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Association of IL-18 (-607) C/A and (-137) G/C gene polymorphism with primary varicose vein disorder and its clinical implication in diagnosis and severity of primary varicose vein disorder

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Background:
Varicose veins are one of the most common long-term symptoms of vascular pathology and are related to various degrees of chronic venous insufficiency (CVI). Although it has been demonstrated that inflammation is induced in varicose veins, it is still unclear how inflammation contributes to vein wall remodelling. **Objective:** To evaluate the association of IL -18 (-607) C/A and (-137) G/C gene polymorphisms with primary varicose disease. **Methods:**
In this study, a total of 73 patients and 73 healthy volunteers served as controls. All demographic and medical information was documented. Using PCR-RFLP, the gene polymorphisms IL -18 (607) C/A and IL -18 (137) G/C were found. **Results:** The proportion of age groups ≤20 years, 21-30 years, 31-40 years, 41-50 years, 51-60 years, and > 60 years was 4.11%, 24.66%, 28.77%, 17.81%, 16.44%, and 8.22%, respectively. The proportion of males and females was 79.45% and 19.18%, respectively. The genotypes of IL -18 (-607) C/A and IL -18 (-137) G/C were not significantly different in the case and control groups. **Conclusion:** The prevalence of primary varicose disease increases with age. The IL -18 (-607) C/A and (-137) G/C gene polymorphism was not associated with susceptibility to primary varicose disease and its clinicopathological development.

Keywords: Varicose vein, inflammatory biomarkers, IL-18, Chronic venous insufficiency

Introduction

Up to 30% of adults in affluent countries suffer from varicose veins, with prevalence increasing with age [1]. Heredity, age, female gender, obesity, pregnancy, prolonged standing (work), and height are risk factors for varicose veins with chronic venous insufficiency (CVI). Although varicose veins typically affect only older people, this is not always the case, as they can affect younger people as well. This syndrome is characterised by an accumulation of dedifferentiated smooth muscle cells (SMCs) in hypertrophic segments and significant disorganisation of the extracellular matrix (ECM) architecture and composition, as shown by histological and proteomic studies [2, 3]. Primary varices (PVVs) are defined as varices unrelated to deep venous thrombosis. Despite the clinical importance of PVVs, the exact nature of their aetiology is still unknown.

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Venous hypertension causes venous dilation, distortion, leakage, and inflammation, leading to valvular and migratory disease and reflux. Varicose veins, a form of CVD and the most common manifestation of CVI, are caused by a loss of homeostasis of the vessel walls [4-6]. Although occasionally caused by PTS, varicose veins usually have a primary cause. They are often inherited and are the result of severe extracellular matrix (ECM) remodelling that weakens or impairs the function of the vein walls [7-9]. Primary varicose veins (PVVs) are defined as varicose veins that are not associated with deep venous thrombosis. Despite the clinical importance of PVVs, the exact nature of their aetiology is still unknown. There is now increasing evidence that the main abnormality is caused by structural and metabolic abnormalities in the vein wall, leading to its weakening and altered tone [10,11]. Varicose veins lead to venous hypertension, low shear stress, turbulent flow, and congestion, which increase oxidative stress and inflammation and promote the development of problems such as CVI [12]. A large body of research suggests that heredity is an important factor in the aetiology of varicose veins [13]. Genetic factors may be responsible for the structural deficiencies of the vein wall, decreased contractility and compliance, and decreased ability to withstand shock.

The relationship between the extent of varicosities and persistent inflammation in chronic venous disease is critical to understanding skin changes [14-17]. Recently, research has been conducted on the genetic causes and risk factors of CVD, such as varicose veins and venous ulcers. This research has mainly focused on specific polymorphisms in genes associated with vein wall remodelling. It is already known that genetic risk factors have an impact on wound progression and healing, and screening in this area may help in the development of appropriate individualised treatment and prevention [18].[16] In addition, a number of thrombophilic single nucleotide polymorphisms (SNPs) may be associated with CVI and ulcers by increasing the incidence of DVT.

Interleukin-18 (IL -18), a member of the IL1 superfamily, which is abundantly expressed in synovial fluids, sera, and synovial tissue of patients with rheumatoid arthritis, can enhance both acquired and innate immune responses [19]. Another proinflammatory cytokine, IL -18, also contributes significantly to the Th1 response and has the ability to activate T cells and natural killer cells to produce interferon (IFN) [20]. In addition, the IL -18 gene polymorphism contributes significantly to hepatitis C virus infection in the Han Chinese population as well as in Americans, Europeans, and Indians [21]. It is well known that the IL -18 gene polymorphism has an impact on the expression and outcome of infection due to its significant role in the immune response, pathophysiology and pathogenesis of various infectious diseases [22]. Three single nucleotide polymorphisms at positions 607C/A, 656G/T, and 137G/C were discovered in the IL -18 gene promoter and have been associated with a variety of inflammatory diseases [23]. In addition, a study clarified the potential association between two different promoter polymorphisms, 607C/A (rs1946518) and 137G/C (rs187238) in the IL -18 gene, with the prognosis and incidence of prostate cancer in the Han Chinese population [24]. In the aetiology of many inflammation-related diseases, the immune response modulator IL -18 plays a crucial role [25]. In deep vein thrombosis, the serum level IL -18 is elevated, which could damage venous endothelial cells and cause venous thrombosis [26]. In this study we aim to evaluate the association of IL -18 (-607) C/A and (-137) G/C gene polymorphisms with clinical significance for the diagnosis and severity of primary varicose disease.

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Materials and Methods:

After obtaining ethical approval from the research cell of King George Medical University, Lucknow, the cohort study, involving a total of 73 patients with chronic venous insufficiency, was conducted in the Department of General surgery in collaboration with the department of CFAR and the cytogenetics laboratory. the control group consisted of 73 healthy volunteers who visited our medical university with patients or for a basic health examination. The study included all adult patients (> 15 years) with chronic venous insufficiency caused by GSV insufficiency. Individuals with secondary varicosis, congenital varicosis, impaired coagulopathy, and paediatric patients (less than 15 years of age) were excluded. The Institute Ethics Committee followed guidelines to obtain ethical approval. Patient demographic and clinical data were obtained from medical records.

Collection and storage of blood samples:

A venous blood sample (approximately 2 ml) was collected from a peripheral vein from each patient and placed in an ethylenediaminetetraacetic acid vial. Genomic DNA was isolated from the blood samples using a DNA extraction kit and stored at (-20°C) for later use.

Genotyping of IL-18 (-607) C/A and (-137) G/ Gene Polymorphism:

Single nucleotide polymorphism (SNP) genotyping will be examined using sequence-specific primer-polymerase chain reaction (SSPPCR) method. For rs 1946518 and rs187238 polymorphisms, the PCR primers and PCR product lengths are shown in Table 1. For rs 1946518, the PCR mixtures will be prepared in 25 µL of a solution containing 2 µL of gDNA, 0.25 µL of 10 mmol of dNTP, 1.5 µL of 25 mmol/L MgCl2, 2.5 µL of 10× buffer, 0.5 U of Taq polymerase, 0.5 µL of one sequence-specific forward primer (for allele C or -A), 0.5 µL of the common reverse primer, and 0.15 µL of internal positive control primer. For rs187238, the PCR mixtures will be prepared in 25 µL of a solution containing 2 µL of gDNA, 0.25 µL of 10 mmol of dNTP, 1.5 µL of 25 mmol/L MgCl2, 2.5 µL of 10 × buffer, 0.5 U of Taq polymerase (thermoscientific EcoRI), 0.5 µL of reverse primer and sequence-specific forward primer (for allele G or allele C), and 0.5 µL of the internal positive control primer. Agarose gel electrophoresis is used to visualize the PCR products. QIAamp DNA Blood mini kit (50) was used in the procedure.

Table 1: Polymorphisms, the PCR primers, PCR product lengths, and PCR program:

Polymorphisms	Primer sequence PCR	Product Lengths	PCR program	
IL-18 rs187238 (−137G>C)	R: 5′-AGGAGGGCAAAATGCACTGG-3′		94 ^{0}C 2 min	
	G:5′-CCCCAACTTTTACGGAAGAAAAG-3′	261 bp	94 ^{0}C 20 s 68 ^{0}C 60 s 72 ^{0}C 40 s	5 cycles
	C: 5′-CCCCAACTTTTACGGAAGAAAAC-3′	261 bp	94 ^{0}C 20 s	
	CTRL: 5′-CCAATAGGACTGATTATTCCGCA-3′	446 bp	62 ^{0}C 60 s 72 ^{0}C 40 s	25 cycles
IL-18 rs1946518 (−607C>A)	R: 5′-TAACCTCATTCAGGACTTCC-3′		94 ^{0}C 2 min	
	F C:5′-GTTGCAGAAAGTGTAAAAATTATTAC-3′	196 bp	94 ^{0}C 20 s 68 ^{0}C 60 s 72 ^{0}C 40 s	5 cycles
	F A:5′-GTTGCAGAAAGTGTAAAAATTATTAA-3	196 bp	94 ^{0}C 20 s 62 ^{0}C 60 s 72 ^{0}C 40 s	25 cycles
	CTRL: 5′-CTTTGCTATCATTCCAGGAA-3′	301 bp		

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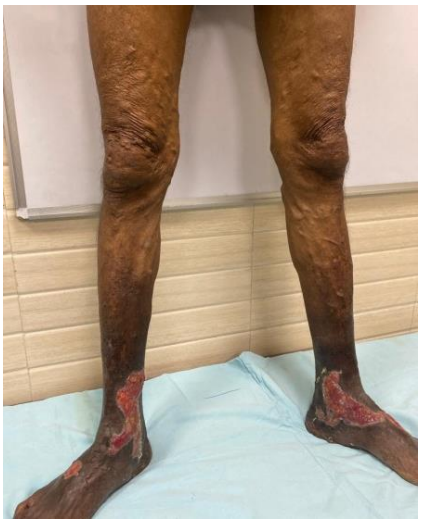
Severity assessment and association:
Severity of primary varicose vein in enrolled patients was clinically assessed using CEAP classification (Clinical etiological anatomical pathological) and VCSS scoring (Venous clinical severity score).



C-6 , VCSS- 9

Statistical Analysis:

SPSS version 21.0 was used for statistical analysis. Data was presented as mean (standard deviation) and percentage (%). The Chi-square test was used to compare the categorical variables and independent t test was used to compare discrete variables between groups. The p value 0.05 was considered significant.

Results:

The percentage of age groups ≤20 years, 21-30 years, 31-40 years, 41-50 years, 51-60 years, and > 60 years were 4.11%, 24.66%, 28.77%, 17.81%, 16.44%, and 8.22%, respectively. The percentage of males and females were 79.45% and 19.18%, respectively. The percentage of C2, C3, C4, C5 and C6 were 23.68%, 28.95%, 26.32%, 9.21% and 11.84%, respectively. The percentage of 4, 5, 6, 7, 8, and 9 were 24.66%, 6.85%, 52.05%, 0.00%, 15.07%, and 1.37%, respectively. The percentage of unilateral and bilateral cases were 68.86% and 30.14%, respectively (Table 2).

Table 2: Baseline characteristics of the patients

	Age Group	**n**	**%**
Age (years)	≤20 years	3	4.11
	21-30 years	18	24.66
	31-40 years	21	28.77
	41-50 years	13	17.81
	51-60 years	12	16.44
	>60 years	6	8.22
Gender	Female	14	19.2%
	Male	59	80.8%
Clinical stage	C2	18	24.66
	C3	22	30.14

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	C4	20	27.40
	C5	7	9.59
	C6	6	8.22
VCSS	4	18	24.66
	5	5	6.85
	6	38	52.05
	7	0	0.00
	8	11	15.07
	9	1	1.37
B/L	Unilateral	51	69.86
	Bilateral	22	30.14

The frequencies of the CC, CA, AA, and CA +AA genotypes of IL -18 (-607) C/A were 19.18%, 69.86%, 10.96%, 80.82% in study cases and 17.81%, 61.64%, 20.55%, and 82.19% in controls, respectively. The frequencies of CC, CA, AA, and CA +AA genotypes of IL -18 (-607) C/A were not significantly different in the case and control groups. The frequencies of C and A alleles were also not significantly different between case and control groups, as shown in Table 3.

Table 3: Association of genotype and allele frequencies of IL-18 (-607) C/A genes polymorphisms in cases and controls.

IL-18 (-607) C/A	Case (n=73)		Control (n=73)		OR (CI95%)	p-Value
	n	%	n	%		
Genotype						
CC	14	19.18	13	17.81	-	ref
CA	51	69.86	45	61.64	0.95 (0.40-2.23)	0.90
AA	8	10.96	15	20.55	1.19 (0.64-6.33)	0.354
CA+AA	59	80.82	60	82.19	1.10 (0.47-2.53)	0.831
Allele						
C	79	54.11	71	48.63	-	ref
A	67	45.89	75	51.37	1.25 (0.79-1.97)	0.212

The frequencies of the GG, GC, CC, and GG+CC genotypes of IL -18 (-137) G/C were 73.97%, 8.22%, 17.81%, and 26.03% in cases and 60.27%, 13.70%, 26.03%, and 39.73% in controls, respectively. The frequencies of GG, GC, CC, and GG+CC genotypes of IL -18 (-137) G/C were not significantly different in the case and control groups. The frequencies of G and C alleles also did not differ significantly between the case and control groups (Table 4).

Table 4: Association of genotype and allele frequencies of IL-18 (-137) G/C genes polymorphisms in cases and controls.

IL18 (-137)G/C	Case (n=73)		Control (n=73)		OR (CI95%)	p-Value
	n	%	n	%		
Genotype						
GG	54	73.97	44	60.27	-	ref
GC	6	8.22	10	13.70	2.05 (0.69-6.07)	0.300
CC	13	17.81	19	26.03	1.79 (0.780-4.03)	0.223
GG+CC	19	26.03	29	39.73	1.87 (0.93-3.78)	0.113
Allele						

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G	114	78.08	98	67.12	-	ref
C	32	21.92	48	32.88	1.44 (0.87-2.37)	0.197

The frequencies of the genotypes CC, CA, AA, and CA +AA of IL -18 (-607) C/A were 23.26%, 69.77%, 6.98%, and 76.74% at the low clinical stage (≤C3) and 13.33%, 70.00%, 16.67%, and 86.67% at the high stage (> C3) of cases, respectively. The frequencies of the genotypes CC, CA, AA, and CA +AA of IL -18 (-607) C/A were not significantly different at different clinical stages. The frequencies of the C and A alleles were also not significantly different between the different clinical stages, as shown in Table 5.

Table 5: Association of genotype and allele frequencies of IL-18 (-607)C/A genes polymorphisms in clinical stage (≤C3) and high stage (>C3) of cases.

IL18 (-607)C/A	**Low stage (≤C3)** (n=43)		**High stage (>C3)** (n=30)		**OR (CI95%)**	**p-Value**
	n	**%**	**n**	**%**		
Genotype						
CC	10	23.26	4	13.33	-	ref
CA	30	69.77	21	70.00	1.75 (0.48-6.34)	0.583
AA	3	6.98	5	16.67	4.17 (0.66-26.30)	0.269
CA+AA	33	76.74	26	86.67	1.97 (0.55-7.00)	0.449
Allele						
C	50	58.14	29	48.33	-	ref
A	36	41.86	31	51.67	1.49(0.76-2.88)	0.317

The frequencies of the GG, GC, CC, and GG+CC genotypes of IL -18 (-137) G/C were 76.74%, 2.33%, 20.93%, and 23.26% at the low clinical stage (≤C3) and 70.00%, 16.67%, 13.33%, and 30.00% at the high stage (> C3) of cases, respectively. The frequencies of the GG, GC, CC, and GG+CC genotypes of IL -18 (-137) G/C were not significantly different at different clinical stages. The frequencies of the G and C alleles also did not differ significantly between the different clinical stages, as shown in Table 6.

Table 6: Association of genotype and allele frequencies of IL-18 (-137) G/C genes polymorphisms in in clinical stage (≤C3) and high stage (>C3) of cases.

IL18 (-137) G/C	**Low stage (≤4)**		**High stage (>4)**		**OR (CI95%)**	**p-Value**
	n	**%**	**n**	**%**		
Genotype						
GG	33	76.74	21	70.00	-	ref
GC	1	2.33	5	16.67	7.86 (0.86-72.07)	0.099
CC	9	20.93	4	13.33	0.70 (0.19-2.56)	0.823
GG+CC	10	23.26	9	30.00	0.84 (0.24-2.98)	0.708
Allele						
G	67	77.91	47	78.33	-	ref
C	19	22.09	13	21.67	1.41 (0.49-4.06)	0.627

Discussion:

These genetic factors are described in the aetiology of primary varicose vein disease. The IL -1 superfamily member interleukin-18 (IL -18), which is abundant in synovial fluid, synovial tissue, and serum of patients with rheumatoid arthritis, may enhance not only the acquired but also the innate immune response of the body. The Th1 response is significantly

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influenced by the proinflammatory cytokine IL -18, which can also trigger the production of interferon by NK cells and T cells. The IL -18 gene polymorphism has been shown to alter both expression and outcome of infection, having a major impact on immunological feedback, disease pathophysiology, and etiopathogenesis of many infectious diseases. 607C/A, 137G/C, and 656G/ are the three single nucleotide polymorphisms discovered among the 14 IL -18 promoter polymorphisms that show significant association with various inflammatory diseases.

In deep vein thrombosis, serum levels IL -18 are elevated, which could damage venous endothelial cells and cause venous thrombosis. Therefore, this work aims to investigate the relationship between varicose veins and the IL -18 gene polymorphism. Therefore, the aim of this study is to demonstrate the association of the IL -18 (-607) C/A and (-137) G/C gene polymorphism with primary varicose veins and its clinical significance for the diagnosis and severity of primary varicose veins.

Innate and acquired immune responses are significantly regulated by the proinflammatory cytokine IL -18. Depending on the immunological milieu, IL -18 is involved in both T helper type 1 (Th1) and Th2 immune responses. IFNG production is stimulated by IL -18 in the presence of IL -12, enhancing Th1-mediated immune responses. In contrast, IL -18 enhances Th2 responses in the absence of IL -12 [27]. IL-18 is essential for host defence against intracellular microbial infections, but also plays a role in the development of autoimmune diseases and the spread of inflammation. Single nucleotide polymorphisms (SNPs) within the gene can affect production of the protein, as is increasingly being demonstrated [28].

In our study, the percentage of age groups ≤20 years, 21-30 years, 31-40 years, 41-50 years, 51-60 years, and > 60 years were 4.11%, 24.66%, 28.77%, 17.81%, 16.44%, and 8.22%, respectively. According to a previous study reported that the prevalence of various diseases of the vascular system increases dramatically with age [29]. Even though a number of severe venous diseases typically occur in childhood, they regularly accumulate and worsen with age. The importance of age as a significant independent risk factor for venous disease has only recently come to light. In our study, a similar age distribution was found with a mean age of 39.93 years. Women are twice as likely to be affected by varicose veins compared with men. In our study, the percentage of men was 79.45% and that of women was 19.18%. Although the incidence of varicose veins is higher in women, our study included more men. It may be because women in our study region are reluctant to go to OPD, wear long dresses (sarees), and ignore treatment, but the exact cause is not known.

In our study the genotype of IL -18 (-607) C/A and IL -18 (-137) G/C were no significant changes in the between the case and control groups. Moreover, these gene polymorphism were also not significantly different severity of the diseased. Chen et al. (2018) reported that the genotype in 607C/A and 137G/C, the IL -18 level was significantly increased in the lower extremity deep vein thrombosis (LEDVT) group compared with the control group, and in the LEDVT group, the IL -18 level in the GG genotype of 137G/C was greater than that in the GC + CC genotype [30]. Significantly, the promoter polymorphisms IL -18-607A/C and IL -18-137G/C, both of which are critical for the production of IL -18, have been associated with susceptibility to penicillin allergy [31].

Serum levels of IL -18 were elevated in patients with deep vein thrombosis, which may have affected venous endothelial cells and caused venous thrombosis. Consequently, IL -18 may represent a new potential target for thromboprophylaxis [32,34]. In addition to LEDVT,

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interleukin polymorphisms have been discovered to play a role in other diseases of a similar nature: IL -6 and its promoter polymorphism 572G/C are associated with an increased risk of venous thromboembolism (VTE), whereas the polymorphism 1082A/G of IL -10 is associated with an increased risk of deep vein thrombosis (DVT) [34,35]. The cytokines IL -22 and IL -17 produced by Th17 cells, which have been suggested as potential indicators of deep vein thrombosis, may both represent a novel biological target for accelerating thrombus clearance in patients with deep vein thrombosis [36]. However, because of the limited amount of data and experimental conditions, the study could be improved in the future.

Conclusion:

The prevalence of primary varicose vein disease increases with age. Our study suggests a similar finding, with the prevalence of primary varicose vein disease increasing with age in our study group. IL -18 (-607) C/A and (-137) G/C gene polymorphism was not associated with susceptibility to primary varicose vein disease and its clinicopathological evolution. Moreover, there is no association between IL -18 (-607) C/A and (-137) G/C gene polymorphism with different clinical stages of primary varicose vein disease.

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