7 (2)	*C = 40 (13)	1.63	0.10
rs429358	*CT*	61 (18)	T = 336 (99)	33 (12)	T = 303 (97)	(1.02-1.98)	
	TT	275 (81)		270 (85)			
MRAS	*CC*	282 (83)	*T = 58 (17)	269 (86)	*T = 41 (13)	1.30	0.79
rs9818870	*CT*	55 (16)	C = 337 (99)	40 (14)	C = 309 (99)	(0.95-1.69)	
	TT	3 (0.9)		1 (0.3)			
CXCL12	*TT*	34 (11)	*C = 306(90)	35 (10)	*C = 275 (88)	1.11	0.06
rs1746048	*CT*	129 (36)	T = 157(46)	127 (48)	T = 162 (50)	(0.85-1.49)	
	CC	177 (53)		148 (42)			
9p21	*GG*	142 (42)	*G = 281 (83)	93 (30)	*G = 254 (82)	1.11	0.77
rs10757274	*GA*	139 (41)	A = 197 (58)	161 (52)	A = 217 (70)	(0.98-1.56)	
	AA	58 (17)		56 (18)			
LPA	*CC*	2 (0.5)	*C = 14 (4)	1 (0.5)	*C = 10 (3)	1.40	0.75
rs3789220	*CT*	12 (3.5)	T = 338 (99)	9 (2.5)	T = 309 (99)	(1.01-1.77)	
	TT	326 (96)		300 (97)			
LPA	*GG*	2 (0.6)	*G = 17 (5)	1 (0.3)	*G = 8 (3)	2.13	0.74
Rs10455872	*GA*	15 (4)	A = 338 (99)	9 (2)	A = 307 (99)	(1.12-2.87)	
	AA	323 (95)		300 (97)			
PCSK9	*GG*	336 (99.2)	*G = 339 (99)	305 (98)	*G = 309 (99)	1.10	0.97
Rs11591147	*GT*	3 (0.4)	T = 4 (1)	4 (1.5)	T = 5 (2)	(0.71-1.55)	
	TT	1 (0.4)		1 (0.5)			
SORT1	*AA*	214 (63)	*A = 326 (96)	172 (53)	*A = 282 (91)	1.15	0.82
rs646776	*GA*	112 (33)	G = 126 (37)	110 (41)	G = 136 (44)	(0.81-1.53)	
	GG	14 (4)		26 (6)			
APOA5	*GG*	10 (3)	*G = 99 (29)	6 (2)	*G = 84 (25)	1.17	0.99
Rs662799	*GA*	89 (26)	A = 330 (97)	78 (25)	A = 304 (95)	(0.89-1.83)	
	AA	241 (71)		226 (73)			
LPL	*CC*	292 (86)	*C = 338 (99)	254 (82)	*C = 306 (96)	1.11	0.35
rs328	*CG*	46 (14)	G = 48 (14)	52 (17)	G = 56 (17)	(0.71-1.53)	
	GG	2 (0.6)		4 (1.2)			
APOB	*GG*	255 (75)	*A = 85 (25)	256 (80)	*A = 54 (17)	1.60	0.49
Rs1042301	*GA*	82 (24)	G = 337 (99)	45 (17)	G = 301 (97)	(1.07-1.98)	
	AA	3 (0.8)		9 (2.8)			

HWE = Hardy-Weinberg Equilibrium; RAF = Risk Allele Frequency; OR = Odds ratio; CI = Confidence Interval.
* $p < 0.05$ by applying Chi-Square test * = Risk allele.

Table 4
Comparison of 13 CAD SNPs Risk allele Frequencies between PCAD patients (n = 340) and controls (n = 310).

Gene	rs number	Patients RAF (95%CI)	Controls RAF (95%CI)	Risk Allele	p-value
MIA3	rs17465367	0.72 (0.69–0.76)	0.66 (0.63–0.71)	C	0.04*
APOE	rs7412	0.05 (0.04–0.07)	0.03 (0.01–0.04)	T	0.03*
APOE	rs429358	0.09 (0.07–0.12)	0.07 (0.05–0.10)	C	0.2
MRAS	rs9818870	0.08 (0.07–0.11)	0.06 (0.05–0.09)	T	0.17
CXCL12	rs1746048	0.71 (0.68–0.75)	0.66 (0.62–0.70)	C	0.04*
9p21	rs10757274	0.63 (0.59–0.67)	0.56 (0.53–0.61)	G	0.02*
LPA	rs3789220	0.02 (0.01–0.03)	0.01 (0.01–0.03)	C	0.63
LPA	rs10455872	0.03 (0.01–0.04)	0.02 (0.01–0.03)	G	0.13
PCSK9	rs11591147	0.999 (0.99–1.0)	0.990 (0.98–1.0)	G	0.05
SORT1	rs646776	0.79 (0.77–0.83)	0.73 (0.70–0.77)	A	0.01*
APOA5	rs662799	0.16 (0.13–0.19)	0.14 (0.11–0.16)	G	0.25
LPL	rs328	0.93 (0.91–0.95)	0.90 (0.88–0.93)	C	0.18
APOB	rs1042301	0.13 (0.10–0.15)	0.10 (0.08–0.12)	A	0.10

RAF = Risk Allele Frequency; CI = Confidence Interval.
* $p < 0.05$ by applying Chi-Square test.

4. Discussion

This is the first study of its kind reporting the effect of the CARDIoGRAMplusC4D SNPs on the inflammatory cytokine serum levels in Pakistani patients suffering from PCAD. The risk allele of MIA3 rs17465367 was the major allele C. The risk allele for MRAS rs9818870 was minor allele T. The TNF-alpha:IL-10 level was raised significantly in the risk allele carriers of the MRAS rs9818870 SNP. These observations point to the new emerging era of integrative genomics where gene variants may actually be tagging the functional SNPs in the transcriptional regulatory regions or gene promoter region of inflammatory or immune biomarkers involved in the pathogenesis of multifactorial diseases [5].

The serum IL-18 and TNF-alpha levels were significantly higher in the CT heterozygotes of the APOE rs7412 SNP in the PCAD patients recruited in the study. This is probably because the risk allele T is masking the protective effect of the protective allele C and promoting an immune-inflammatory state in the PCAD patients. Studies have reported an interaction between the APOE polymorphisms and the serum

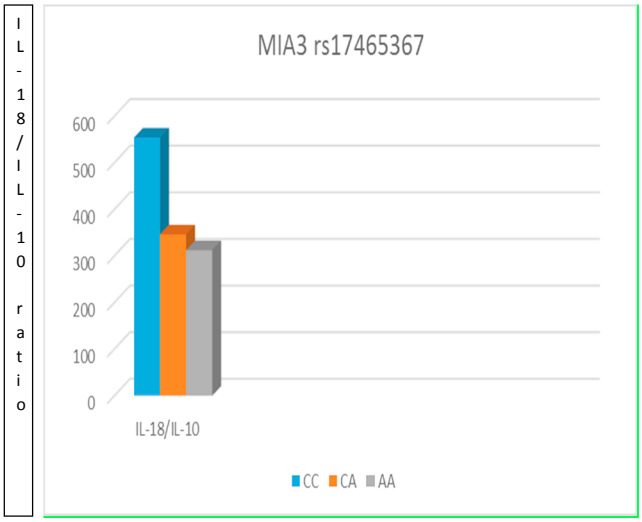

Fig. 1a. Bar chart showing the comparison of serum IL-18/IL-10 ratio with respect to the genotypic distribution of *MIA3 rs17465367* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele is C.

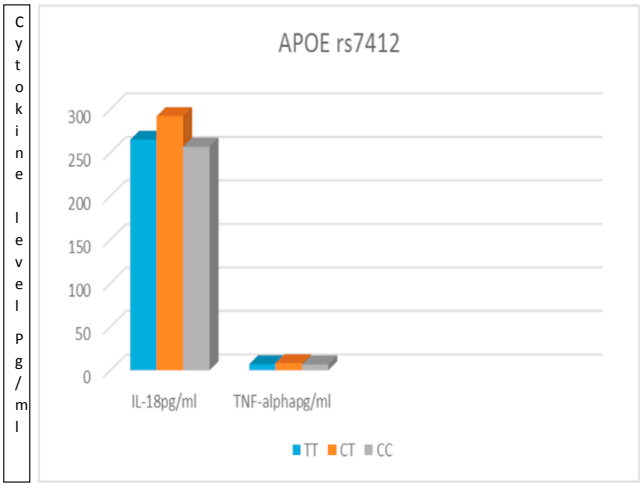

Fig. 1b. Bar chart showing comparison of serum IL-18/IL-10 ratio with respect to the genotypic distribution of *APOE rs7412* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele is T.

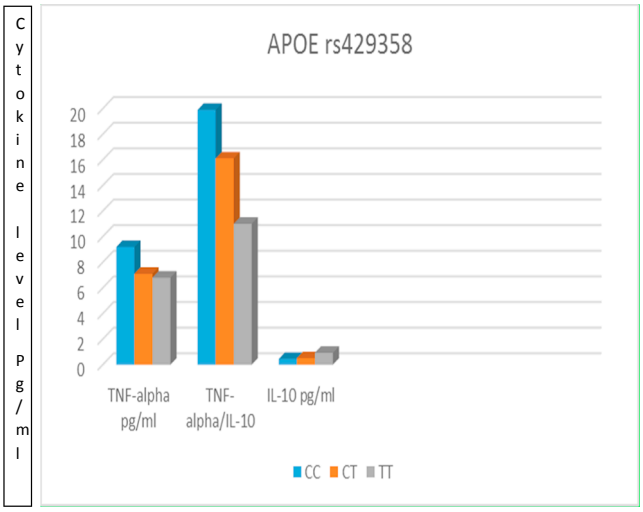

Fig. 1c. Bar chart showing comparison of serum TNF-alpha, TNF-alpha/IL-10 and IL-10 levels with respect to the genotypic distribution of *APOE rs429358* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele is C.

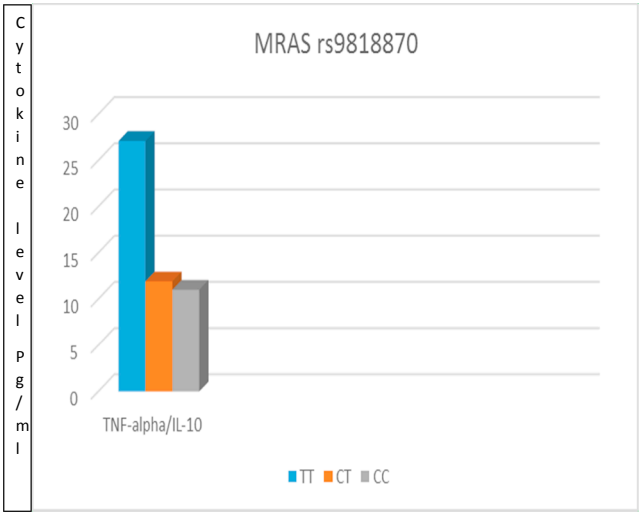

Fig. 1d. Bar chart showing comparison of serum TNF-alpha/IL-10 ratio with respect to the genotypic distribution of *MRAS rs9818870* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele = T.

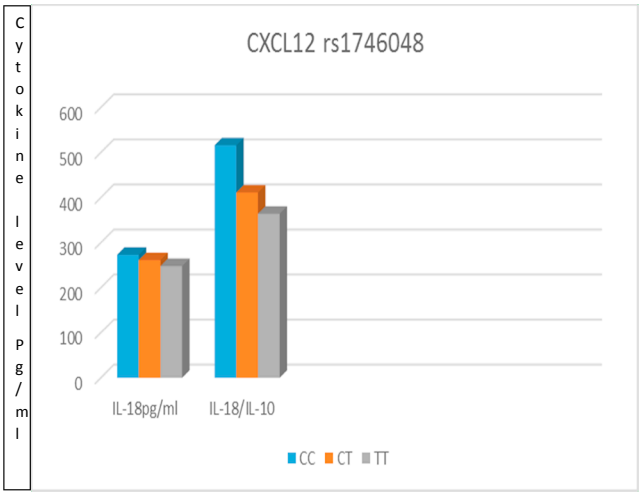

Fig. 1e. Bar chart showing comparison of serum IL-18 and IL-18/IL-10 ratio with respect to the genotypic distribution of *CXCL12 rs1746048* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele = C.

cytokine levels but the mechanism is still unclear [25]. APOE rs429358 CC genotype showed significant association with higher TNF-alpha levels and significantly lower IL-10 levels hence affecting the TNF-alpha:IL-10 ratio also in PCAD patients of the study. The major effect of the APOE SNPs is on the lipid metabolism. Since TNF-alpha is one of the major cytokines involved in mediating the lipid levels therefore the APOE SNPs possibly affect its production also (Urosevic et al., 2008). APOE genotype has been shown to affect the cellular immune response in stably transfected murine macrophages resulting in production of raised levels of pro-inflammatory cytokines including TNF-α [16].

The serum IL-18 level and IL-18:IL-10 ratio was significantly raised in the CC homozygotes as compared to the heterozygotes and the protective allele carriers of the CXCL12 rs1746048 SNP in PCAD patients. Although the mechanism by which CXCL12 rs1746048 SNP influences the serum cytokine levels is still unclear studies suggest that this SNP may function at a nuclear level by affecting the transcription factors like STAT and NF-kB which further regulate the release of pro-inflammatory cytokines [22]. Camargo et al. [7] has shown pro- and anti-inflammatory imbalance leads to plaque instability and that IL-18:IL-10 ratio is an independent predictor of adverse events in stable CAD patients.

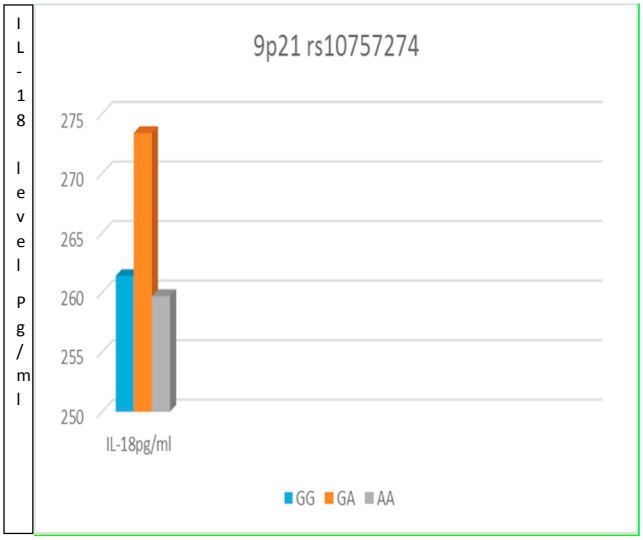

Fig. 1f. Bar chart showing comparison of serum IL-18 level with respect to the genotypic distribution of *9p21 rs10757274* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele = G.

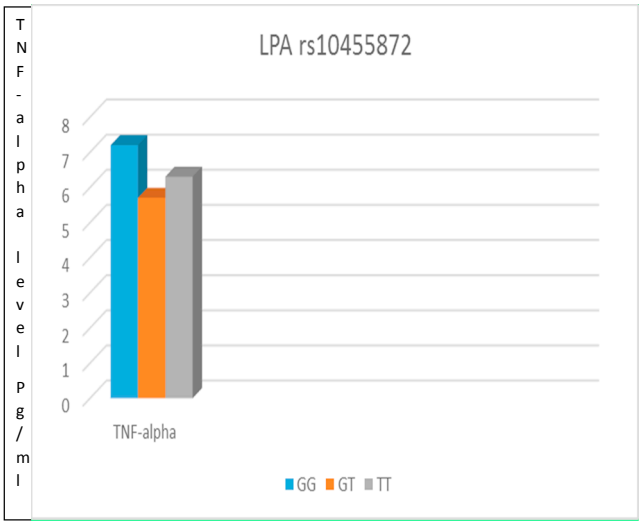

Fig. 1g. Bar chart showing comparison of serum IL-18 level with respect to the genotypic distribution of *LPA rs10455872* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele = G.

The serum IL-18 levels were raised in the risk allele carriers especially the heterozygotes GT for this SNP in PCAD patients. A possible explanation for this rise in the serum IL-18 level could be the differential regulation and expression of IFN-1 in the risk allele carriers. Another study however reports that the 9p21 gene variants can disrupt several transcription factor binding sites leading to altered production of serum cytokines [9].

Serum TNF-alpha was significantly higher in the risk allele carriers of LPA rs10455872 among the PCAD patients in the study. Very limited studies are available which have studied this association so the exact mechanism is still unclear. However, a study suggests that binding of LPA and LDL to toll like receptors activates a signaling cascade that enhances expression of pro inflammatory cytokines IL-6, IL-12 and TNF alpha encoding genes [8].

Serum IL-18 levels were raised in the GG homozygotes of the PCSK9 rs11591147 SNP in PCAD patients of the study. Although the knowledge regarding the effect of this gene polymorphism on cytokine levels is scarce a study suggests that PCSK9 plays a critical part in regulating the innate immune response and PCSK9 gene variants can influence the production serum cytokine levels [23].

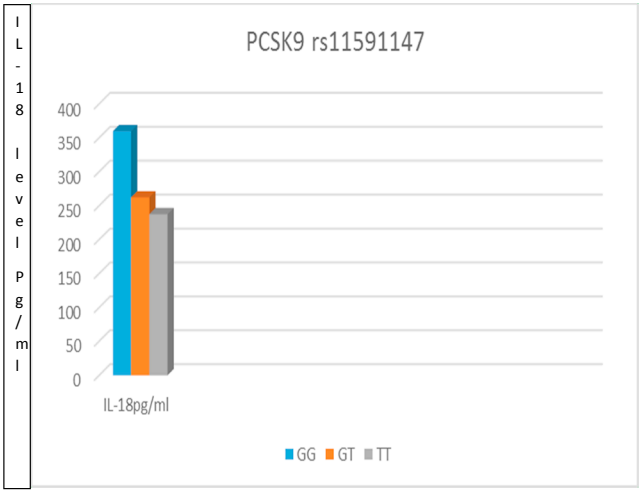

Fig. 1h. Bar chart showing comparison of serum IL-18 level with respect to the genotypic distribution of *PCSK9 rs11591147* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele = G.

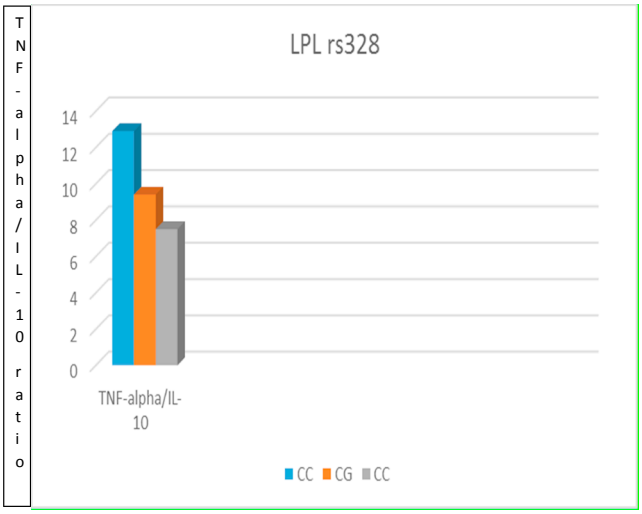

Fig. 1i. Bar chart showing comparison of serum TNF-alpha/IL-10 ratio with respect to the genotypic distribution of *LPL rs328* SNP by applying One-way ANOVA analysis and Post Hoc Tukey's test. Risk allele = C.

The TNF-alpha:IL-10 ratio was raised in the risk allele carriers of the LPL rs328 in the PCAD patients in the study. A possible reason for this association could be the interaction between IFN-gamma and LPL promoter region [15]. Since IFN-gamma potently regulates of the immune-inflammatory response at the transcription level this interaction might result in the pro-/anti-inflammatory cytokine imbalance seen in the pathogenesis of PCAD. Another possible reason could be the location of this polymorphism near splice sites or within splicing regulatory sequences which has altered the splicing patterns. Since phenotypic variability like altered serum cytokine levels result from changes in gene expression, including individual differences in splicing [24]. These changes, in turn, can result in the expression of different mRNA variants that affect disease susceptibility and severity [20]. TNF-alpha:IL-10 ratio was increased considerably in CAD patients of Northern India as compared to the healthy controls [14]. Further studies are required to establish the exact association especially in the South Asian population. The serum cytokine levels did not differ significantly with respect to the genotypic distribution of the 13 CAD SNPs in the disease free controls of the study.

Future recommendations include functional analysis on the SNPs using Electromobility shift assays (EMSA) to identify differences in protein binding between alleles. Luciferase reporter assays should be

Table 5
Comparison of serum cytokine levels with respect to the genotypic distribution of the 13 CAD SNPs.

Cytokine levels	SNP Risk Allele			
MIA3 rs17465637				
Risk allele is C	*CC	CA	AA	p-value
	(n = 180)	(n = 136)	(n = 23)	
	Mean ± SE	Mean ± SE	Mean ± SE	
IL-18/IL-10	554 ± 110.3	346.5 ± 105.1	312.2 ± 65.2	0.04*
APOE rs7412				
Risk allele is T	*TT	CT	CC	p-value
	(n = 6)	(n = 28)	(n = 308)	
	Mean ± SE	Mean ± SE	Mean ± SE	
IL-18 (pg/ml)	264.5 ± 24.4	292.5 ± 25.9	257.2 ± 10.8	0.004*
TNF-alpha (pg/ml)	7.0 ± 0.83	8.1 ± 0.78	6.7 ± 0.35	0.001**
APOE rs429358				
Risk allele is C	CC	CT	TT	p-value
	(n = 4)	(n = 61)	(n = 275)	
	Mean ± SE	Mean ± SE	Mean ± SE	
TNF-alpha (pg/ml)	9.2 ± 1.05	7.1 ± 1.0	6.8 ± 0.39	0.032**
IL-10 (pg/ml)	0.45 ± 0.38	0.51 ± 0.36	0.93 ± 0.14	0.001*
TNF-alpha/IL-10	19.9 ± 6.4	16.1 ± 6.1	11.0 ± 2.4	0.01*
MRAS rs9818870				
Risk allele = T	TT	CT	CC	p-value
	(n = 3)	(n = 55)	(n = 282)	
	Mean ± SE	Mean ± SE	Mean ± SE	
TNF-alpha/IL-10	27.1 ± 6.7	11.9 ± 6.32	11.0 ± 2.72	0.001*
CXCL12 rs1746048				
Risk allele = C	CC	CT	TT	p-value
	(n = 177)	(n = 129)	(n = 34)	
	Mean ± SE	Mean ± SE	Mean ± SE	
IL-18 (pg/ml)	273.0 ± 9.29	261.1 ± 12.4	247.9 ± 13.3	0.003*
IL-18/IL-10	516.1 ± 71.3	411 ± 95.8	364.1 ± 102.2	0.004*
9p21 rs10757274				
Risk allele = G	GG	GA	AA	p-value
	(n = 142)	(n = 139)	(n = 58)	
	Mean ± SE	Mean ± SE	Mean ± SE	
IL-18 (pg/ml)	261.4 ± 9.5	273.4 ± 12.7	259.7 ± 11.9	0.019*
LPA rs10455872				
Risk allele = G	GG	GT	TT	p-value
	(n = 2)	(n = 15)	(n = 323)	
	Mean ± SE	Mean ± SE	Mean ± SE	
TNF-alpha (pg/ml)	7.2 ± 1.40	5.7 ± 1.0	6.3 ± 1.1	0.01*
PCSK9 rs11591147				
Risk allele = G	GG	GT	TT	p-value
	(n = 336)	(n = 3)	(n = 1)	
	Mean ± SE	Mean ± SE	Mean ± SE	
IL-18 (pg/ml)	359.8 ± 28.9	262.1 ± 28.9	237.3 ± 40.4	0.02**
LPL rs328				
Risk allele = C	CC	CG	GG	p-value
	(n = 292)	(n = 46)	(n = 2)	
	Mean ± SE	Mean ± SE	Mean ± SE	
TNF-alpha/IL-10	12.9 ± 2.25	9.4 ± 6.19	7.5 ± 6.45	0.03*

IL-18 = Interleukin-18; IL-10 = Interleukin-10; TNF-alpha = Tumor Necrosis Factor-alpha; SNP = Single Nucleotide Polymorphism.
** $p < 0.01$.
* $p < 0.05$.

performed to assess whether the SNP affects expression of a reporter gene and to assess its definitive effect on the phenotypic traits like serum cytokine levels. A probable limitation of the study is the relatively small sample size therefore replication of the study with a larger sample size and by including other ethnic groups like Sindhis and Baluchis would help to get a broader perspective regarding the ethnic differences in the genotypic distribution and the risk allele frequencies of the selected cytokine SNPs in the Pakistani subjects.

5. Conclusions

The CAD risk SNPs (genotypic trait) have significant influence on the serum cytokine levels and the pro-/anti-inflammatory cytokine imbalance (phenotypic trait) making them important targets for the delivery of epigenetic therapeutics in PCAD patients.

Competing interests

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2017.05.013.

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