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PREMATURE CORONARY ARTERY DISEASE;
POTENTIAL ROLE OF A 13 SNP GENE RISK SCORE IN THE RISK PREDICTION OF PAKISTANI PATIENTS

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ABSTRACT… Introduction: Genetic information which is specific to an individual has the potential to improve Coronary Artery Disease (CAD) risk prediction. 13 CAD risk SNPs were selected by removing SNPs in loci which had not been identified in CARDIoGRAMplusC4D GWAS. Linkage disequilibrium patterns differ between ethnic groups pointing towards the need to investigate how the gene score would perform in different populations which is still largely unknown. Objective of the study was to investigate whether the 13 SNP CAD risk gene score has a role in the risk prediction of Pakistani Premature Coronary Artery Disease (CAD) cases and controls and to compare the CAD risk allele frequency between Pakistanis and Caucasians (samples obtained from the Northwick Park Heart Study II). **Study Design:** Case-control study. **Setting:** Army Medical College, National University of Sciences and Technology (NUST) in collaboration with the Cardiovascular Genetics Institute, University College London, UK. **Materials and Methods:** Total of 650 subjects with a history of chest pain were selected by non-probability convenience sampling. Out of these subjects with > 70% stenosis in at least 1 coronary vessel on angiography were labelled as Premature coronary Artery disease (PCAD) cases (n=340). The 13 SNPs were genotyped in a Pakistani case-control study (n=340 CAD cases, 310 controls) using KASPar and Taqman assays. The use of 13 SNP gene score was tested in the prospective Northwick Park Heart Study (NPHSII) of 2775 healthy UK men (284 cases) and the Pakistani case-control study subjects (n=650). **Results:** Mean ± SD age of CAD patients was 42.7±3.80yrs while in controls it was 39.0±7.8yrs. Complete genotyping was obtained for 635 samples (333 cases, 302 controls). The mean 13 SNP gene score was significantly higher in cases compared to controls (p=0.044). Odds ratio for CAD for each quintile of 13 SNPs gene score showed a trend for higher quintiles of gene score to have increased odds ratio for CAD (p-value for trend=0.01) especially after adjusting for age, sex and ethnicity. There was a significant difference in risk allele frequency between Pakistanis and Caucasians (NPHSII) for all CAD risk SNPs except rs599839 (SORT1) (p=0.08). **Conclusion:** A 13 SNP gene score has significant potential role at differentiating between Pakistani PCAD cases and controls. Risk allele frequencies for CAD differ significantly between Pakistanis and Caucasians stressing the need to develop population specific gene score keeping in view the ethnic stratification.

Key words: Gene Risk Score; Premature Coronary Artery Disease; Case-Control; Single Nucleotide Polymorphism.

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INTRODUCTION

Coronary heart disease (CAD) is the most common cause of the death around the globe.[1] Cardiovascular disease (CVD) leads to 17.1 million deaths (29 per cent of all deaths) worldwide each year.[2] Premature Coronary Artery Disease (PCAD) is the most common reason for death of men and women over 20 years of age.[3] South Asians are at an increased CAD risk especially at a younger age.[4] The Pakistani population has one of the highest risks of CAD in the world attributing 30% to 40% of all deaths to this sole cause.[2] This may be due to increased prevalence of smoking, diabetes, dyslipidemia, fat rich dietary patterns and other conventional risk factors in the South Asians especially in the Pakistanis.[5]

Currently the risk of CAD can be assessed using conventional risk factor (CRF) scores such as the Framingham risk score[6] SCORE[7] and PROCAM.[8]

The potential benefits of risk assessment encompass reassuring low-risk individuals, motivating high-risk individuals to change lifestyles or show better compliance to medical therapy.[9]

The role of genetics in coronary artery disease has been repeatedly emphasized.[10] It has been suggested that CAD is a multifactorial inherited disorder.[11] Susceptibility to atherosclerosis is significantly influenced by the genetic factors beyond the traditional risk factors.[12] There was no marked improvement in risk prediction by the addition of single SNP at 9p21 to the Framingham risk score.[13] However, combining even a small number of variants into a cumulative gene score in certain cases has been shown to improve risk prediction[14] while in others it has not shown any improvement over the conventional risk factors SCORE.[15] A genetic risk score was calculated with 13 CAD risk variants identified from both candidate gene studies and GWAS. It might be expected that those suffering from PCAD have a stronger genetic disposition to CAD and thus the performance of the genetic risk score should be assessed in this group.

The objective of the study was to investigate the potential utility of a 13 SNP risk gene score in a PCAD cases and controls from Pakistan and to determine if the gene score improved risk prediction over and above Conventional Risk Factors (CRFs) in this group which has not been investigated before.

SUBJECTS AND METHODS

The case-control study was performed at Chemical Pathology Department of Army Medical College, National University of Sciences and Technology, Rawalpindi in collaboration with the Cardiovascular Genetics Institute, University College London, UK after approval from the institutional ethical review committees of the participating institutions. The study complies with the Declaration of Helsinki.

Pakistani subjects

Total of 650 subjects aged ≤45 years with a history of chest pain reporting to the National Institute of Heart Diseases, Rawalpindi, Pakistan were selected by non-probability convenience sampling. Out of these subjects patients with > 70% stenosis in at least 1 coronary vessel on angiography were determined to have Premature Coronary Artery Disease (n=340)[16] while those angiographically proven to be disease free were recruited as controls (n=310) after informed consent. Patients with infectious or autoimmune diseases, diabetes, familial hyperlipidemia, diabetes, valvular heart disease, rheumatoid arthritis and congenital heart disease were also excluded. Among the controls those with acute or chronic illness or those on anti-inflammatory drugs were excluded. Demographic characteristics were noted. Blood samples were taken in the morning on the day of angiography of the respective patients. 10ml blood sample was obtained by veni-puncture. 6ml and transferred to a plain vacutainer tube for serum analysis while 4ml was transferred to the EDTA tube for DNA extraction. Genomic DNA was extracted using the QIAmp DNA blood mini kit according to manufacturer protocol (Qiagen, USA). Purified DNA was stored at -20^{0}C till genetic analysis.

The 13 SNPs were genotyped in a Pakistani case-control study (n=650; 340 PCAD cases, 310 controls) using KASPar based on Kompetitive allele specific PCR[17] and Taqman assays according to standard protocols and preformed primers. On multiple occasions Sanger sequencing was performed with the required sequencing primers via Source Bioscience or Eurofins to confirm the genotype of particular samples not called automatically.

Gene Scores

The SNPs included in the gene score are presented in Table I, along with the source publication(s). Gene scores were calculated by multiplying the number of risk alleles by the natural log of the odds ratio for each SNP and adding the products together assuming that all SNPs were acting in an additive manner.[18] Un- weighted Gene scores were calculated by assigning a risk value of, for example, 0 if a subject is a non-carrier of a risk allele, 0.5 or 1 if a carrier, and 1 or 2 if homozygous

for that allele, and then the overall score for each individual was calculated.[19;20]

STATISTICAL ANALYSIS

Analysis for the Pakistani study and genotype frequency comparisons between all studies were carried out using R v3.0.3 (R Core Team 2014) and SPSS version 22.0 (IBM Corp 2013). Variables were compared between the cases and controls using two-sided t-tests for continuous variables and χ^2 tests for categorical variables. Hardy Weinberg equilibrium was assessed using χ^2 tests. The relationship between quintile of gene score and CAD was investigated using logistic regression. Association of GS with individual CRFs was calculated using binary regression analysis. Furthermore the Pakistani subjects were divided on the basis of the Framingham Risk score into four categories of those with <5%, 5-10%, 10-20% and >20% of 10 year CAD risk. The potential of GRS to improve individual risk stratification then was measured using the net reclassification improvement (NRI) method.[21]

RESULTS

The basic characteristics of the participants of the Pakistani study are presented in Table II. As expected, the case group was relatively older and had a significantly higher number of hypertensive patients and smokers. Complete genotyping was achieved for 635 samples (333 cases, 302 controls). Genotypes were confirmed by sequencing. The mean 13 SNP gene score was higher in cases compared to controls (p=0.044) (Table-III). The allele frequencies in Pakistani control group were compared to those in the group in NPHSII who did not go on to develop CAD. The risk allele frequency was statistically significantly lower in the Pakistani group for 8 SNPs and statistically significantly higher for rs10757274 (at the 9p21 locus), rs662799 (APOA5), rs328 (LPL) and rs11591147 (PCSK9) as compared to NPHSII (Table-IV).The 13 SNP GS were found to be statistically significantly lower in the Pakistani controls compared to those in NPHSII who did not go on to develop CAD ($p=4.51 \times 10^{-6}$) (Table-IV).

Gene/Locus	SNP	Risk Allele	Odds Ratio	Reference
MIA3	rs17465637	C	1.14	Samani et al. [34]
9p21	rs10757274	G	1.29	Samani[34], Erdmann [35], McPherson et al. [36]
CXCL12	rs1746048	C	1.17	Samani et al. [34]
APOA5	rs662799	G	1.19	Sarwar et al. [37]
APOB	rs1042031	A	1.73	Casas et al. [38]
LPA	rs3798220	C	1.92	Clarke et al. [39]
LPA	rs10455872	G	1.70	Clarke et al. [39]
MRAS	rs9818870	T	1.15	Erdmann et al. [35]
LPL	rs328	C	1.25	Casas et al. [38]
SORT1+	rs646776+	A	1.19	Samani et al. [34]
PCSK9	rs11591147	G	1.43	Benn et al. [40]
APOE	rs429358	C	1.06	Bennet et al. [41]
APOE	rs7412	T	0.80	Bennet et al. [41]

Table-I. SNPs included in the gene scores.
+rs599839 was genotyped instead of rs646776, r^2=0.95 in Europeans

Trait	PCAD patients n=340 Mean± SD	Controls n=310 Mean± SD
Age (y) Mean ± SD	42.0 ± 3.80	39 ± 7.8
Sex (m/f)	329/11	298/12
Height (m)	1.7 ± 0.12	1.68 ± 0.06
Weight (kg)	76.5 ± 12.7*	69 ± 11.8
BMI (kg/m2)	26.6 ± 6.7	24.1 ± 4.03
Systolic BP(mm of Hg)	124.7±11**	112 ± 5.1
Diastolic BP(mm of Hg)	83 ± 9.6 **	73 ± 3.8
Smokers n (%)	197 (58%)**	81 (26%)
Family history PCAD n(%)	112 (33%)**	28 (9%)
Family history HTN n(%)	136(40%)**	37 (12%)
Family history IHD n(%)	136 (40%)**	31 (10%)
Family history DM n(%)	78 (23%)*	25 (8%)

Table-II. Demographic Characteristics of the Pakistani sample set.

*Categorical variables were compared using a χ^2 test while continuous variables were compared using Welch's t-tests. PCAD: Premature Coronary Artery Disease; BMI: Body Mass Index; CAD: Coronary Artery Disease; HTN: Hypertension; DM: Diabetes Mellitus; BP=Blood Pressure; SD=Standard Deviation.**p<0.01.*

A significant trend (p=0.01) for higher quintiles of gene score to have a greater odds ratio for risk of PCAD compared to the bottom quintile was observed for the 13 SNP GS in the Pakistani sample after adjusting for age, sex and ethnicity (Figure-1).

Multivariate predictive analysis for angiographically ascertained PCAD for conventional risk factors and GS showed that odds ratio of GS for PCAD prediction is greater than that for smoking, hypertension and body mass index (BMI) but lower than the other risk factors taken into account (Table-V).

Linear regression analysis was performed after adjusting for confounding variables like age and sex. It demonstrated that GS is significantly associated with blood pressure and Total cholesterol but it showed no significant association with BMI or number of cigarettes smoked. (Table-VI).

Comparison of risk allele frequencies which were significantly different (p<0.05) between the PCAD Cases (n=315) and Controls (n=305) from the Pakistani study are shown in (Fig-2).

Pakistani Study	**Cases (n=333)**	**Controls (n=302)**	**p-value**
13 CAD Gene Score Mean (SD)	2.73 (0.42)	2.25 (0.35)	0.044*
Unweighted Gene Score (SD)	10.53 (1.69)	9.93(1.39)	9.89×10^{-4} **
NPHSII participants	**NPHSII CHD (n=284)**	**NPHSII No CHD (n=2491)**	**p-value**
13 CAD Gene Score Mean (SD)	2.53 (0.45)	2.43 (0.48)	7×10^{-3} **
p-value	p<0.01	$p=4.51 \times 10^{-6}$	

Table-III. CHD Risk Gene Score in the Pakistani study(n=620) and NPHSII(n=2775)

*CAD: Coronary Artery Disease; SD: Standard Deviation; NPHSII: Northwick Park Heart study.*p<0.05;**p<0.01.*

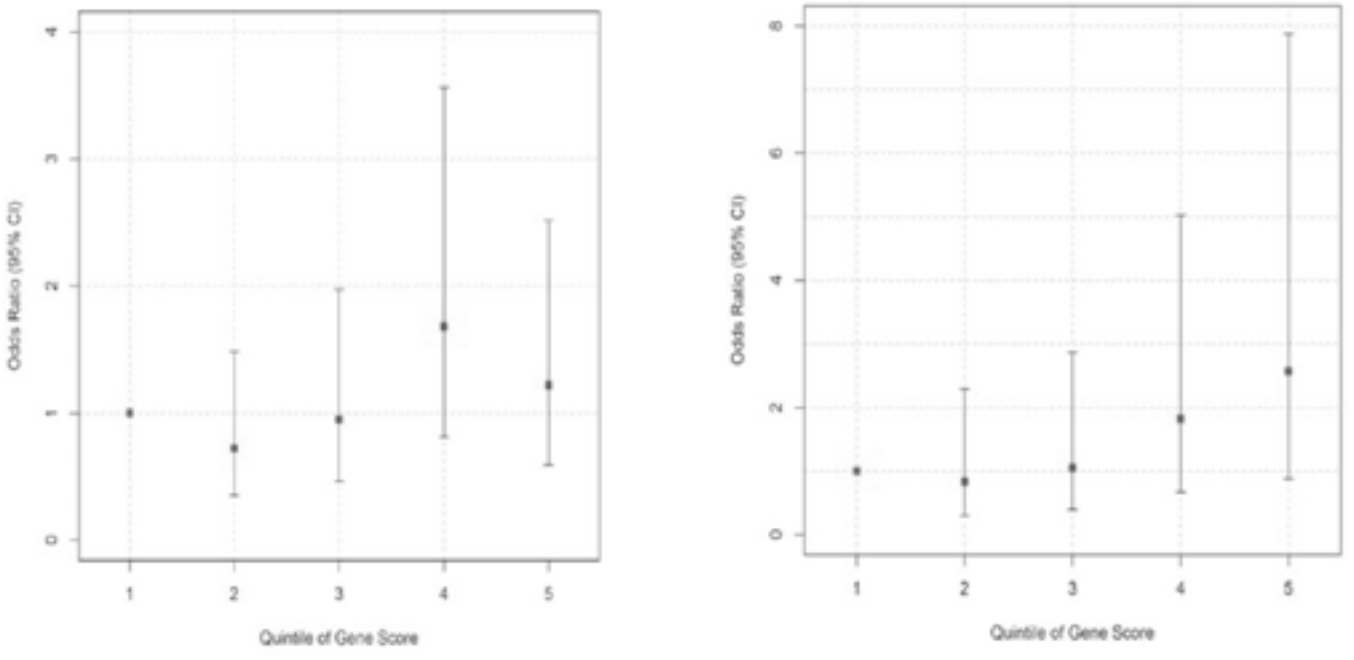
DISCUSSION

We sought to investigate potential use of a 13 SNP score a Pakistani PCAD case-control study, where there may be stronger genetic influence to disease development and where to date the knowledge in literature is very limited.

The risk allele frequency is statistically significantly lower for 8 SNPs out of a total of 13 SNPs in the Pakistani group compared to NPHSII therefore even if many of the SNPs are functional, then the performance of the gene scores based on these SNPs will subdued in this group.

Gene/Locus	SNP	RAF Pakistani Controls	RAF NPSHII No CHD	p-value
MIA3*	rs17465637	0.67*	0.71	0.04
9p21*	rs10757274	0.56**	0.48	$9.02x10^{-5}$
CXCL12*	rs1746048	0.73**	0.86	$1.71x10^{-30}$
APOA5*	rs662799	0.14**	0.06	$1.35x10^{-18}$
APOB*	rs1042031	0.11**	0.18	$1.6x10^{-5}$
LPA*	rs3798220	0.016**	0.02	$6.23x10^{-3}$
LPA*	rs10455872	0.012**	0.08	$1.34x10^{-10}$
MRAS*	rs9818870	0.07**	0.16	$3.26x10^{-8}$
LPL*	rs328	0.93*	0.90	0.02
SORT1+	rs646776+	0.77	0.78	0.08
PCSK9*	rs11591147	1.0*	0.99	0.045
APOE*	rs429358	0.09**	0.15	$5.31x10^{-5}$
APOE*	rs7412	0.03**	0.09	$3.10x10^{-7}$

Table-IV. Comparison of risk allele frequencies between the Pakistani and NPHSII control groups
*Comparisons were performed using tests of proportion. RAF = Risk Allele Frequency. **p<0.01; *p<0.0*



A) Association of quintile of gene score v bottom quintile with MI; unadjusted

p-value trend = 0.05

B) Association of quintile of gene score v bottom qunitile with MI;

Adjusted for age & Sex

Figure-1. Association between gene score and CAD in the Pakistani samples. Logistic regression was performed. Error bars represent 95% confidence intervals.

Variable	Odds ratio	Confidence Interval	p-value
Age	1.32	1.08-1.45	<2.20 x10-16
Male gender	3.35	2.89 -3.95*	0.02
Total cholesterol	2.45	2.23-3.19**	<0.001
Triglycerides	2.56	1.77-2.85**	<0.001
Smoking	3.079	1.89-3.7**	0.004
BMI (kg/m^2)	1.089	0.945-1.345	0.242
PCAD family history	0.138	0.018-1.08	0.057
DM family history	0.204	0.023-1.786	0.139
Systolic BP(mm of hg)	1.832	1.282-2.316**	0.001
Diastolic BP(mm of hg)	1.641	1.251-2.145**	0.000
Genetic Score (13 SNP)	2.55	1.91-2.89	0.09

Table-V. Multivariate regression analysis showing odds ratio for PCAD prediction using Genetic Risk score and Conventional risk factors

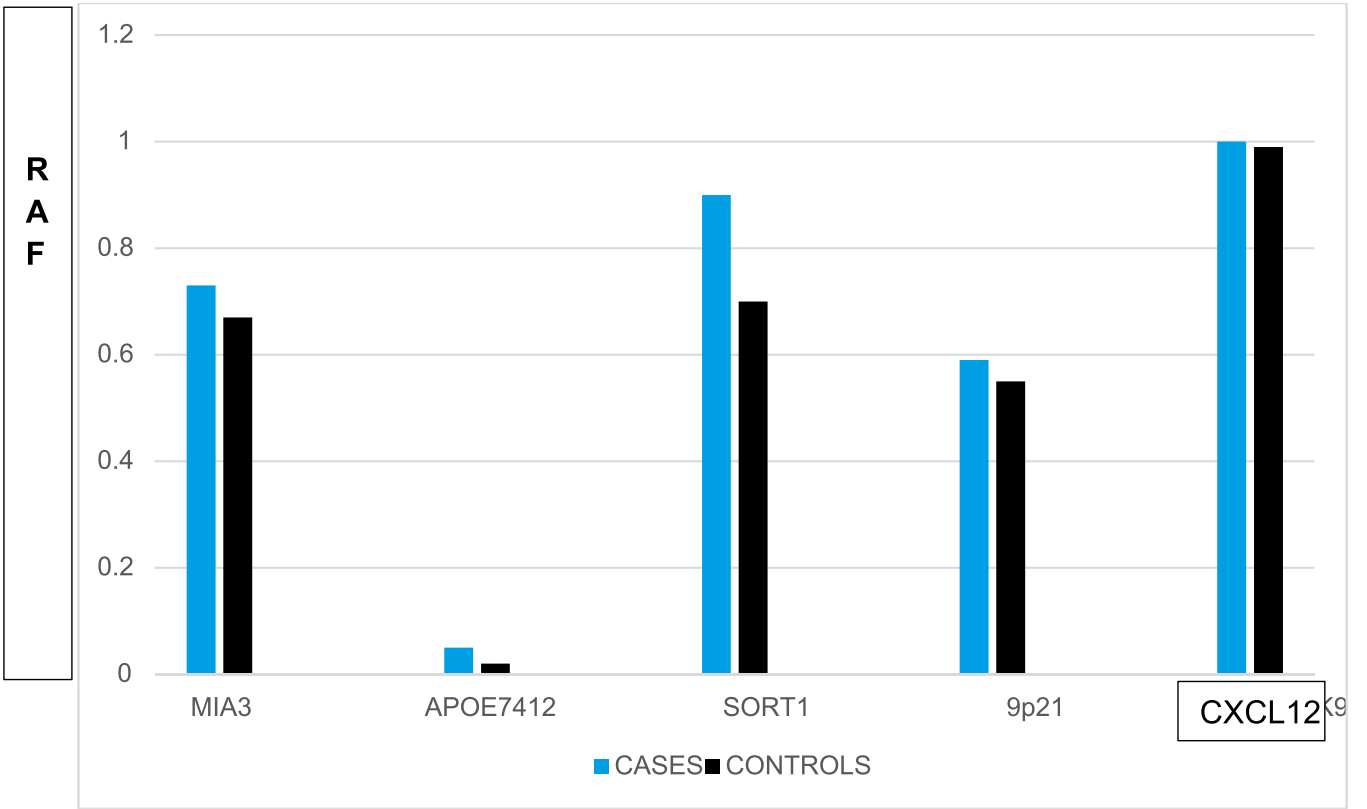
*BMI=Body Mass Index; DM=Diabetes mellitus; BP=Blood Pressure; SNP=single nucleotide polymorphism; PCAD=Premature Coronary Artery Disease; CI=Confidence Interval **p<0.01*

Variable	B	Standard error(SE)	Exp(B) Confidence Interval	p-value
Systolic BP(mm of hg)	0.133	1.715	3.93 (0.552 – 7.30) *	0.023
Diastolic BP(mm of hg)	0.12	1.19	2.46 (0.11-4.81) *	0.041
BMI (kg/m^2)	0.103	0.746	1.32 (0.151-2.78)	0.07
Age (yrs)	0.040	1.121	0.769 (0.040-0.687)	0.49
Number of cigarettes (n)	0.036	1.42	0.867 (0.056-0.98)	0.54
Total cholesterol mg/dl	0.020	0.005	1.010(1.003-1.018)**	0.01

Table-VI. Linear regression analysis showing association of Risk Gene Score with Conventional risk factors

BMI=Body Mass Index; BP=Blood Pressure; CI=Confidence Interval; B= odds ratio
**P<0.05*

Functional SNPs need to be identified In order to optimise the use of the gene scores for subjects with South Asian ethnicity as the functional SNP may differ in the various ethnic groups especially targeting the SNPs with high Risk allele frequencies in this particular ethnic group. The ethnic variation has been highlighted even in case of the conventional risk scoring methods like Framingham and QRISK2.[22] To date, allele frequency information across the Indian Subcontinent is limited for the SNPs used and an excess of homozygotes and consanguinity in the Pakistani sample set may also be a contributing factor to conflicting results. This is likely caused by the presence of population sub-structure.[23] To date there is no specific risk score that has been derived from South Asian populations[4] let alone form the young Pakistani population. This markedly leads to underestimation of the risk prediction of CAD in this population where PCAD is continuously on the rise. Given the comparison between the gene risk score in Pakistanis <45yrs and Caucasians in our study, the observation that the RAF for 8 SNPs out of a total of 13 SNPs selected from GWAS based studies is lower in Pakistanis raises the concern that possibly there are other tagging SNPs or other risk genes in this population subset altogether. Furthermore comparison of risk allele frequen-



MIA3 (rs17465637); APOE (rs7412); SORT1 (rs599839); 9p21(rs10757274); CXCL12 (rs1746048); RAF=Risk allele frequencies.

Figure-2. Risk allele frequencies for CHD risk gene SNPs which were significantly different between the Cases (n=333) and Controls (n=302) in the Pakistani study. (p<0.05)

cies for 13 Risk gene SNPs between Pakistani premature coronary artery cases and disease free controls demonstrated significant differences for MIA3 (rs17465637), APOE (rs7412), SORT1 (rs599839), PCSK9 (rs11591147) and 9p21 (rs10757274). However, the differences in the risk allele frequencies of remaining 8 SNPs were not significantly different between Pakistani PCAD cases and controls. This observation emphasizes the observation that although a risk gene score based on multiple risk gene SNPs has higher clinical utility in differentiation between Pakistani CAD cases and controls as compared to individual gene SNPs[18] but it can be most beneficial for the patient if the polygenic score is made population specific. Even if many of the individual markers have no detected effect, polygenic score could be a strong risk predictor of disease.[24] Genetic risk assessment can become a very useful, non-invasive tool for the risk assessment in young Pakistani subjects especially since preventive measures at young age markedly reduce the incidence of heart disease.[25] Cardiovascular disease at a young age is not only debilitating but is emotionally disturbing for the entire family with the cost of treatment adding to the financial burden especially in developing countries like Pakistan therefore risk assessment in this population subgroup requires its due share of importance.

Risk gene score showed significant association with the systolic and diastolic blood pressures. The probable explanation for this is the strong genetic influence on blood pressure of an individual. Moreover since there is a causal relationship between hypertension and coronary artery disease therefore a significant influence on the variants increasing the risk of PCAD may also have significant influence on the blood pressure. Similar observations have been made in a previous study also.[26] Some studies report that blood pressure genetic risk score is also related to the CAD risk

emphasizing on the inter-relationship between the two conditions.[27] The 13 SNP CAD risk gene score also demonstrated significant association with the total cholesterol level which agrees with the observation made in a previous study highlighting the link between the risk scores of lipid profile and the risk of CAD.[28] However, no significant association was found between risk gene score and BMI or number of cigarettes smoked in our study. The reason for this may be the fact that although height of an individual is genetically influenced but the weight is largely influenced by dietary and environmental factors as well. It has been reported that fish-eaters, vegetarians and especially vegans have lower BMI than meat-eaters[29] therefore probably it is the diverse dietary behavior prevalent in various ethnicities of Pakistan which affects the BMI of the individuals rather than the genetic component of it. Similarly the number of cigarettes smoked or the smoking behavior is also greatly influenced by the environmental, social and emotional conditions of the individual rather than his genetic constitution. While initiation of smoking may have genetic influence the degree of smoking does not have significant association with the polygenic risk score.[30]

Multivariate predictive analysis for angiographically ascertained PCAD for conventional risk factors and GS showed that odds ratio of GS for PCAD prediction is greater than most of the conventional risk factors except for smoking and Total cholesterol. The probable reason for this is that coronary Artery disease has a strong hereditary influence and young individuals are more show stronger genetic predisposition to the diseases therefore the genetic score performs better as compared to some of the traditional risk factors. A number of studies are being performed to assess whether the role of genetic score in prediction of CAD surpasses that of the traditional risk factor scores. However, no definitive conclusion has been made yet and there are conflicting results. Some studies demonstrated that genetic score did not show any improvement in the predictive potential for CAD as compared to the CRFs[31] while others have shown marked increase in the odds ratio for CAD prediction upon addition of GS to the CRFs.[32;33] The results of our study are in agreement to a previous study which shows polygenic risk score to be a promising marker for prediction of CAD.[13;18]

The strength of our study is that it is the first study of its kind to be carried out on premature coronary artery disease patients of Pakistan. The utility of the genetic risk scores has been shown for the Caucasians in a few studies but to date there is very limited knowledge regarding its efficacy in Pakistani CAD patients <45 yrs. Since we have also compared the results with the results obtained in the NPHSII study so it is a comprehensive overview of the predictive capability of GS along with traditional risk factors in these two populations forming a basis of further studies comprising of larger sample sizes. Another strength of our study is that not only the cases but even the controls have been angiographically verified. This minimizes the chance of including diseased subjects in which the symptoms have not yet clinically manifested as controls based merely on their history. The limitation of our study remains the small sample size and it may be one of the contributing factors for variable performance of the risk gene score in Pakistanis and Caucasians. Moreover, ethnic stratification of GS for the Balochis and Sindhis present in Pakistan has not been covered in this study. Furthermore, the functional SNP is yet to be identified for this ethnic group.

Overall this work has demonstrated that the use of a 13 SNP gene score has the potential to improve risk stratification in high risk Pakistani individuals over and above classical CAD risk factors and has considerable utility in differentiating between Pakistani PCAD cases and controls. However, future studies should be targeted in developing population specific risk gene score for favourable results.

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