Article

Clot characterization by multidisciplinary approach: biochemical and imaging parameters in a hypocoagulative setting. A pilot study

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Abstract

Background: Clot characterization is, to the present days, a multimodal approach: scanning the clot by electron microscopy (SEM) is helpful for the visualization of fibrin structure along with laboratory parameters such as the clot waveform analysis (CWA) and thrombin generation in different settings of clot abnormalities. This study aimed to assess whether the coagulative parameters were consistent with the clot images texture acquired by SEM, and therefore to propose a more generalist and integrative approach to clots classification.

Design and methods: In this pilot study, the examined population consists of eight healthy subjects, seven patients affected by Acquired Hemophilia A (AHA) and seven patients treated with Vitamin K Antagonists (VKAs), similar for age and gender. We studied the velocity and acceleration (1st and 2nd derivative of the aPTT) of clot formation (CWA), the thrombin generation, and the clots' scanning by SEM. Images acquired with SEM were then analyzed with the MATLAB software with the "Texture Analysis" methods to perform classification. Among the various texture parameters, we reported Contrast and Energy.

Results: Significant differences among healthy subjects, patients with AHA and those treated with VKAs were detected for the coagulative parameters. We found no differences between VKAs and AHA patients. Contrast and energy highlighted a significant difference among the three groups in agreement with the laboratory's parameters. We found no significant differences between VKAs and AHA patients.

Conclusions: The use of SEM, CWA and thrombin generation parameters may be a starting point for studies aimed to demonstrate the general characteristics of clot formation in different clinical conditions with a multiparametric approach.

Introduction

Blood coagulation is a conventional defensive system versus bleeding, infections and neoplastic cells.[1] The outcome is a clot consisting of a fibrin network whose properties are to form the hemostatic plug along with platelets on one hand and trap, and so kill, pathogens on the other. Blood coagulation activation is triggered by tissue factor (TF), a 47 kDa transmembrane glycoprotein that envelopes all blood vessels ready to activate the blood coagulation cascade when an injury occurs. However, TF can induce both venous and arterial thrombosis since it is expressed by foam cells in the atherosclerotic plaques and monocytes after activation by pathogens during sepsis, thus leading to disseminated intravascular coagulation. Moreover, cancer cells can release micro-vesicles carrying TF, which can provoke venous thromboembolism.[2] Fibrin formation comes from thrombin, the final protease of blood coagulation activation, which is able to cleave fibrinopeptide A and B from the fibrinogen molecule, thus inducing polymerization of fibrin monomers which then lead to fibrin I formation. The next step is the thrombin activation of factor XIII which induces fibrin cross-link at lysine residues of adjacent monomers and an alpha chain of the opposing ones.[3] The clot is then produced. However, several congenital and acquired diseases can affect fibrin formation, so that bleeding occurs. Oral and parental anticoagulants can as well reduce the coagulative potential so limiting the thromboembolic risk but increasing the bleeding one. In other words, the clot becomes weak in all those conditions.

Looking at the clot by scanning electron microscopy (SEM) may be useful for the visualization of the fibrin structure.[4] It has been demonstrated that fibrin structure is associated with venous thromboembolism showing the so-called prothrombotic fibrin clot phenotype.[5] The primary aim of our study was to obtain information on the characteristics of the clot texture by SEM imaging to

Significance for public health

In this pilot study, we propose a multimodal approach to the classification of clot pathologies. To our knowledge, our approach is innovative and describes a powerful and promising method for the classification of clot pathologies, widespread and still poorly understood pathologies.

classify different groups of clot abnormalities and to evaluate whether they could be correlated with the amount of plasmatic thrombin generation, along with the velocity and the acceleration of the clot formation. We focused our attention on two groups of patients at risk of major bleeding, i.e., patients affected by acquired haemophilia A (AHA), a severe life-threatening haemorrhagic disease induced by autoantibodies against the coagulation factor VIII[6] and patients treated with oral anticoagulants such as anti-vitamin K antagonists (VKAs)[7] compared to a healthy subjects control group. In addition, this study was also planned to obtain information on the characteristics of the clot structure aimed at detecting possible anomalies not shown by the laboratory investigation, such as a pro-hemorrhagic fibrin clot phenotype.

Design and methods

Patients

For this first explorative study, we evaluated a total of 22 patients, divided into three groups: eight healthy subjects, seven patients affected by AHA and seven patients treated with VKAs, similar for age and gender. Patients with AHA were studied as they were admitted to our clinical ward of Internal Medicine at the onset of the disease, i.e., when they were bleeding (Table 1). Patients treated with VKAs showed an International Normalized Ratio (INR) between 2.0 and 4.0 (Table 2). Plasma used for this study was a part left from blood sampling from a routine investigation. No further blood sampling was drawn. Patients and healthy subjects gave their informed consent. This study was approved by the Independent Ethical Committee of the Teaching Hospital of Cagliari (Prot. PG/2021/153). All the procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statistical analysis

The data are expressed as median and range because they were not normally distributed. A non-parametric analysis of variance (Kruskal-Wallis test) and the Dunn *post-hoc* test was used for comparison among and between groups. MedCalc software (version 19.0, Ostend, Belgium) was used for statistical analysis.

Clot preparation for clot waveform analysis, thrombin generation and SEM scanning

Blood samples were collected by venous withdrawal using 3 mL vacuum tubes (Venosafe, Terumo Italia, Rome, Italy) containing sodium citrate 0.109 M (3.2%). Plasma was obtained by centrifuging the tubes at 2020 g for 20 min at 20°C. The plasma of patients and controls was distributed into aliquots, which included routine coagulative tests, then immersed in liquid nitrogen and stored at -80°C until analysis. They were then thawed in a thermostatic bath at 37°C and analyzed for Clot Waveform Analysis and Thrombin Generation.

For SEM scanning, thromboplastin, i.e. TF, was added to the plasma, that was mixed for 3 minutes at 37°C. An aliquot of 25 µl of plasma has been added to 50 µl of thromboplastin for clot formation. After 15 min, the clot was placed in a physiological saline solution. The sample was then processed to make it observable by SEM using the following standard protocol: i) fixation in 4% buffered formalin (pH 7) for 1 h; ii) multiple washes in PBS (Phosphate Buffered Saline); iii) dehydration by multiple steps with increasing concentrations of ethanol up to 100%; iv) drying at the critical point, in CO_2; v) mounting on support with double-sided tape; and vi) deposition of 20 nm gold conductive film with sputter coating (Cressington CR10-A). Finally, the samples were observed at SEM (ZEISS SIGMA 300). The observation was conducted with an exploration of some particular region of interest (see below for technical details), to obtain images expression of morphological characteristics of the sample.

Laboratory plasma testing

Thrombin generation

Thrombin Generation (TG) was determined in Platelet Poor Plasma (PPP) using the Calibrated Automated Thrombogram (CAT) method (Diagnostica Stago, Asnières sur Seine France).[8] An aliquot of 80 µl of PPP was pipetted into the well of a microtiter plate together with 20 µl of PPP-Reagent +/- TM (with and without Thrombomodulin) (Thrombinoscope BV, Maastricht, The Netherlands), PPP-Reagent contains a mixture of Tissue Factor (5 pM final concentration) and synthetic phospholipids (4 µM final concentration), PPP-Reagent with Thrombomodulin contains a mixture of Phospholipids, Tissue Factor and Thrombomodulin (in the range of 4-6 nM). The reaction was started with 20 µl of a mixture composed of the fluorogenic thrombin substrate (Z-GlyGly-Arg-AMC, Thrombinoscope BV, 417 µM final concentration) and calcium chloride (15 mM final concentration). The substrate was cleaved by the thrombin formed and liberates a fluorophore, which is converted to thrombin-equivalent concentrations (nM) using a reference curve. Fluorescence was read in a Fluoroscan Ascent® reader (Thermo Fisher Scientific Corporation, Vantaa, Finland), and the thrombin generation curves were calculated using the

Table 1. Characteristic of the patients affected by Acquired Haemophilia A. All the patients showed major bleeding at the admission to the clinical ward.

Sex	Years	aPTT sec	aPTT ratio	Factor VIII %	Inhibitor FVIII (Bethesda Unit)
F	81	86.5	2.85	0.7	48
F	52	51.9	1.71	4.3	38.4
F	47	66.3	2.19	3.5	40
M	75	74.9	2.47	5.9	54
F	61	106.3	3.51	0.0	147
F	76	62.5	2.06	4.5	22.4
M	90	112.7	3.72	0.1	41.6

Table 2. Clinical characteristics of the patients treated with VKA.

Patients treated with VKA	Sex	Years
Ischemic heart disease and intraventricular thrombosis	M	52
Antiphospholipid antibody syndrome	F	77
Mechanical aortic valve replacement	F	42
DVT lower limbs	M	86
Atrial fibrillation with dilative ischemic heart disease	M	52
Atrial fibrillation with cerebral embolic stroke	F	69
Atrial fibrillation	F	90

Thrombinoscope Software. Thrombin generation curves were plotted, and the endogenous thrombin potential (ETP, the area under the curve, nM*min), Peak height (nM), Time to peak (min) and the Velocity index (nM/min) were recorded. The ETP is the area under the curve (AUC) representing all the enzymatic activity of thrombin when it is activated. Therefore, it is the parameter that properly represents the coagulation phase. The Peak height is the maximum amount of thrombin activated in the process, while the Time to peak represents the time that it takes to obtain the peak of thrombin activity from the moment the coagulation starts. The Velocity Index is the slope of the curve from the moment of activation of thrombin to its peak; it represents the speed with which the reaction reaches its maximum thrombin activity.

Clot waveform analysis

CWA detects the hidden parameters of a simple coagulative test, such as the activated Partial Thromboplastin Time (aPTT). It works by means of an automated coagulometer (ACL TOP 500 CTS, Werfen-Instrumentation Laboratory, Barcelona, Spain) employing a turbidimetric method for clot detection. The parameters examined by CWA were the following: i) time at which maximum velocity of clot formation is reached (1st derivative, expressed as mAbs/s), and ii) time at which maximum change in acceleration of clot formation is reached (2nd derivative, expressed as mAbs/s^2) (Table 3). These parameters of the clot formation can be obtained by means of implemented software dedicated to the ACL TOP 500 CTS.[9] This method has been standardized by an official communication of the Subcommittees of the International Society of Thrombosis and Haemostasis (ISTH).[10]

Images from SEM: scanning and image acquisition

All the images from the SEM were taken using standard capture parameters. Magnification: 20.000x - WD (Working Distance): 7.0 mm - Electron High Tension (EHT): 15.00 KV - SE2: Secondary electron detector. The images were captured in 2048x1536 pixels. These conditions allowed to prevent optical distortions and aberrations leading to a misinterpretation of the morphological data, given the considerable variety of samples. Therefore, the care in obtaining the images has been concentrated on the homogeneity of the signal, both at the origin voltage acceleration and enlargement and in the response signal of the sample, due to working distance and brightness and contrast modulations.

Images analysis

Images acquired were analyzed with the Texture Analysis methods to perform classification. Texture definition of an image can be reported as the particular geometric arrangement of grey levels of the pixels defined in a local region of the image. It is important to keep in mind that texture is a property of a region and not a single pixel in the image. Parameters describing the texture of images are calculated by the Grey Level Co-Occurrence Matrix (GLCM) methods, and calculation were performed by homemade routine scripts in MATLAB environment.[11] Texture analysis is used in various contexts, including, for example, the processing of medical mammographic images. The Grey Level Co-Occurrence Matrix (GLCM) is one of the most widely used statistical analysis examining the spatial distribution of pixel grey levels within an image.[12,13] Among the various texture parameters, we reported here *contrast* and *energy* whose detailed meaning is described below.

Table 3. Kruskal Wallis test and *post-hoc* analysis of derivatives of aPTT (1st and 2nd derivative) and parameters of the thrombin generation in the three groups examined. Data are expressed as median and range. Rank is referred to the Dunn *post-hoc* test results.

		AHA n=7	VKA n=7	Healthy subjects n=8
1st aPTT derivative (mAbs/s)	Median, range	123.00, 64.58-256.87	172.83, 137.08	254.48, 188.96-328.08
	Rank	7.29	9.00	17.37
	p < 0.05	vs healthy subjects	vs healthy subjects	vs VKAs and vs AHA
2nd aPTT derivative (mAbs/s^2)	Median, range	205.75, 87.60-869.75	415.65, 315.36-701.48	905.30, 566.28-1124.53
	Rank	6.86	8.86	17.87
	p < 0.05	vs healthy subjects	vs healthy subjects	vs VKAs and vs AHA
ETP (nM*min)	Median, range	327.09, 88.41-1025.50	311.18, 187.55-603.42	1334.93, 1007.91-1562.14
	Rank	7.86	7.29	20.25
	p < 0.05	vs healthy subjects	vs healthy subjects	vs VKAs and vs AHA
Peak (nM)	Median, range	17.82, 5.42-128.79	57.90, 33.36-139.65	221.40, 95.90-308.86
	Rank	5.86	9.43	18.25
	p < 0.05	vs healthy subjects	vs healthy subjects	vs VKAs and vs AHA
Time to Peak (min)	Median, range	16.00, 6.83-26.43	8.00, 4.33-10.00	7.67, 6.00-11.33
	Rank	16.29	9.50	9.06
	p < 0.05	ns	ns	ns
Velocity Index (nM/min)	Median, range	1.21, 0.39-27.36	19.30, 12.08-69.83	57.95, 14.38-132.37
	Rank	4.71	11.71	17.25
	p < 0.05	vs VKAs and vs healthy subjects	vs AHA and vs healthy subjects	vs VKAs and vs AHA

Contrast is a quantity correlated with the visual perception of the brightness of details in the image. The contrast parameter, calculated in the GLCM approach, returns a measure of the intensity of differences between a pixel and its neighbors over the whole image. It has a range depending on the size of the GLCM matrix, but the lower contrast value of 0 is for a "constant" image.

$$Contrast = \sum_{i,j=0}^{N-1} P_{ij}(i-j)^2$$

Formula 1. Contrast calculation formula: Pij is the element i,j of the GLCM matrix.

Energy is a measure of the structures' uniformity in the image. The energy is high when the image has the pixels grey levels distribution very regular and, for this reason, is related to the uniformity of the diagnostic image. It has the range [0/1], and it is 1 for a "constant" image.

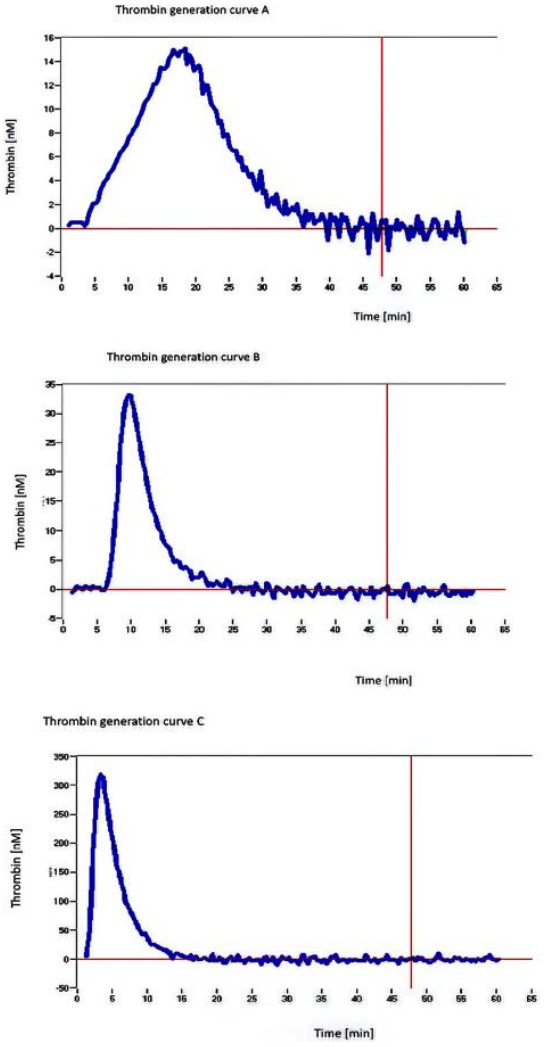

Figure 1. Examples of thrombin generation of a patient with AHA (A), treatment with VKAs (B) and a healthy subject (C).

$$Energy = \sum_{i,j=0}^{N-1} (P_{ij})^2$$

Formula 2. Energy calculation formula: Pij is the element i,j of the GLCM matrix. Results are expressed as arbitrary units.

Results

Results show a significant difference among the three groups of patients as indicated by ANOVA for all parameters examined except time to peak. Dunn's *post-hoc* test shows a significant difference among healthy subjects and both anticoagulated and haemophilic patients for the following parameters: 1st, 2nd derivative of the aPTT, ETP, peak and the velocity index.

No statistically significant differences were found between anticoagulated and haemophilic patients for all the examined parameters. Only the velocity index was significantly lower in AHA patients when compared with both VKAs patients and normal subjects.

Examples of thrombin generation are depicted in Figure 1A for AHA, VKA treatment (Figure 1B) and healthy subjects (Figure 1C); CWA and clot textures for AHA, VKA treatment and healthy subjects are depicted in the Figures 2, 3 and 4.

All the results are shown in Table 3. When contrast and energy were examined, ANOVA highlighted a significant difference between the three groups. In particular, the contrast appeared significantly lower in normal subjects than in anticoagulated patients and those with AHA. No significant differences were found between anticoagulated and haemophilic patients.

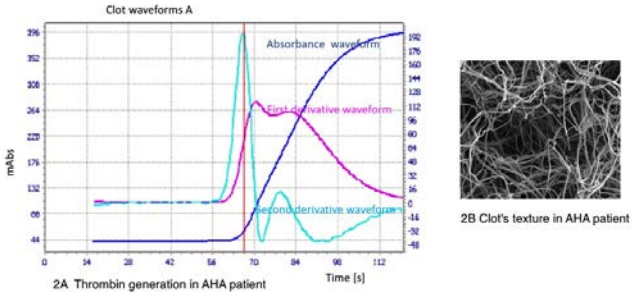

Figure 2. Examples of CWA and clots texture image of patients with AHA.

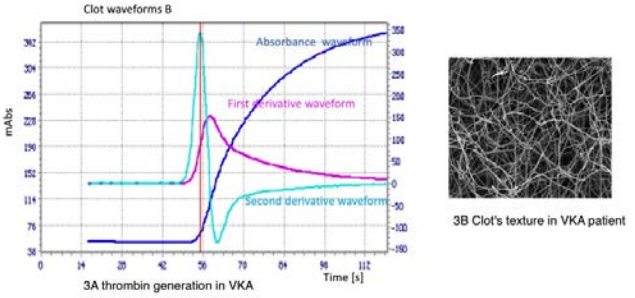

Figure 3. Examples of CWA and clots texture image of patients with treatment with VKAs (B).

Discussion

Clot morphology was used by Undas to identify hypercoagulability conditions through the Ks index and fibrin fiber size.[4] Ks index (Darcy's constant) is a measure of the average pores between fibrin fibers. It shows the fibrin network density in a plasma-based, hydrostatic pressure-driven assay in the presence of thrombin and calcium.[14] In this preliminary study, we decided to use different but simpler methods in order to analyze clot formation and morphology in three categories of subjects: normal, anticoagulated patients with VKAs, and patients with AHA. In other words, we were interested in the study of low coagulative states. Alongside the clot texture, analyzed through MATLAB, we determined some other parameters related to thrombin generation and the velocity and acceleration of the clot formation (Table 4). The primary aim was to assess whether the different laboratory coagulation parameters were consistent with the clot texture. The laboratory results highlight that patients treated with VKAs and patients affected by AHA showed highly significant differences when compared with normal subjects in terms of thrombin generation and velocity and acceleration of clot formation. Moreover, peak, time to peak and velocity index were lower in patients with AHA in comparison with the anticoagulated patients. These findings deserve a dedicated comment. Although the results were significantly different only for the velocity index, it explains what is typical of AHA, which is a disease characterized by a low level of factor VIII, which has a crucial role in the acceleration of the blood coagulation cascade. The physiological behavior of factor VIII explains why in our patients, ETP is not different from healthy subjects because the amount of thrombin is normal in haemophilia, but the critical point is the time to reach an optimal thrombin production. That delay may be critical since the haemostatic system requires an immediate and very rapid reaction if even a minimal vascular injury occurs.[15] Our results confirm that delay in thrombin generation. Both coagulative tests, i.e., thrombin generation and derivatives, appear to be coherent with the clot texture results, thus showing a significantly weaker clot morphology as demonstrated by the values of contrast and energy. Looking more deeply at the results, we see that both clot texture and coagulative parameters were more compromised in patients with AHA than those with VKAs. These findings, although not significant due to the small number of patients examined, were expected because patients with AHA had at the onset a severe haemorrhagic syndrome, typical of the disease.[16] No bleeding was recorded in patients treated with VKAs who were not in an overdose state. However, both factor VIII level and an optimal anticoagulation range are not predictive of no bleeding in these subjects since they can bleed regardless of laboratory tests values.[17,18] Clot morphology studied at SEM can therefore help in examining particular patients who bleed despite their factor VIII value being, for example, above 5%, thus defining mild haemophilia and why patients treated with VKA can bleed despite optimal anticoagulation. The visualization and determination of clot texture by SEM and Texture Analysis could allow to detect additional defects, so explaining the reason of bleeding in these subjects. In fact, the morphological examination can highlight areas of absence of fibrin fibers, as is evident in Figure 3A. The use of SEM should be therefore dedicated only to special and difficult cases for which there is no appropriate explanation for bleeding. SEM may have, if well focused, an important role in the evaluation

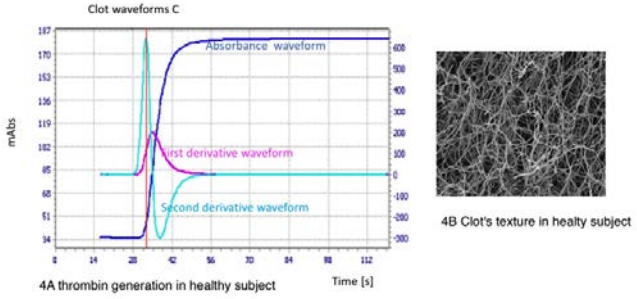

Figure 4. Examples of CWA and clots texture image of healthy subject. The absorbance clot waveform and first and second derivative waveforms describe the process of clot formation; the blue colored trace shows the changes in absorbance; the red-colored curve is the first derivative of the absorbance (coagulation velocity); the light blue colored curve is the second derivative of the absorbance (coagulation acceleration).

Table 4. Kruskal-Wallis test and post-hoc analysis of parameters derived from MATLAB. Data are expressed as median and range. Rank is referred to the Dunn post-hoc test results.

		AHA n=7	VKA n=7	Healthy subjects n=8
Contrast (a.u.)	Median, range	0.44, 0.16-0.65	0.36, 0.14-0.54	0.18, 0.12-0.23
	Rank	15.43	13.71	6.12
	p <0.05	vs healthy subjects	vs healthy subjects	vs VKAs and vs AHA
Energy (a.u.)	Median, range	0.92, 0.88-0.97	0.93, 0.89-0.97	0.97, 0.96-0.98
	Rank	7.57	9.29	16.87
	p <0.05	vs healthy subjects	vs healthy subjects	vs VKAs and vs AHA

a.u., arbitrary units.

of the single patient. The effort made in this work was to demonstrate that a synergy between a blood coagulation laboratory and SEM is possible and desirable. The limitations of this study are represented by the small sample size and the fact that only a few texture parameters resulted statistically significant in our analysis. But this is a pilot study realized to acquire important preliminary information about the application of the SEM and Texture Analysis to the coagulative pathologic conditions. What can we learn from this research experience? The use of SEM seems to be promising in working along with the laboratory dedicated to haemostasis. Other clot strength detection such as fiber size and organization could further emphasize the potential of SEM in the study of hypocoagulability in other clinical conditions such as disseminated intravascular coagulation, defects of other clotting factors and, sometimes, unexplained haemorrhagic conditions. Other fields of interest are those related to the hypercoagulable states which appear to be another topic to be explored with all the techniques employed in this study.

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Key words: clot pathologies, scanning electron microscopy (SEM), texture analysis, clot waveform analysis (CWA).

Contributions: FM, methodology, investigation, data acquisition, writing - original draft; DMN, sample preparation and acquisition, formal analysis; LB, imaging data analysis; MFR, sample preparation and acquisition, data analysis; MSP, formal analysis, supervision; MC, Samples Preparation; LDM, formal analysis, supervision; MM, sample selection; SM, sample selection and preparation; MLV, data acquisition, data analysis; ML, data acquisition; MP and LF, supervision, validation data analysis; CP, formal analysis, data analysis; DB, methodology, supervision, investigation, writing; GF, DF, SEM analysis, supervision and writing; TC, SEM analysis, supervision and writing. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest: The authors declare no conflict of interest.

Funding: This research received no external funding.

Ethical approval: This study was approved by the Independent Ethical Committee of the Teaching Hospital of Cagliari (Prot. PG/2021/153). All the procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients signed a written informed consent to participate to the study.

Availability of data and materials: The data used to support the findings of this study are available from the corresponding author upon request.

Received for publication: 28 April 2021.
Accepted for publication: 17 May 2021.

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Licensee PAGEPress, Italy
Journal of Public Health Research 2021;10:2358
doi:10.4081/jphr.2021.2358
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