The Professional Medical Journal
www.theprofesional.com

DOI: 10.29309/TPMJ/18.3937

ORIGINAL PROF-3937

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Article received on:
21/03/2017
Accepted for publication:
15/01/2018
Received after proof reading:
04/05/2018

PREMATURE CORONARY ARTERY DISEASE;
INTERPLAY OF IMMUNE-INFLAMMATORY GENOMICS IN THE PATHOGENESIS OF PCAD

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ABSTRACT… Introduction: Integrative genomics may help in the identification of novel biological pathways in the pathogenesis of CAD. **Objectives:** To find out the association of 5 Cytokine SNPs and 13 CAD SNPs gene risk scores with serum cytokine levels in Premature Coronary Artery Disease (PCAD). To identify the direct and indirect protein interactions of the 13 CAD risk genes and the 5 cytokine genes in PCAD. **Study Design:** Case-control study. **Setting:** Army Medical College, in association with University College London (UCL), London, United Kingdom (UK). **Materials and Methods:** 340 PCAD patients and 310 age and sex matched controls were recruited. Serum IL18, TNFA, IL6 and IL10 levels were measured using ELISA (Invitrogen). The SNPs were genotyped using TAQMAN and KASPar assays. Data analysis was done using standard SPSS software version-21 (SPSS Inc, Chicago, Illinois, USA). The protein-protein interaction (PPI) network was generated using STRING version 9.0, Genemania and I-Tessar web. **Results:** The patients of PCAD had mean ± SD age of 42 ± 3.80 years consisting of 329 males and 11 females. The 5 SNP cytokine gene risk score correlated significantly with the serum IL-18, IL-6, TNF-alpha, IL-18: IL-10 and TNF-alpha: IL-10 ratios ($p<0.01$). The 13 CAD SNP gene risk score also correlated significantly with the serum IL-18, TNF-alpha, IL-18: IL-10 and TNF-alpha: IL-10 ratios but not with serum IL-6 levels. IL-6 works in close interaction with IL-6R, STAT3 and NFKB1. While MRAS, MIA3 and SORT1 interact with each other CXCL-12 mediates its actions by interacting with IL-18, JAK-2 and CCR4. LPA interacts closely with APOB and LPL acts via interaction with APOA4 and APOA5. **Conclusion:** The correlation between gene risk scores and serum cytokine levels can aid in the analysis of complex networks to understand the pathogenesis of PCAD.

Key words: Interplay; Immune Inflammatory Pathway; Gene Score; Cytokine Levels.

Article Citation: Omer W, Siddiq A, Khan OJ, Khan DA, Khattak EHK, Naveed AK. Premature coronary artery disease; interplay of immune-inflammatory genomics in the pathogenesis of PCAD. Professional Med J 2018; 25(4):784-795. **DOI:**10.29309/TPMJ/18.3937

INTRODUCTION

Premature coronary artery disease is a multi-factorial disease and genetic factors play a vital role in this process.[1] Over the past few years studies have suggested a number of candidate genes, genetic polymorphisms, and other loci which contribute to the development and susceptibility of atherosclerosis. The identification of CAD risk factors has led to the development of CAD risk prediction scores. A number of CAD risk scores have been developed which include QRISK[2] and SCORE.[3] The addition of genetic risk score to the traditional risk factor score significantly improved the CAD risk assessment in Atherosclerosis risk in communities Study.[4] A study performed on a Greek-European cohort has shown that genetic risk score and conventional risk factor scores have additive effects on the risk of CAD.[5]

Despite the advances in genetic risk assessment of CAD the hereditability of CAD still remains a mystery. Therefore, it is being suggested that integrative genomics may help in the identification of novel biological pathways in the pathogenesis of CAD.[6] There is an intricate interplay between the immune inflammatory pathway in the pathogenesis of CAD which may be unraveled by the studying the causal network models of gene-gene interactions.[7] CAD is a multi-factorial disease and a number of processes are operative in disease causation at the same time. It is therefore necessary to use the approach of

integration of the genes and the multiple pathways regulating the disease process. The genes are not only differentially expressed but have multiple functional pathways as well. Genetic approaches based on systems can help to ascertain the complete genetic architecture of frequently occurring disorders.[8] Applying network biology approaches to this pathway has suggested that inflammation and immune response related genes have overlapping signals.[9] This is mainly because the inflammatory pathway is initiated by the pro-inflammatory cytokines.[10] The network analysis can also help in the identification of the transcriptional regulators of genes and their role in the disease process.[11] Hence, objective of the study was to find out the association of 5 Cytokine SNPs gene risk score and the 13 CAD SNPs gene risk score with serum cytokine levels in Premature Coronary Artery Disease. Further, we sought to identify the direct and indirect protein interactions of the 13 CAD risk genes and the 5 cytokine genes in PCAD, generated with the help of bioinformatics tools.

MATERIALS AND METHODS

The study was conducted in the Chemical Pathology department and Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, National University of Sciences and Technology (NUST) Rawalpindi, Pakistan, in association with Cardiovascular Genetics (CVG) Institute, University College London (UCL), London, United Kingdom (UK). Ethical approval was obtained from the Ethical Review Committee Army Medical College, Rawalpindi, Pakistan.

Inclusion Criteria

A total of 650 subjects aged ≤ 45 years who 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 for assessment. All participants were citizens of Pakistan. The sampling technique 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.

Exclusion Criteria

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.Demographic characteristics along with complete history which includes cardiovascular risk factors was noted. Blood samples were obtained once the subject was being prepared for angiography. 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. Four ml whole blood was poured into EDTA tube to extract DNA. Gentra Puregene Blood kit (Qiagen, USA) was used to extract genomic DNA. 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 color imeteric 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 Supplementary Table I(a) and cytokine SNPs Supplementary Table II(b) were geno typed using TAQMAN and KASPar assays according to standard protocols and preformed primers depending on the efficacy and availability of respective assay technique. Fluorescence was detected by 7900HT Real-Time PCR. The CAD risk

SNPs were selected from the Cardiogram plus C4D consortium[11] was studied in detail. These SNPs were further searched for in Cardiogram plus C4D data and finally 13 CAD SNPs in loci meeting the genome wide significance threshold ($p < 5 \times 10^{-8}$) were selected.[12] Details of SNPs selected for the study along with the respective references are shown in supplementary Table-I. The gene risk scores were calculated by multiplying risk alleles number with the natural log of the odds ratio for every SNP. Then the products were added together with the assumption that all SNPs were acting additively.[13] Un-weighted gene scores were calculated by assigning 0 if a subject is not carrying the risk allele, 1 if carrier of a risk allele, and 2 if homozygous for the risk allele, and then calculate the overall score for every subject.[14]

STATISTICAL ANALYSIS

Data analysis was done using standard SPSS software version-21 (SPSS Inc, Chicago, Illinois, USA). 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 (x^2- tests). Pearson's rank correlation coefficients were estimated to see correlation between the gene risk scores and the serum cytokine levels. A two-tailed p value <0.05 was taken as significant. The protein-protein interaction (PPI) network was generated using STRING version 9.0, Genemania and I-Tessar web. This database gives information regarding experimental and predicted interactions from different reference sources based on their neighboring, gene fusions, co-expression, experiments and intense literature search. Only interactions with high level of confidence (Confidence score 0.7) were obtained from the database and considered valid for the PPI network.

RESULTS

The patients of PCAD had mean ± SD age of 42 ± 3.80 years consisting of 329 males and 11 females. Demographic features are given in Table-I. 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 ($p<0.01$). The data showed Gaussian distribution after applying the Kolmogorov-Smirnov test. Serum IL-18, TNF-alpha, IL-6 along with the cytokine ratios were significantly higher in PCAD cases as compared to the controls (Supplementary Table-I). The mean 5 SNP cytokine gene risk score and the 13 CAD SNPs gene risk score were higher in PCAD patients compared to controls (Supplementary Table-II). The 5 SNP cytokine gene risk score correlated significantly with the serum IL-18, IL-6, TNF-alpha, IL-18: IL-10 and TNF-alpha: IL-10 ratios ($p<0.01$). The 13 CAD SNP gene risk score also correlated with the serum IL-18, TNF-alpha, IL-18: IL-10 and TNF-alpha: IL-10 ratios but the correlation was significant ($p<0.05$). The correlation of the 13 CAD SNP gene risk score with serum IL-6 levels was not statistically significant ($p=0.09$). Results are shown in Table-II.

To understand the dynamics of the immune-inflammatory network in PCAD and genes linked to the cytokines IL-18, TNF-alpha, IL-6 and IL-10, protein-protein interaction analysis was performed in silico using STRING version 9, Genemania and I-Tessar web server. IL-6 works in close interaction with IL-6R, STAT3 and NFKB1. The intricate relations are shown by arrows in Figure-1(a). IL-18, IL-6 and IL-10 not only interact with one another but also with the TNF-alpha receptor as shown in Figure-1 (b). The cytokine-cytokine receptor reference pathway is shown in Figure-1 (c).

As far as the 13 CAD SNPs are concerned the protein-protein interaction analysis revealed that while MRAS, MIA3 and SORT1 interact with each other CXCL-12 does not have a direct interaction with them and may be acting independently or through another pathway as shown in Figure-2 (a). The detailed view of Protein-protein interactions of MRAS, MIA3, SORT1 and CXCL12 proteins can be seen in Figure-2 (b) where it can be seen that CXCL12 mediates its actions by interacting with IL-18, JAK-2 and CCR4. LPA interacts closely

with APOB as shown in Figure-2 (c). APOE has an intricate protein-protein interaction network but it also works in close collaboration with APOA5 as shown in Figure-2 (d). LPL acts via interaction with APOA4 and APOA5 as shown in Fig 2 (e). PCSK9 works through an independent pathway but does interact closely with LDL receptor shown in Figure-2 (f). Protein-protein interactions of APOA5, APOB, LPA, PCSK9, APOE and LPL proteins are summarized in Figure-2 (g).

Parameters	PCAD patients n= 340 Mean ± SD	Controls n=310 Mean ± SD	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
BMI (kg/m2)	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

Table-I. Demographic Characteristics of PCAD patients and controls (n=650)
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 **p<0.01; *p<0.05

Parameter	IL-18 r (p-value)	IL-10 r (p-value)	TNF-alpha r (p-value)	IL-6 r (p-value)	IL-18:IL-10 ratio r (p-value)	TNF-alpha:IL-10 ratio r (p-value)
5 SNP Cytokine Gene Risk Score	0.564** (0.0001)	-0.045 (0.53)	0.648** (0.0001)	0.131* (0.04)	0.374** (0.0001)	0.385** (0.0001)
13 CAD SNP Gene Risk Score	0.236** (0.001)	-0.007 (0.91)	0.220** (0.002)	0.103 (0.09)	0.157* (0.027)	0.159* (0.025)

Table-II. Association between gene risk scores and serum cytokine levels.
r= Correlation coefficient; CAD=Coronary Artery Disease; SNP=Single Nucleotide Polymorphism. p<0.05*; p<0.01** (Pearson Correlation test)

Gene	SNP	SNP location	Risk Allele	Odds Ratio	Reference
MIA3	rs17465637	Intergenic	C	1.14	Samani et al.
CXCL12	rs1746048	Intergenic	C	1.17	Samani et al.
APOB	rs1042031	E4181K	A	1.73	Casas et al.
LPA	rs10455872	Intergenic	G	1.70	Clarke et al.
LPL	rs328	S447X	C	1.25	Casas et al.
9p21	rs10757274	Intergenic	G	1.29	Samani, Erdmann., et al.
PCSK9	rs11591147	R46L	G	1.43	Benn et al.
APOA5	rs662799	Promoter	G	1.19	Sarwar et al.
MRAS	rs9818870	Intergenic	T	1.15	Erdmann et al.
LPA	rs3798220	I1891M	C	1.92	Clarke et al.
APOE	rs429358	C112R	C	1.06	Bennet et al.
APOE	rs7412	C158R	T	0.80	Bennet et al.
SORT1	rs646776	Intergenic	A	1.19	Samani et al.

Supplemetary Table-I. Selected CAD SNPs (Beaney et al.,2015)

Gene	SNP	SNP location	Risk Allele	Odds ratio	Reference
IL-18	rs187238	Promoter	G	1.12	Rajesh et al.,2015
IL-18	rs1956519	Promoter	G	1.24	Grisoni et al.,2008
TNF-alpha	rs1800629	Promoter	A	1.13	Wang et al.,2015
IL-10	rs1800871	Promoter	C	1.44	Srikanth et al.,2012
IL-6	Rs1800795	Promoter	C	1.11	Hou et al.,2015

Supplementary Table-I (b). Selected Cytokine SNPs
TNF-alpha= Tumor Necrosis Factor-alpha; IL-18=Interleukin-18; IL-10=Interleukin-10; IL-6= Interleukin-6.

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

Supplementary Table-II. Comparison of Cytokine Levels in PCAD patients and Controls
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.

Pakistani Study	PCAD Patients (n=340)	Controls (n=310)	p-value
13 CAD SNP Gene Risk Score Mean (SD)	2.73 (0.42)	2.25 (0.35)	0.044*
Unweighted Gene Risk Score Mean (SD)	10.53 (1.69)	9.93 (1.39)	9.89×10^{-4} **
5 SNP Cytokine Gene Risk Score (SD)	4.89 (1.77)	4.38 (1.61)	0.06

Supplementary Table-III. 13 CAD SNP gene risk score and Framingham Risk Score in Pakistani patients and controls.
PCAD: Premature Coronary Artery Disease; NPHSII: Northwick Park Heart Study; SD: Standard Deviation; *p<0.05;**p<0.01. (By applying Chi-Square Test). Risk score was calculated for those subjects in whom complete genotyping was achieved (n=635) while Framingham was calculated for all subjects irrespective of complete genotyping information (n=650).

The reference pathway for the 13 CAD SNPs protein-protein interactions in shown in Figure-2 (h).

Categorical variables were compared using a χ^2 test while continuous variables were compared using independent t-tests.

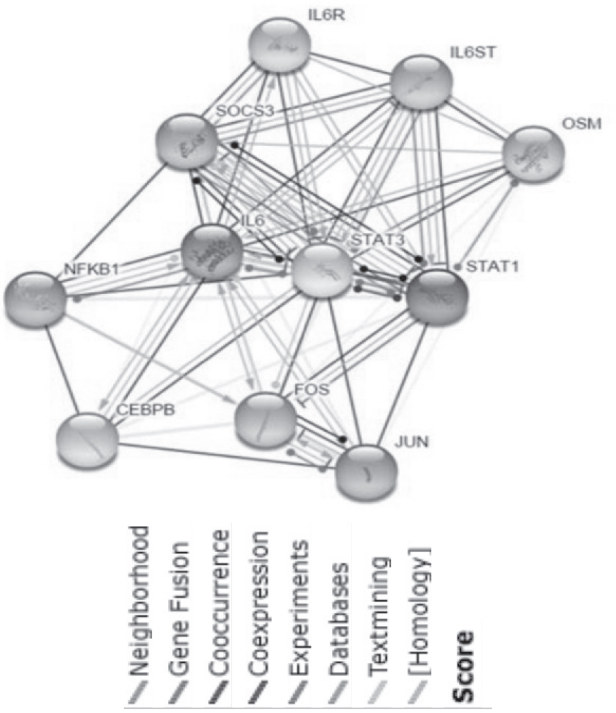

Figure-1 (a). Detail protein-protein Interactions of IL6 proteins (arrows represent actions of proteins)

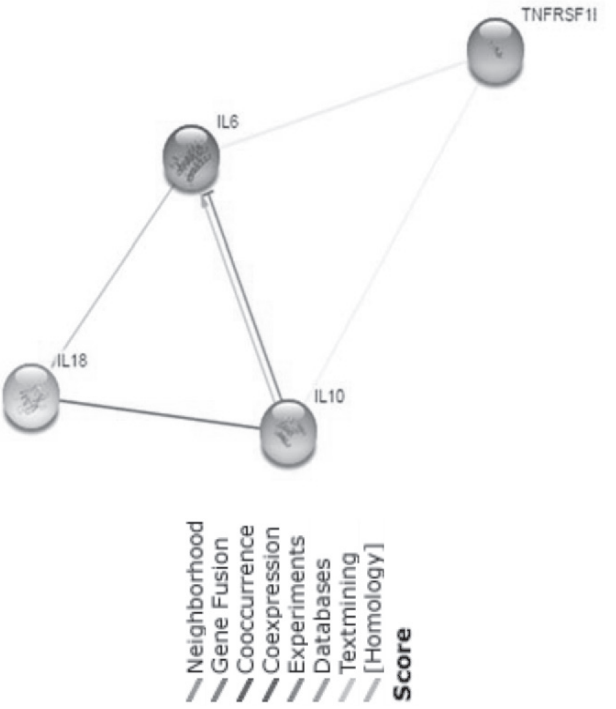

Figure-1 (b). Protein-protein interactions of IL18, IL6, IL10 and TNFRSF1 (TNF-alpha) proteins.

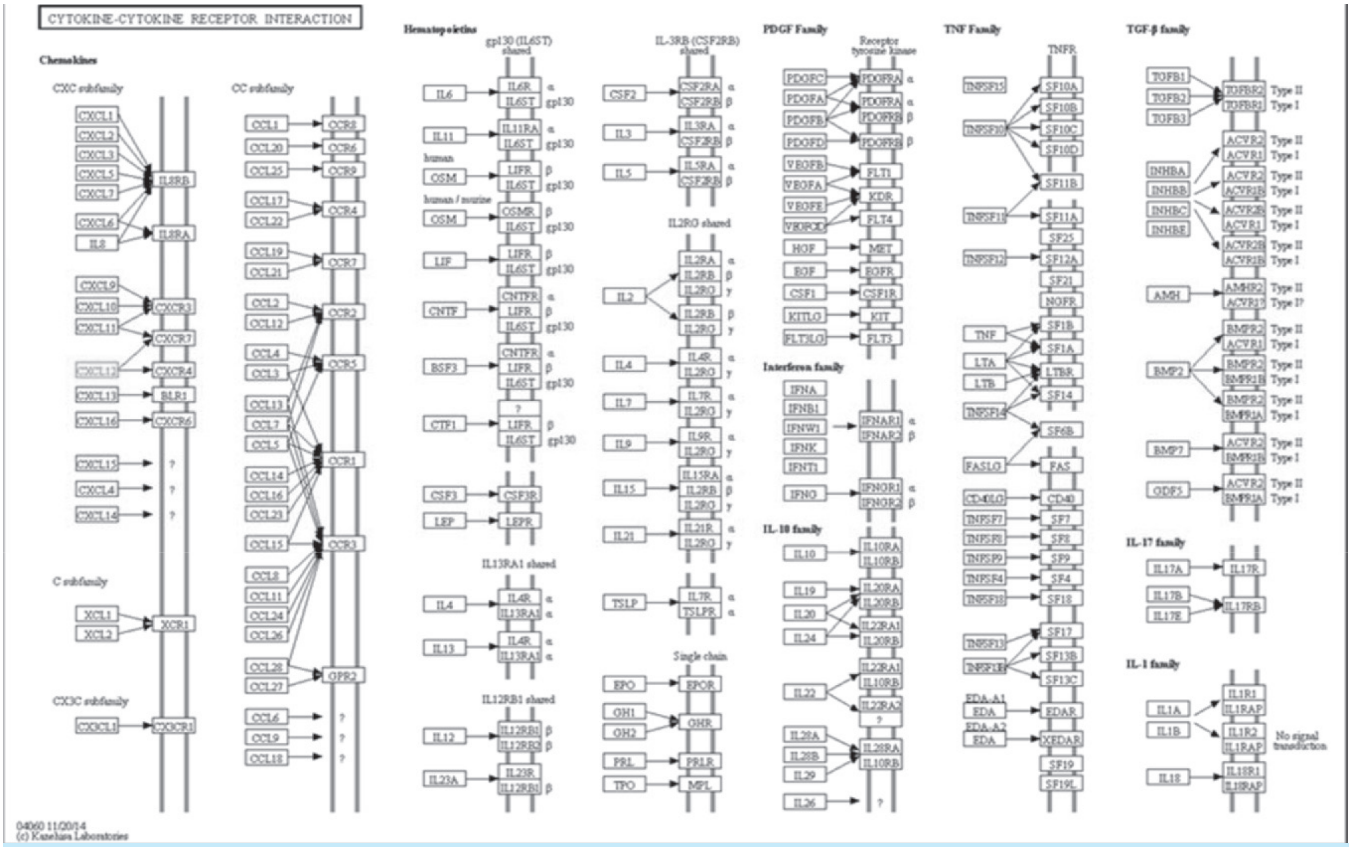

Figure-1 (c). Cytokine-cytokine receptor interaction – Reference pathway

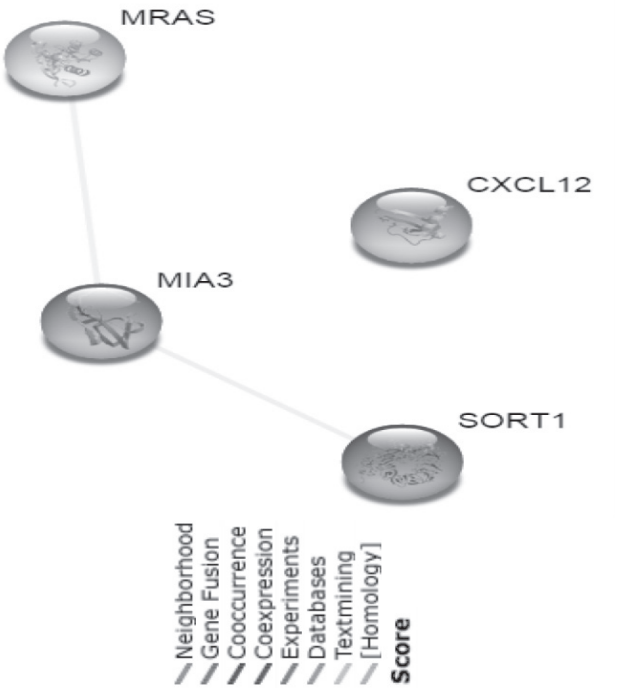

Figure-2 (a). Protein-protein interactions of MRAS, MIA3, SORT1 and CXCL12 proteins (note that CXCL12 has not direct interaction with the other three proteins).

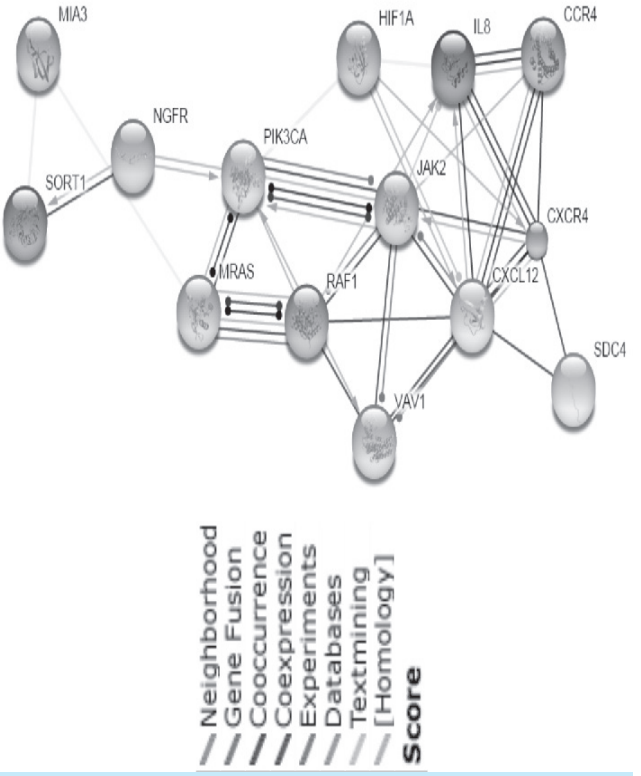

Figure-2 (b). Detailed view of Protein-protein interactions of MRAS, MIA3, SORT1 and CXCL12 proteins

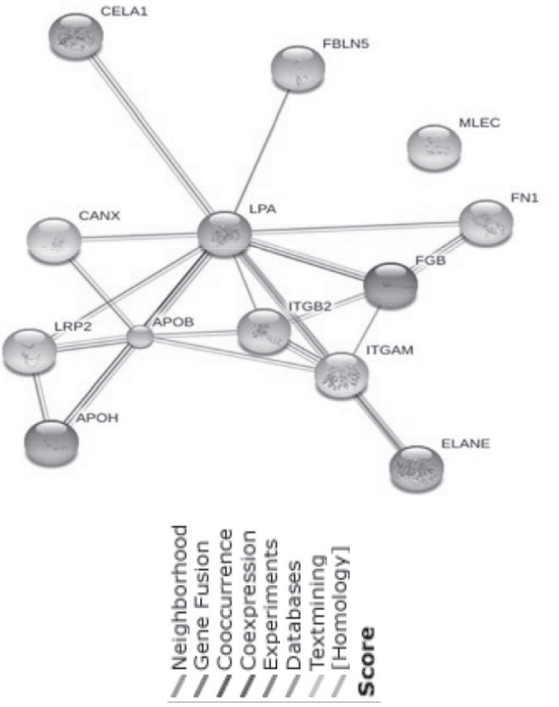

Figure-2 (c). Detailed view of protein-protein interaction of LPA

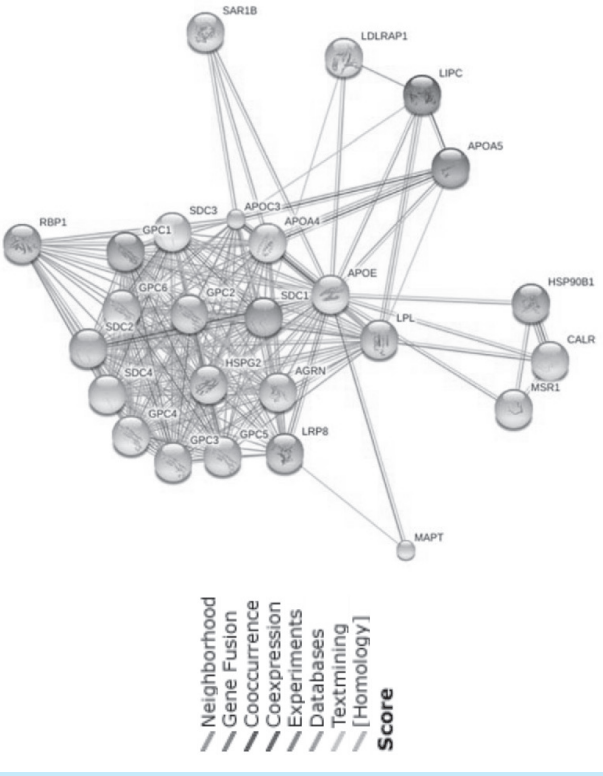

Figure-2 (d). Detailed view of protein-protein interaction of APOE

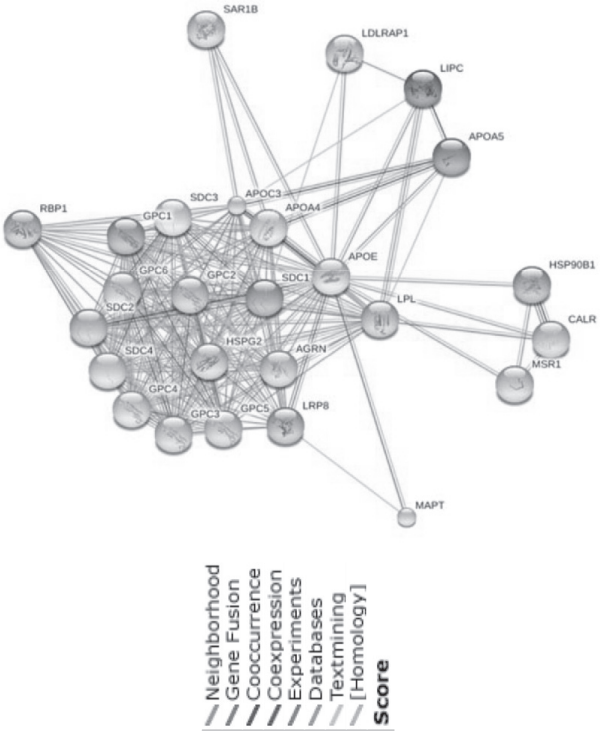

Figure-2 (e). Detailed view of protein-protein interaction of LPL

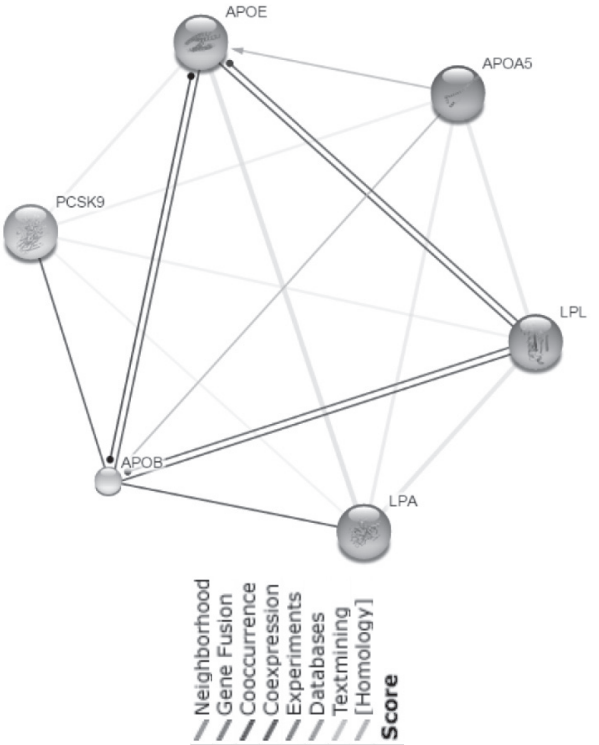

Fig-2 (g). Protein-protein interactions of APOA5, APOB, LPA, PCSK9, APOE and LPL proteins.

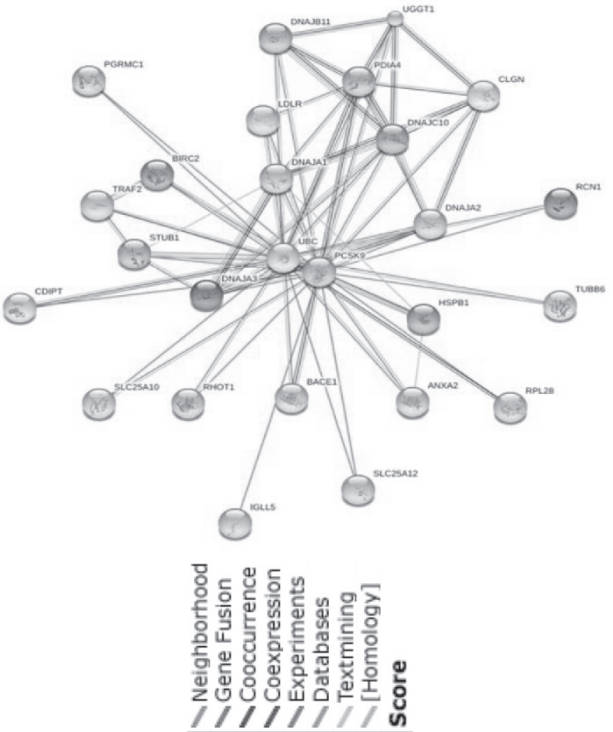

Fig 2 (f). Detailed view of protein-protein interaction of PCSK9

DISCUSSION

The use of 13 CAD SNP gene risk score and a 5 SNP cytokine gene risk score in our study demonstrated that there is an intricate network of immune and inflammatory pathway genomics in the pathogenesis of PCAD. It has been repeatedly emphasized that PCAD is a multi-factorial disease and that a number of factors are working concurrently in the causal pathway of the disease.[15] Studies are underway to study the functional annotation between lipid metabolism, immunity and other gene regulatory networks in the CAD pathogenesis.[6] The reason for this interplay among the immune-inflammatory genomics in CAD could be the complex networks and transcriptional regulators in complex disease.[7] The cytokines play a significant role in connecting the immune-inflammatory pathway though the exact functional pathways are still unclear.[8] Another study is in agreement with our study and has demonstrated that apart from the major pathways involved in the disease pathogenesis there are cross-sub pathways also which link the major pathways.[9]

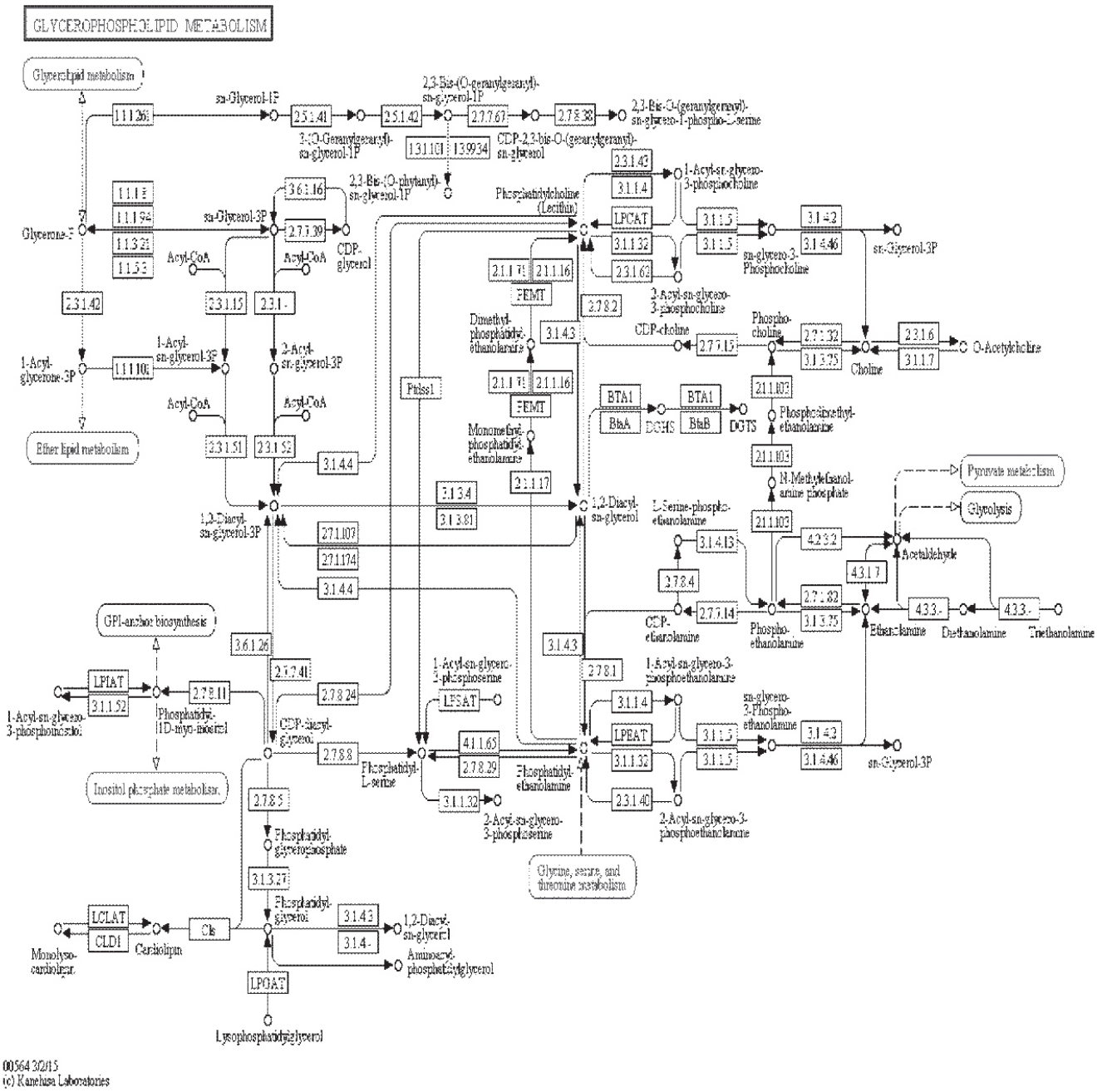

Figure-2 (h). Glycerophospholipid metabolism - Reference pathway

The 5 SNP cytokine gene risk score and the 13 CAD SNP gene risk score correlated significantly with the serum pro-inflammatory cytokine levels and the pro-/anti-inflammatory cytokine imbalance in the study subjects. A previous study has shown that a genetic score based on IL-6 and CRP gene variants significantly influences the serum cytokine levels.[16] IL-18: IL-10 and TNF-alpha: IL-10 ratios correlated significantly with the gene risk score in the study. This is probably because a gene score involves multiple SNPs which influence the transcription regulation of both pro- and anti-inflammatory cytokines having a greater effect on the pro-:anti-inflammatory cytokine imbalance. This observation is supported by another study which have pointed towards the possibility that a shift in the balance of the Th1/Th2 response caused by genetic variants in key cytokine genes

could have important consequences for the pathogenesis of the disease.[17] In silico protein-protein interaction analysis showed that IL-6 works in close interaction with IL-6R, STAT3 and NFKB1.

This is probably because STAT3 is activated in response to the binding of IL-6 to the EGF receptor.[18,19] It was also observed that IL-18, IL-6 and IL-10 not only interact with one another but also with the TNF-alpha receptor. Similar interactions have been observed previously in a different group of cytokines.[20] As far as the 13 CAD SNPs are concerned the protein-protein interaction analysis revealed that while MRAS, MIA3 and SORT1 interact with each other CXCL-12 does not have a direct interaction with them and may be acting independently or through another pathway. This is in agreement with another study on the bioinformatics microarray analysis and identification of gene expression profiles associated with cirrhotic liver.[21] LPA interacts closely with APOB. This is probably because LPA and megalin bind together and then LPA is taken up and degraded in fibroblasts expressing megalin suggesting that uptake of LPA is mediated by APOB partially.[22] LPL acts via interaction with APOA4 and APOA5.

The strength of our study is that it is the first study of its kind to be carried out on Pakistani patients giving a comprehensive overview of the interplay between the immune and inflammatory pathway in PCAD. Moreover, the PCAD patients and the disease free controls were ascertained on angiography which is currently the gold standard for diagnosis of the disease. Most of the studies done earlier recruited patients with multiple disease end points including angina, MI and IHD based merely on the ECG findings or patient history.

The limitation of the study is that due to time and financial constraints all ethnic groups of Pakistan could not be studied which would have strengthened the gene score risk assessment of PCAD even further. Future recommendations include Functional enrichment analysis and promoter analysis of the key inflammatory cytokines to unravel their role in the pathogenesis of PCAD.

CONCLUSION

The correlation between gene risk scores and serum cytokine levels can aid in the analysis of complex networks to assess the interplay between them and help in the prioritization as well as functionality of genes in the pathogenesis of PCAD. This will eventually lead to better therapeutic and preventive measures to be devised against this disease.

Acknowledgements

We wish to acknowledge the efforts of all the laboratory technicians who participated in the study. My sincere gratitude to my colleague Miss Katherine Beaney and Miss Li Kawah at CVG for their valuable guidance, suggestions and input. This study was funded by the International Research Support Initiative Program of Pakistan under the auspices of the Higher Education Commission of Pakistan.

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Children will follow your example more than your advice.

– Millionaire Desires –

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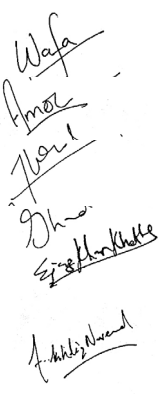
Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Wafa Omer	Concept, Study design, sample collection, Lab analysis, Data collection, Data analysis, drafting.	
2	Amer Siddiq	Sample collection, data analysis, critical review.	
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