



Original Article

Analysis of Genetic Variants of *ANGPTL4* Gene Responsible for Atherosclerosis Severity in Cardiac PatientsKainaat Zafar¹, Amina Shahid¹, Saba Anam¹, Zawar Hussain², Muhammad Saqib Shahzad³, Muhammad Khalil Ahmad Khan⁴, Akram Tariq⁵ and Imrana Tanvir⁶¹Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan²Department of Zoology, University of Education, Lahore, Pakistan³Department of Biology, Government Graduate College, Lahore, Pakistan⁴Department of Zoology, University of Okara, Okara, Pakistan⁵Department of Biology, Higher Education Department, Lahore, Pakistan⁶Department of Pathology, Faculty of Medicine, King Abdul Aziz University, Rabigh, Saudi Arabia

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ABSTRACT

ANGPTL4 gene is a major factor in the onset of atherosclerosis and exacerbation of its severity. *ANGPTL4* regulates lipoprotein lipase (LPL), but its inhibitory effect causes decreased triglyceride clearance. The E40K mutation reduces *ANGPTL4* oligomer formation, reducing LPL activity suppression. **Objectives:** To correlate *ANGPTL4* N-terminal chain variations with atherosclerotic cardiovascular disease severity in Pakistani individuals, enabling diagnosis, treatment, and prevention. **Methods:** A case control study was conducted at Surgimed Hospital, Lahore on 100 Pakistani cardiovascular patients and 50 healthy control subjects. The N-terminal chain of the *ANGPTL4* gene was sequenced revealing 14 individuals (9.33%) were heterozygous carriers of the *ANGPTL4* gene variant (rs116843064; G>A, E40K) in our population (n=150). **Results:** Among the participants, four (2.67%) individuals had severe atherosclerosis with heterozygous genotype (GA), eight (5.33%) had mild atherosclerosis with heterozygous genotype (GA), and two were healthy controls (1.33%) with heterozygous genotype (GA). This study showed the significant association of E40K variant of N-terminal chain of *ANGPTL4* with less likely chance of severe atherosclerosis in our cardiovascular patients. The E40K alters the regulation of lipoprotein lipase, affecting lipid levels and impacting cardiovascular health. **Conclusions:** E40K mutation carriers exhibit a lower risk of severe atherosclerosis in cardiovascular patients due to better lipid profiles as HDL levels were lower in non-carriers and higher in carriers.

INTRODUCTION

Cardiovascular Diseases CVD stand as the primary global cause of mortality and a significant factor in healthcare spending. CVDs are identified to be the main cause of death worldwide, taking toll of mortality up to 17.7 million per year [1-3]. Atherosclerosis is a condition caused by the buildup of cholesterol and low-density lipoproteins (LDL) in the inner walls of blood arteries that thickens and reduces the flexibility of the walls which can make it more difficult for blood to reach organs. In utmost conditions, blood vessels may get completely blocked [4, 5]. Risk factors for the emergence and progression of cardiovascular diseases

may include factors like age, hypertension, obesity, smoking, dyslipidemia and diabetes mellitus Effects and associations of these risk factors in individuals may vary depending on the age and particular genetic structure. That is the very reason due to which specific individuals have greater chances of becoming victim of cardiac diseases [6]. As per findings of different studies, it is concluded that not only traditional but genetic factors also play a vital role in the development of atherosclerosis. A wide range of genes including *ANGPTL4*, *APOB*, *CETP*, *LDLR*, *LIPC*, and *LPL* are directly involved in progression



and extremity of atherosclerosis [7]. Another research reveals that in humans, rare loss-of-function mutations found in the *ANGPTL3* and *ANGPTL4* genes are directly linked with low levels of triglyceride levels. Furthermore, LPL gene mutations are associated with high levels of triglycerides [8]. *ANGPTL4* gene is found on chromosome 19p 13.3, in humans. It has six introns and seven exons in it and encodes for 406-amino-acid glycoprotein. *ANGPTL4* is known to encode glycosylated protein with a coiled-coil N-terminal domain and a fibrinogen-like C-terminal domain. At specific times and in specific tissues, *ANGPTL4*, *ANGPTL3*, and *ANGPTL8* act as significant regulators for LPL to control LPL activity and partitioning of lipid [9]. Catalyzing the breakdown of triglycerides (TG) into glycerol and fatty acids (FAs) is one of the major roles that is played by LPL [10]. It is considered that atherosclerosis gets prevented by this enzymatic nature of LPL. Contrarily, LPL's function is inhibited by *ANGPTL4* that ultimately reduces the number of circulating triglycerides. This inhibition from *ANGPTL4*'s end results in formation of a chronic inflammatory response that is directly linked to atherosclerosis [11]. As per revelations of a population-based study, low levels of triglycerides and high levels of HDL cholesterol are linked directly with E40K, one of the *ANGPTL4*'s mutation [12]. By interrupting the stability of oligomers, this mutation acts directly on *ANGPTL4*'s ability to inhibit LPL. Due to this phenomenon, carriers (either homozygotes or heterozygotes) have better metabolic conditions, such as lower plasma triglyceride levels and greater HDL-C levels than non-carriers. Moreover, another conclusion has also been made that individuals that carry E40K mutation in their *ANGPTL4* gene have lower chances of getting heart related disease [13]. So, N-terminal chain of *ANGPTL4* has this mutation called E40K that may directly have impact on development of atherosclerosis [14].

Atherosclerosis remains a leading cause of cardiovascular morbidity and mortality, with both environmental and genetic determinants influencing disease severity. Although the *ANGPTL4* E40K (rs116843064) variant has been associated with favorable lipid profiles in global populations, there is limited evidence regarding its

distribution and clinical significance in the Pakistani population. Moreover, existing studies have not sufficiently explored its direct relationship with severity levels of atherosclerosis (mild vs severe). The main purpose of this study is to analyze the genetic mutations in N-terminal chain of *ANGPTL4* in Pakistani patients with atherosclerotic cardiovascular disease.

METHODS

A case-control study was conducted from March 2023 to May 2023 to analyze the genetic variants of N-terminal chain of *ANGPTL4* in cardiovascular patients. RaoSoft Sample size calculator was used for calculating the sample size keeping the margin of error as 5% and confidence interval as 95%. A collective of 100 patients from a total of 150 with atherosclerotic cardiovascular conditions as well as age-matched healthy control subjects were thus collected in K3 EDTA vials from Surgimed Hospital Lahore, Punjab. Control samples were also picked from individuals who went to same hospital (Surgimed Hospital, Lahore) and had no history of cardiovascular disease, diabetes or any other major illness and had been for routine checkups. Controls were matched to the patients in terms of age and sex of the patients. Blood samples and data of CVD patients were collected by using a proforma (Annexure 1). A written and verbal consent from the participants and an approval (Reg No: IMBB/BBBC/23.050) from the Institutional Review Board was taken prior to the study. Blood samples were also evaluated for biochemical parameters such as lipid profile including Cholesterol, Triglyceride, HDL, LDL, VLDL and Cholesterol/ HDL Cholesterol ratio biochemistry analyzer as per manufacturer's instructions (CHOLESTECH LDX™ ANALYZER, Abbott, IL, USA). The DNA was extracted from the fresh blood samples by using the DNA extraction kit (QIAamp DNA Blood Mini Kit) as per the manufacturer's instructions. For silico analysis The *ANGPTL4* gene sequence was retrieved from GenBank database of NCBI. Primer3 software was used to design primers for the N terminal chain of the *ANGPTL4* gene (<http://frodo.wi.mit.edu>). The sequences of primers along with their melting temperature (TM) and GC content are given in Table 1.

Table 1: PCR Primers Used for Amplification of N-Terminal Chain of *ANGPTL4* Gene.

Gene name	Primer Name	Maximum	Tm (°C)	GC content
ANGPTL4	F1	ATTCTTTCCAGCGCCTTCTG	61.8	50
	R1	TGCGCCAGGACATTCATCTC	60.5	55
	R2	TGCGCCAGGACATTCATCTT	58.4	50

F1 = Forward Primer for N-terminal Chain of *ANGPTL4* gene R1 = Reverse Primer 1 (having C at 3' end) for binding with Allele G R2 = Reverse Primer 2 (having T at 3' end) for binding with Allele A

The N-terminal chain of *ANGPTL4* gene was amplified from genomic DNA by using primer F1 and R1 and PowerPol 2X PCR mix with dye (RK20719) as per manufacturer's instruction (ABclonal, Inc. MA, USA). For amplification of the N-terminal chain, a reaction mixture comprising 10 ng

of genomic DNA, 0.8µM of each oligonucleotide primer, and a 1X PCR mix was used, resulting in a total volume of 25µl. The PCR cycling parameters were as follows: an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles consisting of denaturation at 95°C for 30 seconds,

annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds. This was followed by a final extension step at 72°C for 2 minutes before holding at 72°C. The purified DNA samples isolated from the blood samples of CVD patients were electrophoresed on 0.8% of agarose gel for quality check of DNA. Moreover, the amplified PCR products of N-terminal chain of ANGPTL 4 were analyzed on 2 % agarose gel to confirm the fragment length in base-pair (bp) and specificity of N-terminal chain product. The 100bp DNA marker (ZOKEYO) was used as a size standard to validate the fragment length of PCR product (264bp). The prominent bands of PCR product of N-terminal chain of ANGPTL 4 were observed at their respective size on agarose gel, when visualized in UV gel documentation system. The purified products were subjected to sequencing using forward primers, following the manufacturer's instructions from Applied Biosystems' Big Dye Sequencing Kit. DNA sequencing was conducted through the Sanger sequencing method using an ABI PRISM 3100 Automated sequencer (Applied Biosystems), with sequencing services provided by Macrogen, Inc. Subsequently, the sequencing results were compiled using ABI PRISM sequencing analysis software version 3.7 (Applied Biosystems), and chromatograms were examined using Chromas software, accessible at www.technelysium.com.au/chromas.html. Following the sequencing process, chromatograms were scrutinized using Chromas version 2.5.1 and Chromas pro, available at (<http://technelysium.com.au/wp/chromas/>). Multiple alignments were carried out on the sequences extracted from atherosclerosis patients (CVD) and individuals with normal gene sequences obtained from Ensemble. Variants were detected by analyzing the multiple aligned sequences and confirmed by manual inspection of the sequencing chromatograms.

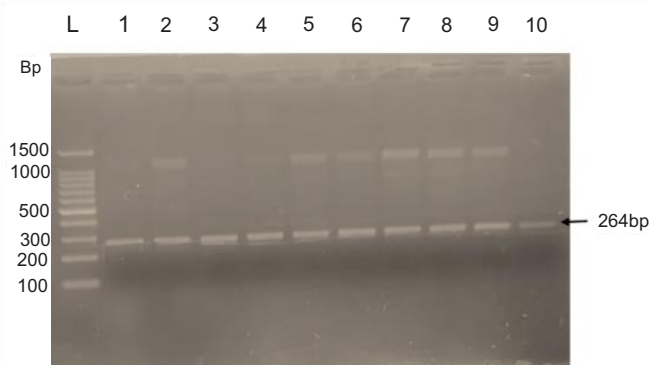


Figure 1: Agarose Gel (2%) Showing the Amplified PCR Products (264bp) from DNA Samples of Cardiovascular Patients; Lane L: 100bp DNA Ladder (SOLIS BIODYNE), 1-10: Amplified PCR Product of Samples(1-10)

RESULTS

The study comprised 100 male and female cardiovascular patients with atherosclerosis, who were visiting Surgimed Hospital, Lahore from various regions of Pakistan. Additionally, 50 healthy control subjects of the same age

group were randomly selected and included. DNA was extracted from the blood samples to sequence variants in N-terminal chain of ANGPTL4 gene responsible for severity in atherosclerosis in Pakistani patients. The extracted DNA was electrophoresed on 1% agarose gel to check the quality of DNA as shown in gel image as an example for 5 samples. The N-terminal chain of ANGPTL4 gene of patients and the control subjects' samples were subjected to amplification using specific primers. The PCR samples were examined through agarose gel electrophoresis (2%) to confirm the specific amplification. The gel image in figure 1 shows the amplified PCR product size (264bp) of N-terminal chain of ANGPTL4 gene. The amplified PCR products of N-terminal chain of ANGPTL4 gene from all cardiovascular patients and control subjects were sequenced by utilizing Sanger sequencing method. The sequencing chromatograms for selected patients (having G or A allele) and control subjects are shown as an example in Figure 2. The following sequencing chromatogram is showing two variants (variant G & A of rs116843064) found in N-terminal chain of ANGPTL4 gene of Pakistani cardiovascular patients.

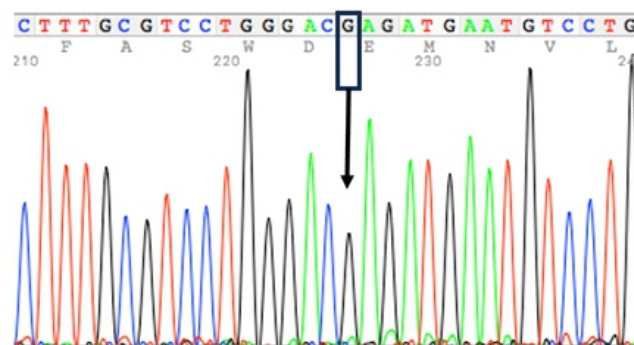


Figure 2(A): Selected Chromatogram of PCR Product From N-Terminal Chain of ANGPTL4 Gene Amplified of Sample CAD-06 (Variant G of Rs116843064)

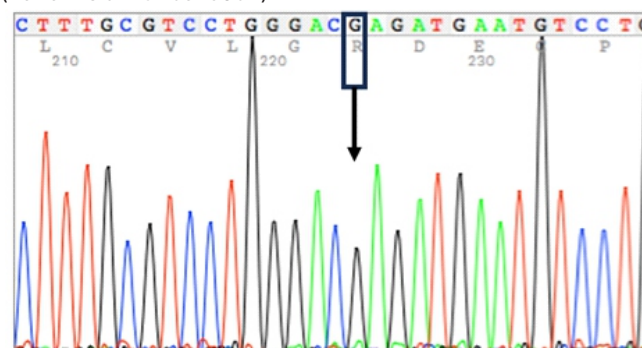


Figure 2(B): Selected Chromatogram of PCR Product From N-Terminal Chain of ANGPTL4 Gene Amplified of Sample CAD-17 (Variant G of Rs116843064)

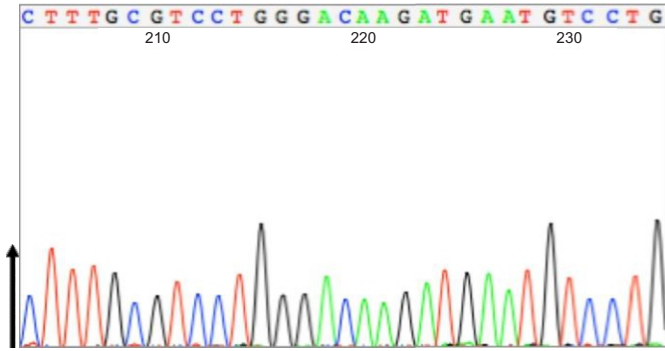


Figure 2(D): Selected Chromatogram of PCR Product From N-Terminal Chain of *ANGPTL4* Gene Amplified of Sample CAD-10 (Variant A Of Rs116843064)

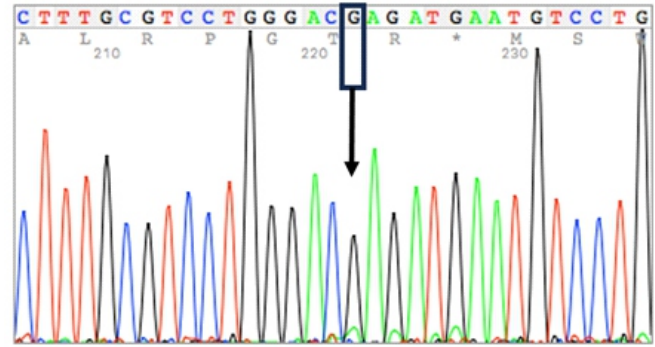


Figure 2(C): Selected Chromatogram of PCR Product From N-Terminal Chain of *ANGPTL4* Gene Amplified of Sample CAD-29 (Variant G Of Rs116843064)

The genotypes of N-terminal chain of *ANGPTL4* gene in 100 atherosclerotic cardiovascular patients and 50 normal healthy control subjects in Pakistani population were observed. Fourteen individuals (14) were heterozygous carriers of variant (rs116843064; G>A) in N-terminal chain of *ANGPTL4* gene out of total 150 individuals, including four individuals having severe atherosclerosis (2.67%), eight individuals having mild atherosclerosis (5.33%), and two healthy control subjects (1.33%). A list of genotypes of all cases and control samples for variant (rs116843064) found in N-terminal chain of *ANGPTL4* gene are shown (Table 2).

Table 2: List Of Genotypes for Variant Rs116843064 of N-Terminal Chain of *ANGPTL4* Gene Found in Atherosclerotic Cardiovascular Patients and Control Subjects from Pakistan.

Sample Ids	Controls		Sample Ids	Mild Atherosclerosis		Sample Ids	Severe Atherosclerosis	
	Genotypes			Genotypes			Genotypes	
CAD-04	G	A	CAD-01	G	G	CAD-03	G	G
CAD-06	G	G	CAD-02	G	G	CAD-10	G	A
CAD-13	G	G	CAD-05	G	G	CAD-15	G	G
CAD-16	G	G	CAD-07	G	A	CAD-18	G	G
CAD-19	G	G	CAD-08	G	G	CAD-23	G	G
CAD-22	G	G	CAD-09	G	G	CAD-28	G	G
CAD-24	G	G	CAD-12	G	G	CAD-29	G	G
CAD-25	G	G	CAD-14	G	G	CAD-33	G	G
CAD-27	G	G	CAD-17	G	G	CAD-34	G	G
CAD-37	G	G	CAD-32	G	A	CAD-39	G	A
CAD-38	G	G	CAD-35	G	G	CAD-40	G	G
CAD-45	G	G	CAD-36	G	G	CAD-41	G	G
CAD-47	G	G	CAD-43	G	G	CAD-42	G	G
CAD-48	G	G	CAD-46	G	G	CAD-44	G	G
CAD-50	G	G	CAD-51	G	G	CAD-49	G	G
CAD-59	G	G	CAD-52	G	G	CAD-54	G	G
CAD-60	G	G	CAD-53	G	G	CAD-55	G	G
CAD-61	G	G	CAD-58	G	A	CAD-56	G	G
CAD-62	G	G	CAD-66	G	G	CAD-57	G	G
CAD-63	G	G	CAD-67	G	G	CAD-68	G	G
CAD-64	G	G	CAD-71	G	G	CAD-69	G	G
CAD-65	G	G	CAD-72	G	G	CAD-70	G	G
CAD-79	G	G	CAD-74	G	A	CAD-73	G	G
CAD-80	G	G	CAD-77	G	G	CAD-75	G	G
CAD-81	G	G	CAD-78	G	G	CAD-76	G	G
CAD-86	G	G	CAD-82	G	G	CAD-83	G	G
CAD-87	G	G	CAD-85	G	G	CAD-84	G	G
CAD-88	G	G	CAD-96	G	A	CAD-100	G	G
CAD-89	G	G	CAD-97	G	G	CAD-101	G	G

CAD-90	G	G	CAD-98	G	G	CAD-103	G	A
CAD-91	G	G	CAD-99	G	G	CAD-104	G	G
CAD-92	G	G	CAD-102	G	G	CAD-105	G	G
CAD-93	G	G	CAD-107	G	G	CAD-106	G	G
CAD-94	G	G	CAD-108	G	A	CAD-109	G	G
CAD-95	G	G	CAD-110	G	G	CAD-111	G	G
CAD-112	G	G	CAD-113	G	G	CAD-114	G	G
CAD-117	G	G	CAD-116	G	G	CAD-115	G	G
CAD-118	G	G	CAD-124	G	G	CAD-125	G	G
CAD-119	G	G	CAD-126	G	G	CAD-129	G	G
CAD-120	G	G	CAD-127	G	G	CAD-130	G	G
CAD-121	G	G	CAD-128	G	G	CAD-131	G	G
CAD-122	G	G	CAD-132	G	G	CAD-133	G	G
CAD-123	G	A	CAD-135	G	G	CAD-134	G	A
CAD-136	G	G	CAD-11	G	A	CAD-143	G	G
CAD-137	G	G	CAD-150	G	G	CAD-144	G	G
CAD-138	G	G	CAD-151	G	G	CAD-145	G	A
CAD-139	G	G	CAD-152	G	G	CAD-146	G	G
CAD-140	G	G	CAD-153	G	G	CAD-147	G	G
CAD-141	G	G	CAD-154	G	A	CAD-148	G	G
CAD-142	G	G	CAD-155	G	G	CAD-149	G	G

The angiographic images determined severe, mild and low atherosclerosis based on stenosis levels with $\geq 70\%$ stenosis classifying as severe while 30-69% stenosis was mild and $< 30\%$ stenosis or no plaque buildup indicated low severity. Chi-square analysis was conducted for SNP rs116843064 genotypes in both atherosclerosis patients and control groups using the online software SHEsis. The genotypes of a total of 50 samples of cardiac patients with severe atherosclerosis along with 50 healthy control subjects were utilized for calculation of p-values. Similarly, the genotypes of a total of 50 samples of cardiac patients with mild atherosclerosis along with 50 healthy control subjects were also utilized for calculation of P-values separately. p-values for the variant rs116843064 and its association with Atherosclerosis severity is shown in Table 2. Carriers of the E40K variant (A of rs116843064 of *ANGPTL4*) significantly showed a less likely chance of atherosclerosis severity than non-carriers (Pearson's p-value=0.045518).

Table 3: Association of variant (rs116843064) of N-terminal chain of *ANGPTL4* with Extent of Atherosclerosis in Cardiovascular Patients

Gene	rs116843064 Genotypes	Frequency (Cases)	Frequency (Controls)	95% CI	Fisher's p-value	Pearson's p-value
<i>ANGPTL4</i> Mild Atherosclerosis	A	0.080	0.020	[0.919362~22.730947]	0.04555	0.04551
	G	0.920	0.980			
<i>ANGPTL4</i> Severe Atherosclerosis	A	0.040	0.020	[0.364506~11.948753]	0.39974	0.39970
	G	0.960	0.980			

The lipid parameters of randomly selected male and female atherosclerotic cardiovascular patients and healthy controls are shown in Table 4 (Severe Atherosclerosis), 5 (Mild Atherosclerosis) and 6 (Healthy Controls Subjects). Mean of circulating triglyceride levels were 200.3 mg/dl in severe atherosclerotic patients, 160.7 mg/dl in patients with mild atherosclerosis. However, the mean of triglycerides in healthy control subjects was 148.9 mg/dl. The patients with severe atherosclerosis showed increased levels of VLDL in both severe and mild cases, mean of which was 41mg/dl and 36.7 mg/dl respectively, in comparison to healthy control subjects with mean value of 22.5 mg/dl. The decreased HDL level was also observed in both severe and mild patients (mean values; 40.2 mg/dl and 42.3 mg/dl respectively). While healthy control subjects showed HDL levels in normal range with mean value of 83.7 mg/dl.

Table 4: The Lipid Profiles of Randomly Selected Male and Female Cardiovascular Patients Having Severe Atherosclerosis

Sample Ids	Total Cholesterol (mg/dl)	Total Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (Cholesterol) (mg/dl)	Cholesterol/ HDL Cholesterol ratio	Age (years)
Normal	< 200*	< 150*	> 60*	< 100*	< 30*	3.5 to 5.0*	25 to 80*
CAD-03	194	236	31	121	42	6.26	35
CAD-10	212	182	33	104	44	6.45	47

CAD-15	230	190	37	114	33	6.23	43
CAD-18	214	174	39	102	48	5.50	28
CAD-23	244	222	40	98	39	6.12	39
CAD-28	239	187	43	110	47	5.57	70
CAD-34	213	192	48	102	42	5.09	65
CAD-39	237	214	44	110	41	5.39	51
CAD-44	219	196	36	111	40	6.11	54
CAD-49	240	210	51	93	34	4.70	72
Mean	224.2	200.3	40.2	106.5	41	5.74	50.4

HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, VLDL: Very Low-Density Lipoprotein

Table 5: The Lipid Profiles of Randomly Selected Male and Female Cardiovascular Patients Having Mild Atherosclerosis

Sample Ids	Total Cholesterol (mg/dl)	Total Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (Cholesterol) (mg/dl)	Cholesterol/ HDL Cholesterol ratio	Age (years)
Normal	< 200*	< 150*	> 60*	< 100*	< 30*	3.5 to 5.0*	25 to 80*
CAD-01	209	158	41	107	40	5.11	60
CAD-02	129	169	51	45	31	2.53	53
CAD-05	218	159	38	112	35	5.75	42
CAD-07	215	155	40	109	39	5.39	39
CAD-08	195	170	39	117	48	5.01	35
CAD-09	239	164	49	102	19	4.88	51
CAD-14	220	157	41	114	44	5.37	69
CAD-17	237	168	42	103	32	5.65	47
CAD-30	219	152	40	114	38	5.47	54
CAD-46	210	155	42	108	41	5.00	62
Mean	209.1	160.7	42.3	103.1	36.7	5.016	51.2

HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, VLDL: Very Low-Density Lipoprotein

Table 6: The Lipid Profiles of Randomly Selected Male and Female Healthy Control Subjects

Sample Ids	Total Cholesterol (mg/dl)	Total Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (Cholesterol) (mg/dl)	Cholesterol/ HDL Cholesterol ratio	Age (years)
Normal	< 200*	< 150*	< 60*	< 100*	< 30*	3.5 to 5.0*	25 to 80*
CAD-04	116	125	44	51	21	2.64	44
CAD-13	88	106	49	20	19	1.8	53
CAD-19	170	105	55	87	18	3.09	66
CAD-22	183	174	47	102	22	3.89	29
CAD-25	162	124	52	86	22	3.12	48
CAD-27	169	91	42	110	16	4.02	59
CAD-38	129	169	51	45	31	2.53	71
CAD-47	181	154	40	110	31	4.5	41
CAD-45	131	141	44	77	19	2.98	55
CAD-50	160	98	52	61	26	3.07	47
Mean	148.9	128.7	83.7	74.9	22.5	3.164	51.2

HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, VLDL: Very Low-Density Lipoprotein

The circulating triglycerides and VLDL levels were significantly lower in mutated carriers (SNP rs116843064; G>A, E40K) as compared with noncarriers as shown in table 6. Meanwhile, significantly higher levels of HDL, the good cholesterol were observed in mutated carriers (SNP rs116843064; G>A, E40K) showing deviated values from the normal ones (Table 7).

Table 7: Comparative Mean values of lipid profiles of atherosclerotic cardiovascular patients (Severe Atherosclerosis (n=10), Mild Atherosclerosis(n=8)and Healthy Control Subjects(n=10)

Atherosclerosis	Total Cholesterol (mg/dl)	Total Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (Cholesterol) (mg/dl)	Cholesterol/ HDL Cholesterol ratio
Severe Atherosclerosis (Mean Lipid Profiles)	224.2	200.3*	40.2	106.5	41	5.74
Mild Atherosclerosis (Mean Lipid Profiles)	209.1	160.7	42.3	103.1	36.7	5.01
Healthy Control Subjects (Mean Lipid Profiles)	148.9	128.7	83.7	74.9	22.5	3.16
Normal Values (Ranges)	< 200	< 150	> 60	< 100	< 30	3.5 to 5.0

HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, VLDL: Very Low-Density Lipoprotein

DISCUSSION

The primary outcomes of this research involve computing allele frequencies and validating a link between the E40K variation in the N-terminal segment of *ANGPTL4* and a reduced likelihood of severe atherosclerosis in individuals with cardiovascular conditions. This study found 9.33% of heterozygous genotype (GA) of variant (rs116843064; G>A) in cardiovascular patients and control subjects of this population (n=150) i.e., 2.67% heterozygous genotype (GA) in severe atherosclerosis patients, 5.33% in mild atherosclerosis patients and 1.33% in healthy control subjects. Furthermore, this research unveiled that individual who carried the E40K variant (SNP rs116843064; G>A) in the N-terminal segment of *ANGPTL4* exhibited a 30.3% reduction in triglyceride levels and a 19.1% decrease in very-low-density lipoprotein (VLDL) levels compared to those who did not carry the variant (E40K, SNP rs116843064; G). Additionally, carriers of the E40K variant demonstrated a 2.1% increase in high-density lipoprotein (HDL) cholesterol levels. In addition to serving as components for energy preservation, fatty acids (FA) and glucose are the primary fuel sources for satisfying the body's requirements for energy. Fatty acids are carried by triglycerides, which are produced by the liver or diet and are utilized to create triglyceride rich lipoproteins (TRL) [15]. The lipoprotein lipase (LPL) enzyme aids in the hydrolysis of the circulating TG. Cardiomyocytes and adipocytes produce LPL, and the endothelium-derived protein GPIGBPI helps it adhere to the capillary surface [16]. It has been already proven that increased LPL levels are due to truncating mutations that are very much linked with and a lower risk of cardiovascular disease and the lower triglyceride levels. On the contrary, mutations with decreased LPL levels become cause of increased triglycerides levels. In humans, at start *ANGPTL4* gene was known as fasting induced adipose factor (FIAF) and is situated on chromosome 19p13.3. *ANGPTL4* is helpful in controlling LPL production. The angiopoietin-like 4

proteins, which is produced by the gene *ANGPTL4*, inhibits endothelial-bound LPL activity that resultantly impact levels of serum triglycerides [17]. *ANGPTL4* is direct controller of LPL that further controls triglyceride levels. With the help of LPL, these triglycerides (TG) and very low-density lipoproteins (VLDL) are broken down into glycerol and fatty acids (FFAs) [18]. As per related data, *ANGPTL4* prevents the elimination of circulating triglycerides by limiting LPL function, being the inhibitor of LPL. This is how this gene exerts influence on the occurrence and progression of atherosclerosis [19]. In cardiovascular patients with cardiac diseases, E40K (SNP) alters *ANGPTL4*'s capacity to inhibit lipoprotein lipase (LPL), which impacts triglyceride clearance. The single nucleotide polymorphism, E40K, alters *ANGPTL4*'s ability to inhibit lipoprotein lipase (LPL). In cardiovascular patients, LPL then further influences the clearance process of triglyceride. *ANGPTL4*'s N terminal segment has the mutation E40K that is possibly involves in occurrence of Atherosclerosis [20]. E40K mutations are associated with fluctuated levels of triglycerides and high-density lipoprotein (HDL) cholesterol, resulting in a decreased risk or severity of CAD [21]. As per the statistical data of research conducted, atherosclerotic patients have higher chances of carrying the E40K mutation as compared to the controls. Occurrence of A/G and G/G in sever atherosclerotic patients is 0.080 and 0.920, respectively. On the other hand, the ratio of prevalence in the normal subjects are 0.020 and 0.980 for alleles A/G and G/G. Allelic frequencies for mild atherosclerotic groups in A/G and G/G are 0.160 and 0.840, respectively. For control group it is 0.040 and 0.960 for A/G and G/G, respectively. It is like the findings of the study conducted for European population. E40K variation of the *ANGPTL4* gene is correspondent with suppressed levels of triglycerides, HDL cholesterol levels and VLDL as per preliminary studies [22]. According to data, people who carried the E40K variation were less likely

than non-carriers to develop coronary artery disease (CAD). Thus, it is proved that chances of occurrence of severe atherosclerosis are limited in carriers of E40K mutation of N-terminal chain of *ANGPTL4*.

This study is limited by a relatively small sample size and single-center design, which may affect the generalizability of findings. The absence of homozygous mutant (AA) genotype and lack of longitudinal follow-up further restrict causal inference. Additionally, potential confounders such as diet, lifestyle, and other genetic factors were not extensively controlled. Future studies should include larger, multi-center cohorts with diverse populations, incorporate longitudinal designs, and explore gene-gene and gene-environment interactions to better understand the role of *ANGPTL4* variants in cardiovascular disease progression.

CONCLUSIONS

This study concluded that individuals with SNP rs116843064 of E40K variant of *ANGPTL4* gene owned favorable lipid profiles that lower the risks of atherosclerosis. Fourteen individuals were heterozygous carriers of the variant E40K in the N-terminal chain of the *ANGPTL4* gene, out of 100 cardiac patients. Therefore, these observations point to the role that carriers of the E40K variant (A of rs116843064 of *ANGPTL4*) showed a less likely chance of atherosclerosis severity significantly than non-carriers. Therefore, assessing genetic connections with atherosclerosis in *ANGPTL4* gene will be a useful tool for diagnosis, treatments and prevention CVDs. It also concluded the findings that non-carriers have higher triglyceride (TG) and VLDL levels but lower HDL levels compared to carriers. So, the heterozygous genotype of the *ANGPTL4* variant (SNP rs116843064; E40K) was observed in the Pakistani population, while the homozygous genotype (A/A) was absent.

Authors' Contribution

Conceptualization: AT

Methodology: KZ, AS, SA

Formal analysis: KZ, AS, SA, ZH, MSS, MKA

Writing and Drafting: KZ, ZH, MSS, MKA, IT

Review and Editing: KZ, ZH, MSS, MKA, IT

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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