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Lack of Association between the Prothrombin gene rs1799963 G/A polymorphism and atherosclerosis risk in non-smokers from Sargodha, Pakistan

Farrakh Ali Abbas

Alifarrakh92@gmail.com

University of Sargodha https://orcid.org/0000-0002-6171-9559

Shazia Batool

University of Sargodha https://orcid.org/0009-0000-4118-9952

Moazma Hina

University of Sargodha https://orcid.org/0009-0008-7786-2561

Amna Aman

University of Sargodha https://orcid.org/0009-0004-8540-5281

Tayyba Khalid

University of Sargodha https://orcid.org/0009-0003-3885-8395

Fazli Rabbi Awan

awan.fr@gmail.com

National Institute for Biotechnology and Genetic Engineering https://orcid.org/0000-0002-8210-705X

Misbah Hussain

misbah.hussain@uos.edu.pk

University of Sargodha https://orcid.org/0000-0001-8825-917X

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Abstract

Background

Atherosclerosis is a leading cause of cardiovascular related morbidity and mortality worldwide. Although smoking is a well-established risk factor, the genetic and environmental factors contributing to atherosclerosis in non-smoking populations remain less understood. This study aimed to investigate the association between the prothrombin F2 gene rs1799963 G/A polymorphism and atherosclerotic risk in non-smoking Pakistani adults.

Results

Family history of cardiovascular diseases ($p < 0.001$), high stress level ($p = 0.001$) and low physical activity ($p = 0.002$) showed significant association with atherosclerosis. The rs1799963 GG genotype (90% vs. 74%, $p = 0.213$) and G allele (95% vs 85%, $p = 0.126$) were more prevalent in patient group as compare to healthy controls. However, association between atherosclerosis and rs1799963 G/A in non-smokers was not significant.

Discussion

The findings suggest that family history, stress level, and lifestyle-related factors may contribute more strongly to atherosclerotic risk in non-smoking individuals than the studied F2 rs1799963 polymorphism. Although a higher frequency of the GG genotype and G allele was observed among patients, the lack of statistical significance may be due to the small sample size. Further studies with larger cohorts and multiple genetic markers are required to better understand genetic and environmental interactions in non-smoking populations.

Conclusion

In summary, risk of atherosclerosis increases in subjects with family history of cardiovascular diseases, high stress level and low physical activity. However, no influence of F2 rs1799963 polymorphism for the risk of atherosclerosis was found in non-smokers in the studied cohort.

1. INTRODUCTION

Atherosclerosis is the most common cause of cardiovascular disease (CVD) [1]. Lipid accumulation and inflammation of large arteries are hallmark of atherosclerosis which eventually lead to its clinical complications including myocardial infarction (MI) and stroke [2]. Atherosclerosis is a serious health threat to scores of populations worldwide. During atherosclerosis fats are gradually deposited as fibrous material on the internal walls of arteries, which leads to stenosis or occlusion of these blood vessels.

Atherosclerosis is a major cause of cardiovascular morbidity and mortality worldwide [3]. Being a chronic and age-related disease, atherosclerosis often affects older people [4]. It is characterized by lifelong progressive build-up of lipids, concentration of inflammatory cells and damage of smooth muscle cells in the inner lining of blood vessels, which leads to growth of lesion [5]. This growth can impede the blood flow and cause angina. Lesions with high fat and inflammatory content are prone to rupture, potentially causing cardiovascular events such as heart attack or stroke [3].

F2 also called coagulation factor II or prothrombin plays an important role in the coagulation of blood [6]. It forms activated thrombin from inactivated prothrombin in case of any injury or cut. This activated thrombin then converts fibrinogen into fibrin, the protein which clots the blood [7]. Recent studies in the genetic epidemiology have indicated the impact of genetic polymorphism in modulating the risk associated with environmental factors like smoking [8, 9]. One such polymorphism is in rs1799963 G/A of the *F2* gene, which is present at the 3' UTR at chromosome 11 [10]. It is implicated in different thrombotic processes, cardiovascular diseases like coronary artery disease and stroke due to raised levels of the prothrombin levels in the blood [11] [12].

Studying the risk of genetic variants for atherosclerosis in non-smokers is of importance in health status monitoring. Although smoking is an established risk factor for atherosclerosis and other cardiovascular diseases, yet, a wide range of cardiovascular events occur in non-smokers as well [13]. Study of genetic factors which contribute to atherosclerosis in non-smoking subjects is important for several reasons. It allows to explore the genetic effects without any influence from the environmental risk factors as smoking has strong influence and it potentially masks the effects of genetic variants [9]. Study of non-smoker subjects for cardiovascular diseases could evaluate clearly the genetic contribution to the disease development and progression [14]. It also provides more personalized prevention planning and strategies for atherosclerosis [15]. Additionally, it may also help in identifying new pathways involved in atherosclerosis progression and lead to new therapeutic targets [16].

As a result of public awareness and government efforts, smoking rates are declining in many countries. However, prevalence of CVDs has not shown a corresponding decline [17]. This emphasizes a need to explore the genetic basis of atherosclerosis in non-smokers for planning and healthcare resource allocations [18]. Therefore, the aim of this study was to investigate the independent link for *F2* rs1799963 polymorphism with atherosclerosis in a cohort of non-smoker Pakistani Punjabi subjects.

2. MATERIALS AND METHODOLOGY

2.1 Selection of Subjects and Sample Collection

This study consists of 62 adults from both genders. Samples were collected from individuals with age > 20 years. All the enrolled subjects were divided into control group (n = 31) without any metabolic disorder and chronic infection; and patient group (n = 31) having atherosclerosis as confirmed through angiography. Patient sampling was done from the DHQ Hospital, Sargodha, Pakistan. Atherosclerosis

was diagnosed by specialist cardiologist on the basis of physical examination and clinical findings (e.g. angiography). Subjects with aged ≤ 20 years, not giving consent for participation, suffering from serious infections and other chronic diseases, pregnant and lactating mothers and cancer patients were not included in this study.

A detailed questionnaire comprised of biodemographic information, disease history, physical activities, stress level and dietary habits was filled for each recruited subject after oral/written consent. The stress level was measured by using Patient Health Questionnaire-9 (PHQ-9) [19]. Declaration of Helsinki guidelines for human research were followed. The Institutional Ethics Review Committee of the University of Sargodha, Sargodha, Pakistan approved this study (Reference No. SU/ORIC/399 dated: 29/02/2024).

Five millilitre (5 ml) blood was drawn from each subject aseptically from their cubital vein by an expert phlebotomist in EDTA containing vacutainers to prevent blood clotting. After sampling, each vial was inverted for 7 to 8 times to ensure mixing of blood with EDTA. These blood samples were further used for genetic analysis.

2.2 Genetic Analysis

The organic extraction method was used to extract genomic DNA from blood samples [8, 20]. Extracted DNA was resolved on 1% agarose through gel electrophoresis to assess the quality of genomic DNA.

For genotyping of these DNA samples, primers were designed for Tri-Amplification Refractory Mutation System-Polymerase Chain Reaction (Tri-ARMS-PCR) using Primer 1 software (http://primer1.soton.ac.uk/primer1.html). These primers were tested for quality parameters like melting temperature, hairpin loop. Heterodimer, homodimer and GC content through a freely available tool Oligoanalyzer ((https://www.idtdna.com/pages/tools/oligoanalyzer). To obtain the specific amplification of designed primers and their product size, In-silico PCR of UCSC genome browser (https://genome.ucsc.edu/cgibin/hgPcr) was used. Details of primers are given in Table 1.

Table 1
F2 rs1799963 Primers used for DNA Amplification

Primer	Sequence (5'→3')	Length	%GC	Tm	Product Size
FI_G allele	GGTTCCCAATAAAAGTGACTCTCATCG	27	44	57.4	**402 bp**
FI_A allele	GGTTCCCAATAAAAGTGACTCTCATCA	27	40.6	56.8	
Reverse	CCAAATCTCAGTTTACTCACCTACCTC	27	44	56.7	

Tri-ARMS-PCR was optimized for efficient and specific genotyping of rs1799963 G/A SNP. PCR reaction mixture was prepared in two PCR tubes in which reverse primer was added to both tubes while forward G allele (FI_G allele) primer was added to one tube and forward A allele (FI_A allele) primer was added to a separate tube. This reaction was performed in a PCR tube having total mixture of 25 µl. This 25 µl

mixture contains 3 µl Taq buffer, 1.8 µl of 0.2 mM dNTPs, 0.6 µl of 10 pmol forward primers (FI_A allele / FI_G allele), 0.6 µl of 10 pmol reverse primer, 0.3 µl Taq polymerase (5 U/µl), 50–100 ng genomic DNA and 17.2 µl double distilled sterile water.

Bio-Rad T100™ Thermal Cycle (Thermo Fischer Scientific, USA) was used for amplification. Amplification protocol was: initial denaturation for 5 minutes at 95^{o}C, 30 cycles of denaturation for 45 seconds at 95^{o}C, annealing for 30 seconds at 64.5^{o}C, extension for 45 seconds at 72^{o}C and final extension for 5 minutes at 72^{o}C. The amplified product was resolved in Ethidium Bromide added 1.5% agarose gel. The gel was visualized under MERADD (ICCC) GelDoc-100. Each allele showed a band of 402 bp. Appearance of single band in A-allele containing tube indicates the presence of AA genotype, bands in A and G allele containing tubes indicate the presence of AG genotype. While, appearance of band in G allele containing tube showed the presence of GG genotype (Fig. 1).

Figure 1: Tri-ARMS-PCR based genotyping of F2 rs1799963 polymorphism.

Lane L: indicates the 50 bp DNA marker. F1 sample: showed amplification in both A and G lanes which indicates the presence of AG genotype. F2 sample: showed amplification in G lane which reveals the presence of GG genotype. F3 sample: showed amplification in A lane only, which indicates the presence of AA genotype.

2.3 Statistical Analysis

Statistical analysis was performed in SPSS version 20. All continuous variables are presented as mean ± standard deviation, while categorical variables are presented as frequency (percentage). Frequency of minor genotype was very low. For this reason, analysis was conducted on alleles only. Alleles were calculated by counting method. Chi-square test was used to check the association of categorical variables with smoking status, while one-way ANOVA was used for continuous variables.

3. RESULTS

The genetic analysis revealed that the GG genotype of *F2* rs1799963 SNP was more frequent in the patient group (90%) as compared to control group (74%), while AA genotype was only 3% in the control subjects with none of the patients showed its presence. Although, there were differences in the prevalence of rs1799963 genotypes between control and patient groups, however, these were not statistically significant ($\chi^2 = 3.090$; $p = 0.213$). It shows that there is no strong association between the GG genotype and atherosclerosis in non-smokers. Similarly, the frequency of G allele was higher in the patient group (95%) relative to control group (85%). This data shows a trend for association of G allele and GG genotype with atherosclerosis (Table 1), yet, it is not supported by statistical analysis ($p > 0.05$), mostly likely due to small sample size.

Analysis of clinical parameters demonstrated higher systolic blood pressure in patients (124 ± 34 mm/Hg vs. 121 ± 3 mm/Hg), while obesity was more common in control group (13% vs. 9%). A

significant association was found between family history of cardiovascular disease and the presence of atherosclerosis, as none of the control subjects had family history of CVD while 39% of the patients reported positive family history of CVD ($\chi^2 = 14.880$, $p < 0.001$). This indicates genetic predisposition to CVDs even non-smokers with a family history of CVD are still at increased risk of developing atherosclerosis (Table 2).

Furthermore, stress levels and physical activity were also significantly different between control and patient groups. Patient group exhibited higher stress levels, with 58% of subjects experiencing high stress compared to only 16% in the control group ($\chi^2 = 14.524$, $p = 0.001$). This suggests that stress may play a critical role in the onset and/or progression of atherosclerosis.

These analyses highlight the significance of certain clinical parameters, such as age, family history of CVD, stress levels, and physical activity, in the development of atherosclerosis in non-smokers. However, the *F2* rs1799963 polymorphism itself does not show a significant association with the disease in our studied cohort. These findings suggest that a multifactorial approach is essential for understanding the development of atherosclerosis, particularly in non-smoking subjects.

Table 2
Genetic, Anthropometric and Clinical Parameters of Non-Smoker Subjects

Parameters			Non-Smoker Subjects (n = 62)		Significance
			Control (n = 31)	Patients (n = 31)	
A. Genetic Parameters					
F2 rs1799963	Genotypes	GG	23 (74%)	28 (90%)	$\chi^2 = 3.090$ p = 0.213
		GA	7 (23%)	3 (10%)	
		AA	1 (3%)	0	
	Alleles	G	53 (85%)	59 (95%)	$\chi^2 = 3.321$ p = 0.126
		A	9 (15%)	3 (5%)	
B. Anthropometric Parameters					
Age (in years)			38 ± 12	56 ± 14	p < 0.001
Gender		Male	12 (39%)	13 (42%)	$\chi^2 = 0.067$ p = 1.000
		Female	19 (61%)	18 (58%)	
C. Clinical Parameters					
Blood Pressure (mmHg)		Systolic	121 ± 3	124 ± 34	p = 0.637
		Diastolic	80 ± 1	79 ± 19	p = 0.850
Obesity		Normal (BMI = 18.5–24.9)	18 (58%)	21 (68%)	$\chi^2 = 0.624$ p = 0.732
		Overweight (25.0–29.9)	9 (29%)	7 (23%)	
		Obese (BMI = 30.0 and above)	4 (13%)	3 (9%)	
Family History with CVD			0	12 (39%)	$\chi^2 = 14.880$ **p < 0.001**
Stress Level		High (score ≥ 16)	5 (16%)	18 (58%)	$\chi^2 = 14.524$ **p = 0.001**
		Medium (score 11–15)	16 (52%)	7 (23%)	
		Low (score ≤ 10)	10 (32%)	3 (9%)	

Parameters		Non-Smoker Subjects (n = 62)		Significance
		Control (n = 31)	Patients (n = 31)	
Physical Activity	High	0	5 (16%)	$\chi^2 = 12.913$ **p = 0.002**
	Medium	9 (29%)	17 (55%)	
	Low	22 (71%)	9 (29%)	

4. DISCUSSION

This study investigated the association of the prothrombin *F2* rs1799963 G/A polymorphism alongside environmental and lifestyle factors with atherosclerotic risk in a non-smoking Pakistani cohort.

The family history with cardiovascular diseases has shown a significant association for the development of atherosclerosis ($p < 0.0001$), suggesting a potential genetic contribution beyond the *F2* rs1799963 polymorphism, or shared environmental factors within families as supported by the findings by Taylor, Wang (21). Furthermore, a higher stress level was observed among patients, aligning with the findings of Kivimäki and Steptoe (22) who demonstrated that stress is a notable risk factor for cardiovascular disease. Association of lower physical activity emphasizes the protective role of exercise for cardiovascular health. Although physical activity is protective against CVD, the higher medium activity among patients likely reflects post-diagnosis lifestyle changes and reporting bias rather than a causal effect.

Previous study by our group indicated a strong association between *F2* rs1799963 polymorphism and atherosclerosis in smokers [8]. While, contrary to some previous studies, this study does not support the association between the *F2* rs1799963 polymorphism and atherosclerosis in non-smokers. The GG genotype and G allele were more prevalent in-patient group (90% vs. 74% in controls) and (95% vs. 85% in controls) respectively. However, GG genotype and G allele did not show any statistical significance. These findings are not aligned to those earlier studies about association of rs1799963 with CAD and stroke [12]. Current study shows that influence of rs1799963 polymorphism on non-smokers for atherosclerotic risk may be less pronounced than the findings by Levin, Klarin (23). It emphasizes the consideration of smoking status for studying genetic association with cardiovascular diseases.

Findings of current study have impact for understanding the atherosclerotic risk in non-smoking populations. There is a lack of genetic association with *F2* rs1799963 polymorphism in this study which suggest the contribution of other genetic factors or gene-environment interactions in non-smokers. This research also highlights the importance of life-style factors in prevention of atherosclerotic risk. Family history, stress and physical activity were significantly associated with the cardiovascular disease.

This study has several limitations. The recruited samples size (n = 62) was relatively smaller which has limited our capacity to detect smaller effect sizes specially for genetic association. This study focused

on one single nucleotide polymorphism, hence a future study should be conducted by considering multiple genetic polymorphisms and their interactions. Further, the recruited samples were from Punjabi Pakistani adults, which may limit the generalizability to other ethnic groups.

Future research directions could include the larger-scale genetic association studies in non-smoking populations, possibly using genome-wide association studies. Investigation of gene-environment interactions, specially focusing on established life-style factors including stress, physical activity etc. would be highly valuable. Longitudinal studies to track the atherosclerotic development and progression in association with genetic and environmental factors could provide more significant evidences.

5. CONCLUSION

In addition to highlighting the significant effect of age, family history, higher stress level and lower physical activity in atherosclerotic risk, this study could not establish a significant association between the *F2* rs1799963 polymorphism in non-smoker atherosclerotic patients. These findings add to our understanding of cardiovascular disease risk in non-smoking populations. It may help to pave the way for new targeted strategies to prevent atherosclerotic risk. Further research with large samples is necessary to explore the association of genetic and environment factors in development and progression of atherosclerosis specially in non-smoking populations.

Declarations

Ethical Approval and Consent to Participate: This research work is approved by the ethical review committee of the University of Sargodha, Pakistan (Reference No. SU/ORIC/399 dated: 29/02/2024). The sampling process was completed after obtaining informed consent from the recruited subjects.

Availability of Supporting Data: Not Applicable

Competing Interest: All the authors declare that they have no competing interests.

Funding: Not Applicable

Authors' Contributions: F.A.A: Data Collection, Data Analysis, Methodology, Investigation, Visualization, Writing the paper, Writing – Original Draft Preparation; **S.B.**, **M.Hina.**, **A.A.**, and **T.K.** helped in Methodology; **M.Hussain:** Conceptualization, Study Design, Data Curation; **M.Hussain** and **F.R.A.**: Writing – Reviewing and Editing, Submission

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Figures

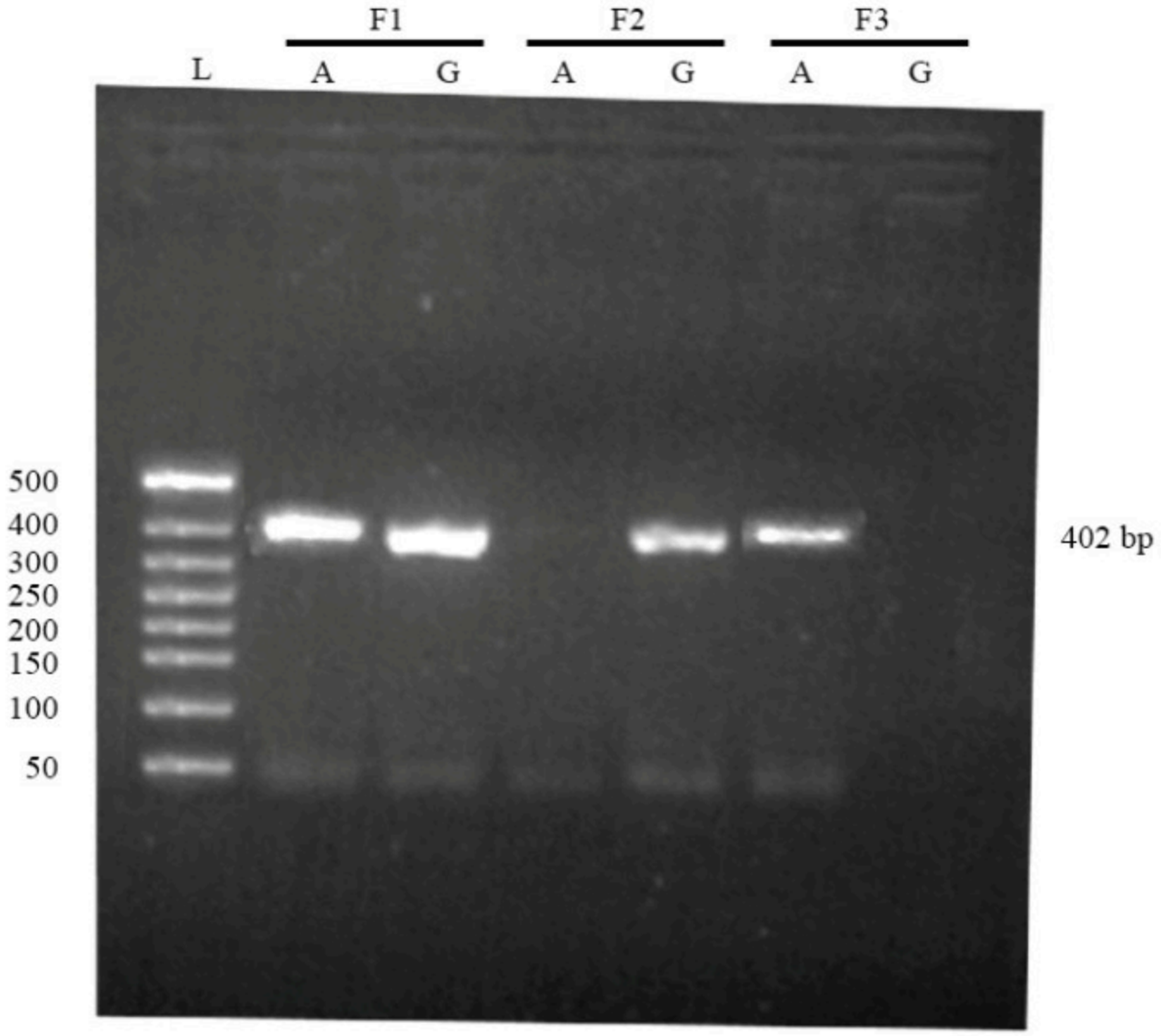


Figure 1

Tri-ARMS-PCR based genotyping of *F2*rs1799963 polymorphism.

Lane L: indicates the 50 bp DNA marker. F1 sample: showed amplification in both A and G lanes which indicates the presence of AG genotype. F2 sample: showed amplification in G lane which reveals the presence of GG genotype. F3 sample: showed amplification in A lane only, which indicates the presence of AA genotype.