



# Association of *ACE* and *NOS3* Gene Polymorphism with Blood Pressure in a Case Control Study of Coronary Artery Disease in Punjab, Pakistan

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## ABSTRACT

Single nucleotide polymorphisms (SNPs), *ACE* rs4341 and *NOS3* rs1799983 have been reported to be associated with coronary artery disease (CAD) and blood pressure (BP) in many but not all studies. We aimed to investigate the effect of these SNPs on BP and CAD in people from Punjab, Pakistan. A total of 650 subjects (430 CAD cases and 220 controls) were genotyped by TaqMan/KASPar allelic discrimination technique. Two BP measurements were reordered and their mean was calculated. The results showed that the risk allele frequencies (RAFs) of both SNPs were higher in cases than controls but the difference was not statistically significant. For rs4341, RAF in cases and controls was 0.577 vs. 0.525,  $p = 0.08$  and for rs1799983, the RAF was 0.202 vs. 0.178,  $p = 0.31$ . The SNPs were not associated with CAD. The CAD odds ratio of rs4341 (1.22, 0.97-1.53,  $p = 0.09$ ) and that of rs1799983 (1.15, 0.86-1.54,  $p=0.33$ ) was not statistically significant. Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) were significantly higher in cases than controls ( $p<0.05$ ) and the SNPs showed a significant association with BP. Each risk allele of rs4341 (G) increased SBP by 10.04±0.8 mmHg and DBP by 2.5±0.6mmHg, while risk allele of rs1799983 (T), increased SBP and DBP by 16.4±0.9mmHg and 8.8±0.6mmHg respectively, all were statistically significant ( $p<0.05$ ). When a combined effect of genotypes of both SNPs was examined, a significant effect on CAD outcome ( $p=0.01$ ) was observed when GG of rs4341 and GT of rs1799983 co-existed. Similarly, maximum elevation in BP was observed when risk alleles of both SNPs in homozygous form (GG and TT) appeared together. In conclusion, the SNPs were not independently associated with CAD but were associated with BP in Pakistani subjects under study and may be causing CAD by modulating BP.

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### Authors' Contribution

SUS performed experiments, analysed the data and wrote the manuscript. S helped in performing experiments and assisted in writing manuscript. JAC helped in statistical analysis. AR and SEH designed and supervised the study.

### Key words

Coronary artery disease, Blood pressure, TaqMan, KASPar.

## INTRODUCTION

Coronary artery disease (CAD) remains the leading cause of death worldwide and Pakistan is among the highest risk countries. Both environmental and genetic factors have role in development of CAD and hypertension is an independent CAD risk factor. Hypertension (HTN) is caused by behavioural factors like increased saturated fat and dietary sodium intake, physical inactivity, mental stress as well as genetic factors (Perry *et al.*, 1994; Williams *et al.*, 1994; Freitas *et al.*, 2007). The heritability of HTN has been estimated to be about 30% and its genetic nature is polygenic involving multiple genes (Lifton, 1996; Morris and Griffiths, 1998; Rice *et al.*, 2000). Mutations or common variants in the genes regulating BP and vascular homeostasis may have a role in predisposition to HTN (Dickson and Sigmund, 2006; Butler, 2009). Pakistan is emerging as an epicentre of the global HTN epidemic. A

survey conducted by the Pakistan Medical Research Council showed that 16.2% of the rural and 21.6% the urban Pakistani population is hypertensive ([www.heartfile.org/pdf/surveillance](http://www.heartfile.org/pdf/surveillance)). Keeping in view that the prevalence of HTN and CAD in Pakistan is different than that of western countries from where most of the studies have been reported, we examined the association of two SNPs, *ACE* rs4341 and *NOS3* rs1799983 with BP and CAD in a small group of Pakistani people.

The *NOS3* gene consists of 26 exons and gives rise to a 1203 amino acids long protein (Miyahara *et al.*, 1994; Nadaud *et al.*, 1994). The *NOS3* is second most reported gene for association with CAD and fourth most important for association with myocardial infarction (MI) ([www.hugenavigator.net](http://www.hugenavigator.net)). Hingorani *et al.* (1995) first described a point mutation in this gene at nucleotide 1917. The polymorphism rs1799983 (Glu298Asp or E298D) is a G>T missense variation located in the exon 7 of the gene (Hingorani *et al.*, 1995). The substitution of the codon GAG to GAT in the 894 position (G894T), changes glutamate with aspartate (Glu298Asp) in the protein. A possible cause of reduction in NOS activity due to this polymorphism is that the 894Asp substitution

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might cause an increase in the susceptibility of the protein for degradation due to a tight run of alpha helix (Kelm, 2006). This amino acid change may also affect protein-protein interaction and interaction of the protein with chaperon (Monti *et al.*, 2003). The protein variant with this change has different susceptibility to cleavage, that is an amino terminal 35kDa and carboxy terminal 100 kDa fragments (Tesauro *et al.*, 2000). A meta-analysis of 42 published studies comprising 13876 cases and 13042 controls revealed significant association of this polymorphism with CAD (OR 1.17, C.I 1.07-1.28,  $p=0.001$ ) (Casas *et al.*, 2006a). Similarly, the SNP was significantly associated with MI in Asians (Luo *et al.*, 2014). In a recent large meta-analysis, including 132 studies from all over the world, the polymorphism was associated with CAD (OR range 1.28-1.52,  $p<0.00001$ ) (Rai *et al.*, 2014). This polymorphism was also associated with essential hypertension in two independent Japanese populations and the allele frequency of 298Asp was significantly high in hypertensive than normotensive subjects (Miyamoto *et al.*, 1998).

The renin angiotensin system (RAS) maintains body fluid levels and cardiovascular functioning (Abdollahi *et al.*, 2008). The angiotensin converting enzyme (*ACE*) is a major player of this system and converts angiotensin I to angiotensin II and inactivates bradykinin which is a vasodilator. Strong genetic control of *ACE* regulation has been reported (Cambien *et al.*, 1988). The *ACE* gene is 21 kb in length consisting of 26 exons and 25 introns. Most of the common variants reported in this gene to date are in the noncoding region (Sayed-Tabatabaei *et al.*, 2006). The gene contains an important polymorphism consisting of an *ALU* repeat of 287 base pairs in intron 16. The polymorphism is characterized by either an insertion (I) or deletion (D) of the repeat (Eisenmann *et al.*, 2009). The DD genotype is associated with higher levels of the enzyme as compared to the II and ID genotype (Rigat *et al.*, 1990). The D allele of the SNP was found to be significantly associated ( $p=0.0005$ , OR 4.157) with hypertension in a study on Indian people (Choudhury *et al.*, 2012). In a meta-analysis of 46 studies comprising of 32715 white individuals, the ID and DD allele conferred greater susceptibility ( $p<0.001$ ) to ischemic disease than II allele (Agerholm-Larsen *et al.*, 2000). Since the I/D polymorphism is in the non-coding/intronic region, it is less likely to be a functional variant (Rigat *et al.*, 1990). The polymorphism *ACE* rs4341 is in complete linkage disequilibrium (LD) with the I/D polymorphism of this gene and can be a best proxy for I/D polymorphism (Domingues-Montanari *et al.*, 2010).

Both of these SNPs were identified in candidate gene studies predating genome wide association study (GWAS), however, these SNPs have now been studied in

GWAS but were not found to be associated with CAD in GWAS (Casas *et al.*, 2006). The reasons may be false associations in candidate gene studies, publication bias or the effect of the SNPs may be too small to reach GWAS significance threshold. The objective of the current study was to examine whether the common variants in the above mentioned genes having potential role in regulation of vascular physiology and RAS system are associated with HTN and CAD in Pakistani people.

## MATERIALS AND METHODS

### *Study subjects*

The study consisted of 430 CAD cases and 220 controls. The CAD cases were reported as ischemic heart disease patients based upon ECG, cardiac echo, radiological and biochemical markers of cardiac ischemia (CK-MB and troponinT/I levels). All cases were newly diagnosed and were not taking any lipid lowering or anti-hypertensive drugs. Controls were age, sex and ethnicity matched individuals without any history of cardiovascular disease. The exclusion criteria included the presence of chronic diseases like cancer, kidney or liver disease and any on-going acute infection. All the procedures were in compliance with the Helsinki declaration. The case history and other details were collected after obtaining an informed written consent. The study was approved by the institutional ethical committee, University of the Punjab, Lahore.

### *Genotyping*

Genomic DNA was extracted from whole blood leucocytes using Promega Wizard® Genomic DNA purification kit. DNA was quantified using nanodrop (ND-8000, USA). The DNA samples were diluted to a fixed concentration (1.25ng/μl). A 4μl of this dilution was used to get a final working concentration of 5ng of DNA. The DNA arraying into 384 well plates was done by a liquid handling robotic system (Biomerk FX, Beckman Coulter). Specially designed plates (Micro Amp) suitable for fluorescence amplification were used. The *NOS3* rs1799983 was genotyped by TaqMan allelic discrimination technique. The TaqMan reaction mixture consisted of 1X KAPA qPCR master mix (KAPABiosystems, USA), 100nM of each primer, 100nM of each probe, ROX high (0.4μl/20μl reaction mixture), 5ng DNA and PCR grade water as needed. The PCR program consisted of an initial temperature of 50°C for 2 min, denaturation/enzyme activation at 95°C for 10 min followed by amplification for 40 cycles each consisting of denaturation at 95°C for 15 sec and amplification at 60°C for 1 min. The programme was run on BioRad C1000TM thermal cycler.

**Table I.- Sequence of primers and probes used for rs179983 assay.**

SNP	40x	Primer and probe	Sequence of primers and probes
rs179983	NOS3_G894T_F	Primers	GGCTGGACCCCAGGAAA
	NOS3_G894T_R	Primers	CACCCAGTCAATCCCTTTGGT
	NOS3_G894T_VIC	Probe VIC =T	CCCAGATGATCCCCCA
	NOS3_G894T_FAM	Probe FAM=G	CCAGATGAGCCCCCA

**Table II.- Sequence of primers used for rs4341 assay.**

SNP	Sequence, 50bp either side of the polymorphism [C/G].	FAM	VIC
rs4341	TCTCTGAGCTCCCCTTACAAGCAGARGTGAGCTAAGGGCTGGARCTYAAG[C/G]CATT CMAMCCCCTACCAGATSTGACGAATRTGATGGCCRCRTCCCGGAAA	C	G

The ACE rs4341 was genotyped by KASPar (KBiosciences Competitive Allele Specific PCR) technique. The assay mixture per plate consisted of 950µl of each of KASPar master mix and Sigma water and 26.4µl of SNP assay (consisting of allele specific primers and labelled probes). The touchdown thermal cycler program consisted of an initial denaturation/enzyme activation at 94°C for 15 min, then 10 cycles comprising of 94°C for 20 sec and annealing/extension temperature reducing from 65°C to 57°C lowering 0.8°C per cycle. The final round consisted of 26 cycles each consisting of 94°C for 20 sec and annealing/extension at 57°C for 60 sec. The information about the primers and probes used in TaqMan and KASPar genotyping is available in Tables I, II.

After amplification, the results were analysed on ABI Prism 7900HT (Applied Biosystems/Life Technologies) and the genotypes were called using sequence detection software (SDS) version 2.0. The genotypes were also confirmed randomly by conventional direct DNA sequencing (source biosciences, UK) to check the accuracy of techniques and the results were always concordant.

#### Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS version 22, IBM statistics). The student *t*-test was used to compare continuous variables between case and control groups. The normality of outcome variable (blood pressure) was checked by drawing normal distribution curve. The studied population was checked for Hardy Weinberg equilibrium (HWE) by a Chi-square goodness of fit test. Allele and genotype frequencies were calculated by an excel spread sheet (Johnson, 1975) and also confirmed by direct counting. Allele frequencies were compared

between cases and controls using chi-squared test. As CAD is a binary variable, the association of genotypes with CAD (OR) were obtained by binary logistic regression taking “CAD status” as dependent variable and the “genotypes” as covariate. Similarly, the association of blood pressure with CAD was also examined using binary logistic regression. The mean blood pressure levels across different genotypes were calculated by one way analysis of variance (ANOVA) taking blood pressure as the dependent variable and genotype as the factor. The effect sizes of the SNPs on blood pressure ( $\beta$  effect/ $\beta$  coefficients), which is the increase in blood pressure for each additional risk allele held were obtained by linear regression taking blood pressure as dependent variable and genotype as independent variable.

The power of the sample size used in study was calculated using an online calculator (<https://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx>). A *p*-value <0.05 was considered significance cut-off for all measurements.

## RESULTS

The general characteristics of the cases and controls are shown in Table III. As expected, cases had higher percentage of hypertension and diabetes than controls and smoking rate was also higher in cases than controls. The SBP and DBP were normally distributed and significantly different among cases and controls. The cases had higher blood pressure values as compared to the controls. Both systolic and diastolic blood pressures were significantly associated with CAD status as shown by their OR (Table IV).

Both SNPs showed good genotyping results call

rates (~98%) and for both SNPs the population examined was in Hardy Weinberg equilibrium (Table VI). For the SNP *ACE* rs4341, the common allele G is the risk allele and the risk allele frequency (RAF) was higher in cases than controls but the difference was not significant statistically (0.57 vs. 0.525,  $p = 0.079$ ). For *NOS3* rs1799983, the minor allele T is the risk allele which was non-significantly (0.202 vs. 0.178,  $p = 0.332$ ) higher in cases than controls (Table VI). Although the cases had higher number of risk allele for both SNPs but the association with CAD was not significant in the studied samples in either of additive, dominant and recessive models examined. The per allele CAD odds ratio (OR) of both the SNPs was not statistically significant (Table VI).

**Table III.- Basic characteristics of the subjects under study.**

Variables	Cases	Controls	<i>p</i> -value
Sample No.	430	220	
Age (years)	59.1±12.6	56±10.5	0.002
Sex			
Males (n)	230	120	0.27
Females (n)	200	100	
Diabetes (%)	64.6	13.6	5.1x10 <sup>-34</sup>
Hypertension (%)	62.1	16.4	8.9x10 <sup>-28</sup>
Smoking (%)	29.5	10.5	7.3x10 <sup>-08</sup>

Continuous variables are expressed as Mean±SD. Categorical variables are expressed as (%).

**Table IV.- Blood pressure levels in cases and controls and association with CAD.**

	Systolic (mmHg)	Diastolic (mmHg)
Cases	139.9±16.1	88.0±9.6
Controls	128.7±14.8	85.3±11.3
<i>P</i> -value	<0.001	0.002
CAD OR*	1.05 (1.04-1.06)	1.03 (1.01-1.05)
<i>P</i> -value	<0.001	0.002

\*Odds ratio for 1 SD increase in blood pressure.

The presence of risk alleles of both SNPs increased SBP and DBP quantitatively. For rs4341, each 'G' allele increased SBP and DBP by 10.04±0.8 and 2.5±0.6 mmHg respectively. While rs1799983, per risk allele elevation in SBP and DBP was 16.4±0.9 and 8.8±0.6 mmHg, respectively (Table VII).

In order to examine the combined effect of both SNPs on CAD, the effect of the combined genotypes was examined where common homozygous genotype of rs4341 was examined against common homozygous,

heterozygous and risk homozygous genotypes of rs1799983. The association of these genotypic combination was significant when risk homozygous of rs4341 (GG) and heterozygous of rs1799983 (GT) co-existed (Table VIII). Similarly, mean SBP and DBP increased by the addition of risk alleles of both SNPs and maximum elevation was observed when both risk homozygous genotypes (GG and TT) existed simultaneously (Table IX).

## DISCUSSION

Hypertension, a potential CAD risk factor has strong genetic basis. We have studied two SNPs in the *ACE* and *NOS3* genes to examine their association with HTN and CAD in Pakistani population. Our results showed that the *ACE* rs4341 was associated with elevation in blood pressure. This is consistent with two other studies where this variant was found to be associated with HTN in Pakistani and Indian people (Sameer *et al.*, 2010; Choudhury *et al.*, 2012). However, in contrast to our findings, in another study carried out on Indian people, this polymorphism was not associated with HTN (Gupta *et al.*, 2009). The polymorphism was not associated with CAD in our study, which is consistent with another study conducted by Iqbal *et al.* (2004). Whereas, this polymorphism was found to be associated with CAD in a study by Masud and Qureshi (2011) on Pakistani people. In a large meta-analysis carried on white Caucasians, the *ACE* polymorphisms were found to be associated with *ACE* levels and CAD but not with the HTN (Agerholm-Larsen *et al.*, 2000). Whereas, another study reported a modest association of the *ACE* polymorphisms with CAD emphasizing the need of bigger study groups with inclusion of more SNPs (Samani *et al.*, 1996; Keavney *et al.*, 2000). According to Sayed-Tabatabaei *et al.* (2003) the association between risk alleles of the gene and atherosclerosis was stronger in the high risk individuals (having other risk factors like diabetes and hypertension) than in low risk individuals.

The risk allele of *NOS3* rs 1799983 SNP was found to be significantly associated with elevation in BP in the current study. However, in another study in China, the risk allele was not found to be associated with essential hypertension in northern Han Chinese but a subsequent meta-analysis in southern Han Chinese proved the association. So, in Chinese, the association of this SNP with BP was ethnicity specific (Liu *et al.*, 2014). We could not find a significant association of the risk allele of rs1799983 with CAD which is consistent with Nassar *et al.* (2001), whereas Nawaz *et al.* (2015) found a strong association of the risk allele with CAD in Pakistani people. Similarly, the association of this polymorphism

**Table V.- Basic features of the SNPs under study.**

Gene	CHR	SNP	Call rate %	HWE <i>p</i> -value		
				Cases	Controls	Total
ACE	17q23,3	rs4341	98	0.593	0.128	0.162
NOS3	7q36,1	rs1799983	97	0.159	0.158	0.05

CHR, location of the SNP on chromosome; HWE, Hardy Weinberg equilibrium.

**Table VI.- Risk allele frequencies and association of the studied SNPs with CAD.**

SNP	Alleles	RAFs			CAD OR (CI), <i>p</i> -value		
		Cases (C.I)	Controls (C.I)	<i>p</i>	Additive	Recessive	Dominant
rs4341	C>G*	0.577 (0.54-0.61)	0.525 (0.48-0.57)	0.079	1.22 (0.97-1.53) 0.089	1.19 (0.83-1.70) 0.337	1.47 (0.99-2.18) 0.056
rs1799983	G>T*	0.202 (0.17-0.23)	0.178 (0.14-0.21)	0.312	1.15 (0.86-1.54) 0.332	1.15 (0.53-2.48) 0.74	1.20 (0.85-1.71) 0.30

\* is the risk allele; RAF, risk allele frequency; C.I, confidence interval; OR, odds ratio.

**Table VII.- Systolic and diastolic blood pressure values by genotypes.**

SNP	Genotype	SBP (mmHg)	DBP (mmHg)
rs4341	CC	124.2±13.2	83.5±10.7
	CG	135.4±12.9	87.4±8.9
	GG	144.5±18.3	88.7±11.4
	β±Se	10.04±0.8	2.5±0.6
	<i>p</i> -value	<0.001	<0.001
rs1799983	GG	129.8±14.1	83.8±9.4
	GT	145.6±11	91.5±6.8
	TT	164±19	103.9±12
	β±Se	16.4±0.9	8.8±0.6
	<i>p</i> -value	<0.001	<0.001

β±Se is effect size with standard error which is increase in blood pressure for each additional risk allele held.

with CAD has also been reported in other studies (Hibi *et al.*, 1998; Shimasaki *et al.*, 1998; Ben Ali *et al.*, 2015). In other two studies the authors could not detect any risk allele in heterozygous or homozygous forms in a Pakistani and Indian subjects (Nishevitha *et al.*, 2009; Taqddus *et al.*, 2014).

The difference with respect to CAD association of our results with other studies conducted in the same region may be due to the genotyping techniques. We have genotyped the SNPs with fully automated high

**Table VIII.- Coronary artery disease by combined genotype groups.**

ACE	NOS3	N controls/ cases	OR (95% CI)	<i>p</i> - value
CC	GG	40/51	1.00	-
	GT	14/19	1.06 (0.48-2.38)	0.88
	TT	1/5	3.88 (0.41-190.0)	0.38
CG	GG	63/127	1(0.95-2.64)	0.08
	GT	30/56	1.46 (0.80-2.69)	0.22
	TT	5/9	1.41 (0.39-5.78)	0.78
GG	GG	48/84	1.37 (0.80-2.37)	0.26
	GT	14/46	2.58 (1.25-5.33)	0.01
	TT	4/7	1.37 (0.32-6.83)	0.88

**Table IX.- Blood pressure by combined genotype groups.**

ACE	NOS3	N	SBP	DBP
CC	GG	91	117.8 (8.1)	80.4 (10.4)
	GT	33	136.1 (8.4)	90.3 (7.2)
	TT	6	154.8 (4.5)	93.8 (6.6)
CG	GG	190	130.9 (12.3)	85.6 (9.2)
	GT	86	142.6 (7.9)	90.0 (6.5)
	TT	14	152.0 (11.1)	97.4 (7.1)
GG	GG	132	136.4 (14.6)	83.7 (8.4)
	GT	60	155.0 (9.1)	94.5 (6.3)
	TT	11	184.2 (14.2)	117.6 (4.4)

throughput fluorescence based techniques and randomly confirmed the results by direct DNA sequencing; hence the results are more reliable. The studied population was also in Hardy Weinberg equilibrium collectively as well as among cases and controls separately. The allele frequencies in our samples were also comparable to the allele frequencies of Pakistani Punjabi population from Lahore (PJL), a group of 96 subjects from Pakistan genotyped in 1000 genomes project phase three.

The study has a limitation which is the small sample size so modest effects of these SNPs on CAD could not be detected. The study power, as calculated, came out to be 81.7% with a sample size of 650. In order to have 100% power, the sample size needed was 1068. It is because of a small sample size than required, that we couldn't detect some significant associations evident from the nominal *p*-value for CHD association of the selected variants. While *NOS3* variant is functional the *ACE* variant is not and may not be in such strong LD with the functional variant elsewhere in this gene as in UK Caucasians. It is concluded that for both the SNPs, risk alleles were associated with BP which may suggest that the SNPs are acting through controlling HTN in studied Pakistani population.

### CONCLUSION

In conclusion, the SNPs *ACE* rs4341 and *NOS3* rs1799983 were significantly associated with blood pressure in a dose response model in Pakistani subjects under study. The SNPs were not independently associated with CAD. The effect of SNPs was synergetic on blood pressure CAD.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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