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Original Article

Molecular signatures of varicosity: Diagnostic insights from ten biomarkers

Apurba Ganguly (PhD) [1*]
Sudip Kumar Banerjee (PhD) [2]
Vidya Sagar Gubby Venkatesh (PhD) [3]
Dinesh Kumar Singal (MD) [4]
Anondeep Ganguly (BBA) [5]

1. Department of Biochemistry, Techno India University, Kolkata, India
2. Department of Environmental Science, University of Calcutta, Kolkata, India
3. Department of Biochemistry, Kempegowda Institute of Medical Sciences, RGUHS, Bangalore, India
4. Department of Cardiac Surgery, Somerset, New Jersey, USA
5. CEO Nano Phyto Care Pvt Ltd, Harvard Medical School, Boston, USA

*** Correspondence:**
Ganguly Apurba, 1.Department of Biochemistry, Techno India University, Kolkata, India

E-mail:
apurbaganguly15@gmail.com
Tel: +91 9830389616

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Abstract

Background: Varicose veins are a chronic vascular disorder influenced by factors such as inflammation, fibrosis, endothelial dysfunction, and vascular cell activation. This study evaluates the association between ten molecular biomarkers and these risk factors in patients with lower extremity varicosity to enhance understanding of the condition's pathophysiology and highlight potential diagnostic biomarkers.

Methods: A cross-sectional study was conducted involving 126 patients diagnosed with lower extremity varicosity, confirmed by duplex ultrasound, and 108 age- and sex-matched control subjects. Serum levels of ten biomarkers C-reactive protein, interleukin-6, tumor necrosis factor-alpha, and tissue inhibitor of metalloproteinases-1, transforming growth factor-beta 1, procollagen type I N-terminal propeptide, vascular endothelial growth factor, malondialdehyde, endothelin-1, and matrix metalloproteinase-9 were quantified using ELISA. ROC curve analyses assessed the predictive value of these biomarkers.

Results: Patients with varicosity displayed significantly elevated levels of all biomarkers compared to controls, with strong associations to varicosity risk ($p < 0.0001$). ROC curve analysis revealed high predictive values, with AUCs ranging from 0.858 to 0.939.

Conclusion: The elevated biomarker levels suggest mechanisms including inflammation, fibrosis, endothelial dysfunction, and venous pressure in varicosity. Biomarker monitoring may support early diagnosis and management of varicose veins, enhancing patient outcomes. Future longitudinal studies are advised to further validate these associations.

Keywords: Varicose veins, Molecular biomarkers, Inflammation, Endothelial dysfunction fibrosis, Vascular cell activation, Elevated venous pressure.

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Varicose veins are a prevalent chronic vascular disorder characterized by the dilation,enlargement, and tortuosity of veins,predominantly affecting the lower extremities. This condition is intricately linked to venous insufficiency and involves a multifaceted interplay of biological and mechanical risk factors, including chronic inflammation, fibrosis, endothelial dysfunction, vascular cell activation, and elevated venous pressure. Despite its high prevalence, the precise pathophysiological mechanisms underlying varicose veins remain only partially understood, with many aspects still being actively investigated (1-5).

Recent advancements in molecular biology have brought to light a range of biomarkers that are integral to the development and progression of varicose veins (6).These biomarkers offer diagnostic insights into the underlying pathophysiology and can be categorized into four major domains:

1. Inflammation-driven mechanisms: C - reactive protein (CRP), Interleukin-6 (IL-6), and Tumor Necrosis Factor-alpha (TNF-α) are key mediators in inflammatory processes that can deteriorate venous wall integrity. CRP and IL-6 are instrumental in recruiting and activating inflammatory cells. At the same time, TNF-α promotes the expression of adhesion molecules, encouraging immune cell migration to venous tissues, thereby worsening inflammation and causing structural damage (7).

2. Fibrosis and collagen deposition mechanisms: Transforming Growth Factor-beta 1 (TGF-β1) stimulates fibroblast activity, leading to excessive collagen deposition and fibrosis, resulting in vein stiffening and compromised function. Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) regulates matrix remodelling by inhibiting matrix degradation enzymes, leading to matrix accumulation and venous wall thickening. Procollagen Type I N-terminal Propeptide (PINP) reflects increased collagen synthesis and fibrotic activity, reducing venous elasticity (8).

3. Endothelial dysfunction mechanisms: Vascular Endothelial Growth Factor (VEGF) contributes to abnormal vessel permeability and pathological neovascularization, undermining endothelial integrity and accelerating varicosity. Malondialdehyde (MDA), a lipid peroxidation product of oxidative stress, causes direct endothelial cell damage, further impairing the vascular barrier and contributing to disease progression (9).

4. Vascular cell activation and venous hypertension mechanisms: Endothelin-1 (ET-1) acts as a potent vasoconstrictor, increasing venous pressure and contributing to the pathophysiology of varicose veins. Elevated venous pressure exacerbates stress on the vein walls. Matrix Metalloproteinase-9 (MMP-9) breaks down extracellular matrix proteins, which weakens venous walls and facilitates dilation under high pressure (10).

Understanding the molecular signatures of these biomarkers offers a window into the pathological mechanisms of varicose veins. Monitoring these biomarkers not only provides early diagnostic insight but also has the potential to shape personalized therapeutic strategies aimed at halting or reversing disease progression. Current systems of biomarker diagnostics emphasize the utility of these molecular signatures in enhancing clinical assessments and tailoring interventions (11-14).

To establish the clinical relevance of these biomarkers, we employed rigorous statistical methods, including ROC curve analysis to assess their diagnostic accuracy, a meta-analysis with standardized mean differences (SMDs) to confirm their consistency across studies, and correlation analysis to examine the interrelationships among key biomarkers. Radar chart analysis was utilized to visualize the multifaceted roles of these biomarkers in varicosity pathogenesis. These analytical approaches provide a robust foundation for the hypothesis that these biomarkers are responsible for the progression of varicosity and may inform future diagnostic and therapeutic strategies.

The present study builds on these emerging diagnostic frameworks, analyzing serum biomarker levels in patients with clinically confirmed lower extremity varicosity and comparing them to age- and gender-matched controls (15-16). By exploring the intricate relationship between molecular biomarkers and the severity of varicosity, this study seeks to deepen our understanding of the underlying pathophysiological mechanisms driving the condition. This investigative approach paves the way for the discovery of predictive biomarkers, while simultaneously refining diagnostic and therapeutic frameworks, with the ultimate goal of enhancing patient outcomes in managing varicose veins. While previous studies have identified individual biomarkers linked to varicose vein pathophysiology, this study is novel in its comprehensive approach to simultaneously examining multiple biomarkers across inflammation, fibrosis, endothelial dysfunction, and venous hypertension pathways. By integrating rigorous statistical analyses, including ROC curve analysis, meta-analysis, and correlation, this research uniquely positions these biomarkers as potential diagnostic and prognostic tools in clinical settings. Our findings aimed to advance the current understanding of varicosity mechanisms and to provide a foundation for personalized therapeutic strategies, offering new insights for improving early detection and targeted treatment options for patients with varicose veins.

Methods

Study design and setting: This cross-sectional study was conducted from June 24, 2023, to June 30, 2024, at OPTM Health Care Pvt Ltd and Nano Phyto Care Pvt Ltd, located in Kolkata and Delhi. The study aimed to investigate the association between ten molecular indicators and risk factors for varicosity in patients with lower extremity varicosity. A total of 126 (59% female) patients with clinically confirmed varicose veins, diagnosed via duplex ultrasound, were enrolled in the experimental group. An additional 108 matched control subjects without venous disorders were included.

To ensure a representative sample, the following steps were undertaken:

• **Geographic diversity:** Participants were recruited from two distinct geographical locations (Kolkata and Delhi) to

account for regional differences in lifestyle, socio-economic status, and venous health outcomes. This diversity enhances the generalizability of the results across different populations.

• **Systematic random sampling:** A systematic random sampling method was employed to select participants from clinic records, ensuring that every eligible individual had an equal chance of being included.

• **Matching control group:** The control group (108 subjects) was matched to the experimental group (126 patients with varicosity) based on age, gender, and socio-economic status to minimize potential confounders."

Power calculation: A sample size calculation was performed to ensure that the study had sufficient power to detect significant differences in biomarker levels between varicosity patients and control subjects. Based on an expected moderate effect size (Cohen's d = 0.5), a power level of 0.80, and a significance level of 0.05, it was determined that a minimum of 100 participants per group would be required. Our final sample included 126 patients with varicosity and 108 matched controls, exceeding this minimum requirement and thus providing sufficient statistical power to detect meaningful associations.

Inclusion and exclusion criteria:

Inclusion criteria:

- Clinically confirmed lower extremity varicosity, as verified by duplex ultrasound.
- Age between 35 and 75 years.
- History of varicosity for more than 3 years.

Exclusion criteria:

- Presence of other chronic vascular diseases or systemic conditions affecting biomarker levels (e.g., peripheral arterial disease, deep vein thrombosis, or other systemic diseases such as diabetes and cardiovascular conditions). These conditions were excluded as they may interfere with the biomarkers of interest, such as CRP, IL-6, TNF-alpha, and MMP-9, leading to confounding effects that could skew the study's focus on varicosity-related biomarkers.
- Recent surgery or trauma affecting the lower extremities.
- Pregnancy or lactation.

Sample collection: Fasting venous blood samples were collected from all participants in the morning to minimize diurnal variations. Samples were processed to obtain serum within 2 hours of collection and stored at -80°C until analysis for both groups.

Biomarker measurement: The study focused on the following ten molecular indicators, chosen for their established association with inflammation, tissue remodelling, oxidative stress, and vascular function all key processes implicated in the pathophysiology of varicosity:

1. **C-reactive protein (CRP):** CRP is a well-known marker of systemic inflammation, elevated in various inflammatory conditions, including chronic venous disease, and serves as an indicator of ongoing inflammation that may contribute to venous insufficiency.
2. **Interleukin-6 (IL-6):** This pro-inflammatory cytokine is central to the inflammatory cascade, promoting endothelial dysfunction and vascular inflammation, both of which are linked to venous disease progression.
3. **Tumor necrosis factor-alpha (TNF-α):** TNF-α is another key inflammatory cytokine that exacerbates vascular inflammation and contributes to the degradation of vascular extracellular matrix, impacting vein structure and function.
4. **Tissue inhibitor of metalloproteinases-1 (TIMP-1):** TIMP-1 regulates matrix metalloproteinase activity, balancing tissue remodelling processes. Altered TIMP-1 levels may influence venous wall integrity, thus affecting varicosity.
5. **Transforming growth factor-beta 1 (TGF-β1):** TGF-β1 is involved in tissue fibrosis and remodeling. Elevated levels may contribute to vein wall thickening and stiffness, common in varicose veins.
6. **Procollagen type I N-terminal propeptide (PINP):** PINP reflects collagen synthesis and is used to assess fibrotic changes. Increased collagen turnover is a hallmark of venous remodelling in varicosity.
7. **Vascular endothelial growth factor (VEGF):** VEGF plays a crucial role in angiogenesis and endothelial repair. In varicosity, elevated VEGF levels are indicative of abnormal vessel formation and permeability changes.
8. **Malondialdehyde (MDA):** MDA is a marker of oxidative stress, which contributes to endothelial damage and inflammation, playing a role in varicose vein pathogenesis.
9. **Endothelin-1 (ET-1):** ET-1 is a potent vasoconstrictor associated with vascular tone regulation. Dysregulation of ET-1 contributes to venous hypertension and vascular dysfunction.
10. **Matrix metalloproteinase-9 (MMP-9):** MMP-9 is involved in extracellular matrix degradation, and elevated levels are linked to tissue remodelling and vein wall weakening, which are critical in varicose vein formation.

These biomarkers were selected to capture a comprehensive profile of the inflammatory, oxidative, and tissue remodelling processes relevant to varicosity, providing a robust foundation for understanding the molecular dynamics underlying the condition.

Assay methods: The details of the Human Assay Kits utilized for biomarker evaluation, including manufacturers

and geographical locations, are summarized in table 1. Receiver operating characteristic (ROC) curve analysis was conducted to determine the diagnostic performance of each biomarker. Sensitivity and specificity were assessed, and the area under the curve (AUC) values were computed, accompanied by 95% confidence intervals. These intervals were established using the bootstrap method as per established protocols (17-18) and in line with previous publications (19-20).

Table 1. Details of Human Assay Kits Used in the Study, Including Manufacturer Names and Locations

Biomarkers	Human assay Kit	Manufacturer	Place
CPP	CRP ELISA Kit	Nirmal Bioscience Pvt. Ltd.	Haryana 134113,
Il-6	IL-6 ELISA Kit	RayBiotech Life, Inc.,	New Delhi 110008,
TNF-α	TNF-α ELISA Kit	Elite Biotech	New Delhi 110044
TIMP-1	TIMP-1 ELISA Kit	Immuno Diagnostic India Pvt. Ltd.	New Delhi 110058
TGF-β1	TGF-β1 ELISA Kit	Diacron International	Mumbai 400093
PINP	PINP ELISA Kit	Rad Laboratories India Pvt. Ltd.	Pune 411014
VEGF	VEGF ELISA Kit	Genetix Biotech Asia Pvt. Ltd.	Haryana 134109
MDA	Colorimetri c Assay Kit	Labcare Diagnostics	New Delhi 110005
ET-1	VEGF ELISA Kit	Genetix Biotech Asia Pvt. Ltd.	Haryana 134109
MMP-9	MMP-9 ELISA Kit	Biobasic India	Bangalore 560095.

CRP: C-Reactive Protein, Il-6: Interleukin-6, TNF-α: Tumor Necrosis Factor-alpha, TIMP-1: Tissue Inhibitor of Metalloproteinases-1, TGF-β1: Transforming Growth Factor-beta 1, PINP: Procollagen Type I N-terminal Propeptide, VEGF: Vascular Endothelial Growth Factor, MDA: Malondialdehyde, ET-1: Endothelin-1, and MMP-9: Matrix Metalloproteinase-9.

Meta-analysis and heterogeneity tests: A meta-analysis was conducted to aggregate data from multiple studies, enhancing reliability. Heterogeneity across studies was assessed using Cochran's Q-test and the I-squared index to determine consistency. Effect-size indices quantified the results within the meta-analysis (21).

Radar chart analysis: Radar charts were used to visualize standardized mean differences and percentage changes in biomarkers between experimental and control groups, focusing on the severity of varicosity (22).

Correlation coefficient analysis: To elucidate the intricate relationships among key molecular biomarkers governing inflammation, vascular function, and tissue remodelling, a comprehensive Pearson correlation coefficient analysis was evaluated. The analysis was conducted for both the experimental and control groups, assessing the strength and direction of associations between biomarkers, including CRP, IL-6, VEGF, MDA, ET-1, MMP-9, TNF-alpha, TIMP-1, PINP, and TGF-beta1. Pearson's correlation coefficients (r-values) were computed for each biomarker pair, accompanied by p-values to assess statistical significance. A two-tailed test with a significance level of $p < 0.05$ was used to identify meaningful correlations. The strength of correlation was classified as strong ($r > 0.7$), moderate (r between 0.3 and 0.7), or weak ($r < 0.3$). Statistical analyses were conducted using appropriate software, ensuring the robustness and reproducibility of results.

Statistical analysis: Descriptive statistics, including means, standard deviations, and ranges, were calculated for both demographic and biomarker data. Independent t-tests were used for normally distributed data, while Mann-Whitney U tests were applied for non-normally distributed data when comparing serum biomarker levels between varicosity patients and control subjects. ROC curves were constructed to evaluate the diagnostic performance of each biomarker, with AUC values calculated to determine a predictive value for varicosity. Risk ratios and 95% confidence intervals were computed to assess associations between biomarker levels and varicosity risk. Data were analysed using IBM SPSS Statistics Version 29.0 (2022), with statistical significance set at $p < 0.05$. Data accuracy and completeness were verified before analysis.

Ethical considerations: The study received approval from the OPTM Health Care Pvt Ltd Review Board with ethical code No. OPTM/EC/VIBIO/2022. Additionally, informed consent was obtained from all participants before their inclusion in the study.

Results

Enrolment and baseline characteristics of patients: A total of 324 participants meeting the inclusion criteria were enrolled in the study of these, 216 (63.08% females) participants with varicosity lasting more than 3 years were assigned to the experimental group, while 108(60.87%

females)participants without varicosity were assigned to the control group. Demographic and baseline analysis indicated that both groups were predominantly females, with similar profiles in terms of age, weight, height, and BMI. The intervention group exhibited a marginally higher prevalence of hypertension. Across both groups, there were comparable distributions of ethnicity, dietary habits, varicose vein symptoms, occupational roles, marital status, comorbidities, and strategies for managing pain and inflammation.

ROC curve analysis: ROC curve analysis demonstrated that several molecular markers exhibited high diagnostic accuracy in distinguishing the experimental group from the control group. Notably, TNF-α achieved the highest area under the curve (AUC) of 0.921 (95% CI: 0.87, 0.97), with a sensitivity of 85.71% and a specificity of 87.76%. Other significant markers included PINP with an AUC of 0.939 (95% CI: 0.89, 0.98), and TIMP-1 with an AUC of 0.921 (95% CI: 0.87, 0.97). These biomarkers demonstrated strong sensitivity, specificity, and predictive values, highlighting their potential for effective varicosity identification (figures 1A to 1D and table 2).

Table 2. Analysis of important parameters of receiver operating characteristic curves of the Molecular Markers Experimental group (n=126) versus control group (n=108)

BIOMARKERS	AUC (95%CI), *p*-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Cut-off value
MOLECULAR INDICATORS OF INFLAMMATION IN VARICOSITY							
CRP	0.858 (0.79, 0.93),p<0.00001	66.67	79.63	80.77	53.85	59.83	2 mg/L
IL-6	0.862 (0.8, 0.93), p<0.00001	90.48	51.86	72.88	65.52	69.23	8.5pg/mL
TNF-α	0.921 (0.87, 0.97), p<0.00001	85.71	87.76	83.02	70.31	76.07	6 pg/mL
MOLECULAR INDICATORS OF FIBROSIS AND COLLAGEN DEPOSITION IN VARICOSITY							
TGF-β 1	0.896 (0.84, 0.95), p<0.00001	95.24	59.26	80.85	64.29	70.94	500 pg/mL
PINP	0.939 (0.89, 0.98), p<0.00001	96.72	66.67	87.76	70.59	77.76	100 ng/mL
TIMP-1	0.921 (0.87, 0.97), p<0.00001	96.83	70.59	82.98	65.71	72.65	250 ng/mL
MOLECULAR INDICATORS OF ENDOTHELIAL DYSFUNCTION IN VARICOSITY							
VEGF	0.881 (0.82, 0.94), p<0.00001	87.3	62.96	79.07	60.81	67.52	150 pg/mL
MDA	0.891 (0.83, 0.95), p<0.00001	90.48	61.11	80	62.5	69.23	5 µmol/L
MOLECULAR INDICATORS OF VASCULAR CELL ACTIVATION AND VENOUS PRESSURE IN VERICOSITY							
ET-1	0.923 (0.87, 0.97). P<0.00001	93.65	64.81	86.36	65.75	73.5	15.05 pg/ml
MMP-9	0.907 (0.85, 0.96), p<0.00001	93.65	61.11	82.22	63.89	70.94	312.4 ng/ml

CRP: C-Reactive Protein, Il-6: Interleukin-6, TNF-α: Tumour Necrosis Factor-alpha, TIMP-1: Tissue Inhibitor of Metalloproteinases-1, TGF-β1: Transforming Growth Factor-beta 1, PINP: Procollagen Type I N-terminal Propeptide, VEGF: Vascular Endothelial Growth Factor, MDA: Malondialdehyde, ET-1: Endothelin-1, and MMP-9: Matrix Metalloproteinase-9.

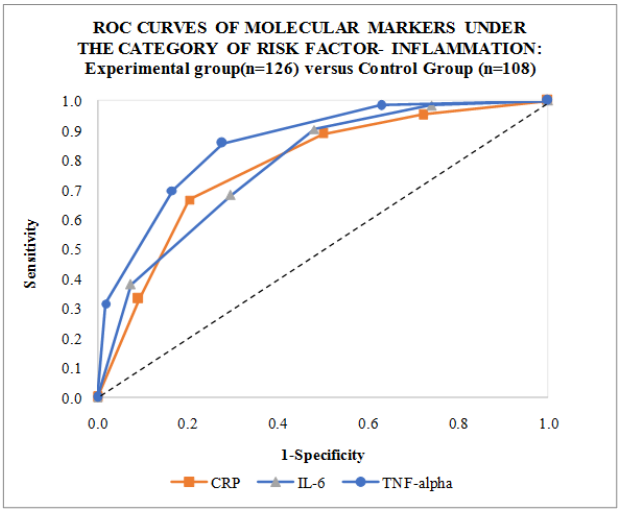

Figure 1A. ROC curves of molecular markers under the category of risk factor- Inflammation: Experimental group (n=126) versus control group (n=108)

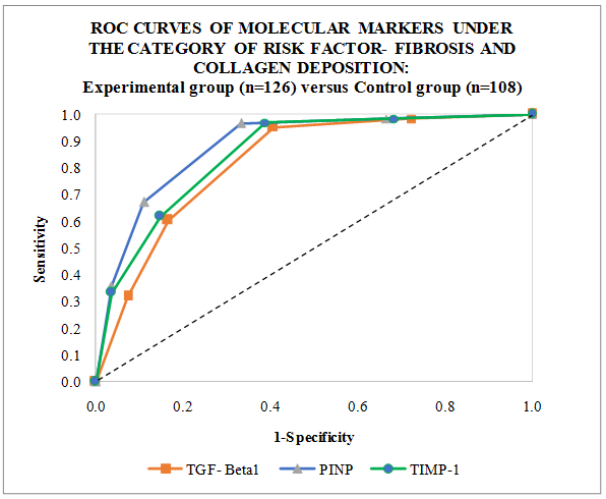

Figure 1B. ROC curves of molecular markers under the category of risk factor fibrosis and collagen deposition: Experimental group (n=126) versus control group (n=108)

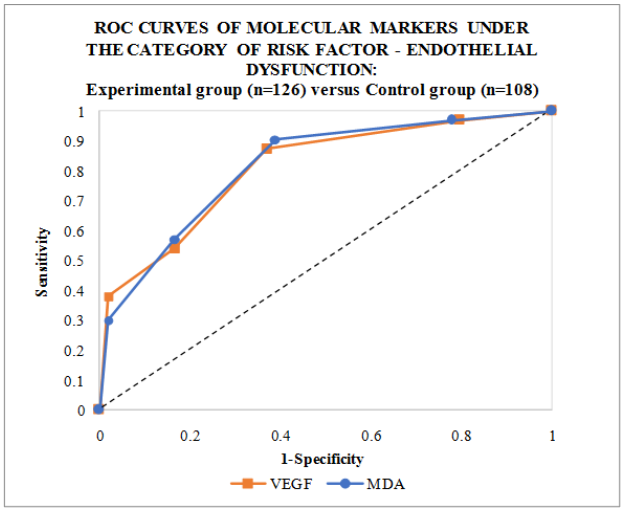

Figure 1C. ROC curves of molecular markers under the category of risk factor - endothelial dysfunction: Experimental group (n=126) versus control group (n=108)

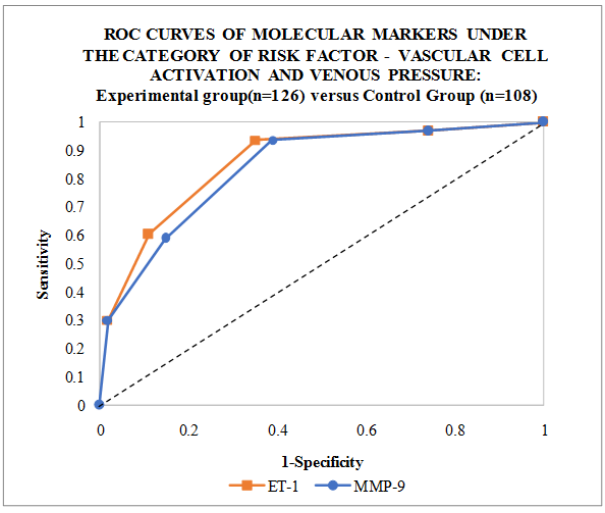

Figure 1D. ROC curves of molecular markers under category of risk factor - vascular cell activation and venous pressure: Experimental group (n=126) versus control group (n=108)

Meta-analysis and heterogeneity Tests: Meta-analysis revealed significant elevations in molecular biomarkers in the experimental group compared to the controls. Inflammatory markers exhibited standardized mean differences (SMDs) of 3.09 for CRP, 4.44 for IL-6, and 7.12 for TNF-α. Fibrosis markers, including TGF-β1, PINP, and TIMP-1, showed SMDs of 11.3, 5.56, and 6.6, respectively. Markers of endothelial dysfunction and vascular activation, such as VEGF, MDA, ET-1, and MMP-9, also showed notable increases, with SMDs ranging from 1.91 to 9.55 (figure 2).

Radar chart analysis: Radar chart analysis revealed significant increases in the standardized mean differences (SMDs) of molecular biomarkers in the experimental group compared to the control group. Inflammatory markers demonstrated substantial elevations, with CRP increased by 145.16%, IL-6 by 150.98%, and TNF-α by 251.22%. Fibrosis and collagen deposition markers, including TGF-β1, PINP, and TIMP-1, showed increases of 66.5%, 89.44%, and 72.57%, respectively. Additionally, endothelial dysfunction and vascular cell activation markers, such as VEGF, MDA, ET-1, and MMP-9,

demonstrated increases of 98.14%, 86.49%, 125.25%, and 126.33%, respectively (figure 3).

Overall, the elevated levels of these ten molecular indicators were significantly associated with varicosity, reflecting underlying pathophysiological mechanisms. This comprehensive biomarker assessment offers valuable insights into risk factors and may inform strategies for the early detection and management of varicosity.

Correlation coefficient analysis: Pearson's correlation analysis of the experimental group (n=126) revealed significant relationships between key biomarkers (Table 3A). CRP demonstrated strong positive correlations with TNF-alpha ($r = 0.506$, $p < 0.001$) and moderate correlations with IL-6 and TIMP-1, highlighting its role in inflammation. IL-6 is strongly associated with VEGF, PINP, and TGF-beta1, indicating a link between inflammatory and tissue repair pathways. VEGF and TGF-beta1 also showed high correlations with PINP, emphasizing their involvement in tissue remodelling. Moreover, MDA, ET-1, MMP-9, and TIMP-1 exhibited moderate correlations with key inflammatory and tissue remodelling biomarkers, further emphasizing the intricate interplay between inflammation, vascular dynamics, and tissue repair mechanisms.

Similarly, Pearson's correlation analysis of the control group (n=108) (table 3B) revealed strong correlations among key biomarkers. CRP displayed strong positive correlations with IL-6 ($r = 0.75$, $p < 0.001$), VEGF ($r = 0.68$, $p < 0.001$), and TGF-beta1 ($r = 0.64$, $P = 0.001$). IL-6 also correlated strongly with VEGF ($r = 0.71$, $p < 0.001$), TGF-beta1 ($r = 0.59$, $P = 0.002$), and MMP-9 ($r = 0.6$, $P = 0.002$). Notably, VEGF had strong associations with TGF-beta1 ($r = 0.67$, $p < 0.001$) and MMP-9 ($r = 0.56$, $P = 0.004$). TGF-beta1 was also significantly correlated with PINP ($r = 0.58$, $P = 0.002$). These findings suggest robust interrelationships between inflammatory, vascular, and tissue remodelling biomarkers in the control group, similar to those observed in the experimental group.

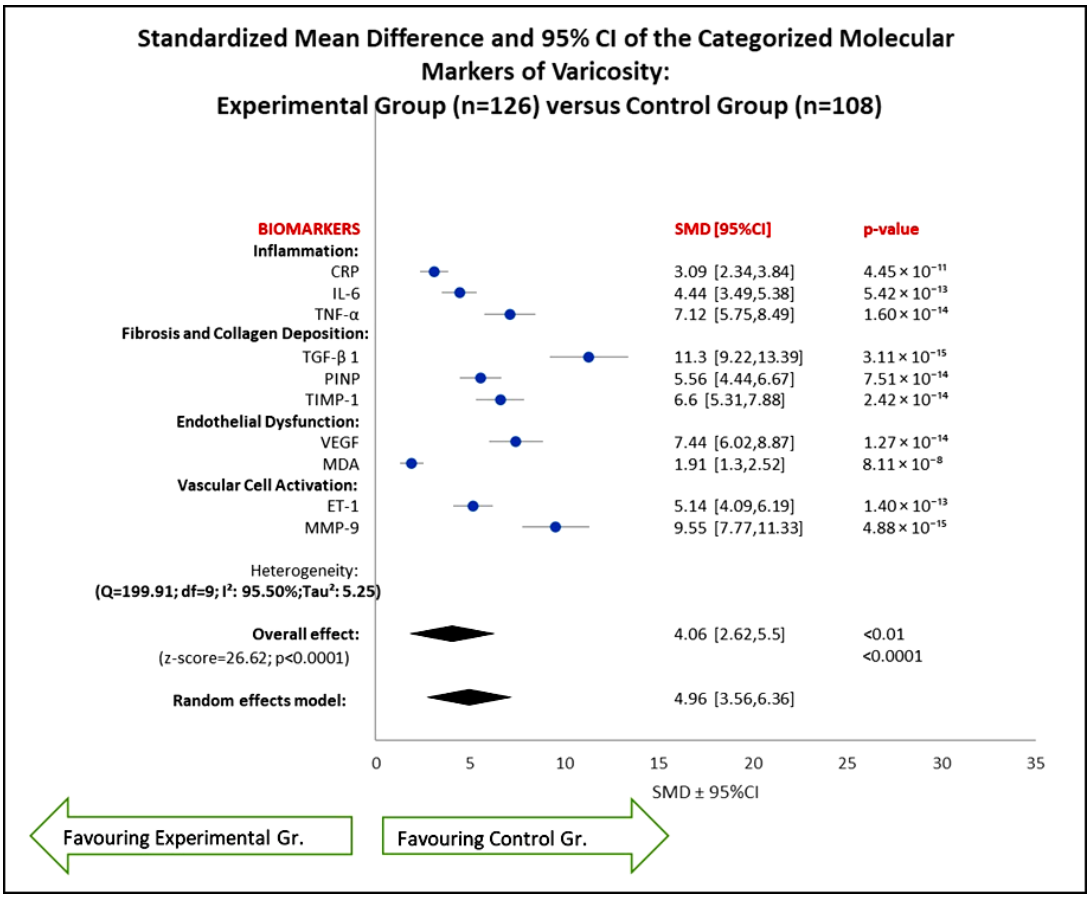

Figure 2. Standardized mean difference and 95% CI of the categorized molecular markers of varicosity, experimental Group (n=126) versus Control Group (n=108).

CRP: C-Reactive Protein, Il-6: Interleukin-6, TNF-α: Tmor Necrosis Factor-alpha, TIMP-1: Tissue Inhibitor of Metalloproteinases-1, TGF-β1: Transforming Growth Factor-beta 1, PINP: Procollagen Type I N-terminal Propeptide, VEGF: Vascular Endothelial Growth Factor, MDA: Malondialdehyde, ET-1: Endothelin-1, and MMP-9: Matrix Metalloproteinase-9

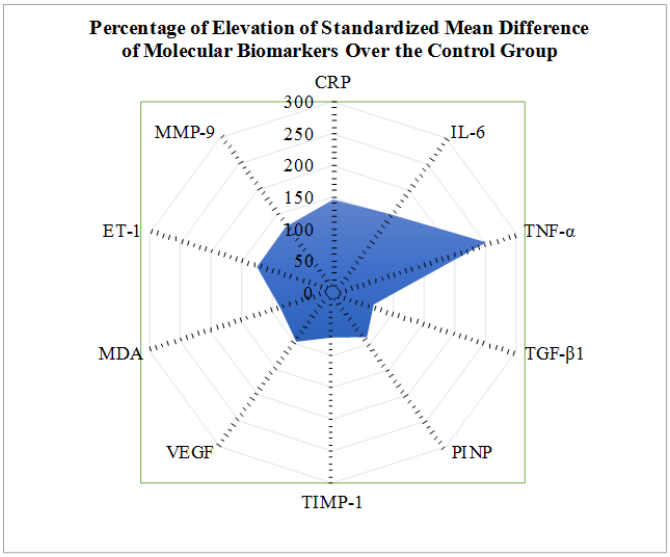

Figure 3. Percentage of elevation of standardized mean difference of molecular biomarkers over the control group. CRP: C-Reactive Protein, Il-6: Interleukin-6, TNF-α: Tumor Necrosis Factor-alpha, TIMP-1: Tissue Inhibitor of Metalloproteinases-1, TGF-β1: Transforming Growth Factor-beta 1, PINP: Procollagen Type I N-terminal Propeptide, VEGF: Vascular Endothelial Growth Factor, MDA: Malondialdehyde, ET-1: Endothelin-1, and MMP-9: Matrix Metalloproteinase-9

Table 3A. Pearson's correlation between the molecular biomarkers of the experimental Group (n=126)

Biomarkers	Correlation coefficient & p-value	CRP	IL-6	VEGF	MDA	ET-1	MMP-9	TNF-α	TIMP-1	PINP	TGF-β1
CRP	r-value	1	0.489	0.273	0.183	0.279	0.224	0.506	0.324	0.148	0.2
	p-value		<0.01	0.034	0.191	0.027	0.091	<0.001	0.009	0.3	0.163
IL-6	r-value	0.489	1	0.431	0.246	0.0306	0.223	0.412	0.232	0.526	0.621
	p-value	<0.001		<0.001	0.053	0.013	0.097	<0.001	0.083	<0.001	<0.001
VEGF	r-value	0.273	0.431	1	0.224	0.208	0.313	0.286	0.124	0.542	0.672
	p-value	0.034	<0.001		0.093	0.121	0.012	0.027	0.31	<0.001	<0.001
MDA	r-value	0.183	0.246	0.224	1	0.284	0.165	0.171	0.246	0.569	0.641
	p-value	0.191	0.053	0.093		0.029	0.214	0.2	0.055	<0.001	<0.001
ET-1	r-value	0.279	0.306	0.208	0.284	1	0.249	0.372	0.364	0.573	0.641
	p-value	0.027	0.013	0.121	0.029		0.049	0.002	0.003	<0.001	<0.001
MMP-9	r-value	0.284	0.255	0.289	0.314	0.379	1	0.356	0.396	0.421	0.274
	p-value	0.027	0.051	0.024	0.014	0.003		0.005	0.001	0.001	0.036
TNF-α	r-value	0.506	0.412	0.286	0.171	0.372	0.224	1	0.253	0.59	0.632
	p-value	<0.001	<0.001	0.027	0.2	0.002	0.097		0.004	<0.001	<0.001
TIMP-1	r-value	0.189	0.232	0.124	0.246	0.364	0.398	0.329	1	0.59	0.275
	p-value	0.162	0.083	0.31	0.055	0.003	0.001	0.009		<0.001	0.035
PINP	r-value	0.071	0.094	0.12	0.079	0.097	0.048	0.054	0.062	1	0.596
	p-value	0.636	0.528	0.394	0.598	0.508	0.755	0.724	0.677		<0.001
TGF-β1	r-value	0.319	0.198	0.225	0.226	0.21	0.191	0.284	0.284	0.596	1
	p-value	0.01	0.134	0.095	0.091	0.115	0.155	0.086	0.028	<0.001	

CRP: C-Reactive Protein, Il-6: Interleukin-6 ,TNF-α :Tumor Necrosis Factor-alpha, TIMP-1: Tissue Inhibitor of Metalloproteinases-1, TGF-β1: Transforming Growth Factor-beta 1, PINP: Procollagen Type I N-terminal Propeptide, VEGF: Vascular Endothelial Growth Factor, MDA: Malondialdehyde, ET-1: Endothelin-1, and MMP-9: Matrix Metalloproteinase-9.

Table 3B. Pearson's correlation between the molecular biomarkers of control Group (n=108)

Biomarker	Correlation coefficiant& p-value	CRP	IL-6	VEGF	MDA	ET-1	MMP-9	TNF-α	TIMP-1	PINP	TGF-β1
CRP	r-value	1	0.75	0.68	0.55	0.49	0.5	0.56	0.46	0.52	0.64
	p-value	-	<0.001	<0.001	0.002	0.01	0.01	0.005	0.02	0.01	0.001
IL-6	r-value	0.75	1	0.71	0.5	0.58	0.6	0.52	0.39	0.47	0.59
	p-value	<0.001	-	<0.001	0.01	0.005	0.002	0.01	0.05	0.02	0.002
VEGF	r-value	0.68	0.71	1	0.48	0.55	0.53	0.5	0.37	0.44	0.67
	p-value	<0.001	<0.001	-	0.02	0.01	0.01	0.01	0.05	0.03	<0.001
MDA	r-value	0.55	0.5	0.48	1	0.47	0.4	0.39	0.35	0.36	0.44
	p-value	0.002	0.01	0.02	-	0.02	0.05	0.05	0.06	0.05	0.02
ET-1	r-value	0.49	0.44	0.52	0.55	1	0.48	0.45	0.39	0.4	0.42
	p-value	0.01	0.02	0.01	0.006	-	0.01	0.02	0.03	0.04	0.03
MMP-9	r-value	0.5	0.47	0.56	0.5	0.48	1	0.44	0.47	0.45	0.42
	p-value	0.01	0.02	0.004	0.01	0.01	-	0.02	0.02	0.02	0.03
TNF-α	r-value	0.56	0.45	0.52	0.44	0.43	0.47	1	0.37	0.41	0.49
	p-value	0.005	0.02	0.01	0.02	0.03	0.02	-	0.05	0.03	0.01
TIMP-1	r-value	0.46	0.37	0.42	0.35	0.38	0.36	0.37	1	0.36	0.41
	p-value	0.02	0.05	0.03	0.06	0.05	0.05	0.05	-	0.05	0.04
PINP	r-value	0.52	0.47	0.55	0.48	0.4	0.45	0.41	0.36	1	0.58
	p-value	0.01	0.02	0.005	0.01	0.04	0.02	0.03	0.05	-	0.002
TGF-β1	r-value	0.64	0.59	0.67	0.44	0.42	0.45	0.49	0.41	0.58	1
	p-value	0.001	0.002	<0.001	0.02	0.03	0.02	0.01	0.04	0.002	-

CRP: C-Reactive Protein, Il-6: Interleukin-6, TNF-α Tumor Necrosis Factor-alpha, TIMP-1: Tissue Inhibitor of Metalloproteinases-1, TGF-β1: Transforming Growth Factor-beta 1, PINP: Procollagen Type I N-terminal Propeptide, VEGF: Vascular Endothelial Growth Factor, MDA: Malondialdehyde, ET-1: Endothelin-1, and MMP-9: Matrix Metalloproteinase-9.

Discussion

This study delivers an in-depth exploration of ten molecular biomarkers, providing critical insights into the pathophysiology of varicosity and their diagnostic and therapeutic implications. The ROC curve analysis demonstrates the robust utility of these biomarkers in diagnosing varicosity. Notably, TNF-α emerged as a particularly potent marker of inflammation, with a high AUC, sensitivity, and specificity, underscoring its pivotal role in the inflammatory processes associated with venous insufficiency (23) (figures 1A-1D, table 2). This aligns with meta-analysis findings, which show TNF-α as one of the most significant inflammatory markers, further validating its importance in varicosity pathology (figure 2).

The analysis of fibrosis and collagen deposition markers, including PINP and TIMP-1, reveals their exceptional diagnostic performance. Elevated levels of PINP and TIMP-1, along with TGF-β1, highlight their crucial roles in fibrotic activity and extracellular matrix remodelling within venous walls. These biomarkers are instrumental in understanding the structural alterations that characterize varicosity progression. The substantial elevations observed in TGF-β1 and PINP emphasize their roles in fibrotic processes, reinforcing their relevance in the disease mechanism (24-26) (figures 1A-1D, figure2).

Endothelial dysfunction and vascular cell activation are also critical components of varicosity, as evidenced by elevated VEGF, MMP-9, and ET-1 levels. VEGF and

MMP-9 indicate significant endothelial dysfunction and increased angiogenesis, while ET-1 reflects heightened vascular cell activation, contributing to venous hypertension. These findings illustrate the complex interplay of molecular factors driving varicosity, suggesting that these biomarkers are valuable for identifying therapeutic targets (27-29) (figures 1A-1D, figure 2). The correlation analysis (Table 3A) revealed that CRP demonstrated moderate positive correlations with IL-6 and TNF-α, indicating a meaningful association with systemic inflammation. The weaker correlation of CRP with VEGF suggests a limited role in angiogenic processes linked to venous and epidermal healing.

IL-6 exhibited a moderate positive association with VEGF and PINP, highlighting its contribution to tissue repair and angiogenesis. VEGF's moderate correlation with PINP and strong correlation with TGF-β1 underscores its critical involvement in extracellular matrix remodelling and vascular growth. MDA's strong correlation with TGF-β1 and moderate correlation with PINP (Table 3A) supports the hypothesis that oxidative stress exacerbates inflammation and tissue damage. ET-1 demonstrated weak correlations with VEGF and MMP-9, suggesting a limited role in vascular dysfunction and tissue remodelling.

In the control group (Table 3B), CRP displayed strong correlations with IL-6, VEGF, and TGF-β1, highlighting an amplified inflammatory and angiogenic response. The moderate correlation between MDA and TGF-β1 further implicates oxidative stress in chronic inflammation and fibrosis. IL-6's moderate associations with MMP-9 and VEGF align with its dual role in inflammation and extracellular matrix remodelling. Overall, the data emphasize the interconnected roles of inflammation (CRP, IL-6, TNF-α), oxidative stress (MDA), and tissue remodelling markers (VEGF, MMP-9, TGF-β1) in the pathophysiology of venous and epidermal ailments. These findings identify potential therapeutic targets for improved management strategies.

Despite its contributions, this study has several limitations. The cross-sectional design restricts the ability to establish causal relationships between biomarkers and varicosity, limiting conclusions to associations only ally, potential confounding variables, such as lifestyle factors, underlying health conditions, and medication use, may influence biomarker levels and were not fully controlled in this study. While includes patients from two distinct geographic locations, it may not fully represent the broader population of individuals with venous insufficiency. Variability in measures and potential measurement errors could also affect the results. Future research using longings, with broader, more diverse populations and comprehensive control of confounding factors, is recommended to validate these findings and further elucidate the role of these biomarkers in varicosity (30-32).

This study underscores the critical role of molecular biomarkers in diagnosing and managing varicosity. Specifically, markers such as TNF-α, PINP, and TIMP-1 emerge as valuable indicators of inflammation and fibrosis in venous insufficiency. The findings support the clinical potential of these biomarkers in improving diagnostic accuracy and guiding treatment strategies for varicosity-related pathologies. By highlighting the interconnected roles of inflammation, tissue remodelling, and vascular dysfunction, this research provides a foundation for integrating these biomarkers into diagnostic and therapeutic frameworks, enhancing early intervention and individualized treatment approaches in clinical practice.

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