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Lipoprotein Oxidation, Genetic Expression and Polymorphism of *Apo A* and *B* Gene in Patients with Coronary Artery Disease in a Pakistani Punjabi Population

(Pengoksidaan Lipoprotein, Ekspresi Genetik dan Polimorfisme Gen *Apo A* dan *B* pada Pesakit Penyakit Arteri Koronari dalam Kalangan Penduduk Pakistani Punjabi)

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ABSTRACT

Oxidative alterations of lipoproteins and numerous polymorphism locations in the *apolipoprotein A1 (APOA1)* and *B (APOB)* genes have been identified as risk factors for cardiovascular events. The aim of the study was to investigate the gene expression and the role of *APOA* and *B* gene polymorphisms in coronary artery disease (CAD) and their relationship with lipid profiles, oxidized High Density Lipoprotein (oxHDL), and oxidized Low Density Lipoprotein (oxLDL) in the Punjabi Pakistani population. A total of 200 subjects were included, of which 135 were diagnosed as having CAD and 65 were not. The lipid profile, oxHDL, and oxLDL were measured through the calorimetric method and ELISA, respectively. Quantitative variables represented by mean ± SD for normally distributed data. Gene expression was done on cDNA through Real-time PCR. Genotyping of *APOA1* (rs670, rs5069) and *APOB* (rs693, rs11279109) were performed by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism), and gel electrophoresis was used for band visualization. Our findings showed that the oxHDL, oxLDL, *APOA*, *B* gene expression and Del genotype of *APOB* rs11279109 was significantly increased in CAD cases. In addition, significant high levels of oxHDL, oxLDL, serum cholesterol, VLDL, TG, and cholesterol/HDL ratio has been shown to be associated with CAD. *APOB* (rs11279109) has a significant impact on dyslipidemia and exhibit a higher risk of CAD (OR=3.97) while, *APOB* (rs693), *APOA1* (rs670), (rs5069) possesses a lower risk of CAD in the population. Hence, levels of oxHDL and oxLDL as well as *ApoA1* and *APOB* genotyping could be useful in identifying individuals who are at risk of CAD.

Keywords: *APOA1* gene; *APOB* gene; coronary artery disease; dyslipidemia; oxHDL; oxLDL

ABSTRAK

Perubahan oksidatif lipoprotein dan banyak lokasi polimorfisme dalam gen apolipoprotein *A1* (*APOA1*) dan *B* (*APOB*) telah dikenal pasti sebagai faktor risiko gangguan kardiovaskular. Matlamat penyelidikan ini adalah untuk mengkaji pengekspresan gen dan peranan polimorfisme gen *APOA* dan *B* dalam penyakit arteri koronari (CAD) dan hubungannya dengan profil lipid, lipoprotein ketumpatan tinggi (oxHDL) teroksida dan lipoprotein ketumpatan rendah (oxLDL) teroksida dalam kalangan penduduk Punjabi Pakistan. Sebanyak 200 subjek telah dimasukkan dalam kajian ini dengan 135 daripadanya telah didiagnosis sebagai mempunyai CAD dan 65 tidak. Profil lipid, oxHDL dan oxLDL diukur masing-masing melalui kaedah kalorimetrik dan ELISA. Pemboleh ubah kuantitatif diwakili oleh min ± SD untuk data taburan normal. Pengekspresan gen dilakukan pada cDNA melalui PCR masa nyata. Genotaip *APOA1* (rs670, rs5069) dan *APOB* (rs693, rs11279109) telah dilakukan oleh PCR-RFLP (Tindak Balas Berantai Polimerase - Polimorfisme Pemotongan Panjang Cebisan (PCR-RFLP)) dan elektroforesis gel digunakan untuk visualisasi jalur. Penemuan kami menunjukkan bahawa pengekspresan gen oxHDL, oxLDL, *APOA*, *B* dan genotip Del *APOB* rs11279109 meningkat dengan ketara dalam kes CAD. Di samping itu, paras tinggi oxHDL, oxLDL, kolesterol serum, VLDL, TG dan nisbah kolesterol/HDL yang tinggi telah terbukti dikaitkan dengan CAD. *APOB* (rs11279109) mempunyai kesan yang ketara terhadap dislipidemia dan menunjukkan risiko CAD yang lebih tinggi (OR=3.97) manakala, *APOB* (rs693), *APOA1* (rs670), (rs5069) mempunyai risiko CAD yang lebih rendah dalam populasi. Oleh itu, tahap oxHDL dan oxLDL serta genotaip *ApoA1* dan *APOB* boleh berguna dalam mengenal pasti individu yang berisiko mendapat CAD.

Kata kunci: Dislipidemia; gen *APOA1*; gen *APOB;* oxHDL; oxLDL; penyakit arteri koronari

Introduction

The most prevalent cardiovascular disease is coronary artery disease (CAD). There is growing evidence that oxidative alteration of lipoproteins is crucial to the development and progression of atherosclerosis (Itabe & Obama 2023). Oxidized low density lipoprotein (oxLDL) has been implicated in CAD progression, promoting inflammation and atherosclerosis. Oxidized high density lipoprotein (oxHDL) can lose its protective function when oxidized, exacerbating CAD severity (Chen et al. 2014). In addition to known multiple factors, genetic factors have a great role in the onset and progression of CAD. Epidemiologists have recognized for decades that 40-60% of predisposition for CAD is genetic (Roberts, Chang & Hadley 2021). The highly implicated risk factors for CAD pathogenesis are increased levels of cholesterol and low-density lipoproteins (LDL) and/or decreased levels of high-density lipoproteins (HDL). Lipids are well known to play a major role in the development of type 2 diabetes mellitus (T2DM), obesity, and hypertension, collectively known as the metabolic syndrome that subsequently could result in heart disease. Therefore, it is important to characterize genes responsible for lipid transport and metabolism including the Apolipoprotein family. Mutations in these polymorphic genes may alter the protein function thereby affecting the transport and metabolism of lipoproteins. Such variants may also interact with common risk factors leading to atherosclerosis and consequently to the manifestation of CAD (Al-Bustan et al. 2013). Two of these genes are *APOA1* and *APOB* genes.

Apolipoprotein A1 (APOA1) makes up the majority of the high-density lipoprotein (HDL) component. It is engaged in reverse cholesterol transportation (Segrest et al. 2000). Increased expression of *APOA* in the blood may be protective but numerous single nucleotide polymorphisms (SNPs) have been discovered on the long arm of chromosome 11 in the *APOA1* gene (Dawar, Gurtoo & Singh 2010). Various studies have already been done on a G to A transition of -75 base pairs (bp) and the transition at +83 bp from C to T and its association with HDL and *APOA1* levels (Casillas-Munoz et al. 2018; Hosseini-Esfahani et al. 2017).

Chylomicrons, very low-density lipoprotein (VLDL), and low-density lipoproteins (LDL) are atherogenic metabolites that contain *APOB*, which also serves as the primary ligand for hepatic LDL receptors. *APOB* contain numerous polymorphic sites (Xiao et al. 2015). The XbaI variant, also known as the rs693 single nucleotide polymorphism (SNP), is composed of a silent transition in exon 26 (ACC - ACT) (Srivastava et al. 2013). Additionally, this polymorphism has been linked in several studies (Hassan et al. 2015; Liu et al. 2014; Niu et al. 2017) to CAD, elevated APOB, Total Cholesterol (TC), triglycerides (TG) and LDL levels, and decreased HDL levels. However, various research (Bogari et al. 2015; Casillas-Munoz et al. 2018; Srivastava et al. 2013) showed no correlation between these characteristics. Rodrigues et al. (2013) noted that individuals with the T allele had a risk of dyslipidemia that was on average two times higher than that of controls, whereas homozygotes had a risk that was four times higher. It has also been noted that T homozygotes had an increased frequency of atherosclerotic plaques in their carotid arteries (Nikolajevic Starcevic et al. 2014).

The first exon contains the *APOB* Insertion/Deletion Polymorphism (rs11279109). The normal allele (Ins) and the polymorphic allele (Del) express a 27- and 24-amino acid peptide, respectively. The shorter peptide lacks the Ala-Leu-Ala segment (Xiao et al. 2015). Numerous investigations (Rebhi et al. 2008; Tsunoda et al. 2012) have shown a potential connection between the *APOB* Ins/Del polymorphism and lipid profile, making this polymorphism a candidate for the pathophysiology and etiology of dyslipidemia and CAD. According to a study by Lamia et al. (2012) on 316 patients, people with the Del-allele carrier genotype (Ins/Del + Del/Del) have greater LDL-C levels than people with the Ins/Ins genotype. Cavalli et al. (2000) on the other side, found no association between the *APOB* Ins/Del polymorphism and lipid profile.

This study is designed to investigate the gene expression and the role of *APOA1* and *B* gene polymorphism with CAD and their relationship with oxLDL, oxHDL and lipid profile in the Pakistani Punjabi population. Punjabis are well known for eating a high fat diet and are genetically predisposed to developing CAD (Hanif et al. 2022). Although previous literature shown presence of *APO* gene polymorphism among CAD (Hassan et al. 2015; Liu et al. 2014; Niu et al. 2017), however, in some studies there were no such association (Bogari et al. 2015; Casillas-Muñoz et al. 2018; Srivastava et al. 2013). To the best of our knowledge, there has no study done for oxidative alterations, gene expression, *APOA* and *B* gene polymorphism in CAD and healthy adults among native Pakistani Punjabi population.

Materials and Methods

Participants were selected from the Outpatient Department of Punjab Institute of Cardiology Hospital, Lahore, Pakistan by using non-probability convenient sampling technique. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of The Institute of Molecular Biology and Biotechnology, The University of Lahore (IMBB/UOL/21/1033). The present study involved 200 participants with 135 patients with CAD which is defined as the presence of at least one coronary artery with greater or equal to 50% stenosis. While remaining of 65 healthy non-CAD controls were also included. A questionnaire that asked about sociodemographic information, medical history and physical activity, medication, alcohol and cigarette use was completed by the participants. Both patients and controls were evaluated according to their clinical, biochemical, anthropometric, and CAD risk variables (hypertension, diabetes mellitus). CAD patients suffering from thyroid disease, rheumatoid arthritis, acute infections, unstable angina pectoris, before resuscitation of cardiopulmonary arrest, cerebrovascular accidents in the previous six months, and constant sepsis were excluded. All women included in the study were non-pregnant and non-lactating. Informed consent was obtained from all subjects involved in the study.

Biochemical Measurements After at least a 12-h overnight fast, blood samples were obtained. It was centrifuged and kept for further laboratory examination. Using the Randox Kits, the levels of TC, TG, and HDL-C were assessed. The Friedwald formula was used to determine LDL-C levels (Friedewald, Levy & Fredrickson 1972). Both oxLDL and HDL were determined through an enzyme-linked immunosorbent assay (ELISA) Bioassay laboratory kit (Cat.No E1521Hu, Cat. No E4091hu).

Genetic assessment RNA was isolated from whole blood vacutainers through GeneJET RNA Purification Kit (Cat. No. K0732). It was then converted to cDNA and expression analysis was done through Real-time PCR. The primer sequence used was F 5'-AGGGACAGAGCTGATCCTTGAACTCTTAAG-3'. Genomic DNA was extracted from peripheral leukocytes using the typical proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation. The rs670, rs5069, rs693, sp ins/del polymorphism was genotyped by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) analysis. PCR reactions were performed in a BioRad thermal cycler, using Taq polymerase (Thermo Scientific), and restriction fragments were analyzed by 2% agarose gel migration. Primers, PCR conditions, and RFLP analysis were performed according to a previously published protocol (Al-Bustan et al. 2014; Chen et al. 2009).

The restriction endonuclease MspI was used to digest the amplified PCR products overnight. For UV-light visualization, 3% agarose gel was used to digest the fragments. The presence of the MspI restriction site at -75 bp (G allele) and at +83 bp (C allele) in the 433 bp product leads to four fragments of 45, 66, 113, and 209 bp. 45, 179, and 209 bp fragments were produced because the restriction site at -75 bp (A allele) was absent. Instead of two pieces of 45 and 209 bp, a bigger fragment of 254 bp was produced (T allele). More than 10% of the samples were subjected to double sampling PCR-RFLP for quality control, and no discrepancies were discovered. This was later validated by direct automated Sanger sequencing using capillary-based ABI-3730, ABI-3130xl and ABI-3100 DNA Analyzers.

The segment of the *APOB* gene was amplified using 5'– GGAGAC-TATTCAGAAGC TAA-3' and 5' -GAAGAGCCTGAAGACTGACT-3' primers (Chen et al. 2009). The device from Applied Biosystems was used for thermocycling which started at 80 °C for 1 min and 94 °C for 2 min were used to first denaturize the DNA during amplification. This was followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 45 s, and extension at 72 °C for 50 s, before the final extension cycle at 72 °C for 5 min. Thermo Scientific›s XbaI restriction enzyme (Thermo Scientific, USA) was used to digest the PCR-generated products (amplicons) at 37 °C for 10 h. 2% agarose gel electrophoresis with direct visualization examination under UV light was used to confirm each anticipated restriction pattern (710, 433, and 277bp in T/C patients) (Chen et al. 2009). *APOB* Ins/Del polymorphism was also genotyped by PCR, followed by 3% polyacrylamide gel electrophoresis according to the previous study protocol (Al-Bustan et al. 2014).

SPSS software (SPSS 22.0 SPSS Inc., Chicago, IL). was used for analysis. Normal distribution was assessed using the Shapiro-Wilk test and mean ± SD was taken for quantitative variables. The gene counting technique was used to calculate allele frequencies. Student t tests were used to compare differences between continuous variables, whereas x2 tests were used to compare differences between categorical variables. The x2 test was

used to see whether there were any variations in genotypic frequencies between groups. Additionally, the odds ratio (OR) was calculated, confidence intervals (CI) was 95%. $p \leq 0.05$ was considered statistically significant.

Results

The mean HDL was significantly lower (35.75±3.09 mg/dl) in CAD as compared to the control (41.31±3.16 mg/dl) (p-value 0.001) (Table 1). However, there is not statistically significant difference in mean levels of Cholesterol, LDL, VLDL, and TG in all groups. OxHDL was significantly increased in cases (762.92±951.65 ng/L) as compared to controls (458.38±425.22 ng/L) in the population (p-value =0.019). The levels of oxLDL in cases (40.53±31.16 ng/mL) were higher than in control (32.21±25.53 ng/mL) but not significant. *APOA* and *B* gene expression were also significantly raised in CAD (p<0.001) (Table 1).

The frequencies of *APOA* rs670 AA genotype (OR= 0.171, p=0.042) and A allele were significantly higher in CAD than in the control (Table 2). oxHDL was also significantly raised in AA genotype (p=0.03). No significant association was seen in lipid profile (Table 3). The frequencies of *APOA* rs5069 TT genotype and T allele (OR=0.53, p=0.35) were raised in CAD but not statistically significant (Table 2). Serum VLDL and cholesterol/HDL ratio were significant (p= 0.023 and 0.039), respectively (Table 4).

The TT genotype frequencies of *APOB* rs693 and T allele were increased in CAD but not statistically significant (OR=0.53, p=0.23) (Table 2). Serum VLDL and cholesterol/HDL ratio were significant (p= 0.023 and 0.039), respectively (Table 4). Significant association was seen with lipid profile in control but no significant association of *APOB* rs693 with lipid profile was seen in CAD cases (Table 5).

The Del/Del genotype of *APOB* rs11279109 and Del allele was significantly increased (OR=3.94, p<0.001) in CAD cases (Table 2). Significant increase in oxHDL (p=0.04), oxLDL (p=0.002), serum cholesterol (p=0.003), VLDL (p=0.001) and borderline p-value of serum triglycerides (p=0.050) was seen with Del/Del in CAD cases. Also in CAD cholesterol/HDL ratio (p=0.051) result tend to be significant (Table 6). Increased expression of *APOA* and *B* genes were observed in CAD as compared to controls (Figure 1).

A significant weak positive correlation of serum VLDL was observed with *APOB* (p=0.045). Cholesterol/HDL ratio was positively and significantly correlated with *APOB* (p=0.059). TGs were negatively correlated with *APOA* and the results were statistically significant (p=0.011). Negative correlation of oxLDL was observed with total lipids (p=0.043) (Figure 2).

TABLE 1. Comparison of gene expression, lipid profile and oxidized HDL and LDL in study groups

Parameters	Control	CAD	p-value
Serum cholesterol (mg/dl)	171.49±30.17	179.58±32.64	0.085
Serum LDL (mg/dl)	102.38±25.58	108.98±25.96	0.090
Serum HDL (mg/dl)	41.31±3.16	35.75±3.09	0.001*
Serum VLDL (mg/dl)	31.94±7.04	33.09±5.62	0.263
Triglycerides (mg/dl)	156.60±36.09	163.88±27.81	0.164
Cholesterol/HDL ratio	4.30±0.81	4.48±0.78	0.164
oxdHDL (ng/L)	458.38±425.22	762.92±951.65	0.019*
oxdLDL (ng/mL)	32.21±25.53	40.53±31.16	0.127
APOA	1	2.03±0.43	0.000*
APOB	1	2.95±1.07	0.000*

Independent sample t-test. *p-value < 0.05 significant. oxdHDL, *APOA* and *APOB* are significantly increased in CAD

TABLE 2. Genotype and allelic distribution in the study groups

cSNP	Genotype/Allele	Control	CAD	p-value	Adjusted Odds ratio	95% Cl	
						Lower	Upper
APOA1 rs670 (-75G/A)	GG	27(41.5%)	33(24.4%)	0.079	-	-	-
	GA	36(55.4%)	88(65.2%)	0.074	.452	.189	1.079
	AA	2(3.1%)	14(10.4%)	0.042*	.171	.025	1.171
APOA1 rs5069 (+83C/T)	CC	23(35.4%)	35(25.9%)	0.283	-	-	-
	CT	32(49.2%)	73(54.1%)	0.443	1.482	.542	4.049
	TT	10(15.4%)	27(20%)	0.352	.534	.143	2.001
APOB rs693 (XBa1)	CC	29(44.6%)	45(33.3%)	0.493	-	-	-
	CT	21(32.3%)	49(36.3%)	0.591	.775	.306	1.961
	TT	15(23.1%)	41(30.4%)	0.235	.538	.194	1.495
APOB rs 11279109 (sp Del/Ins)	Ins/Ins	44(67.7%)	113(83%)	0.000*	-	-	-
	Del/Ins	18(27.7%)	9(6.7%)	0.356	2.044	.448	9.338
	Del/Del	3(4.6%)	13(9.6%)	0.000*	3.937	1.578	8.663

Odds ratio by Logistic regression analysis. *p-value < 0.05 significant. Del/Del genotype and Del allele significantly increased in CAD

TABLE 3. Comparison of *APOA1* rs670 genotypes with lipid profile between study groups

APOA1 rs670	Control			CAD			
Parameters	GG	GA	p-value	GG	GA	AA	p-value
Serum Cholesterol (mg/dl)	176.07±30.69	182.08±34.14	0.469	172.82±32.08	172.59±30.46	161.43±22.76	0.992
Serum LDL (mg/dl)	107.33±21.56	110.16±28.91	0.699	106.85±27.76	101.83±24.84	95.29±24.70	0.651
Serum HDL (mg/dl)	41.22±3.46	41.37±2.99	0.856	39.94±2.41	39.73±3.44	39.50±2.21	0.990
Serum VLDL (mg/dl)	32.63±6.24	33.40±5.26	0.601	31.15±5.00	32.58±7.87	29.86±5.14	0.939
Total Lipids	727.29±79.40	735.52±85.51	0.708	711.55±86.64	702.08±126.42	650.30±49.80	0.451
Triglycerides (mg/dl)	162.63±31.00	164.70±25.94	0.778	154.30±22.70	159.05±41.04	146.71±27.04	0.902
Cholesterol/ HDL ratio	4.49±0.68	4.49±0.86	0.974	4.45±0.85	4.28±0.83	3.97±0.41	0.647
oxdHDL (ng/L)	438.57±39.18	376.77±25.41	0.901	322.97±13.14	617.87±84.89	619.46±72.14	0.03*
oxLDL (ng/mL)	36.56±28.73	38.82±31.59	0.59	24.48±7. 63	51.22±15.10	69.16±25.62	0.733

values generated by ANOVA. p-value <0.05 significant. Significantly raised oxHDL in AA genotype in CAD

TABLE 4. Comparison of *APOA1* rs5069 genotypes with lipid profile between study groups

APOA1 rs5069	Control				CAD			
Parameters	CC	CT	TT	p-value	CC	CT	TT	p-value
Serum Cholesterol (mg/dl)	176.57±28.51	182.75±37.65	176.40±25.22	0.749	170.06±33.78	171.92±27.87	172.19±32.29	0.803
Serum LDL (mg/dl)	104.13±25.51	114.00±27.42	104.10±20.89	0.313	101.11±29.85	102.48±24.22	103.74±24.07	0.632
Serum HDL (mg/dl)	40.78±2.25	41.56±3.85	41.70±2.58	0.616	40.06±2.11	39.51±3.70	40.04±2.26	0.993
Serum VLDL (mg/dl)	31.65±4.69	35.18±6.01	30.60±4.81	0.023*	31.23±7.36	32.66±7.36	30.96±5.61	0.973
Total Lipids	711.21±74.82	759.78±86.65	706.20±71.60	0.062	696.63±101.59	700.13±120.25	708.55±89.65	0.596
Triglycerides (mg/dl)	157.91±23.60	172.61±30.31	153.20±24.10	0.069	155.00±35.64	158.00±39.17	154.93±28.14	0.875
Cholesterol/HDL ratio	4.29±0.71	4.76±0.84	4.18±0.55	0.039*	4.22±0.92	4.34±0.80	4.32±0.57	0.627
oxdHDL	335.60±10.04	413.80 ±26.30	446.98±42.69	0.357	472.53±56.06	744.72±88.44	746.41±98.99	0.287
oxLDL	47.19±34.84	30.09±22.62	39.28±34.53	0.108	30.01±20.40	55.32±17.41	65.24±24.91	0.737

values generated by ANOVA. *p-value <0.05 significant

TABLE 5. Comparison of *APOB* rs693 genotypes with lipid profile between study groups

APOB rs693	Control				CAD			
Parameters	CC	CT	TT	p-value	CC	CT	TT	p-value
Serum cholesterol mg/dl	172.93±33.54	181.14±32.19	190.27±30.39	0.002**	168.67±24.88	171.18±30.35	174.95±35.22	0.293
Serum LDL mg/dl	105.10±24.17	108.86±30.71	116.67±21.73	0.004**	101.18±20.41	103.29±26.45	102.61±29.85	0.602
Serum HDL mg/dl	41.90±3.89	40.43±2.42	41.40±2.32	0.247	40.18±2.46	39.37±2.73	39.76±4.00	0.782
Serum VLDL mg/dl	32.28±5.07	31.86±5.48	36.20±5.92	0.001**	32.56±7.40	31.33±6.38	32.02±7.49	0.514
Total Lipids mg/dl	722.28±79.55	721.24±84.44	766.50±81.33	0.002**	677.52±153.30	699.30±87.26	713.87±106.08	0.441
Serum Triglycerides	161.32±25.50	158.67±27.35	175.47±30.57	0.002**	159.78±34.33	156.63±30.98	153.10±43.50	0.480
Cholesterol/HDL ratio	4.37±0.58	4.49±1.05	4.67±0.64	0.003**	4.24±0.61	4.26±0.76	4.39±0.97	0.665
oxdHDL	467.99±350.04	384.99±375.26	388.27±295.70	0.442	462.39±133.46	571.42±821.54	739.03±967.99	0.451
oxLDL	46.78±35.89	29.19±18.15	31.93±27.26	0.086	27.52±20.77	35.47±23.26	96.22±307.06	0.136

values by ANOVA. *$p < 0.05$; ** $p < 0.01$. Significant association with lipid profile in control but no significant association was seen in CAD

TABLE 6. Comparison of *APOB* Ins/Del genotypes with lipid profile in study groups

APOB rs11279109	Control				CAD			
Parameters	Ins	Ins/Del	Del	p-value	Ins	Ins/Del	Del	p-value
Serum Cholesterol (mg/dl)	183.55±36.94	164.67±16.26	178.98±31.81	0.447	168.93±29.86	193.67±28.79	178.38±28.40	0.003**
Serum LDL (mg/dl)	114.89±24.60	96.00±13.08	107.45±26.95	0.473	101.12±25.16	119.22±27.45	101.61±25.84	0.875
Serum HDL (mg/dl)	41.67±3.83	40.67±0.58	41.20±3.00	0.760	39.46±2.92	42.00±4.87	40.77±2.31	0.128
Serum VLDL (mg/dl)	34.78±5.56	28.00±4.00	32.98±5.59	0.139	31.05±6.30	34.00±4.97	38.31±10.59	0.001**
Total Lipids	771.39±82.51	669.00±51.74	722.73±80.68	0.264	696.19±89.95	771.00±81.47	677.64±224.09	0.651
Triglycerides (mg/dl)	173.83±27.50	139.67±19.55	161.18±26.89	0.182	153.04±32.73	158.78±34.28	186.15±51.94	0.05
Cholesterol/HDL ratio	4.79±0.67	4.00±0.44	4.41±0.80	0.388	4.24±0.78	4.86±0.90	0.88±0.26	0.05
oxdHDL	428.28±37.39	337.86±79.39	316.37±83.14	0.793	399.30±50.44	548.45±75.99	650.98±79.65	0.04*
oxLDL	35.63±27.07	27.17±21.94	39.04±31.68	0.767	45.72±14.07	24.74±9.69	134.11±41.52	0.002*

value ANOVA-test. * $p < 0.05$; ** $p < 0.01$. Significant association was seen with lipid profile in CAD

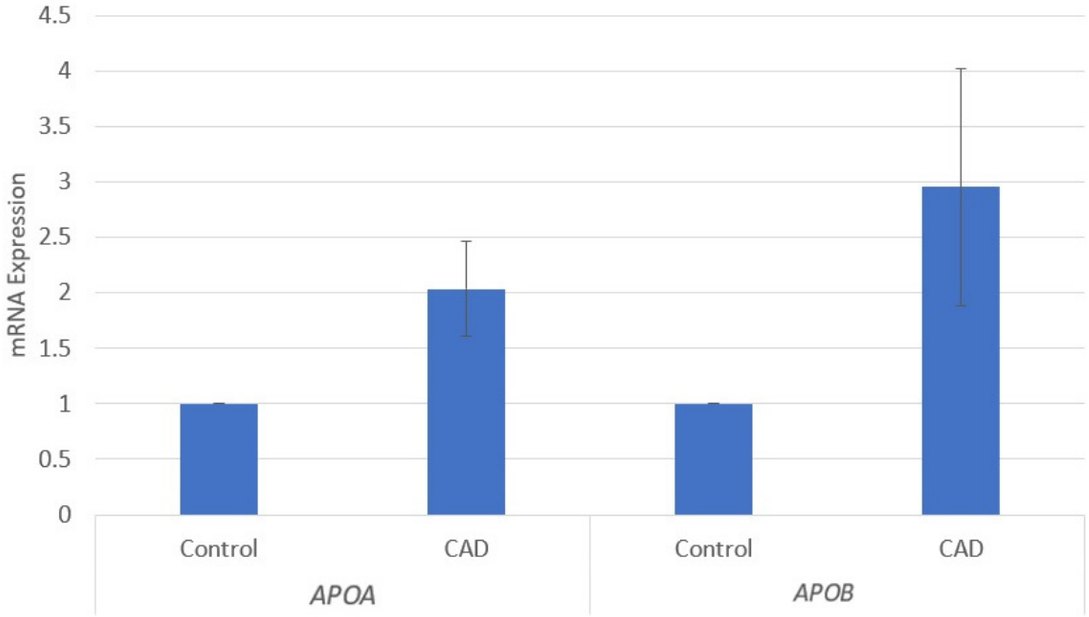


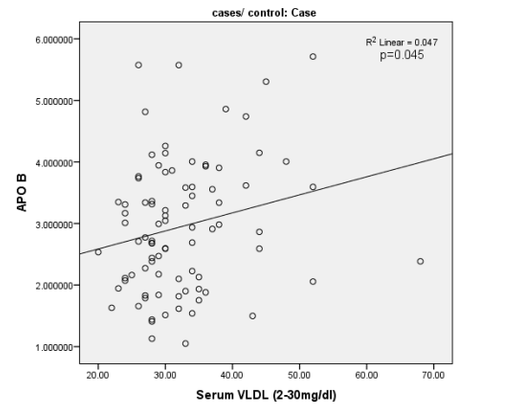
FIGURE 1. *APOA* and *B* mRNA expression between study groups represented as fold change. Increased expression of *APOA* and *B* genes were observed in CAD as compared to controls

DISCUSSION

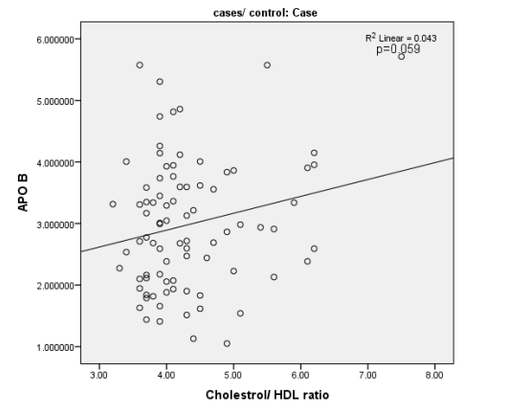
For early prediction and identification of at-risk patients and for better clinical management, a better understanding of the pathogenesis of CAD is of great importance. Genetic polymorphisms and diseases of lipid metabolism have been tightly linked, and plasma components are regulated by a complicated interplay between heredity and environment. In this sense, oxidation of lipoproteins and their link with the *APOA* and *APOB* genes (and their polymorphisms) are of particular interest. Since its end product is crucial for the metabolism of circulating lipoprotein particles. The goal of the current study was, therefore, to investigate the role of lipoprotein oxidation, gene expression and association of *APOA1* (rs670 and rs5069) and *APOB* (rs693 and rs11279109) polymorphism with dyslipidemia and CAD susceptibility in Pakistani Punjabi CAD patients and healthy controls.

Oxidized HDL was significantly increased in CAD as compared to controls in the population. An observational cohort study by Sorokin et al. (2018) found higher levels of oxHDL in cases compared to controls. A study done by Pagonas et al. (2020) showed patients with CAD had higher levels of oxHDL compared to patients with no CAD and controls. OxHDL can undergo structural changes that make it less effective at transporting cholesterol away from arterial plaques. This can lead to the accumulation of cholesterol within atherosclerotic lesions, exacerbating plaque formation and CAD progression.

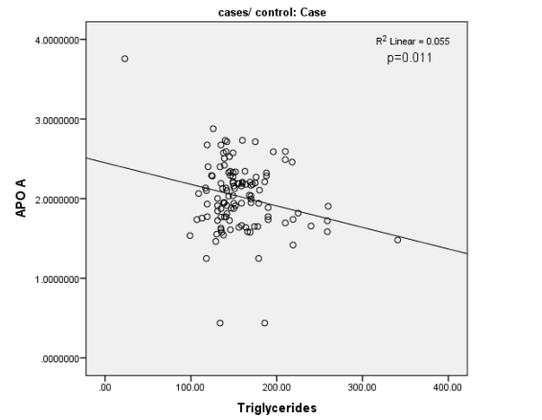
In rs670 polymorphism of *APOA1*, controls have a high frequency of normal wild genotype GG of 27(41.5%) compared to CAD 33(24.4%). While the mutant genotype AA is present in CAD which is statistically significant. Plasma HDL was lowered in GA polymorphism but not statistically significant and oxHDL was significantly raised in AA genotype. The rest of the lipid profile is not altered much as our patients were on lipid-lowering drugs, which might influence the levels of lipid profile in their blood. Reduced plasma HDL-C and elevated oxHDL are related to the *APOA1* gene polymorphism. Our results are supported by other studies such as Rader and Hovingh (2014) which found that homozygous mutations result in normal plasma levels of LDL and TG with the absence of *APOA1*, and HDL-C levels of less than 5 mg/dL. A missense mutation in the *APOA1* gene results in low plasma levels of ApoA1 and HDL-C, which alter the ApoA1 protein's structure.



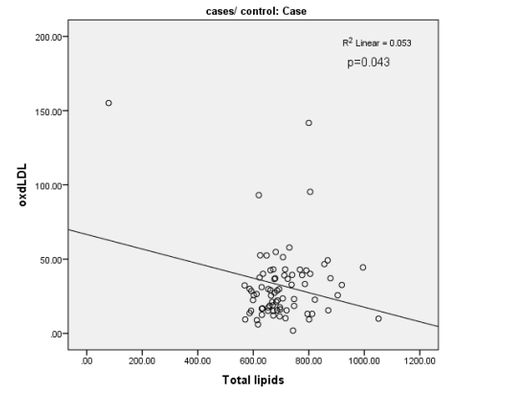
Serum VLDL and *APOB*



Cholesterol/HDL ratio and *APOB*



Triglycerides and *APOA*



Total lipids and oxdLDL

FIGURE 2. Significant correlation of *APOA* and *B* genes with lipid profile and oxLDL. A significant positive correlation of serum VLDL and Cholesterol/HDL ratio with *APOB*. Triglycerides and *APOA* as well as Total lipids and oxdLDL showed negative correlation

A study conducted on Asian Indians also noticed a positive association between *APOA1* polymorphism and CAD (Shanker et al. 2008). A pilot study in North India by Dawar, Gurtoo and Singh (2010) found a positive association of G/A polymorphism and myocardial infarction. Chen et al. (2009) conducted a study on Brazilian cohort patients, and found G/A polymorphism associated with hypertension. A meta-analysis of 6 studies

conducted by Dong and Da Huang (2018) found on the basis of ethnicity that GG was linked with a significantly low risk of CAD in Caucasians and a high risk in Asians. The significantly raised levels of oxHDL in individuals with the AA genotype could indicate that this genetic variant may lead to oxidative stress or alterations in HDL composition, making it more prone to oxidation. Oxidized lipoproteins, including oxHDL, are known to be pro-inflammatory and pro-atherogenic, potentially contributing to CAD risk.

In *APOA1* rs5069 polymorphism, the frequency of wild genotype CC was found greater in control 23(35.4%) than in CAD 35(25.9%) but not statistically significant. And mutant genotype TT frequency was found greater in cases 27 (20%) than in control 10 (15.4%). No significant association was found between lipid profiles, oxHDL and oxLDL. Our results are supported by a study done by Liao et al. (2015). It has been shown that no association between CT polymorphism and CAD risk in Han Chinese. A meta-analysis demonstrated no association between CT polymorphism with CAD. On subgroup analysis by ethnicity, the CC genotype had a low risk of CAD for Caucasians (Dong & Da Huang 2018). In the North Indian population, a positive association of C/T polymorphism was found with MI (Dawar, Gurtoo & Singh 2010). In the Brazilian cohort study, a significant association between CT polymorphism was found with hypertension and obesity (Chen et al. 2009). These findings emphasize the complexity of the genetic and environmental factors that contribute to CAD risk. While the *APOA1* rs5069 polymorphism may play a role, it appears to be a relatively weak contributor in this specific population.

In *APOB* rs693 polymorphism, the frequency of wild genotype CC was higher in control 29(44.61%) than in CAD 45(33.3%). The mutant genotype TT was higher in CAD than in control (30.4% vs 23.1%) but not statistically significant. There is an association of T allele in Asians may be caused by raised cholesterol, triglycerides and LDL-C and decreased HDL-C levels. There was no association between this dyslipidemia and the APOB XbaI (rs693) genotypes among cases. The results of numerous investigations on the connection between high lipid levels and the XbaI rs693 polymorphism were debatable. The same results were obtained by Sharma et al. (2011). They also found no association of *APOB* with lipid profile. Another study by Casillas-Muñoz et al. (2018) also found no association between lipid profile and XBa1 polymorphism. A study done on Egyptian patients by El-Sayed et al. (2022) also reported the same. However, studies done on the Chinese population by Gu et al. (2015) reported a significant association between XBa1 and dyslipidemia in CAD patients. Niu et al. (2017) in a meta-analysis also reported an association between rs693 and altered lipid profile. The observed changes in these results are thought to be caused by gene-environment interaction and genetic heterogeneity, presumably as a result of population variances in risk variables (Chen et al. 2016). Sotos-Prieto and Peñalvo (2013) concluded that CC allele frequencies are very close in the Caucasian populations and ranged from a minimum - 43.0 % in the Danes to the maximum - 56.0% in the Italians. The CC allele frequency in the Indians is much higher - 75.0%. This parameter reaches a maximum in the Mongoloids - 90.0%: in the Chinese - 92.0%, in the Japanese - 95.0%, to the total mono-morphism in the Koreans - 100%. According to studies on epigenetics by Casillas-Muñoz et al. (2018), food exposure and other environmental stressors might modify some cellular pathways. Numerous factors, such as increased lipid concentration and poor consumption of vegetables and fruits, might interpolate lipid density (Xiao et al. 2015). Studies on the interactions between genes and the environment shows that a person's diet may affect their genetic susceptibility to cardiovascular risk. Study by Abd El-Aziz and Mohamed (2016) found that *APOB* had increased the risk of CAD by affecting dyslipidemia. Another study Ma, Zhang and Wang (2011) suggested that *APOB* is the risk factor for CAD. While the study among Turkish population done by Duman and the study on Chinese population done by Pan found no significant association of XBa1 among cases and control (Duman et al. 2005; Pan et al. 1995). Study done by Sharma et al. (2011) found out that their CAD patients had significant higher levels of *APOB* as compared to controls. They determined *APOB* gene (c.12669G>A,p. Gln4154Lys) in Indian Punjabi patients suffering from CAD while we determined *APOB* gene rs693 and sp Ins/Del polymorphism in Pakistani CAD patients and its comparison with healthy controls. The lack of significant association between rs693 genotypes and dyslipidemia among CAD cases indicates that other factors may be more influential in driving lipid abnormalities in this population.

In *APOB* Ins/Del polymorphism, frequency of wild genotype Ins was 44 (67.7%) in controls and 113 (83%) in cases. The mutant gene Del/Del frequency was 3 (4.6%) in controls and 13 (9.6%) in cases. We found a significant association of the Del allele with serum cholesterol, TGs, VLDL, oxHDL and oxLDL. Dyslipidemia

was increased with Del allele carrier. The same results were obtained in other studies. According to research from Lamia et al. (2012) the del allele was linked to a higher risk of developing CAD. Zhang et al. (2017) performed a meta-analysis, and found Ins/Del was significantly linked with a greater risk of CAD. In Caucasians, Boekholdt et al. (2003) and Chiodini et al. (2003) also found similar findings. An observational cohort study found higher levels of oxLDL in CAD compared to controls (Sorokin et al. 2018). Another study also found higher oxLDL in their population (Burgos Alves et al. 2010; Honda et al. 2010). Having the sp Ins/Del D allele homozygously elevate LDL-C, oxHDL, and oxLDL levels which may influence the structure and function of LDL particles, leading to alterations in lipid metabolism and hence is a risk factor for coronary artery disease.

Studies done by Al-Bustan et al. (2014) on the Kuwait population found a significant association between *APOB* single peptide polymorphism and TG. Heterozygous samples were significantly associated with low TG levels. In a study on 700 diabetic subjects, Rafiee et al. (2019) found a significant relation between Ins/Del polymorphism and serum LDL levels. As the *APOB* gene is located on chr 2P, and contains 29 exons, the Ins/Del polymorphism is within 1st exon. The abnormalities in the lipid profile linked to the *APOB* might be caused by a variation in the degree of hydrophobicity and effectiveness of *APOB* processing as a result of either the presence or lack of the hydrophobic amino acid residue. The absence of leu-ala-leu in the del allele alters the rate of translocation of *APOB*. Thus, increasing the rate at which *APOB* containing lipoproteins are secreted (Lamia et al. 2012). Inconsistencies seen in the literature may be related to intra- and inter-population variations in nutrition and lifestyle, as well as the well-known genetic variability between groups and gene-environment interaction.

Expression of *APOB* gene was significantly raised in CAD. A positive correlation between *APOB* gene expression and VLDL and cholesterol/HDL ratio was found in cases. A study done by Han et al. (2017) indicates that *APOB* could predict the vulnerability of Coronary Artery plaques. Work done by Liting, Guoping and Zhenyue (2015) also indicates that *APOB* predict the severity of CAD which provides more prognostic data than other routine lipid profiles. A review of numerous studies conducted by Zhang et al. (2017) showed a clear association between greater *APOB* levels and CVD patients with T2DM, which is consistent with the findings of the current study. This suggests that inflammatory pathophysiology may be linked to *APOB* monitoring.

There are a few limitations of the current study. First of all, the present study did not examine the interaction between internal and external variables that affect CAD which consider as a complex illness. Second, Punjabi was the only Pakistani ethnic group included. Future research with comparable findings in various populations would be captivating. Thirdly, cholesterol levels were impacted by the use of statins in the population. Although confounders of the study have been controlled but still we were unable to get data on the subjects' initial lipid levels. Lastly, as this is a hospital-based case control research and the individuals may not be typical of the general community, it was not possible to prevent the potential selection bias.

Conclusions

APOB gene expression is positively correlated with VLDL while *APOA* has significant negative correlation with TG. *APOB* (rs11279109) has a significant impact on lipid profile, oxHDL and oxLDL and has a higher risk of CAD (OR=3.97). Hence, serum oxHDL and oxLDL, *APOB* gene expression as well as *APOA1* and *APOB* genotyping could be useful in identifying individuals who are at risk of CAD and suggesting strategies for preventing disease linked to increased HDL-C levels and decreased LDL levels. We suggest further studies to identify the physiological impact of allelic variations of *APOA* and *APOB* with environmental factors.

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