

Investigation of Genetic Variations in APLN and APLNR Genes and Their Potential Role in Cardiovascular Diseases

Nabaa Azhar Abdulmuttaleb¹, Abdelhameed Abdelkhaliq Oliwi Nasir²,
Sami awad Alkubaisy³, Osama Akram Mohsein^{*4,5}

Abstract

Background: Apelin is a naturally produced ligand for G protein-linked receptors derived from a 77-amino acid pre-propeptide. The effect of apelin on the development of cardiovascular diseases and the relationship between the apelin gene and the apelin receptor.

Methods: The case-control study included 100 participants of people suffering from cardiovascular diseases. Samples were collected from patients hospitalized at the Nasiriyah Heart Center between November 10, 2023, and February 15, 2024. The study also included 50 healthy people who did not suffer from cardiovascular disease. The lipid profile was measured by spectrophotometer, and the Apelin level was measured by enzyme-linked immunosorbent assay. Four single nucleotide polymorphisms for Apelin (APLN) and Apelin receptor (APLNR) were chosen, and Sanger sequencing was used to genotype them accurately.

Results: The findings indicated that there was no statistically significant difference in age between the two groups. Upon comparing the age demographics of the two groups in the study, the results indicated a lack of statistical significance in the levels of APLN or the lipid profile, despite the case group exhibiting markedly elevated Apelin and lipid levels compared to the control group. After multiple test adjustments ($P < 0.05$), neither the APLN rs2235310T allele nor the APLNR rs9943582 allele demonstrated an association with an elevated risk of coronary heart disease.

Conclusion: The investigation revealed no significant age variations or genetic correlations associated with CHD risk. However, rather than age or genetic differences, elevated apelin and cholesterol levels in the case group indicate these factors as primary contributors to cardiovascular risk.

Keywords: Apelin Receptors, Cardiac, Lipid Metabolism, Polymorphism, Risk factors.

Introduction

Adipokines are involved in several bodily processes, such as energy metabolism, inflammation, heart health, and cancer (1). Apelin (APLN) was discovered in 1998 as a substance released by fat tissue within the body. It is also present in the human circulatory system and is critical for regulating endothelial cell function. It assists these cells in growing,

migrating, and creating new blood vessels (2). There is increasing research on the link between apelin receptor (APJ) system and endothelial cell (EC) dysfunction related chronic inflammatory diseases such as atherosclerosis, obesity and rheumatoid arthritis (3). Apelin is a natural peptide that interacts with APJ receptors, a type of receptor found in

1: Department of Clinical Laboratories, College of Applied Medical Sciences, University of Kerbala, Iraq.

2: Al Iraqia University, College of Dentistry, Iraq.

3: Center of Desert Studies, University of Anbar, Iraq.

4: Department of Medical Laboratory Techniques, Mazaya University College, Thi-Qar, Iraq.

5: Thi-Qar Health Directorate, Al Habbobi Teaching Hospital, Thi-Qar, Iraq.

*Corresponding author: Osama Akram Mohsein; Tel: +96 47806864295; E-mail: osamaakram889@gmail.com.

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the heart and vasculature. The APJ receptors have extensive sequence homology with the angiotensin II type 1 (AT1) receptor, sharing 40% to 50% similarity in the hydrophobic transmembrane domains (4). APJ receptors do not bind angiotensin II (Ang II) (5). This has led to the assumption that apelin functions as an antagonist of Ang II on AT1 receptors in the renin-angiotensin system. Dysregulation of Apelin is associated with several clinical conditions such as obesity, diabetes, hypertension, cardiac hypertrophy, and heart failure (6). APLN -36, APLN -17, and APLN -13 are among the numerous fragments cleaved at the C-terminal from a 77 amino-acid preproapelin encoded by the apelin gene (7). APLN-13, known for its high binding affinity to APJ receptors, is the most biologically active form of apelin. The pyroglutamyl derivative, [Pyr1]-APLN-13, exhibits resistance to enzymatic degradation, resulting in a longer half-life compared to other isoforms (8). Intravenous apelin-13 treatment has shown in past research to have a beneficial inotropic impact on the heart and a transient depressing effect on blood pressure, or BP. APLN is thought to be protective in heart failure and hypertension among other cardiovascular disorders (9). Under regular settings, apelin and APJ are found in heart muscle cells. APLN has a positive inotropic effect in vitro and is involved in lowering arterial blood pressure, causing arterial dilation, and improving of cardiac output (10). The link between apelin and heart problems was first discovered in 2003. Földes and others found that apelin mRNA levels were higher in heart tissue from people with heart failure than in healthy tissue. They suggested that apelin may play a role in the development of heart failure in humans (11). Apelin has been shown to enhance cardiac output while reducing blood pressure and peripheral vascular resistance in individuals with heart failure (12). Additionally, administration of [Pyr1]-APLN -13 in a rat model of myocardial infarction led to a reduction in infarct size, an increase in heart rate, and elevated serum nitric oxide

levels over consecutive days, indicating a sustained cardioprotective effect against myocardial infarction (13). Further research demonstrated a significant association between improved cardiac function and the marked upregulation of stromal cell-derived factor 1 (SDF-1) and C-X-C chemokine receptor type 4 (CXCR4) expression in post-myocardial infarction mice treated with APLN -13 (14). Preventing the formation of reactive oxygen species (ROS), reducing oxidative stress, and preventing heart enlargement are all accomplished with this peptide. In mice lacking apelin, more heart attacks occurred, and the damage caused by the heart attacks was worse. This was associated with increased inflammation and a reduction in a protective pathway in the heart (13,14).

Materials and Methods

The case-control study included 100 participants suffering from cardiovascular diseases who were diagnosed by specialist doctors. Samples were collected from patients hospitalized at the Nasiriyah Heart Center between November 10, 2023, and February 15, 2024. The study also included 50 healthy individuals who did not suffer from cardiovascular disease. The participants' ages ranged from 35 to 45 years. Included individuals who were diagnosed with cardiovascular disease by a specialist. Healthy individuals without any cardiovascular disease, aged between 35 and 45 years. Excluded were individuals with other chronic diseases or conditions that could affect lipid profiles or the study outcomes, as well as individuals who have previously undergone surgery, those who are taking medication, and individuals aged over 45 or under 35 years. A five ml blood sample was drawn from each participant, placed in a gel tube, and left for 30 min at room temperature until clotting, after which it was quickly separated using a centrifuge at 3000 RCF for 15 minutes, and the blood serum was separated and frozen until use. The lipid profile level was quantified using a

spectrophotometer based on the recommendations of the manufacturer Biolabo (France), and the Apelin level was assessed using an enzyme-linked immunosorbent assay based on the recommendations of the manufacturer Bio-Techne (R&D Systems, USA).

Single Nucleotide Polymorphism (SNP) Selection

A thorough review was conducted on studies examining genetic variations in the APLN/APLNR signaling system. Next, we focused on SNPs that have already been researched. These SNPs had to be associated with functional alterations and susceptibility to coronary heart disease and psychiatric disorders. We used a tag SNP approach to enhance detection efficiency. All chosen SNPs had a minor allele frequency of greater than 0.1 across Chinese or Asian populations. Only one SNP was selected as a representative when multiple SNPs were in linkage disequilibrium (LD), defined as having similar allele statuses within the same haplotype ($r^2 > 0.8$). For example, rs2235309 showed a strong association with rs2235310, and rs7119375 with rs9943582. The selected SNPs were rs3115757 and rs2235310 in APLN, and rs9543582 and rs2282623 in APLNR.

DNA Extraction and Genotyping

A four mL blood sample was collected from a peripheral vein into tubes containing sodium citrate to prevent clotting. Genomic DNA was extracted using the TIANamp Blood DNA Kit (Tiangen, China), following the manufacturer's instructions. Primers for PCR amplification and sequencing were designed accordingly. The final PCR mixture consisted of 2.5 μ L of 10 \times PCR buffer (TaKaRa Bio Inc., Japan), 2 μ L of dNTPs (TaKaRa Bio Inc.), 2 μ L of genomic DNA, 0.2 μ L of Taq polymerase (TaKaRa Bio Inc.), 0.5 μ L of each primer, and 17.3 μ L of water, resulting in a total volume of 25 μ L. The PCR products were analyzed using Sanger sequencing by

Shanghai Majorbio Bio-pharm Technology Co., Ltd.

Statistical analysis

We performed statistical studies using SPSS Statistics version 24.0. Demographic, clinical, and genetic information were presented as averages with standard deviations or as numbers and percentages, depending on what was appropriate. We compared the features of patients and healthy controls using t-tests for continuous data and chi-squared tests for categorical data. A chi-squared test was performed to check if the genetic differences follow Hardy-Weinberg equilibrium. SHEsis platform was used to perform linkage disequilibrium analyses on the pairs of data. We used the chi-squared test to compare the genotype distributions and gene rates between cases and controls. The Bonferroni correction was used to account for multiple comparisons. We used a statistical method called MANOVA with a Bonferroni correction to compare the risks of coronary heart disease and mental health problems among different groups. P-values were adjusted when necessary. To assess the associations, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

Results

Socio-demographic characteristics among the study groups

The results revealed socio-demographic differences between the two groups. There was no significant difference in age ($P=0.60$) and BMI ($P=0.78$). The proportion of smokers was higher in the case group (35%) compared to the control group (20%), but this was not significant ($P=0.06$). A significant decrease in physical activity was observed in the case group (2.1 ± 1.3 hours/week) compared to the control group (3.5 ± 1.2 hours/week) ($P=0.02$). A higher proportion of individuals in the case group had a family history of cardiovascular disease (45% vs. 10%, $P<0.001$) and hypertension (50% vs. 15%, $P<0.001$). The case group (65%) also had a

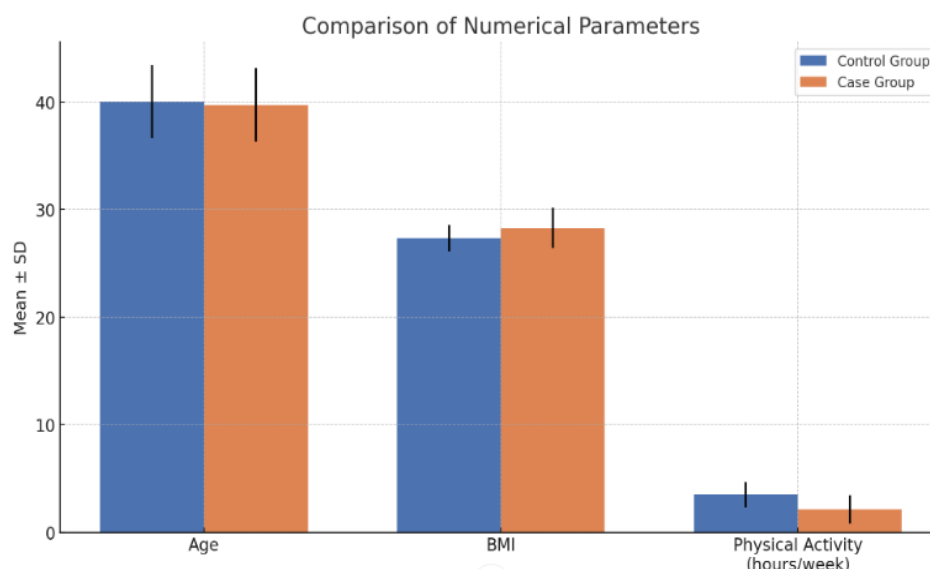
higher tendency to use medication compared to the control group (25%) (P=0.03). While 40% of the case group ate a balanced diet compared to 60% in the control group, this

difference was not significant (P=0.05). There was no significant difference in education level (P=0.08), with more university-educated individuals in the case group (30% vs. 16%).

Table 1. Differences of the socio-demographic characteristics among the study groups.

Parameters	Control group (n=50) Mean±SD	Case group (n=100)	P. value
		Mean±SD	
Age	40.02 ± 3.40	39.71 ± 3.40	0.60
BMI	27.33± 1.22	28.28± 1.87	0.78
Smoking Status	10 smokers	35 smokers	0.06
Physical Activity Level (hours/week)	3.5 ± 1.2	2.1 ± 1.3	0.02
Family History of CVD	5 cases	45 cases	<0.001*
Hypertension	8 cases	50 cases	<0.001*
Medication Use	13 using medication	65 using medication	0.03
Dietary Habits	30 balanced diet	40 balanced diet	0.05
Education Level	8 with college education	30 with college education	0.08

*p-value<0.05 (statistically significant).



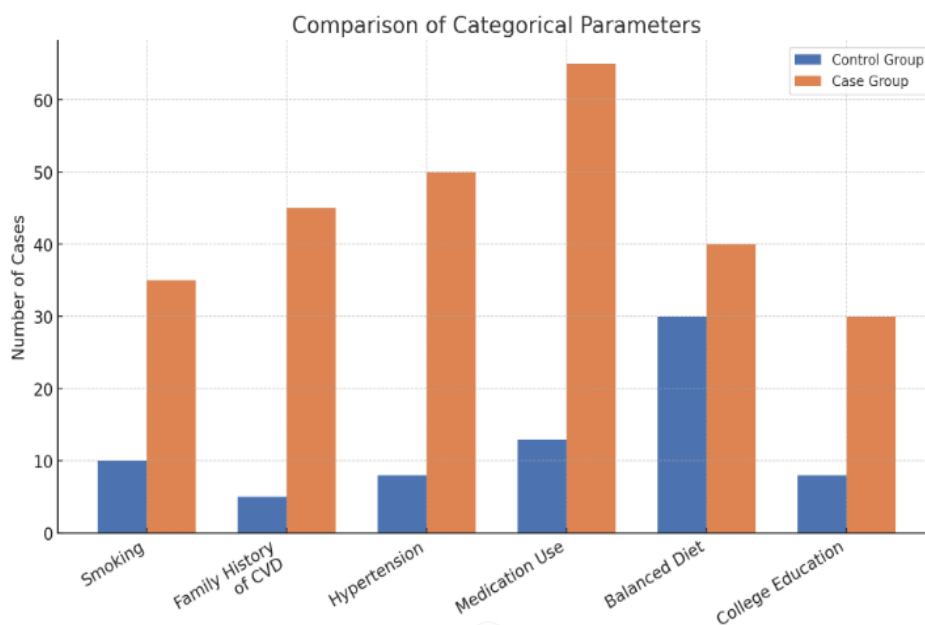


Fig. 1. A. Comparison of Mean Values of Numerical Parameters Between Control and Case. B. Distribution of Categorical Variables in Control and Case Groups. *p-value<0.05 (statistically significant).

Differences of the Apelin, Lipid profile levels between control and Case group

The study shows significant differences in apelin, triglycerides (TG), LDL, total cholesterol (T.C.), and VLDL between the control group (50 participants) and the case group (100 participants). Apelin increased in the case group (0.39 ± 0.03) compared to control (0.32 ± 0.01), $P < 0.001$. Triglycerides

were higher in the case group (277.09 ± 65.75) compared control (120.01 ± 19.21), $P < 0.001$. LDL also increased in the case group (140.27 ± 12.75) compared to control (47.60 ± 15.23), $P < 0.001$. Total cholesterol was higher in the case group (142.41 ± 6.88) compared to control (125.44 ± 5.98), $P < 0.05$. VLDL was higher in the case group (55.41 ± 13.15) compared to control (24.02 ± 3.84), $P < 0.001$.

Table 2. Differences in apelin and lipid profile between control subjects and case group.

Parameters	Control group (n=50) Mean±SD	Case group (n=100)	P. value
		Mean±SD	
Apelin	0.32 ± 0.01	0.39 ± 0.03	<0.001*
TG	120.01 ± 19.21	277.09 ± 65.75	<0.001*
LDL	47.60 ± 15.23	140.27 ± 12.75	<0.001*
T.C	125.44 ± 5.98	142.41 ± 6.88	<0.05*
VLDL	24.02 ± 3.84	55.41 ± 13.15	<0.001*

*p-value<0.05 (statistically significant).

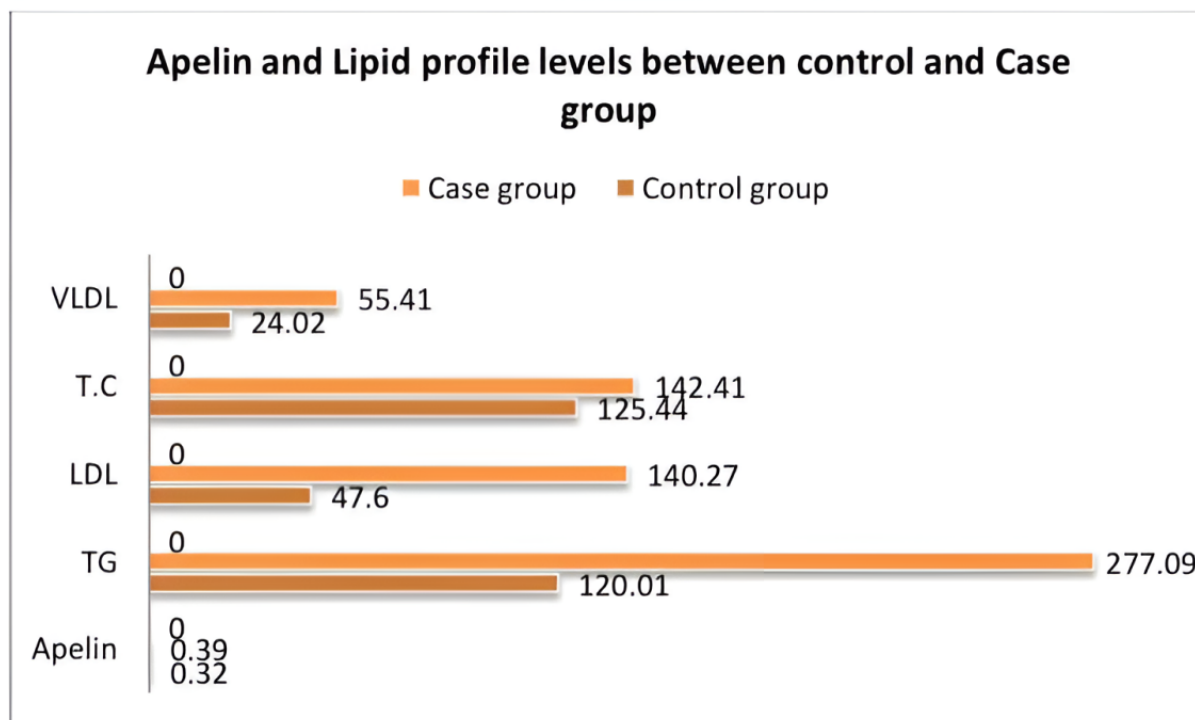


Fig. 2 . Differences in apelin and lipid profile levels between control and case groups. *p-value<0.05 (statistically significant).

Differences of the Apelin, Lipid profile levels between two age group.

A study comparing biomarkers (apelin, triglycerides, LDL, total cholesterol, and VLDL) between two age groups (34-40 years, 56 participants; 41-46 years, 44 participants) found no significant differences. The results

showed similar concentrations for apelin (P = 0.77), triglycerides (P = 0.78), LDL (P = 0.41), total cholesterol (P = 0.62), and VLDL (P = 0.54) between the two groups, indicating no statistical significance in these markers across age groups.

Table 3. Differences in Apelin, Lipid profile between two age group

Parameters	(35-40) years group (n=56) Mean±SD	(41-46) years group (n=44)	P. value
		Mean±SD	
Apelin	0.39 ± 0.02	0.39 ± 0.03	0.77
TG	278.69 ± 65.83	275.05 ± 66.36	0.78
LDL	141.20 ± 13.02	139.10 ± 14.43	0.41
T.C	141.66 ± 3.21	138.83 ± 2.87	0.62
VLDL	55.73 ± 13.16	55.01 ± 13.26	0.54

*p-value<0.05 (statistically significant).

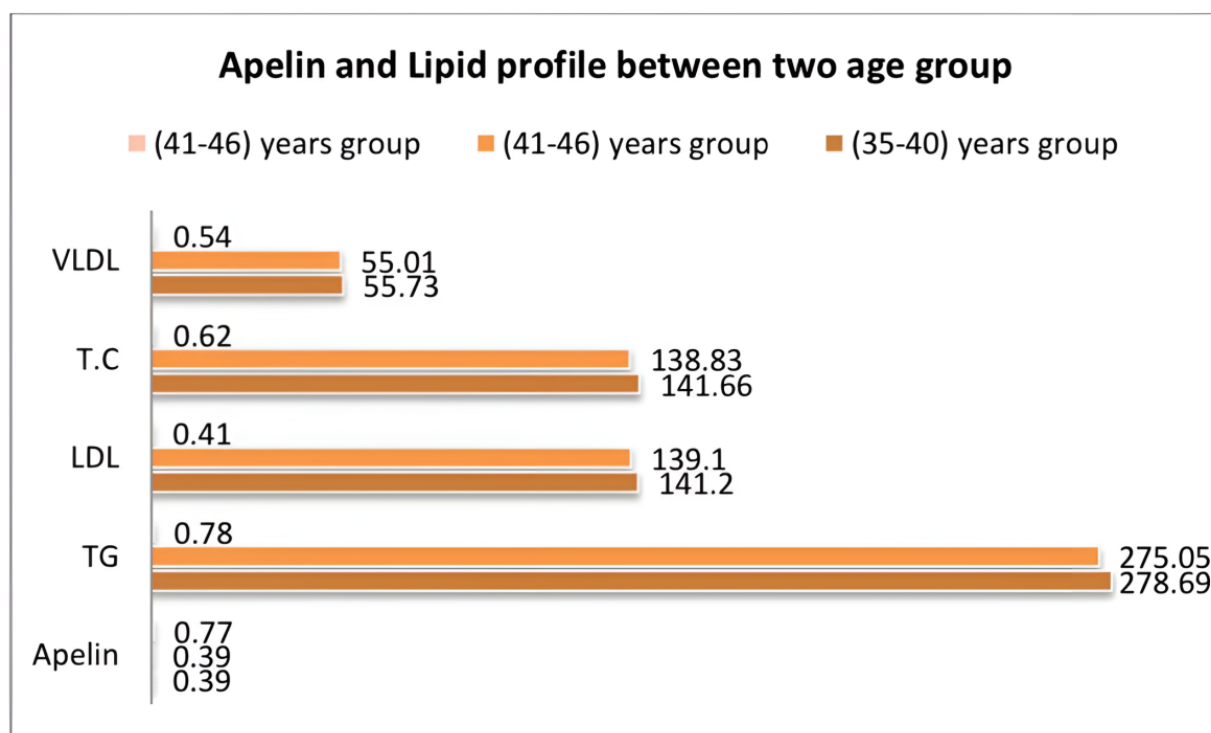


Fig. 3. Apelin and Lipid profile levels in two age group. *p-value<0.05 (statistically significant).

Pearson correlation analysis was conducted between all biomarkers in the study group.

The Pearson correlation coefficient between age and triglycerides (TG) is -0.034, showing a very weak negative relationship, with a significance value (Sig.2-tailed) of 0.812, indicating no statistically significant

correlation. Similarly, the correlation coefficient between age and LDL is -0.016, also showing a very weak negative relationship, with a significance value of 0.899, further confirming no statistically significant correlation.

Table 4. Pearson correlation between all biomarkers in the study group.

Parameters		Age	Apelin	TG
Apelin	Pearson Correlation	-.095		
	Sig. (2-tailed)	.349		
TG	Pearson Correlation	-.034*	.089	
	Sig. (2-tailed)	.736	.380	
LDL	Pearson Correlation	-.086	.237	.014
	Sig. (2-tailed)	.394	.018*	.891

*p-value<0.05 (statistically significant).

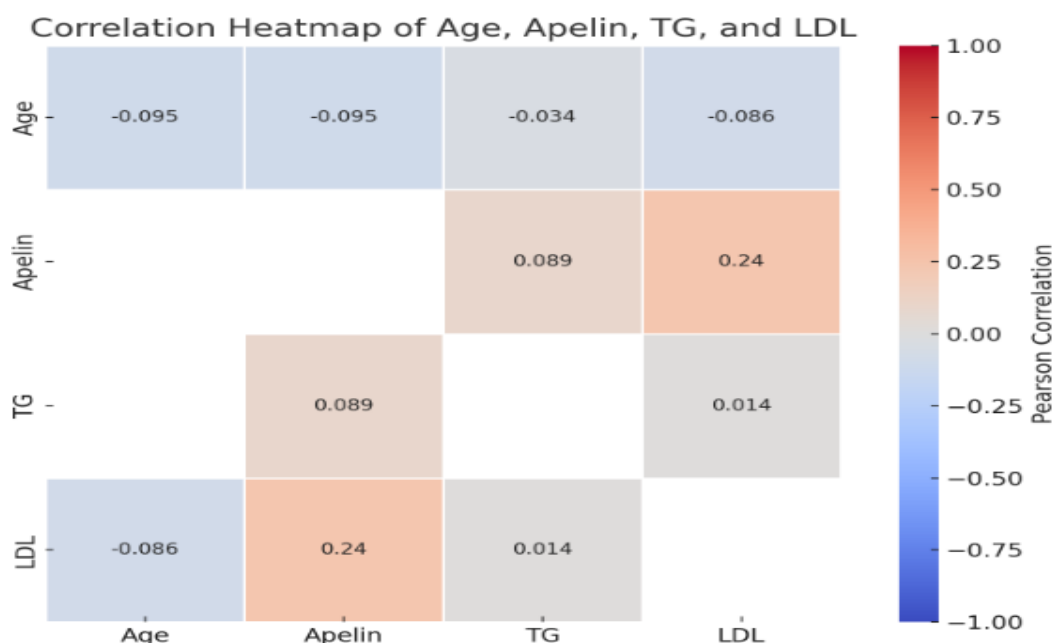


Fig. 4. Pearson correlation analysis was performed for all biomarkers in the study group.

The genetic diagnosis of patients with cardiovascular diseases was compared with the control group for several SNPs. For APLN: rs3115756, The GG genotype was more prevalent in the control group (0.63) than in the patient group (0.31), while the GC and CC genotypes showed varying frequencies between the two groups. For APLN: rs2235310, the TT genotype carriage ratio was significantly higher in the patient group (1.53)

compared to the control group (0.17). Differences were also noted for TC and CC genotypes. Additionally, for APLNR: rs9943582 and rs8222623, significant differences in the frequencies of TT, TC, and CC genotypes were observed, with certain patterns more frequent in the patient group and others in the control group, indicating potential associations between these genotypes and cardiovascular disease.

Table 5. Hardy-Weinberg equilibrium analysis was conducted for single nucleotide polymorphisms (SNPs) in cardiovascular disease (CVD) patients and controls.

SNP-ID	Genotype	Cardiovascular patient			Control		
		Number	X_1^2	P_1	Number	X_2^2	P_2
APLN: rs3115756 NCBI Gene ID: 11808	GG	51	0.31	0.63	26	0.19	0.59
	GC	35			15		
	CC	14			9		
APLN: rs2235310 NCBI Gene ID: 11808	TT	48	1.53	0.28	24	0.17	0.71
	TC	36			17		
	CC	16			9		
APLNR: rs9943582 NCBI Gene ID: 57320	TT	49	1.43	0.31	22	2.24	0.11
	TC	32			16		
	CC	19			12		
APLNR: rs8222623 NCBI Gene ID: 57320	CC	50	3.12	0.084	24	0.31	0.55
	CT	32			17		
	TT	18			9		

*p-value<0.05 (statistically significant).

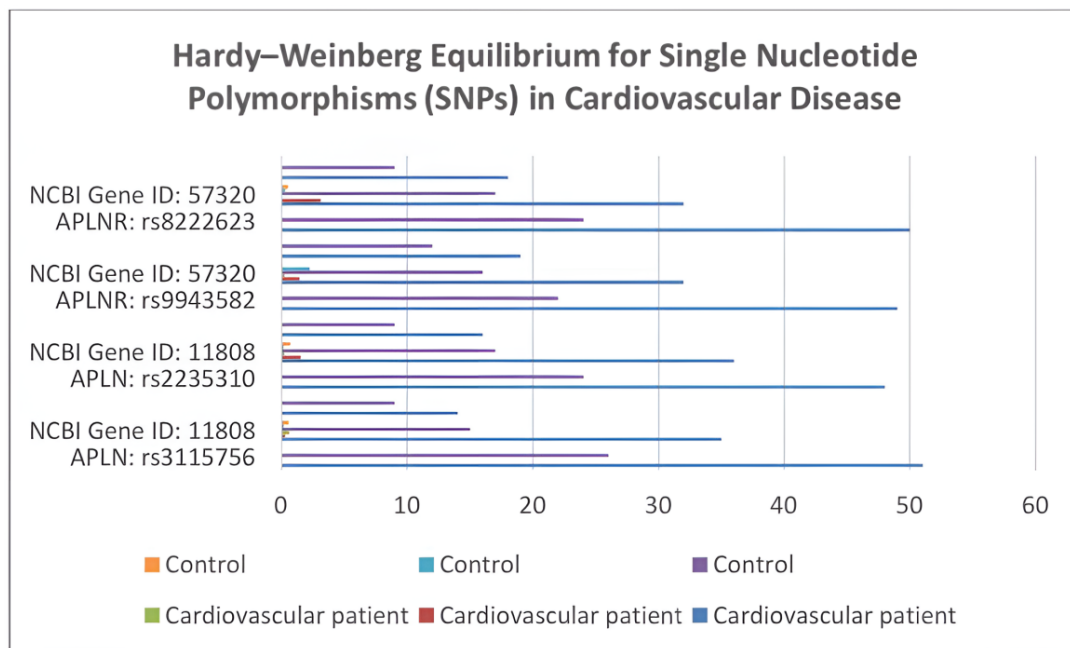


Fig. 5. Hardy-Weinberg Equilibrium Analysis.

Association Between Genetic Polymorphisms and Cardiovascular Disease (CVD) Risk

The study compared SNPs in the APLN and APLNR genes between cardiovascular patients and controls. For APLN rs3115756, 51% of patients and 52% of controls carried the GG genotype. The G allele was more frequent in patients (137%) and the C allele in controls (66%). The P-values (0.67, 0.86) indicated no

significant difference. For APLN rs2235310, the TT genotype was present in 48% of both groups, with the T allele slightly more frequent in patients (132%) than controls (130%) (P=0.43). Similarly, APLNR gene mutations showed no significant differences, with P-values ranging from 0.49 to 0.87. Overall, the study found no significant differences between the groups.

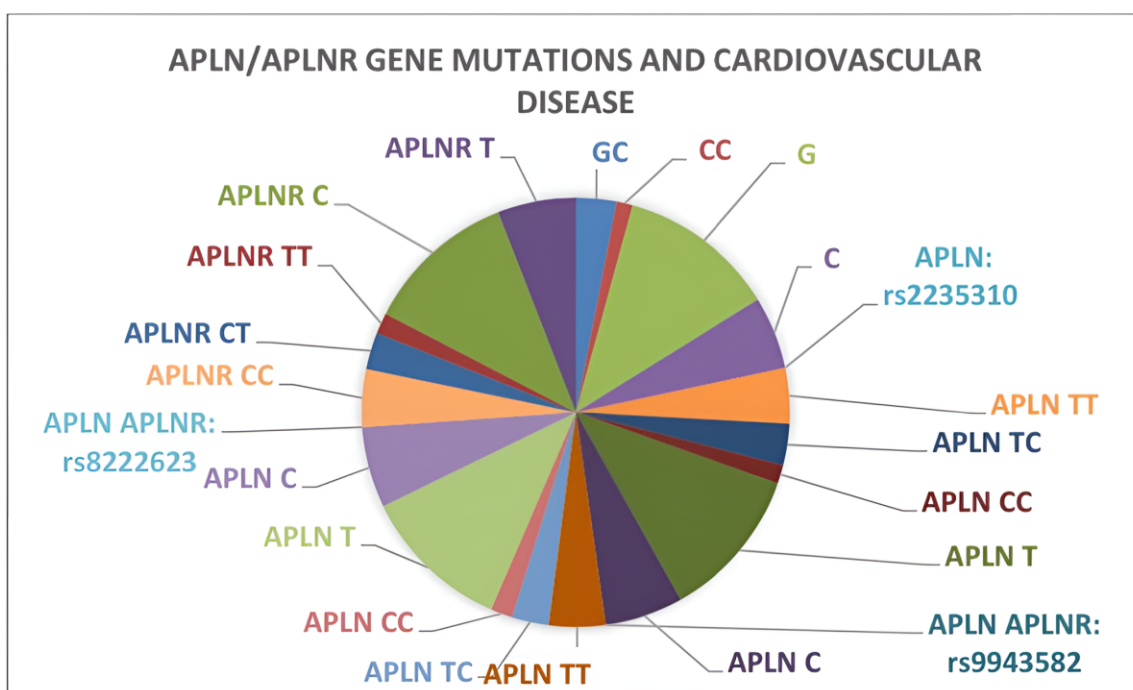


Fig. 6. Genetic Polymorphisms and Cardiovascular Disease.

Table 6. Association Between APLN/APLNR Gene Mutations and Cardiovascular Disease (CVD) Patients.

Gene	SNP-ID	Cardiovascular patient (N-100)	Percentage	Control (N-50)	Percentage	P-value
APLN	APLN: rs3115756					
	GG	51	51.0%	26	52.0%	0.67
	GC	35	35.0%	15	30.0%	
	CC	14	14.0%	9	18.0%	
	G	137	137.0%	67	134.0%	0.86
	C	63	63.0%	33	66.0%	
	APLN: rs2235310					
	TT	48	48.0%	24	48.0%	0.43
	TC	36	36.0%	17	34.0%	
	CC	16	16.0%	9	18.0%	
T	132	132.0%	65	130.0%	0.53	
C	68	68.0%	35	70.0%		
APLNR	APLNR: rs9943582					
	TT	49	49.0%	22	44.0%	0.87
	TC	32	32.0%	16	32.0%	
	CC	19	19.0%	12	24.0%	
	T	130	130.0%	60	120.0%	0.57
	C	70	70.0%	40	80.0%	
	APLNR: rs8222623					
	CC	50	50.0%	23	46.0%	0.77
	CT	32	32.0%	17	34.0%	
	TT	18	18.0%	10	20.0%	
C	132	132.0%	63	126.0%	0.49	
T	68	68.0%	37	74.0%		

*p-value<0.05 (statistically significant).

Discussion

This study highlights the significant variations between groups, particularly in physical activity, CVD family history, hypertension, and medication use. The P-values for age (0.60) and BMI (0.78) suggest no significant association with the study outcome, consistent

with Akbari et al. (2022) (15), as BMI alone does not predict CVD. A strong association between CVD family history and cases ($P < 0.001$) aligns with Brown et al. (2020), confirming genetic predisposition as a key risk factor. Conversely, Li et al. (2021) (17) suggests that environmental factors may play a

greater role, particularly in populations with lower genetic diversity. The higher hypertension rates in cases ($P < 0.001$) correspond with Hu et al. (2021) (18), reinforcing its role as a major CVD risk factor. Additionally, increased medication use in the case group ($P = 0.03$) may reflect the need for managing comorbidities, as noted by Chapman et al. (2023) (19). Dietary habits and educational levels showed some tendencies ($P = 0.05$ and $P = 0.08$, respectively), but were not statistically significant enough to indicate independent associations, as noted by Czarzasta et al. (2019) (20). This study highlights the importance of modifiable risk factors, particularly physical activity, while reinforcing the impact of non-modifiable factors like genetic predisposition. Further research is needed to explore targeted interventions (21). Table 2 reveals significant differences in Apelin and lipid profile parameters between the case and control groups. Apelin levels were higher in cases (0.39 ± 0.03) than controls (0.32 ± 0.01), $P < 0.001$, aligning with Haffd et al. (2022) (22), who suggested its role as a compensatory response to vascular dysfunction in metabolic syndrome patients. Additionally, lipid profile parameters (TG, LDL, T.C, and VLDL) were significantly elevated in the case group ($P < 0.05$), consistent with Lateef et al. (2024) (23), who linked increased TG and LDL levels to higher CVD risk. The rise in VLDL aligns with Balling et al. (2023), emphasizing its role in atherosclerosis (24), while Islam et al. (2019) attributed lower total cholesterol differences to dietary factors or population-specific effects (25). The observed lipid elevation in cases may be linked to metabolic disturbances, as suggested by Ndzie Noah et al. (2020) (26), reinforcing the role of dyslipidemia and Apelin dysregulation in disease mechanisms and their potential as therapeutic targets. No significant differences in Apelin or lipid profiles were found between ages 35–40 and 41–46 ($P > 0.05$), with Apelin levels remaining stable (0.39 ± 0.02 vs. 0.39 ± 0.03 , $P = 0.77$). This finding supports Bellissimo et al. (2021), who reported minimal age-related variation in

Apelin levels among middle-aged individuals (27). Similarly, TG, LDL, T.C., and VLDL levels showed no significant differences between age groups, indicating stable lipid metabolism within this range. This contrasts with Hirano et al. (2022), who observed age-related lipid increases in broader age spans, suggesting that the narrow age range in this study may have limited trend detection (28). The lack of significant differences suggests that factors like lifestyle or genetics may have a greater impact on Apelin and lipid levels in this age range. Future studies with larger sample sizes and broader age ranges are needed to explore age-dependent effects (29). Table 4 shows no correlation between age and Apelin ($r = -0.095$, $P = 0.349$) or age and TG ($r = -0.034$, $P = 0.736$), confirming age does not significantly affect these parameters. These findings align with El Wakeel et al. (2022) (30). However, a significant positive correlation was found between Apelin and LDL ($r = 0.237$, $P = 0.018$), suggesting higher Apelin levels are linked to elevated LDL, consistent with Wu et al. (2020) (31). There were no associations between TG to LDL ($r = 0.014$, $P = 0.891$), indicating differential regulation of these lipid parameters in the studied population. These results emphasize the relationship between Apelin and lipids, suggesting the need for further studies to explore the underlying mechanisms (32). Regarding Hardy-Weinberg Equilibrium (HWE) for SNPs in CVD cases and controls, deviations were observed in all genetic variants. For APLN: rs3115756, the P-values for patients ($P_1 = 0.63$) and controls ($P_2 = 0.59$) indicate equilibrium in both groups, consistent with Rasool et al. (2024), who also found no significant deviation in CVD patients, suggesting stability across populations (33). For APLN: rs2235310, the P-values for patients ($P_1 = 0.28$) and controls ($P_2 = 0.71$) indicate equilibrium, similar to Ilandari Dewage et al. (2024), who identified this polymorphism as stable in populations without significant environmental or genetic drift (34). For APLNR, rs9943582, the P_1 value of 0.31 in patients suggests equilibrium, but the P_2

value of 0.11 in controls indicates a slight deviation, possibly due to population-specific differences or small sample effects, as noted by Yoshikawa *et al.* (2020) (35). Additionally, APLNR: rs8222623 showed a borderline P1 value of 0.084 in patients, suggesting some departure from equilibrium, which may indicate selective pressure or inheritance in the CVD group. The control group remained balanced ($P_2 = 0.55$), aligning with Alieva *et al.* (2024), who proposed selective pressure at this locus in disease-prone populations (36). Overall, SNPs exhibited equilibrium in controls with slight deviations in patients, potentially reflecting genetic factors linked to CVD. Further research with larger, diverse populations is needed to confirm these findings (37). Table 6 shows that no statistically significant differences were found between the patient and control groups for any of the analyzed SNPs. Genotype frequencies (GG, GC, CC) for APLN: rs3115756 were similar ($P = 0.67$), and allele frequencies (G and C) were not significantly different ($P = 0.86$). These findings align with Chen *et al.* (2024), who also found no direct association between rs3115756 and elevated CVD risk (38). Similarly, for APLN: rs2235310, both genotype ($P = 0.43$) and allele ($P = 0.53$) distributions were similar between groups, consistent with Kamińska *et al.* (2020), who found no significant association between this variant and cardiovascular conditions, suggesting this locus may not play a key role in disease pathogenesis (39). For APLNR, rs9943582, genotype ($P = 0.87$) and allele ($P = 0.57$) frequencies were consistent with those reported in Zhang *et al.* (2024), who reported that rs9943582 is likely neutral for susceptibility to CVD. Finally, APLNR, rs8222623 demonstrated no difference in genotype ($P = 0.77$) or allele ($P = 0.49$) frequencies. Zhu *et al.* (2024) also found this SNP in equilibrium with an absence of a robust association with CVD (40,41). None of the 30 SNPs was universally significant with respect to CVD risk, suggesting that the APLN and APLNR mutations tested here may

contribute to CVD risk in the studied population, but not independently. But role of potential gene-environment interactions, greater samples, and haplotype analyses may help uncover the genetic etiology of CVD (42,43).

The results showed a significant increase in apelin levels in the patient group compared to the control group, and this indicates the role that apelin plays in causing the development of cardiovascular diseases. In addition, lipid levels were significantly high in the patient group, and this indicates an imbalance in fat metabolism that leads to It accumulates in the blood vessels, which increases the risk of cardiovascular disease. In addition, the relationship between fats and epilein is still in doubt and requires more research to find out the relationship between them and their effective role in causing cardiovascular disease.

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Ethical approval

All participants were provided with detailed information and verbal consent was obtained prior to the blood draw. Approval for the study was granted by the Thi Qar Health Directorate, Al-Habboubi Teaching Hospital, under committee code 2243 on November 10, 2023.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this research.

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