Utility of routine laboratory tests in the assessment of chronic venous disease progression in female patients

SERGIU-CIPRIAN MATEI[1,2], MERVAT MATEI[1], FLAVIA MEDANA ANGHEL[1], EMMA CARABENCIOV[1], MARIUS-SORIN MURARIU[1,2] and SORIN OLARIU[1,2]

[1]Department X of Surgery, 'Victor Babeș' University of Medicine and Pharmacy Timișoara, Timișoara 300041; [2]1st Surgical Clinic, 'Pius Brînzeu' University Clinical Hospital Timișoara, Timișoara 300723, Romania

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Abstract. Chronic venous disease (CVD) is a frequently encountered disease that progresses with age. Although the principal method of evaluation and diagnosis is Doppler ultrasound, routine laboratory tests may be an easier and more accessible way to evaluate CVD progression. The present retrospective study evaluated the laboratory results of 256 patients diagnosed with CVD. According to the Clinical, Etiological, Anatomical and Pathophysiological classification, depending on the CVD stage, patients were stratified into three groups: Group 1 (C2-C3; mild disease), Group 2 (C4; moderate to severe disease) and Group 3 (C5-C6; severe disease). The considered parameters were age, red blood cell count (RBC), white blood cell count (WBC) and platelet count (PLT), percentage of neutrophils and lymphocytes, neutrophil-to-lymphocyte ratio (NLR), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen, prothrombin time (in percentages and seconds), internal normalized ratio, activated partial thromboplastin time, creatine kinase (CK), CK myocardial band, alanine transaminase, aspartate transaminase, total bilirubin and urea. No significant differences among the groups were noted in RBC, WBC, PLT and coagulation factors; on the other hand, inflammatory markers exhibited differences among the groups. Several differences were observed in hepatic, metabolic and muscle tissue markers. Intraluminal thrombus formation in the case of varicose veins (thrombophlebitis) may be due to conditions of turbulent flow, stasis and endothelial inflammation, rather than hypercoagulability. The results of the present study confirmed the implication of inflammatory factors in pathophysiological modifications, including thickening of venous walls and valvular modification, as well as the appearance of intraluminal thrombi and trophic lesions. NLR, ESR, CRP and fibrinogen were increased with CVD progression and may be considered useful markers in evaluating CVD progression. Simple blood tests may provide phlebologists with additional insight for the management of those patients.

Correspondence to: Professor Marius-Sorin Murariu, Department X of Surgery, 'Victor Babeș' University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Sq. Timișoara 300041, Romania
E-mail: murariu.marius@umft.ro

Abbreviations: CVD, chronic venous disease; RBC, red blood cell count; WBC, white blood cell count; NLR, neutrophil-to-lymphocyte ratio; PLT, platelet count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; PT, prothrombin time; INR, international normalized ratio; aPTT, activated partial thromboplastin time; CK, creatine kinase; CK-MB, CK myocardial band; ALT, alanine transaminase; AST, aspartate transaminase

Key words: chronic venous disease, laboratory tests, neutrophil-to-lymphocyte percentage ratio, fibrinogen, ESR, CRP, venous disease progression assessment

Introduction

The prevalence of chronic venous disease (CVD) is high worldwide, affecting 60-70% of the population and having a negative impact on the quality of life of these patients (1,2). A recent study determined that the prevalence of CVD in Romania is 68,4% (3), double the frequency of that reported by a similar study from 10 years ago (4). Considering these local and worldwide statistical data, CVD is a common pathology. Although the diagnosis of CVD is currently made in a clinical setting, it may be considered that paraclinical investigations provide valuable additional information allowing for disease progression monitoring.

From the paraclinical investigation perspective, colour-Doppler ultrasound is the currently preferred tool used in the assessment of blood flow for various diseases (5), including venous insufficiency diagnosis (6). Despite the fact that this technique is highly accurate in the diagnosis of CVD and its progression (7), it may be time-consuming. In addition, the device is relatively expensive and the results are operator-dependent.

Routine laboratory tests are relatively inexpensive and are performed for almost all in- and outpatients (8). Regarding CVD, blood tests are usually performed in the preoperative context or when thromboembolic complications occur (9). However, simple blood tests may also be used as predictive markers of disease severity and clinical status in patients with venous insufficiency. The fibrinogen-to-albumin ratio is a significant predictor of disease severity (10), but albumin is not a commonly performed

blood test for outpatients. Laboratory tests have also been used to determine the aetiology of CVD. Oestradiol, homocysteine and vascular endothelial growth factor variations were indicated to be associated with primary chronic venous insufficiency (11).

The present study examined the application of routine laboratory tests (complete blood count, inflammatory markers, coagulation factors, biochemistry) as markers of CVD progression.

Materials and methods

Patients and laboratory tests. The present study was an observational, retrospective cohort study that monitored the variations of laboratory parameters during CVD progression. Blood test results and clinical charts of 587 patients that presented at the Phlebology Department (1st Surgical Clinic, 'Pius Brînzeu' University Clinical Hospital Timișoara, Timișoara, Romania) with CVD between January 2017 and December 2020, inclusive, were evaluated (all patients who presented with CVD during the defined time period were initially included). Venous blood samples were collected from the elbow or anterior antebrachial region veins. All of the analysed data refer to the first ambulatory presentation of each patient. The parameters considered were age, red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), percentage of neutrophils and lymphocytes, neutrophil-to-lymphocyte ratio (NLR), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen, prothrombin time (in percentages and seconds), internal normalized ratio (INR), activated partial thromboplastin time (aPTT), cytokeratin (CK) and CK myocardial band (CK-MB), glycaemia, alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin and urea. Considering the paraclinical parameters studied, the laboratory reference values were as follows: RBC, 4.5-5.9x10^6/μl; WBC, 4-9.5x10^3/μl; neutrophil percentage, 45-70%; lymphocyte percentage, 20-40%; PLT, 150-400x10^3/μl; ESR, 0-15 mm/h; CRP, 0-10 mg/l; fibrinogen, 200-393 mg/dl; prothrombin time (PT) percentage, 90-148%; PT, 9.4-12.5 sec; INR, 0.85-1.14; aPTT, 25.1-36.5 1/s; CK, 30-170 U/l; CK-MB, 0-16 U/l; glycaemia, 74-106 mg/dl; ALT, 0-55 U/l; AST, 5-34 U/l; total bilirubin, 0.2-1.2 mg/dl; and urea, 15-45 mg/dl.

Enrolment criteria. In order to establish uniformity among and between the groups, the following data were excluded from the study: Male patients (95 cases); patients with incomplete data; patients who did not sign the written informed consent form to participate in future studies (111 cases); patients aged <45 or >80 years (79 cases); and patients with confirmed diabetes mellitus/patients with blood sugar values >140 or <50 mg/dl (46 cases). By applying these criteria, 256 patients were finally included in the study. Subsequently, patients were assigned to three groups according to the stage of the disease, using the clinical aspect (C) from the Clinical, Etiological, Anatomical, and Pathophysiological classification (12): Group 1 (mild disease)-included 91 female patients in stages C2 and C3; Group 2 (moderate to severe disease)-included 84 female patients in the C4 stage; Group 3 (severe disease)-included 81 female patients in stages C5 and C6.

Statistical analysis. For comparative statistical analysis among the groups, Microsoft Excel 2019 (v 16.0; Microsoft Corporation) was utilized, using predefined functions, such as the Data Analysis module. Measures of central tendency (mean), minimum and maximum were determined, with data reported by rounding to two decimal places. The normality of distribution of the variables was analysed using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and, subsequently, comparisons of these parameter variations among the three severity cohorts were performed using ANOVA with Tukey's post-hoc test for between-group comparison. $P<0.05$ was considered to indicate statistical significance.

Results

Statistical analysis results. For statistical analysis, the following parameters of interest were grouped according to the pathophysiological mechanisms that maybe implicated in CVD progression: Age, complete blood cell count and inflammatory markers (RBC, WBC with the percentage of neutrophils and lymphocytes, NLR, PLT, ESR, CRP), coagulation factors (fibrinogen, PT%, PT, INR and aPTT), characteristic markers for muscle tissue (CK, CK-MB), and hepatic and metabolic markers (glycaemia, ALT, AST, total bilirubin, urea). The studied parameters are presented in Table I as the mean ± standard deviation.

The P-values resulting from the comparative statistical analysis among all three study groups are presented in Table II, individually for each parameter evaluated in this study.

Age analysis. In terms of age, a highly significant difference was noted between the first two groups and Group 3 ($P<0.0001$ in both cases), although no statistically significant difference was observed between Groups 1 and 2. Age distribution was 55±8.40 years for Group 1, 57.04±10.11 years for Group 2 and 65.75±10.12 years for Group 3.

Complete blood cell count and inflammatory marker analysis. RBC count analysis revealed significant differences between Group 1 and Group 3 ($P=0.025$), as well as between Group 2 and Group 3 ($P=0.0058$). However, no statistically significant difference was observed between Group 1 and Group 2.

WBC count analysis did not reveal any statistically significant differences among groups. Despite this, the percentage of neutrophils increased with disease progression and exhibited statistically significant differences among groups: For Group 1 and Group 2, $P=0.0042$; for Group 2 and Group 3, $P=0.0008$; for Group 1 and Group 3 $P<0.0001$. With regard to the percentage of lymphocytes, a decrease with disease progression was noted, with a statistically significant difference between Groups 1 and 2 ($P=0.0247$) as well as Groups 1 and 3 ($P=0.0004$). When analysing the NLR, values increased with disease progression and statistically significant differences were obtained among all three groups: For Group 1 and Group 2, $P=0.019$; for Group 2 and Group 3, $P<0.0001$ and for Group 1 and Group 3, $P<0.0001$.

PLT analysis revealed a statistically significant difference between Groups 2 and 3 ($P=0.0384$).

In the present study, ESR and CRP parameters were increased, with statistically significant differences among all three groups ($P<0.0001$).

Table I. Mean ± standard deviation values of the studied parameters: Age, RBC, WBC, neutrophil and lymphocyte percentages, NLR, PLT, ESR, CRP, fibrinogen, PT%, PT, INR, aPTT, CK, CK-MB, glycemia, ALT, AST, total bilirubin and urea.

Group	Age, years	RBC, x$10^6/\mu$l	WBC, x$10^3/\mu$l	Neutrophils, %	Lymphocytes, %	NLR	PLT, x$10^3/\mu$l	ESR, mm/h	CRP, mg/l	Fibrinogen, mg/dl	PT, %	PT, sec	INR	aPPT, 1/sec	CK, U/l	CK-MB, U/l	Glycaemia, mg/dl	ALT, U/l	AST, U/l	Total bilirubin, mg/dl	Urea, mg/dl
Group 1	55±	4.62±	6.96±	57.16±	30.56±	1.87±	249.35±	9.93±	3.398±	317.18±	108±	12.40±	1.02±	31.79±	129.61±	18.01±	101.78±	32.06±	23.91±	0.53±0.24	33.47±
	8.40	0.42	1.85	11.28	9.52	1.18	71.76	4.80	3.30	80.32	17.40	9.90	0.17	5.45	61.56	10.51	18.77	22.15	19.21		10.75
Group 2	57.04±	4.67±	7.33±	61.66±	27.59±	2.23±	235.61±	18.23±	8.65±	368.48±	109.54±	12.08±	1.07±	31.38±	117.10±	18.07±	100.10±	31.52±	23.70±	0.58±	34.29±
	10.11	0.45	2.31	8.57	7.80	1.09	72.35	11.95	7.18	104.90	40.39	5.69	0.46	4.63	63.54	14.17	13.11	15.43	8.69	0.26	11.38
Group 3	65.75±	4.44±	7.54±	66.98±	23.47±	2.85±	262.15±	26.77±	20.31±	412.69±	99.86±	12.37±	1.12±	30.92±	101.9±	20.38±	110.14±	23.61±	20.45±	0.49±	39.64±
	10.12	0.57	2.34	11.54	9.64	1.19	89.60	14.53	16.71	111.29	18.37	4.69	0.42	6.46	69.46	15.03	18.52	9.19	6.87	0.25	13.54

Normal ranges (laboratory reference values): RBC, 4.5-5.9x$10^6/\mu$l; WBC, 4-9.5x$10^3/\mu$l; neutrophil percentage, 45-70%; lymphocyte percentage, 20-40%; PLT, 150-400x$10^3/\mu$l; ESR, 0-15 mm/h; CRP, 0-10 mg/l; fibrinogen, 200-393 mg/dl; PT percentage, 90-148%; PT, 9.4-12.5 sec; INR, 0.85-1.14; aPTT, 25.1-36.5/sec; CK, 30-170 U/l; CK-MB, 0-16 U/l; glycaemia, 74-106 mg/dl; ALT, 0-55 U/l; AST, 5-34 U/l; total bilirubin, 0.2-1.2 mg/dl; urea, 15-45 mg/dl. RBC, red blood cell count; WBC, white blood cell count; NLR, neutrophil to lymphocyte ratio; PLT, platelet count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; INR, internal normalized ratio; PT, prothrombin time; aPTT, activated partial thromboplastin time; CK, creatine kinase; CK-MB, CK myocardial band; ALT, alanine transaminase; AST, aspartate transaminase.

Coagulation factor analysis. Fibrinogen analysis revealed that the average level increased with disease progression and exhibited a statistically significant difference between Groups 1 and 2 (P=0.0004), Groups 2 and 3 (P=0.0095), and a highly significant difference between Groups 1 and 3 (P<0.0001).

PT% analysis revealed a statistically significant difference between Groups 1 and 3 (P=0.0031), as well as between Groups 2 and 3 (P=0.048). There were neither statistically significant differences between Groups 1 and 2, nor for the absolute value of PT. Among all three groups, no statistically significant difference was noted for the INR and aPTT.

Characteristic markers for muscle tissue analysis. CK analysis revealed a statistically significant difference between Groups 1 and 3 (P=0.0208). However, there was no statistically significant difference between groups in terms of the CK-MB.

Hepatic and metabolic markers analysis. Glycaemia values were slightly increased in the groups. Analysis of glycaemia revealed a statistically significant difference between Groups 1 and 3 (P=0.0038), as well as between Groups 2 and 3 (P<0.0001).

Transaminases analysis revealed a statistically significant difference between Groups 1 and 3 (P=0.0011), as well as between Groups 2 and 3 (P<0.0001) for ALT and a statistically significant difference between Groups 2 and 3 (P=0.0085) for AST.

Total bilirubin analysis revealed a statistically significant difference between Groups 2 and 3 (P=0.0227), while urea analysis revealed a statistically significant difference between Groups 1 and 3 (P=0.0013), as well as between Groups 2 and 3 (P=0.0069).

Discussion

CVD progresses with age (13). In accordance with the previous literature, the present results confirmed the aetiological hypothesis that, even for healthy subjects, aging appears to be a risk factor for CVD onset.

It has been indicated that the RBC generally decreases after 60 years of age (14), and, particularly in female patients, the RBC count tends to decrease beginning with the seventh decade of age (15). Due to the fact that, generally speaking, patients with venous ulcers, which represent the most severe stage of chronic venous insufficiency, have an average age of 59.67±11.95 years (16), 65.75±10.12 years according to the present results, and since no statistically significant differences were observed between Groups 1 and 2, which comprised subjects of similar age groups, it may be considered that the alterations of this parameter in the present study were not a result of CVD, but are related to advancing age.

The WBC count did not exhibit any statistically significant difference between groups, whereas the percentages of neutrophils and lymphocytes revealed a statistically significant difference. The present study demonstrated that the NLR may be a useful parameter in the evaluation of CVD progression. Literature data also described the NLR as a useful predictor of the severity of chronic venous insufficiency (17). To the best

Table II. Results of the comparative statistical analysis between the study groups.

Parameter	Group 1 vs. Group 2 comparison P-values	Group 1 vs. Group 3 comparison P-values	Group 2 vs. Group 3 comparison P-values
Age	0.2349	<0.0001	<0.0001
RBC	0.4570	0.0252	0.0058
WBC	0.2512	0.0752	0.5548
Neutrophil %	0.0042	<0.0001	0.0008
Lymphocyte %	0.0247	0.0004	0.0533
NLR	0.0190	<0.0001	<0.0001
PLT	0.2096	0.3064	0.0384
ESR	<0.0001	<0.0001	<0.0001
CRP	<0.0001	<0.0001	<0.0001
Fibrinogen	0.0004	<0.0001	0.0095
PT%	0.7613	0.0031	0.0485
PT	0.2756	0.0757	0.7227
INR	0.4499	0.0685	0.4473
aPPT	0.3821	0.3439	0.2779
CK	0.0955	0.0208	0.1447
CK-MB	0.9746	0.2380	0.3114
Glycaemia	0.4896	0.0038	<0.0001
ALT	0.8503	0.0011	<0.0001
AST	0.9229	0.1103	0.0085
Total bilirubin	0.1579	0.3140	0.0227
Urea	0.6234	0.0013	0.0069

PLT, platelet count; RBC, red blood cell count; WBC, white blood cell count; NLR, neutrophil to lymphocyte ratio; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; INR, internal normalized ratio; aPTT, activated partial thromboplastin time; CK, creatine kinase; CK-MB, CK myocardial band; ALT, alanine transaminase; AST, aspartate transaminase.

of our knowledge, no previous study has indicated the value of WBC in terms of disease progression.

Despite statistically significant differences between Groups 2 and 3 (P=0.0384) resulting from the PLT count analysis, it was not possible to consider this result as being associated with CVD progression, as no differences between the mild disease group and the other two groups were obtained. Literature data also revealed that the PLT count cannot be used to assess CVD progression (10).

ESR and CRP are used as inflammatory markers (18). As ESR is not a disease-specific marker, increased values of this parameter maybe observed in several conditions, such as inflammatory diseases, infections or tumours (19). CRP is considered an early indicator of inflammatory conditions as well (20). The role of CRP in cardiovascular events and as a marker of chronic inflammation is well known (21). Due to the association of high CRP levels with the development of atherosclerotic lesions (22) and because CRP mediates tissue fibrosis in several cardiovascular diseases (23), it may also be suspected that this characteristic may have an impact on the vasa vasorum from the venous adventitia, accelerating the processes of phlebosclerosis (24). Despite certain data suggesting that inflammation may not be an important component of the pathogenesis of varicose veins (25), most of the sources indicate that CVD is an inflammatory disease (26). The present results revealed a direct involvement of ESR and CRP in venous disease progression (P<0.0001) and confirm the inflammatory nature of CVD (2). Other markers commonly used in tumour evaluation, such as tumour necrosis factor-α or lactate dehydrogenase, are additional parameters that may be useful in highlighting inflammatory processes in other diseases (27,28), like CVD; however, they are not performed as routine laboratory tests.

Certain literature data revealed that in comparison to the systemic blood markers of inflammation and endothelial damage, inflammatory markers are increased in varicose vein blood (29). Even though in the present study, certain blood samples were collected from antebrachial veins and those results refer to systemic blood markers, statistically significant differences were observed among the groups.

Serum fibrinogen levels may be a useful parameter in determining disease severity in patients with CVD (10). Fibrinogen has an important role in the formation of intraluminal thrombus (30), with values increasing with the progression of CVD, an aspect that explains the occurrence of phlebothrombosis, a pathological aspect of advanced disease stages. In the case of venous thrombosis, increased fibrinogen levels are not consequences of acute phase reactions but are in fact increased in the context of chronic inflammation (31). The present results revealed that increased fibrinogen levels were directly correlated with disease progression (P≤0.0095 among all the groups), confirming this aetiological hypothesis. In addition, in

the venous leg ulcer, the predominant histological features are represented by pericapillary fibrin cuffs with an asymmetric capillary distribution (32). As fibrin is formed after thrombin cleavage of fibrinopeptide A from fibrinogen (33), the association between increased levels of fibrinogen and advanced cases of CVD maybe easily explained. In those cases, a defective fibrinolysis may also be responsible (34).

PT is the most commonly used laboratory coagulation test. It is mathematically converted to the INR (35). Even though by analysing PT%, significant differences between the first two groups and the third group were noted, no other significant differences were determined among the groups for other coagulation tests and average values were normal for all coagulation factors.

The incidence of superficial thrombophlebitis is higher than that of deep vein thrombosis (>1/1,000) (36) and it usually occurs in patients with varicose veins (37). Hypercoagulability, stasis and endothelial injury are the three factors according to Virchow's triad, and they may predispose a patient to venous thrombosis (38). The present observations suggested that hypercoagulability is not mandatory for the occurrence of thrombi in phlebological patients. Inflammation of the venous wall (39), damage to the vein endothelium, valvular dysfunction and venous stasis remain the main factors involved in acute thrombophlebitis in patients with varicose veins of the lower limbs.

CK levels decreased with increasing group number, but statistical analysis only revealed a significant difference between Group 1 and Group 3. Low serum CK levels maybe a consequence of decreased serum muscle enzyme outflow due to reduced physical activity caused by disease or advanced age (40). The present observations also confirm the hypothesis that CK is inversely and independently associated with CRP (41). The absence of statistically significant differences between groups in terms of CK-MB suggests no coexisting heart damage, which may have altered the values of other parameters (CRP, fibrinogen).

Alterations of hepatic markers (glycaemia, AST, ALT, total bilirubin and urea) indicate an association between CVD and altered hepatic function. It may also be acknowledged that increased urea maybe associated with a muscle tissue-affecting process, possibly of venous aetiology, an aspect that suggests a link between degeneration of the venous wall and hepatic function.

Diabetic patients are predisposed to developing chronic leg ulcerations (42). The present results on blood sugar levels revealed statistically significant differences between the first two groups and the third one ($P \leq 0.0038$ between the groups). This observation may suggest that the association between varicose veins and high blood sugar levels increases the chances of leg ulcer occurrence and poor wound healing, albeit not being connected with diabetes. Regarding the blood sugar range established for the present study, the upper limit was set to 140 mg/dl because the possibility that certain patients had an ingestion less than two hours before blood sampling could not be excluded (43).

The main limitation of the present study is the small number of patients. The fact that male patients we excluded may also be considered a limitation of this study. The reasons for implementing these exclusion criteria were the relatively small number of male patients, particularly for the severe groups (67 for Group 1, 10 for Group 2 and 18 for Group 3), the differences between the normal laboratory reference values that may occur between the sexes for certain parameters (e.g., RBC) or that may be influenced by male gender (e.g., CK) and the fact that female patients are more frequently affected by CVD (according to our observation, 65-80% of our patients are female each year-unpublished data). It may be considered that further studies on large groups of patients and mixed groups (males and females) maybe useful.

In addition, we consider a further study to demonstrate the usefulness of blood tests in assessing the ongoing condition of the patient once treatment has been initiated and for assessing the evolution of chronic venous disease during and after treatment. As we do not have a follow-up for all of the patients included in the present study, it was not possible to include all of these data in this manuscript. When a post-treatment follow-up for at least 50 patients for each group is available, we may consider analysing the results in order to present them in a future paper.

The present results confirmed the implication of inflammatory factors in pathophysiological alterations of CVD, such as the thickening of venous walls, valvular modification, the appearance of intraluminal thrombi and trophic lesions. These processes are exacerbated by impaired liver function and atherosclerotic phenomena. The parameters NLR, ESR, CRP and fibrinogen maybe considered useful markers in the evaluation of CVD progression. CK values had a slightly decreasing tendency, while glycaemic and urea values had a slightly increasing tendency with CVD progression. These results may provide phlebologists and clinicians with additional insight for the management of patients suffering from CVD.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SM was responsible for protocol design, data collection, obtaining ethical approval, data analysis and interpretation, manuscript drafting and revision. MM was involved in data collection, data analysis and interpretation, and critical revision. FMA performed statistical analysis and data interpretation, as well as manuscript drafting. EC was responsible for data collection, analysis and manuscript drafting. MSM was responsible for data analysis and interpretation, manuscript revision and approval of the final version of the manuscript, as well as being a corresponding author. SO was involved in data analysis and interpretation, manuscript revision and approval of the final version of the manuscript, as well as being a PhD supervisor and guarantor. SM and MSM confirm the

authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol, in compliance with the principles of the Declaration of Helsinki, was approved by the 'Pius Brînzeu' University Clinical Hospital Timișoara Ethical Committee (Timișoara, Romania; REC no. 258/2021).

Patient consent for publication

All of the patients included in the study provided written informed consent agreeing to the publication of the medical data for research and scientific purposes.

Competing interests

The authors declare that they have no competing interests.

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