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Apolipoprotein E Polymorphism And Dyslipidemia In Elderly Patients Of Calcific Aortic Stenosis

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Abstract

Objective: This study aimed to investigate the impact of the Apo E polymorphisms on plasma lipid profile and identify the apo-E gene's polymorphism as a genetic predictor of calcific AS in the Pakistani population.
Methodology: This was a case-control study conducted at Dow University of Health Sciences and the National Institute of Cardiovascular Disease, Karachi. It included a total of 100 individuals, 50 echocardiographically identified calcific AS cases and 50 age and gender matched controls. Apo E allele frequencies were computed, lipid profiles were estimated and Apo E gene polymorphism was identified by the techniques of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).
Result: Apo E 2, 3, and 4 allele frequencies were 16%, 52%, and 32% in calcific AS cases, and 10%, 52%, and 28% in controls respectively (p=0.622). Out of 50 cases, 18% presented with mild AS, 22% with moderate AS and 60% lied in severe calcific AS. It was observed that levels of total cholesterol (TC), triglyceride (TG) and low-density lipoprotein (LDL) were higher in the Apo E4 allele as compared to other genes in both cases and control.
Conclusion: The findings of this study suggested that the Apo E4 allele of the Apo E gene is an important risk factor for dyslipidemia while the Apo E4 allele is not associated with calcific AS and contemplates distinctive genetic backgrounds of CAD and AS.
Keywords: Apolipoprotein E gene, polymorphism, dyslipidemia, Aortic stenosis

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1. Introduction

Calcific aortic stenosis (AS) is the most common valvular heart disease (VHD) known. (1) The prevalence of developing aortic valve disease (AVD) tends to be higher in elderly people and the incidence of calcific AS is high as the age expectancy of the general population is increasing worldwide. (2) Among cardiac procedures, valve replacement ranks as the second most common procedure, surpassed only by coronary bypass surgery, (3) it has been proven by the evidence that the degeneration of the aortic valve is identical to the process of atherosclerosis to a certain extent and the origin of sclerotic and calcific aortic valve follows same pathophysiology of vascular atherosclerosis, so the treatment of the disease would be same as of the chronic vascular atherosclerosis. (4)
Elevated low-density lipoprotein (LDL) cholesterol levels, hypertension (HTN), smoking, diabetes mellitus (DM), and male gender are traditional possible risk factors for AS. (5)
Patients suffering from familial hypercholesterolemia would be at high risk for atherosclerosis and as well as calcific AS. The older population with calcific AS is also at increased possibility of myocardial infarction and death from cardiovascular causes. Apolipoprotein E (Apo E) is a protein complex that performs a crucial part in metabolizing cholesterol and triglycerides by clearing up the residual particles from the liver. Apo E is a 34kDa molecular weight protein comprised of 299 amino acids. Isoelectric focusing investigations have revealed that the Apo E gene predominantly possesses three allelic variations, namely Apo E2, Apo E3, and Apo E4. Three key patterns of isoforms contribute to three heterozygous and three homozygous physical compositions, namely E4/3, E4/2, E2/2, E4/4, E3/2. E3/3, differ at position 158 and 112. Arginine and cysteine are present in Apo E3, on the contrary, apo E4 possesses

two arginines and Apo E2 possesses two cysteines at the mentioned locations in a mature apo E polypeptide state. [7]

Compared to the apo E3 allele, Apo E4 is related to greater total and LDL cholesterol levels and lower high-density lipoprotein (HDL) cholesterol levels; the apoE2 allele has the reverse impact.

Along with many risk factors dyslipidemia is the important one, high serum levels of low-density lipoprotein (LDL) and lipoprotein A are established clinical risk factors for calcific AS. Histologically prominent lipo-calcific changes on the aortic side of valve cusps can be seen in the calcific aortic valve. Several studies assessed the role of apo E polymorphism with dyslipidemia and AS and observed the effect of lipid-lowering agents in these patients. However, no published study has determined the association of apo E polymorphism with dyslipidemia and AS in Pakistan. So major emphasis of this study was to detect the role of Apo E and its isoforms in the development of dyslipidemia in calcific AS, since Apo E gene polymorphism may affect the metabolic pathway of lipids and lipoproteins.

2. Materials & Methods

This case-control research was performed in the Department of Physiology, Institute of Basic Medical Sciences (IBMS), Dow University of Health Sciences (DUHS). The sample size had been calculated using the Open-Epi online calculator. With the hypothesized proportion of the control group (patients not affected by AS) with the revelation of the apoE4 allele (27%) and the hypothetical proportion of cases (AS patients) with the revelation of the apoE4 allele (40%), [8] with 95% confidence interval and 80% power of the test, the estimated targeted population size, due to time and budget constrain using finite correction factor is 44 subjects. [9]

However, 50 patients with calcific AS were diagnosed on echocardiogram and a total of 50 gender and age-matched individuals without AS had been identified. Patients having a history of endocarditis, rheumatic heart disease, rheumatic fever or rheumatoid arthritis and echocardiographic evidence of rheumatic valvular stenosis had been excluded. Patients with a prosthetic valve, severe aortic regurgitation, chronic renal failure patients, and detected with cancer or familial hypercholesteremia were also omitted from the research. AS patients were categorized according to echocardiographic findings. AS is known as calcified and/or thickened aortic leaflets with restricted systole, a calculated aortic valve area ≤ 2.0 cm^2 and a transaortic mean gradient <25 (mild AS) to >40 mmHg (severe AS) and peak pressure gradient <35 (mild AS) to >60 mm Hg (severe AS). [10]

Cases were registered from echocardiography and the outpatient department (OPD) of NICVD, following inclusion and exclusion criteria. Written consent was taken, and blood samples were collected along with detailed medical history. Laboratory work was conducted in the biochemistry and molecular biology section at Dow Diagnostic Reference & Research Laboratory (DDRRL), DUHS. We used new Asian criteria for the body mass index (BMI) classification to assess the BMI. Blood pressure was also measured. A blood sample for lipid profile was taken after 12 hours of fasting and lipid levels were estimated by using photometry technique. (HITACHI 902 analyzer) Reference values for lipid profiles were taken according to the national cholesterol education program.

Blood for DNA analysis was taken, thoroughly mixed, centrifuged and stored at a 4-degree temperature. Blood extraction was performed from 200 µl of blood using Thermo Scientific Gene JET Genomic DNA Purification Kit (#K0721, #K0722 User Manual Version A. USA 2012 pg7) as per protocol. Genotyping for Apo E polymorphism was carried out through polymerase chain reaction (PCR). β Globin gene (housekeeping gene) amplification PCR was used to verify the purity of extracted DNA. A DNA fragment of length 244bp was obtained by using forward primer Sequence 5′to 3′ (ACAGAATTCGCCCCGGCCTGGTACAC) and reverse primer Sequence 5′ to 3′ (TAAGCTTGGCACGGCTGTCCAAGGA). Each reaction mix of PCR comprised of 2.5µl 10× AccuPrime PCR master mix, 1µl forward and reverse primers each, 0.3µl AccuPrime™ Taq DNA Polymerase, 16.2 µl of autoclaved distilled water and 5 µl DNA template. Reaction conditions of PCR were programmed at thermocycler as denaturation at 95 °C for 5 minutes, the amplification cycles were followed by 35 cycles, with one-minute annealing at 75°C, one-minute extension at 63°C, and one-minute denaturation at 72°C. The reaction was completed after a 10-minute final extension at 72 °C. (Invitrogen. AccuPrime™ Taq DNA

Polymerase System. Cat. No. 12339-016. Life Technologies Corporation.2010). Electrophoresis was performed. The final product was loaded on 2% agarose gel along with the loading dye. A band of 244 bp in length was interpreted as a positive reaction. **(**Thermo scientific 100 bp ladder, ready to use, 2011**)**. (Figure 1)

The PCR product (10µl) was digested with restriction enzymes Hhal (1µl), 10X Tango buffer (2µl), and nuclease-free water (18µl) for Apo-E restriction fragment length polymorphism analysis (RFLP) at 37ºC and the mixture was incubated for 24 hours. After that, to stop the digestion 1.24µl of EDTA was added. (Thermo scientific Hhal #ER1851 2000u Lot: 00112662, 2011**).** Electrophoresis was implemented on 4% agarose gel along with 6×Orange DNA loading dye. O'RangeRuler 10 bp DNA Ladder (Thermo Scientific) had been used for identifying restricted fragments. The results were documented by gel documentation system photography. (Invitrogen Ultra Pure™. Agarose). (Figure 2)

Statistical analysis:

Data was analyzed by using Statistical Package for the Social Sciences (SPSS Version 21.0). Descriptive statistics for continuous variables were presented as medians with interquartile ranges (non-parametric) as appropriate, while frequencies and percentages were reported for all categorical characteristics. The comparison of continuous characteristics like age, BMI, total cholesterol, TG, LDL cholesterol and high-density lipoprotein (HDL) cholesterol was assessed between both groups by using the Mann-Whitney U test. Pearson chi-square test was used to evaluate the association of categorical variables between both groups. Kruskal–Wallis test was performed to compare the influence of Ape E gene polymorphism on lipid profile. A value of $p<0.05$ was believed to be statistically significant.

3. Results

A total of 100 patients, 50 calcific AS cases while 50 patients in the control group were taken. The median age of the calcific AS patients was 66(IQR: 65-70; Range: 60-85) years while median age of the control group was 65(IQR: 64-68; Range: 63-80) years ($p>0.05$). There was a significant difference in TG level according to case and control groups ($p<0.05$) while no significant differences were observed in other lipid profile parameters like TC, HDL and LDL between both groups ($p>0.05$).

The prevalence of smokers, HTN and DM was significantly higher in AS patients as compared to controls while the prevalence of CAD was significantly higher in controls as compared to cases ($p<0.05$). Out of 50 AS patients, 18% had mild AS, 22% moderate AS and 60% had severe AS. To examine the association between Apo E genotypes with cases, the individuals are allocated into three groups ApoE2 (ε2/ε3 individuals), ApoE3 (ε3/ε3 subjects) ApoE4 (ε3/ε4 and ε4/ε4 persons) groups while ε2/ε2 and ε2/ε4 genotypes were not identified. The prevalence of Apo E ε2, ε3 and ε4's alleles were 16%, 52%, 32% in calcific AS and 10%, 52%, and 28% in control ($p>0.05$). The detailed comparison of patient characteristics concerning both groups is summarized in Table 1.

Table 1 Comparison of patient's characteristics between calcific AS cases and controls

Characteristics	**Cases**	**Controls**	**p-value**
	(n=50)	**(n=50)**	
	Median (IQR)	**Median (IQR)**	
Age (years)	66.0 (65.0-70.0)	65.0 (64.0-68.0)	0.843
BMI (kg/m2)	22.0 (20.4-26.7)	21.6 (19.1-23.6)	0.182
TG (mg/dl)	114.5 (98.0-114.7)	140.0 (106.5-163.7)	0.013*
Total cholesterol (mg/dl)	160.0 (123.5-194.0)	151.5 (133.5-200.2)	0.866
HDL Cholesterol (mg/dl)	38.5 (33.8-45.0)	40.0 (32.0-48.3)	0.720
LDL Cholesterol (mg/dl)	93.0 (79.5-100.0)	87.0 (70.5-98.5)	0.229
	n (%)	**n (%)**	
Gender			
Male	31 (62)	33 (66)	0.419
Female	19 (38)	17 (34)	
Smokers			
Yes	20 (40)	10 (20)	
No	30 (60)	40 (80)	0.016*
HTN			

Yes	32 (64)	20(40)	0.017*
No	18 (36)	30(60)	
DM			
Yes	32 (64)	17 (34)	0.003*
No	18 (36)	33 (66)	
CAD			
Yes	17 (34)	31 (62)	0.005*
No	33 (66)	19 (38)	
Apo E allele			
e2	8 (16)	5 (10)	
e3	26 (52)	26 (52)	0.622
e4	16 (32)	19 (28)	
* p-value calculated by using Mann-Whitney U test and Chi-square test			

Where **n:** Numbers, **%:** Percent

5. Discussion

Apo E gene has an important role in metabolizing lipid accumulated in the liver. [11] Dyslipidemia is among the overlapping risk factors shared by calcific aortic stenosis and atherosclerosis. [8, 12] The role of Apo-E gene polymorphism in altered lipid levels is well recognized in many researches,[13] by considering this, Apo E gene was selected as a candidate gene for this research to observe the direct association of Apo E polymorphism to dyslipidemia and calcific AS. In our study we found the calcific AS patient's age ranges between 60-85 years, this shows that it's a progressive disease and its severity increases as the age advances. [5] In contrariety to other research steered in the Western world the minimum age for calcific AS is 65 years. [14]

Prevalence of calcific AS occurs comparatively at an early age in our study may be because most of the AS patients belong to a low socioeconomic background from a developing country. [15]The conducted research shows that 62% of the male population had calcific AS in contrast only 38% of the female population had the disease. This result was in agreement with the study showing similar results, demonstrating male gender is one of the risk factors for calcific AS. [16] Mean BMI in both cases and controls showed an insignificant difference in our study this result differs from the results of other studies conducted previously; this may be in consequence of that the older population from a developing country with a low socioeconomic status is represented in the data.

Mean total cholesterol, LDL cholesterol and HDL cholesterol did not show any significant difference and TG was high in the control group, which may be for the reason that the control group included CAD patients. The decline in the level of HDL-cholesterol may be linked with calcific AS and its advancement, as a histological study displayed HDL-cholesterol deposition in the aortic stenosed valve in the early stage of stenotic changes. [17] HDL-cholesterol also have antioxidant, anti-inflammatory and anti-atherogenic effect. It helps lower inflammation and apoptotic changes in cells. Lowering HDL-cholesterol levels reduces its protective effects. [18]

Comorbid like smoking and hypertension showed a positive association with AS in our study. Smoking is a well-recognized risk factor in the progression of AS, it may cause cellular oxidative stress by inhibiting nitric oxide [19] elevating the plasma lipoprotein (a) and decreasing HDL- cholesterol. [20] HTN is related to the exaggeration of the degeneration in aortic valve stenosis and sclerosis, owing to the reason that they share a common pathophysiological pathway. [21] In this research there were more CAD patients in the control group. The reason may be that we did not exclude CAD cases from the control group as it is a hospital-based study and CAD and AS has few of the similar risk factors and pathophysiology. [22]

Apo-E is a distinctive candidate gene in cholesterol metabolism. Novaro 2003 identified a significant association between apo-E alleles polymorphism and AS.[8] Ortlepp 2006 however did not attain any relationship of calcific AS to Apo-E polymorphism. [23] These contrary results urged us to propose our study to reassure that polymorphism of Apo E gene is one of the genetic causes of calcific AS. The study conducted by us showed insignificant relationship between Apo E polymorphism with our cases.

On the contrary Apo E gene is remarkable candidate for dyslipidemia [5] and its association with altered lipid profile was significant in our study.

Table-2 Comparison of lipid profile and apo E allele in cases and controls

Lipid profile	**Apo E allele**	**Cases**	**Controls**	**p-value**
		Median (IQR)	**Median (IQR)**	
TG (mg/dl)				
	e2	82.5 (71.0-125.5) ##	135.0 (117.0-155.5)	0.056
	e3	105.5 (98.0-117.5) ###	122.5 (93.0-143.0) ###	0.305
	e4	149.5 (113.5-164.7) ##, ###	181.0 (141.0-248.0)###	0.104
	p-value	0.001*	<0.001*	
TC(mg/dl)				
	e2	121.0 (113.7-141.5) ##	126.0 (116.5-210.5) ##	0.373
	e3	149.5 (122.5-173.5) ###	143.0 (133.5-163.5)	0.545
	e4	207.5 (162.2-226.5) ##, ###	200.0 (151.0-230.0) ##	0.654
	p-value	<0.001*	0.007*	
HDL-C (mg/dl)				
	e2	42.7 (27.7-48.1)	44.3 (32.3-47.5)	0.825
	e3	40.4 (33.7-45.1)	41.4 (34.2-50.4)	0.582
	e4	35.3 (33.8-40.0)	39.0 (29.0-45.0)	0.881
	p-value	0.60	0.342	
LDL-C (mg/dl)				
	e2	76.5 (59.2-91.5) ##	88.0 (64.5-122.5) ##	0.622
	e3	92.5 (79.0-98.2) ###	79.5 (67.2-94.0)	0.058
	e4	98.0 (90.5-142.2) ##, ###	98.0 (75.0-115.0) ##	0.461
	p-value	0.030*	0.048*	
Where IQR: Inter quartile range (Q1-Q3) *p-value calculated by using Kruskal–Walli's test and post-hoc by Mann-Whitney U test For post hoc test, the # depicts the e2 compare with e3 and the ## depicts e2 compare with e4, and the ###depicts e3 compare with e4.				

Table 2 depicts two types of comparison; (1) lipid levels according to Apo E genotypes in cases and control separately (2) lipid levels according to Apo E genotypes in cases and controls. The TG, TC and LDL-C of calcific AS group showed significant difference according to Apo E alleles ($p<0.05$) while each Apo E allele was evaluated separately in patients and controls, no significant differences were found in lipid profiles paramters ($p>0.05$).

We found remarkable high median level of TG, total cholesterol and LDL cholesterol in Apo E 4 gene as compared to other alleles in both cases and control. These findings showed that the presence of the apo E4 allele suggests a greater risk of dyslipidemia.

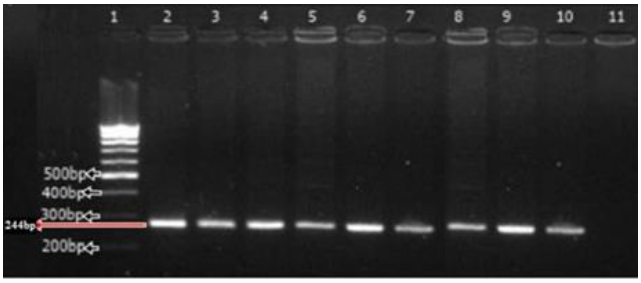

Figure-1 Gel photograph of Apo E amplified gene. 2% agarose gel showing a 244 bp amplified product of Apo E gene. Lane 1 =100 bp ladder, lane 2,3,4,5,6,7,8,9 and 10 = Undigested amplified apo E gene, lane 11= - ve control (without genomic DNA)

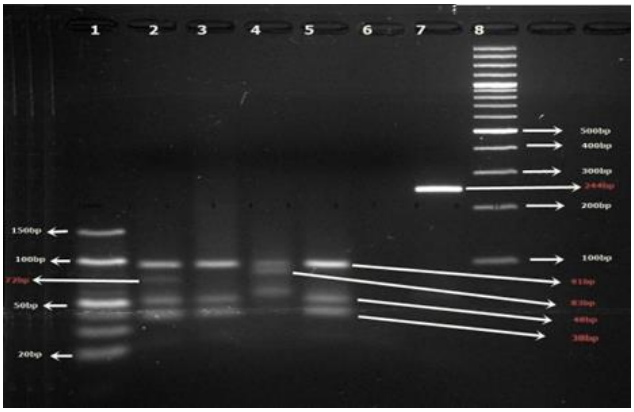

Figure-2 Gel photograph of Apo E gene. 4% agarose gel showing Lane 1= 10bp ladder, lane 2= HhaI digested amplified Apo E gene E3/E4 genotype, lane 3and 5= E3/E3 genotype, lane 4=E2/E3 genotype, lane 6= Negative control, lane 7= Undigested amplified apo E gene, Lane 8= 100bp ladder.

It is in agreement with the study which witnessed E4 allele is more common in hyperlipidemic patients, [24] this is because Apo-E variants affects triglyceride rich lipoproteins metabolic pathways. It increases total cholesterol, LDL cholesterol and remnant cholesterol levels. LDL cholesterol is increased because of downregulation of expressed LDL receptors in liver. ApoE4 isoform effects LDL and VLDL particle sizes and their composition increasing their concentrations of TG and free/esterified and total cholesterol along with phospholipids. [25] There are few limitations to present study as it had small sample size. It had higher frequency of coronary artery disease in our both study groups as it was a hospital-based study in which we selected only echocardiographically diagnosed calcific AS cases, and controls were also selected on the bases of echocardiography, who did not have any valvular disease. The strength of this research was that we excluded familial hypercholesterolemia patients, as well as patients having history of rheumatic fever. As they would have been a confounding factor due to development of valvular heart disease as a complication, since rheumatic fever is highly prevalent in developing country like Pakistan. End point PCR assay and RFLP for Apo E polymorphism was presented and standardized as a sensitive method for detection of said gene polymorphism.

5. Conclusion

The conclusive findings of this study shows that Apo-E polymorphism is not related to calcific AS, this is in contrary to its relationship with CAD, this fact could potentially lead to distinct genetic background. Where as BMI, HTN and smoking had significant impact on calcific AS. Furthermore, it was evident from this study that, Apo E polymorphism was considerably coexisting with dyslipidemia. People having Apo E 4 allele possessed more chances of having altered lipid profile. This fact could potentially lead to a redirection of diverse genetic backgrounds.
This fact could potentially influence diverse genetic backgrounds in a different direction.

CONFLICTS OF INTEREST- None

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Contributions:
E.A - Conception of study
E.A, A.I.K - Experimentation/Study Conduction
E.A, H.F.W - Analysis/Interpretation/Discussion
E.A, M.K.N, M.I.A.Y - Manuscript Writing
M.K.N, M.S, M.I.A.Y - Critical Review
A.I.K - Facilitation and Material analysis

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