Assessing coagulopathy and endothelial dysfunction in paediatric venous malformation: A thromboelastometry and syndecan-1 study

Francisco Nava y Hurtado[1], Elena Monzon Manzano[1], Vanesa Viana-Huete[2], Paloma Triana[1], Maria Teresa Alvarez-Roman[1], Elena G. Arias-Salgado[1], Nora Butta[1], and Juan Carlos Lopez-Gutierrez[1]

[1]Hospital Universitario La Paz
[2]Universidad Europea Facultad de Ciencias Biomedicas y de la Salud

November 17, 2023

Abstract

Objective (69) The occurrence of unpredictable pain crises are the principal determinant of the quality of life for patients with venous malformations (VM). A definite coagulation phenomenon, characterized by an increase in D-dimer levels and the presence of phleboliths within the malformation, has been previously reported. By applying Virchow's triad and evaluating intralesional samples, our objective is to delineate the coagulation profile and the extent of endothelial dysfunction within the malformation. Methods (42) With the authorization of the Ethics Committee, a research project was undertaken on intralesional and extralesional blood samples from 30 pediatric patients afflicted with spongiform VM. Thromboelastometry analyses were performed using ROTEM ® Sigma, and the concentration of syndecan-1 was determined by ELISA. Results (80) In the ROTEM ® analyses, the A5, A10, and MCF values were below the established reference ranges in the intralesional samples in both the EXTEM and INTEM assays indicating that intralesional clots had significant instability. Furthermore, during the investigation of the delayed fibrinolysis phase using recombinant tissue plasminogen activator (rtPA) in EXTEM analysis, widespread hyperfibrinolysis was observed intralesional. Additionally, analysis of syndecan-1 showed significant differences between extralesional and intralesional levels ($p<0.026$) and controls ($p<0.03$), suggesting differences in the state of endothelium. Conclusions (39) For the first time, we developed a comprehensive understanding of the coagulopathic profile of VM and the role of endothelial dysfunction in its pathogenesis. These findings will enable the implementation of targeted therapies based on the individual coagulation profiles.

Assessing coagulopathy and endothelial dysfunction in paediatric venous malformation: A thromboelastometry and syndecan-1 study Francisco Nava y Hurtado[a],MD, Elena Monzon Manzano[b,c], PhD, Vanesa Viana-Huete[d], PhD, Paloma Triana Junco[a], MD, PhD, Maria Teresa Alvarez-Roman[b,c], MD, PhD, Elena G. Arias-Salgado[b,c], PhD, Nora Butta[b,c], PhD, Juan Carlos Lopez Gutierrez[a,c], MD, PhD.**Affiliations:** aDepartment of Paediatric Surgery, Hospital Universitario La Paz, Madrid. [b] Department of Haematology, Hospital Universitario La Paz, Madrid, Spain.[c] IdiPAZ, Madrid, Spain, [d]Department of Health Sciences. Universidad Europea de Madrid**Address correspondence to**: Francisco Nava y Hurtado, Department of Paediatric Surgery, Hospital Universitario La Paz, Edificio Infantil, 1º planta; Paseo de la Castellana 261 - 28046 Madrid, Spain

Email: franciscodeborja.nava@salud.madrid.org

Phone: (+34) 91 727 70 19

Word Count:

Abstract: 230

Main text: 2148

Tables: 4

Figures: 1

Short title: Coagulopathy and endothelial dysfunction in paediatric venous malformation**Keywords:** Coagulopathy, venous malformation, intralesional, ROTEM®, syndecan-1, glycocalyx.**Abbreviations:** Venous malformations (VM), syndecan-1 (SDC-1), localised intralesional coagulopathy (LIC), disseminated intravascular coagulopathy (DIC), clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF), clot amplitude at 5 and 10 minutes after CT (A5 and A10), recombinant tissue plasminogen activator (tPA)

Objective (69)

The occurrence of unpredictable pain crises are the principal determinant of the quality of life for patients with venous malformations (VM). A definite coagulation phenomenon, characterized by an increase in D-dimer levels and the presence of phleboliths within the malformation, has been previously reported. By applying Virchow's triad and evaluating intralesional samples, our objective is to delineate the coagulation profile and the extent of endothelial dysfunction within the malformation.

Methods (42)

With the authorization of the Ethics Committee, a research project was undertaken on intralesional and extralesional blood samples from 30 pediatric patients afflicted with spongiform VM. Thromboelastometry analyses were performed using ROTEM® Sigma, and the concentration of syndecan-1 was determined by ELISA.

Results (80)

In the ROTEM® analyses, the A5, A10, and MCF values were below the established reference ranges in the intralesional samples in both the EXTEM and INTEM assays indicating that intralesional clots had significant instability. Furthermore, during the investigation of the delayed fibrinolysis phase using recombinant tissue plasminogen activator (rtPA) in EXTEM analysis, widespread hyperfibrinolysis was observed intralesional. Additionally, analysis of syndecan-1 showed significant differences between extralesional and intralesional levels ($p<0.026$) and controls ($p<0.03$), suggesting differences in the state of endothelium.

Conclusions (39)

For the first time, we developed a comprehensive understanding of the coagulopathic profile of VM and the role of endothelial dysfunction in its pathogenesis. These findings will enable the implementation of targeted therapies based on the individual coagulation profiles.

Introduction (352)

Venous malformations (VM) are vascular anomalies, affecting 1% of the general population, characterized by venous dilatations of dysplastic veins, leading to an increased risk of thrombosis. This condition can result in elevated D-dimer levels in extralesional blood, known as localised intralesional coagulopathy (LIC) .In some cases, a decrease in fibrinogen levels may precede the development of disseminated intravascular coagulopathy (DIC) , although very few cases have been described in the literature. Asymptomatic lesions are common, but they may cause unpredictable pain crises, mostly due to exacerbation of LIC. The emotional distress caused by these episodes can lead to a lower quality of life for patients affected by VM compared with the general population .

To date the most significant discoveries for advancing treatment of patients affected by VM was the identification of a collection of mutated genes and their clinical impact in cellular models .These advances have enabled the application of targeted therapies that inhibit hyperfunctioning signalling pathways, such as sirolimus and mTOR proteins , alpelisib and PIK3CA proteins , and rebastinib and TIE receptors . Despite the identification of the initial genetic error, thrombosis phenomena are still based on Virchow's classical triad.

In this regard, different research groups have analysed conventional coagulation parameters using extralesional blood samples although the use of new tools for a more complete analysis of the clot-formation process has recently been reported .Viscoelastic tests allow us to quickly identify the function of different elements involved in coagulation and fibrinolysis processes, which has popularised their use in situations that require rapid response .

Recently, glycocalyx impairment has been linked to several diseases with significant social impacts involving endothelial dysfunction .This structure, which contains glycosaminoglycans and glycoproteins, mediates various physiological processes, such as angiogenesis , vascular permeability , and coagulation .

To investigate coagulopathic phenomena and endothelial dysfunction in paediatric patients with venous malformations, we conducted thromboelastometry studies using ROTEM® technology (Tem GmBH; Munich, Bavaria, Germany). Additionally, we analysed the intralesional coagulation system and investigated the degree of endothelial dysfunction as a function of syndecan-1 glycoprotein levels.

Methods (547)

Patients and study design

This was an observational, prospective and transversal study approved by the local Research Ethics Committee (PI-5557). Paediatric patients (younger than 18 years old) who presenting with focal skin lesions and/or muscle involvement with slow flow on Doppler ultrasound were considered to have VM by the expertise group of the Vascular Anomalies Unit. All patients were included once parental representative had provided signed informed consent.

Patients who had undergone recent treatment with possible haemostatic modifiers, such as antiplatelets and anticoagulants, and those who had experienced pain in the previous week were excluded from the study. All procedures and actions were conducted in accordance with the World Medical Association Declaration of Helsinki.

Collection data and preparation of samples

The demographic and general characteristics of the patients and their lesions were collected from clinical history.

Extralesional and intralesional venous blood samples were obtained from patients in 3.8% sodium citrate tubes (Vacutainer®, BD, Becton Dickinson and Company, Madrid, Spain). Platelet-poor plasma (PPP) was obtained by centrifuging the whole blood at 2500 g for 15 min at 23ºC. Plasma samples were stored at -80ºC until analysis.

Conventional coagulation assays and rotational thromboelastometry

Traditional coagulation analysis (haemogram, prothrombin time, prothrombin activity, cephalin time, cephalin time ratio, INR and D-dimer and fibrinogen values) as well as SDC-1 levels were performed in whole blood or plasma, as appropriate.

Thromboelastometry, a global coagulation assay describing the dynamics of clot formation and lysis, was performed using ROTEM® sigma with 500 µL of extralesional and intralesional citrated venous blood in accordance with the manufacturer's protocol. The samples were analysed using cartridges containing the EXTEM/INTEM/FIBTEM/HEPTEM reagents. The minimum test duration was 20 minutes, and the following parameters were recorded: clotting time (CT), the time from test start until a clot firmness amplitude of 2 millimetres is reached; clot formation time (CFT), the time between 2 and 20 millimetres clot firmness amplitude is achieved; clot amplitude at 5 and 10 minutes after CT (A5 and A10) and maximum clot firmness (MCF).

A subsequent study was conducted on 9 paediatric patients and 11 healthy controls with demographic and clinical characteristics similar to those of the initial cohort. The aim of this study was to investigate fibrinolytic clot properties. A modified EXTEM analysis was performed with 360 µL of extralesional citrated

venous blood and 125 ng/mL rtPA to accelerate the fibrinolysis process ex vivo, according to the technique described by Kuiper et al. . Values for lysis initiation time (LOT, the time period from clotting time until 15 % of clot lysis is achieved) and lysis time (LT, time from CT until the clot firmness decreases to 10% compared with MCF) parameters were recorded.

Glycocalyx analysis

Levels of SDC-1 in plasma from extralesional and intralesional samples were measured using a human CD138 ELISA kit (Diaclone, Besançon Cedex, France)

Statistical analysis

Quantitative variables were described using mean and standard deviation, and the Wilcoxon paired test was used to compare these variables. Correlation analyses between quantitative variables were carried out using Pearson's "R" correlation coefficient, while the Mann-Whitney U test was utilized for qualitative variables. In this study, statistically significant differences were defined as those with a probability of error of less than 5% ($p<0.05$). Data analysis was conducted using the SAS software version 9.4 (SAS Institute Inc. Cari, NC).

Results (413)

Thirty paediatric patients with spongiform VM who underwent a therapeutic procedure under sedation between February 2019 and March 2023 at the Vascular Anomalies Unit of the Hospital Universitario La Paz were enrolled. The baseline patient characteristics are presented in Table 1. The malformations corresponded to spongiform lesions predominantly located in the upper extremities. Ninety percent of patients experienced episodes of pain, although only 43.6% required pharmacological treatment during their evolution. Thirteen patients had previously undergone sclerotherapy procedures, with an average of 3.4 procedures per patient. Ethoxysclerol was the most commonly used sclerosing agent.

Table 2 lists the analytical parameters of the 30 patients. At the time of analysis, the patients were free of clinically relevant symptomatology and had not recently used drugs that could alter coagulation parameters. Thirty patients (75%) had fibrinogen levels above 500 ng/mL, which is the cut-off point used in deep vein thrombosis diagnostic and treatment algorithms , and only one patient had elevated fibrinogen levels. All other parameters were within normal range.

Table 3 displays the results of the statistical comparisons between extralesional and intralesional samples and the reference values. The A10 and MCF values of the EXTEM test and MCF values of the INTEM test in intralesional blood were found to differ from Oswald's reference values , whereas those from extralesional blood where within the reference range. The results of the EXTEM test showed significant differences between the intralesional and extralesional blood samples in the clot formation process (A5 and A10). When the cohort was analysed based on the D-dimer cut-off point (500 ng/mL), patients with values lower than the cut-off point (25%) did not exhibit significant changes, whereas patients with a D-dimer level $>$ 500 ng/mL (75%) showed statistically significant differences in clot formation in both EXTEM and INTEM between the intralesional and extralesional blood samples. Table 4 and figure 1 show the results of the modified fibrinolysis phase analysis.

SDC-1 exhibited significant variability in the different settings in which it was measured. In the cohort of 15 patients, we observed a substantial decrease in SDC-1 levels within the endothelial lumen (30.6 ng/mL) compared with the levels in extralesional samples (34.9 ng/mL) and controls (35.6 ng/mL).

Possible correlations between D-dimer levels and parameters that have traditionally been associated with the ILC phenomenon (sex, phleboliths, location), together with significant analytical parameters of ROTEM$^{(r)}$ and SDC-1 levels, were analysed. No significant correlation was detected, except for a strong correlation between the intralesional and extralesional SDC-1 levels (correlation coefficient, 0.954).

Discussion (740)

Coagulation and fibrinolysis processes are intricate, and while some of the proteins and enzymes involved

can be measured, these values do not accurately reflect the dynamic functionality of the systems at play. Recent research has relied on biochemical models of HUVEC and analyses of extralesional blood samples. Natynki et al. demonstrated that mutations in TIE-2 produced alterations in the plasminogen-plasmin system. However, studies on patients with extralesional blood samples have been inconclusive .

Rotational thromboelastometry, or ROTEM$^{(r)}$, is an instrument that enables the rapid and straightforward evaluation of coagulation dynamics. Despite its prior utilisation , we used it in the present study to analyse and compare intralesional blood samples from VM with extralesional blood samples from the same patient. In our cohort, we observed a loss of firmness of the intralesional clot, probably caused by hyperfibrinolysis.

During the initial phase, we observed modifications in intralesional blood values relative to the age-specific reference ranges of Oswald et al. . Specifically, the A10 (46.7 and 48 s) and MFC (51.4 and 51 s) parameters were below the normal range in both the EXTEM (extrinsic pathway) and INTEM (intrinsic pathway). Although there were no reference values for A5, they were significantly lower than those for the extralesional samples. These findings suggest that the intralesional clot does not reach its maximum potential relative to the reference values and extralesional samples. Moreover, this effect is more pronounced in patients with higher levels of D-dimer ($>$ 500 ng/ml), significant alterations were observed in both INTEM and EXTEM.

The onset of coagulation, dependent on coagulation factors and anticoagulants is reflected in the CT and CFT variables, was normal in the analyses. The firmness phase, which is dependent on platelets and fibrinogen and was assessed in the extralesional samples, also showed normal values. Aronniemi et al, performed extralesional analyzes in a cohort of patients who predominantly presented a venous endothelium, but also lymphatic and capillary endothelium. According to our analysis, they identified a decrease in the proteins responsible for stabilising the clot in the final phase whereas 54.8% had antithrombin levels above their range . This anticoagulant molecule binds to heparan sulfate of SDC-1, and alterations in the anchoring molecule can cause alterations in the coagulation system .

VMs are characterised by a supposed hyperfibrinolytic component, as they exhibit elevated D-dimer levels and the presence of phleboliths . Viscoelastic studies allow the analysis of the fibrinolysis phase; however, they require a long time and lose their clinical usefulness. To address this issue, we have added recombinant tPA to accelerate fibrinolytic process. Under these circumstances, we detected hyperfibrinolysis in patients with VM. The alterations in the coagulation system that occur in VM are partially reflected in the intralesional samples because, as demonstrated in Figure 1, the hyperfibrinolytic potential is not fully transferred to the extralesional samples.

Endothelial dysfunction, resulting from modifications in the composition of the glycocalyx, is a key factor in certain pathologies with a high incidence and may act as a prothrombotic factor . In our study, we identified a significant decrease in SDC-1 within the endothelial lumen compared to extralesional samples.In the case of a quiescent and unaltered endothelium, SDC-1 levels should be similar, but in line with the hypothesis of Redondo et al., there is a persistent process of seeking quiescence, which could disrupt angiogenic and haemostatic regulatory systems .

ROTEM$^{(r)}$ enables the identification of diverse coagulation patterns in venous, lymphatic, and mixed endotheliums. In addition, we will be able to perform early detection of susceptible patients and the implementation of individualized prevention measures based on their coagulation pattern. Furthermore, ROTEM$^{(r)}$ facilitates the monitoring of anticoagulant treatments and evaluation of the effectiveness of therapies such as sclerotherapy. SDC-1 is the first molecule to show a strong correlation between intralesional and extralesional levels in VMs. This glycoprotein has been proposed as a possible biomarker in different scenarios . In our study, SDC-1 may serve as a novel endothelial biomarker, indicating the stability of the ecosystem and enabling more precise monitoring of treatment, as compared to D-dimer levels. This study has several limitations: Since we did not perform a genetic analysis of the malformations in which blood samples were obtained, we do not know the venous endothelial behaviour under different mutational scenarios, although most of the mutations identified in spongiform VMs are in the TIE-2 gene . Lastly, the control group recruited was of adults, given the difficulty in finding paediatric samples.

Conclusions (96)

The interaction between the endothelium and abnormal venous environment plays a crucial role in determining the clinical and prognostic outcomes of VM. Our findings suggest that endothelial dysfunction of genetic origin can impair basic cellular systems, particularly the coagulation system, which exhibits a notable reduction in clot quality due to excessive fibrinolysis. Syndecan-1 has the potential to serve as a sensor of endothelial well-being, given its strong correlation between intralesional and extralesional levels, which may lead to the identification of a range of values that pose no risk of clinical onset or progression of the lesion.

Conflict of Interest Disclosures (includes financial disclosures): The authors have no conflicts of interest to disclose.

Funding/Support: No funding was secured for this study.

Role of Funder/Sponsor (if any): None

Clinical Trial Registration (if any): None

Acknowledgements: None

Characteristics	**Mean ± SD or%(n)**
Age, yo (±SD)	11 ± 4.7
Sex,% (n)	
Female	53.3 (16)
Men	46.6 (14)
Localization, % (n)	
Head and neck	6.6 (2)
Trunk	3.3 (1)
Extremity	90 (27)
Position, % (n)	
Upper	43.3 (13)
Lower	56.6 (17)
Phleboliths,% (n)	
Yes	64.3 (18)
No	35.7 (10)

Table 1 . General characteristics

Variables	**Mean ± SD**
Platelets (x10e3/µL)	298 (576)
D-dimer (ng/mL)	5041.5 (10939.6)
Fibrinogen (mg/dL)	241.4 (65.2)
Prothrombin time (s)	11.5 (0.7)
Prothrombinactivity (%)	86 (12.3)
Partialthromboplastin time (s)	28.2 (2.6)
INR	1.1 (0.1)

Table 2. Conventional analytical parameters [1] *Based on the parameters of the center.*

Table 2. Conventional analytical

Variables	**Extralesional**	**Extralesional**	**Intralesional**	**Intralesional**	*P*-**value**	*P*-**value**	**References valu**
EXTEM							
CT (s)	64.4 (9.2)	64.4 (9.2)	78.5 (75.8)	78.5 (75.8)	.811	44 - 91	44 - 91

Variables	**Extralesional**	**Extralesional**	**Intralesional**	**Intralesional**	*P*-**value**	*P*-**value**	**References valu**
CFT (s)	87.4 (10.2)	87.4 (10.2)	98.2 (14.7)	98.2 (14.7)	.080	53 - 115	53 - 115
A5 (mm)	42.9 (6.4)	42.9 (6.4)	36.9 (11.9)	36.9 (11.9)	.016*	n/a	n/a
A10 (mm)	52.1 (5.6)	52.1 (5.6)	46.8 (11.8)	46.8 (11.8)	.011*	49 -67	49 -67
MCF (mm)	55.9 (6.3)	55.9 (6.3)	51.6 (11.9)	51.6 (11.9)	.103	53 -72	53 -72
INTEM							
CT (s)	185.6 (26.3)	185.6 (26.3)	193.2 (103)	193.2 (103)	.362	128 - 206	128 - 206
CFT (s)	83.3 (6.8)	83.3 (6.8)	89.7 (10.9)	89.7 (10.9)	.109	45 - 106	45 - 106
A5 (mm)	43.4 (5.1)	43.4 (5.1)	40.4 (8.9)	40.4 (8.9)	.161	n/a	n/a
A10 (mm)	51.4 (5.1)	51.4 (5.1)	48.3 (9.8)	48.3 (9.8)	.323	48 - 67	48 - 67
MCF (mm)	54.4 (6.2)	54.4 (6.2)	51.2 (9.6)	51.2 (9.6)	.174	54 - 71	54 - 71
FIBTEM							
A5 (mm)	10 (2.9)	10 (2.9)	6.5 (3.2)	6.5 (3.2)	.290	n/a	n/a
A10 (mm)	11.1 (3.3)	11.1 (3.3)	10.5 (3.6)	10.5 (3.6)	.432	7 - 22	7 - 22
MCF (mm)	11.7 (3.9)	11.7 (3.9)	11.1 (3.5)	11.1 (3.5)	.356	8 - 24	8 - 24

Table 3 . Results from extralesional compared to intralesional

Note . Values are presented as mean and (standard deviation). *p-value of <0.05 was considered significant.

[1]References values derived by Oswald et al.

Variables	**Extralesional**	**Extralesional**	**Controls**	**Controls**	*P*-**value**	*P*-**value**
Modified EXTEM						
LOT (s)	1174 (362.3)	1174 (362.3)	1522 (286.6)	1522 (286.6)	.0158*	.0158*
LT (s)	2133 (490)	2133 (490)	2702 (767.4)	2702 (767.4)	.036*	.036*

Table 4 . Modified EXTEM analysis performed in presence of tPA.

Note . Values are presented as mean and (standard deviation). *p-value of <0.05 was considered significant.

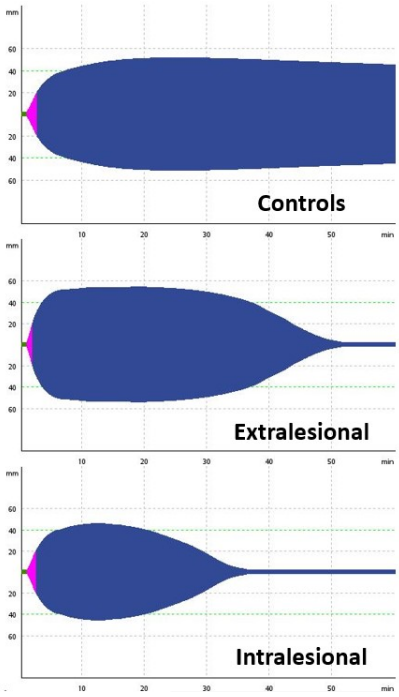

Figure 1. Modified EXTEM/tPA tracing in different environments

Hosted file

`Figures - Pediatric Blood &amp; Cancer.docx` available at https://authorea.com/users/700977/articles/687658-assessing-coagulopathy-and-endothelial-dysfunction-in-paediatric-venous-malformation-a-thromboelastometry-and-syndecan-1-study

Hosted file

`Tables - Pediatric Blood &amp; Cancer.docx` available at https://authorea.com/users/700977/articles/687658-assessing-coagulopathy-and-endothelial-dysfunction-in-paediatric-venous-malformation-a-thromboelastometry-and-syndecan-1-study