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Angiogenic and prothrombotic markers in extensive slow-flow vascular malformations: implications for antiangiogenic/antithrombotic strategies

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Summary

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Background Venous and combined malformations are slow-flow haemodynamically inactive lesions that are present at birth and worsen slowly with advancing age, showing no tendency towards involution. The pathogenesis of vascular anomalies has not been fully elucidated, but their formation and progression are closely related to angiogenesis. Localized intravascular coagulation associated with venous or combined malformations is characterized by low fibrinogen, high D-dimers, and normal platelet count.

Objectives To assess the relationship of angiogenic factors with prothrombotic and endothelial damage/dysfunction markers in patients with extensive slow-flow vascular malformations.

Methods A 2-year study (2005–2007) included 31 consecutive patients with extensive slow-flow vascular malformations from one centre.

Results Serum levels of the endothelial receptor tyrosine kinase TIE-2, matrix metalloproteinase (MMP)-9 and angiopoietin (Ang)-2 and plasma levels of D-dimer, plasminogen activator inhibitor type 1 (PAI-1), tissue-type plasminogen activator and von Willebrand factor (vWf) were significantly increased in patients compared with healthy controls, whereas serum levels of vascular endothelial growth factor (VEGF)-C, VEGF-D, MMP-2, Ang-1, platelet-derived growth factor (PDGF)-AB and PDGF-BB were significantly decreased in patients compared with controls. A strong positive correlation was present between Ang-1 and PDGF-AB levels ($r = 0{\cdot}63$, $P < 0{\cdot}001$), between PDGF-AB and PDGF-BB levels ($r = 0{\cdot}67$, $P < 0{\cdot}001$), and between fibrinogen and PAI-1 levels ($r = 0{\cdot}41$, $P = 0{\cdot}031$). A strong negative correlation was present between Ang-1 and vWf levels ($r = -0{\cdot}48$, $P = 0{\cdot}006$), between D-dimer and fibrinogen levels ($r = -0{\cdot}71$, $P < 0{\cdot}001$), and between PDGF-AB and vWf levels ($r = -0{\cdot}42$, $P = 0{\cdot}017$).

Conclusions These findings suggest that angiogenic, coagulation and endothelial damage/dysfunction markers are possibly linked in pathogenesis of extensive slow-flow vascular malformations, and might have therapeutic implications.

Venous malformations and combined malformations of the Klippel–Trenaunay syndrome (KTS) type are slow-flow, haemodynamically inactive lesions that are present at birth and worsen slowly with advancing age, showing no tendency towards involution.[1] Venous malformations occur in a wide dysmorphic spectrum, including varicosities and ectasias, localized spongy masses, and complex lesions that can permeate any organ system, especially muscle. The thin-walled vascular channels are composed of a deficient layer of smooth-muscle cells and are lined by quiescent endothelium.[1] The endothelial receptor tyrosine kinase TIE-2 signalling pathway is critical for endothelial cell–smooth muscle cell communication in venous morphogenesis. One to two per cent of venous malformations are familial with an autosomal dominant hereditary pattern. Inheritable cutaneomucosal venous malformations are linked to TIE-2/TEK gain-of-function mutations.[2]

KTS is a combined vascular malformation characterized by a triad of port-wine stains of the affected extremity, bony and soft tissue hypertrophy, and varicose veins and/or venous malformations, frequently with persistent embryologic veins.[3,4]

Researchers into vascular morphogenesis have identified the vascular gene of chromosome 5Q (*VG5Q*, *AGGF1*) in patients with KTS,[5] although other authors showed the cause of KTS to be a polymorphism.[6] The VG5Q protein acts as a potent angiogenic factor in promoting angiogenesis, whereas suppression of VG5Q expression inhibits vessel formation. VG5Q is strongly expressed in blood vessels, is secreted with the initiation of vessel formation, and binds to endothelial cells, thereby promoting cell proliferation. Increased angiogenesis is probably a pivotal molecular mechanism in the pathogenesis of KTS.[5]

The pathogenesis of vascular anomalies has not been elucidated, but their formation and progression are closely related to angiogenesis. Angiogenesis, defined as the formation of new vessels from pre-existing vessels, is a complex multistep process consisting of basal membrane breakdown, endothelial cell migration and proliferation, subsequent tube formation and, finally, the establishment of connections between newly formed tubes and the initiation of blood flow. The vascular endothelial growth factor (VEGF) and angiopoietin (Ang) families of ligands are key regulators of blood vessel formation. The Ang/TIE-2 system regulates the growth, formation and maturation of new blood vessels. Ang-2 promotes endothelial cell death and vessel regression if the activity of endogenous VEGF is inhibited. By contrast, in the presence of VEGF, Ang-2 promotes a rapid increase in capillary diameter, a remodelling of the basal lamina, and the proliferation and migration of endothelial cells, stimulating the sprouting of new blood vessels.[7] In mice and humans, Ang-2 is selectively expressed in ovary, uterus and placenta and as an angiogenic switch in tumorigenesis, while Ang-1 is widely expressed in both the embryo and the adult.[8] We previously reported elevated Ang-2, TIE-2 and VEGF levels in the serum of a patient with extensive arteriovenous malformation.[9]

Coagulation abnormalities associated with venous or combined malformations of the extremities are reported as localized intravascular coagulation (LIC). This specific entity, characterized by low fibrinogen, high D-dimers, and normal platelet count, must be distinguished from Kasabach–Merrit syndrome, which markedly differs in clinical and pathological features and treatment.[10,11]

The purpose of this study was to assess the relationship of angiogenic factors with prothrombotic and endothelial damage markers in patients with extensive venous and combined malformations in order to establish whether a systemic proinflammatory/prothrombotic profile can influence the development of the disease and suggest a possible therapeutic approach. Among biomarkers associated with vascular damage, inflammation and coagulation, we selected von Willebrand factor (vWf), a marker of endothelial cell damage/dysfunction, plasminogen activator inhibitor type 1 (PAI-1), the main physiological inhibitor of tissue-type plasminogen activator (t-PA), a serine protease that converts inactive plasminogen into active plasmin, and fibrin fragment D-dimer, a stable terminal product generated during the course of fibrin degradation by plasmin.

Patients and methods

Patients

This 2-year study (2005–2007) included 31 consecutive patients with extensive slow-flow vascular malformations referred to our multidisciplinary centre for vascular anomalies in Pamplona. The study was approved by the institutional review board and written informed consent was obtained from all participants. The diagnosis was made by clinical examination and confirmed by Doppler ultrasonography when necessary. KTS was diagnosed when soft tissue and/or bony hypertrophy, port-wine stain and venous varicosities were all present. Table 1 shows the baseline characteristics of the patients.

The patient sample comprised symptomatic patients with malformations involving at least 15% of their body surface area (equivalent to at least one complete lower extremity) (Fig. 1). All lesions infiltrated the muscular plane as well as involving the skin and subcutaneous cell tissue. Direct multidetector computed tomography venography (Somaton VolumeZoom; Siemens, Erlangen, Germany) or fast three-dimensional magnetic resonance imaging venography (Magnetom Symphony; Siemens) was performed to study the venous malformations and to evaluate the patency of the deep venous system prior to the application of polidocanol microfoam sclerotherapy. Although all lesions were stable, their progression had been slow but disproportionate and had exceeded the growth of the patient in all cases. None of the patients had a malignant tumour or known thrombophilia or was receiving antithrombotic therapy.

Table 1 General characteristics of the patients with vascular malformation and healthy controls

Data variable	Vascular malformations	Controls
Number of patients	31	28
Age (years), mean ± SD (range)	30·5 ± 6·5 (16–43)	31 ± 6·4 (18–45)
Sex, n (%)		
Male	17 (54)	15 (53)
Female	14 (46)	13 (47)
Type of vascular anomaly, n (%)		
Venous	11 (35)	–
Combined (capillary-lymphaticovenous)	20 (65)	–
Location of vascular anomaly, n (%)		
Head and neck	1 (3)	–
Hemicorporal	10 (32)	–
Lower extremity	13 (42)	–
Upper extremity	3 (10)	–
Thorax	2 (6)	–
Pelvis and groin	2 (6)	–

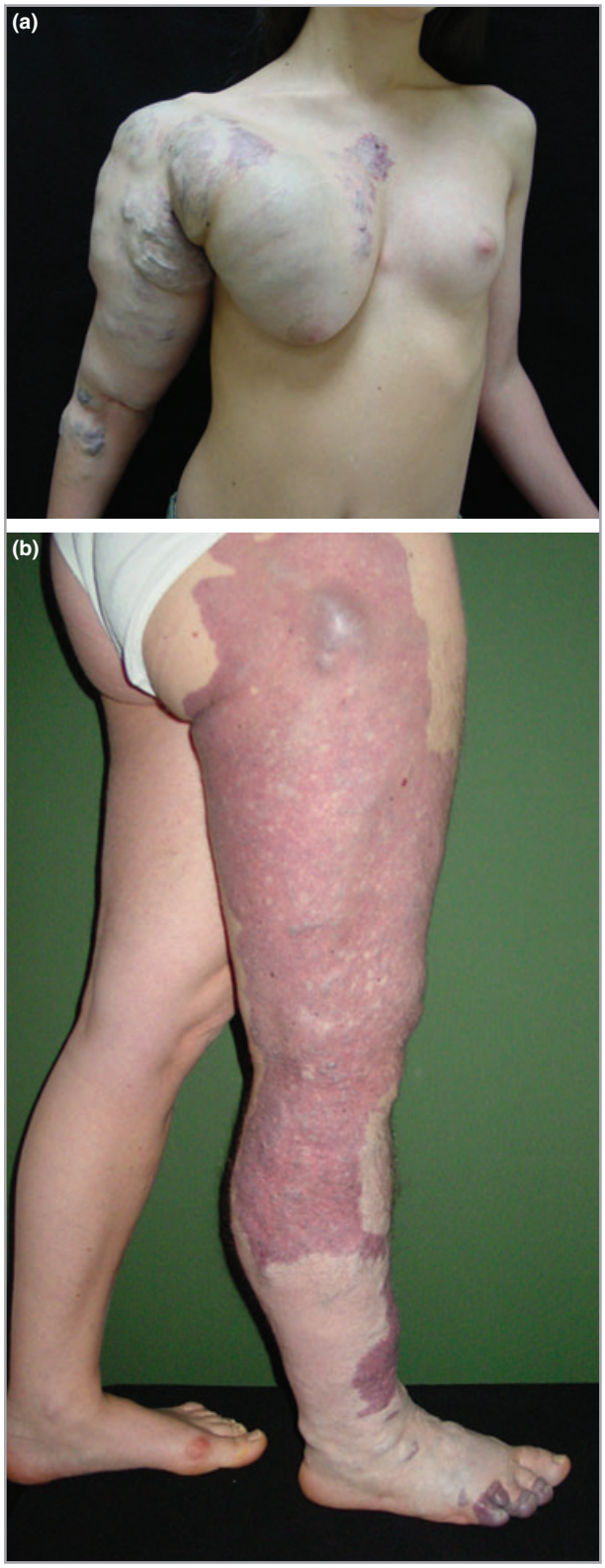

Fig 1. (a) A 16-year-old patient with extensive venous malformation involving right arm and chest. (b) A 34-year-old woman with Klippel–Trenaunay syndrome with venular malformation of geographic morphology and dark red-purple colour, and extensive venous malformation (several phlebectasias in thigh and leg).

Methods

Venous samples were drawn from the cubital vein (n = 31) or local malformation vein (n = 27) before microfoam sclerotherapy was performed, and compared with samples from age- and sex-matched controls. Plasma and serum were removed and stored at −70 °C until analysis. Levels of angiogenic factors were measured with quantitative enzyme-linked immunosorbent assay (ELISA) kits [VEGF, VEGF-C, VEGF-D, matrix metalloproteinase (MMP)-2, MMP-9, Ang-1, Ang-2, TIE-2, platelet-derived growth factor (PDGF)-AB, PDGF-BB; Quantikine; R&D Systems, Minneapolis, MN, U.S.A.]. Levels of t-PA, PAI-1 and vWf were studied as endothelial damage markers (ELISA kits; Diagnostica Stago, Asnières sur Seine, France). Inter- and intra-examiner coefficients of variation for all ELISAs were < 6%.

For prothrombotic/coagulation tests, a blood sample was drawn from a peripheral vein not affected by the vascular malformation. We measured fibrinogen, prothrombin time and platelets by standard automatized techniques, and D-dimer levels (ELISA kit; Siemens) in all patients.

Statistical analysis

Continuous variables were expressed as mean ± SD and compared by Mann–Whitney U-test. Examination of categorical variables was performed using χ^2 analysis. Because data distributions were abnormal, we calculated Spearman's rank correlation coefficients for the relationships between angiogenic factors, and prothrombotic and endothelial damage markers. SPSS version 15.0 (SPSS Inc., Chicago, IL, U.S.A.) was used for the analyses, considering two-sided $P < 0{\cdot}05$ to be statistically significant.

Results

Table 1 shows the clinical characteristics of the study populations. The vascular malformation involved the lower extremity in 24 patients (77%), the upper extremity in five (16%), and was multifocal in six (19%). Patients and controls did not differ in sex ($P = 0{\cdot}102$) or age ($P = 0{\cdot}054$). No differences in serum angiogenic or prothrombotic marker levels were found between cubital and local venous blood samples, which were obtained before sclerotherapy treatment in the patients.

Analytical measurements

Table 2 shows the mean ± SD levels of the angiogenic factors and prothrombotic markers studied in patients and controls, and Figure 2 shows the results as box plots which illustrate better the distribution of the data. Mean ± SD VEGF levels did not differ significantly between patients and controls, whereas they were slightly higher in the venous malformation group than in the combined malformation group and controls.

Table 2 Mean ± SD levels of angiogenic and prothrombotic markers in patients with vascular malformations

Variable	Controls (n = 28)	Venous malformations (n = 11)	Combined malformations (n = 20)	Total malformations (n = 31)
VEGF (pg mL^{-1})	259·52 ± 113·16	315·86 ± 175·28	305·57 ± 118·39	309·22 ± 138·36
VEGF-C (pg mL^{-1})	1346·92 ± 322·39	978·53 ± 437·24	988·94 ± 323·26	985·25 ± 360·46
VEGF-D (pg mL^{-1})	700·40 ± 285·19	415·15 ± 125·64	489·25 ± 147·28	462·96 ± 142·47
TIE-2 (ng mL^{-1})	1·21 ± 0·32	1·75 ± 0·52	1·79 ± 0·47	1·78 ± 0·48
MMP-2 (ng mL^{-1})	30·29 ± 7·22	24·28 ± 6·57	27·49 ± 7·02	26·35 ± 6·93
MMP-9 (ng mL^{-1})	5·45 ± 2·58	13·53 ± 7·24	14·64 ± 6·31	14·24 ± 6·56
ANG-1 (pg mL^{-1})	1245·22 ± 196·57	953·46 ± 176·82	989·86 ± 195·01	976·94 ± 186·60
ANG-2 (pg mL^{-1})	240·19 ± 73·43	301·26 ± 156·25	474·94 ± 435·93	413·31 ± 368·28
PDGF-AB (pg mL^{-1})	552·56 ± 133·21	325·12 ± 94·50	345·28 ± 109·54	338·13 ± 103·31
PDGF-BB (pg mL^{-1})	135·44 ± 47·74	96·73 ± 17·50	109·02 ± 39·87	104·66 ± 33·83
D-Dimer (ng mL^{-1})	171·64 ± 74·02	1352·18 ± 1096·50	1077·10 ± 2219·77	1174·71 ± 1881·31
Fibrinogen (mg dL^{-1})	238·71 ± 54·19	183·18 ± 61·76	222·30 ± 68·95	208·42 ± 68·15
PAI-1 (ng mL^{-1})	75·71 ± 8·54	98·18 ± 50·25	211·60 ± 113·17	171·35 ± 109·53
t-PA (ng mL^{-1})	30·29 ± 9·90	57·00 ± 17·69	80·15 ± 182·69	71·93 ± 146·18
vWf (%)	91·57 ± 25·14	114·36 ± 29·96	124·85 ± 42·83	121·13 ± 38·56

VEGF, vascular endothelial growth factor; TIE, tyrosine kinase with immunoglobulin and epidermal growth factor homology domain; MMP, matrix metalloproteinase; ANG, angiopoietin; PDGF, platelet-derived growth factor; PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue-type plasminogen activator; vWf, von Willebrand factor.

The Mann–Whitney U-test revealed that serum TIE-2 ($P < 0·001$), MMP-9 ($P < 0·001$), Ang-2 ($P = 0·023$), D-dimer ($P = 0·004$), PAI-1 ($P = 0·001$), t-PA ($P = 0·006$) and vWf ($P = 0·007$) levels were significantly higher in patients vs. controls. PAI-1 ($P = 0·001$) and t-PA levels ($P = 0·044$) were significantly higher in the combined malformation group than in the venous malformation group, which did not differ in levels of the other angiogenic factors or prothrombotic markers. VEGF-C ($P < 0·01$), VEGF-D ($P = 0·001$), MMP-2 ($P = 0·042$), Ang-1 ($P < 0·001$), PDGF-AB ($P < 0·001$) and PDGF-BB ($P = 0·019$) levels were significantly lower in patients vs. controls.

The significant differences in MMP-2, PDGF-BB and t-PA levels found between patients and controls remained significant in the comparison between the venous malformation group and controls (MMP-2, $P = 0·038$; PDGF-BB, $P = 0·009$; t-PA, $P < 0·001$), but no significant differences were found between the combined malformation group and controls.

Platelet counts and prothrombin time were within normal ranges. Fibrinogen levels were significantly lower in the venous malformations group vs. controls ($P = 0·021$), but no differences were found between controls and the combined malformation group or the whole group of patients. Plasma D-dimers were elevated in 18 (58%) patients, being fivefold higher in 10 (mean 2540 ng mL^{-1}, normal 150–350), one showing a level > 10 000 ng mL^{-1}. Eight of the 11 patients with venous malformations (73%) and 10 of the 20 with combined malformations (50%) had elevated D-dimer levels.

Univariate analysis

We used Spearman's rank correlation to examine the relationships between angiogenic, prothrombotic and endothelial damage markers.

Ang-1 levels were significantly correlated with PDGF-AB in patients ($r = 0·63$, $P < 0·001$) and controls ($r = 0·63$, $P = 0·016$).

In the patient group, Ang-1 levels were positively correlated with PDGF-BB ($r = 0·37$, $P = 0·036$), yet negatively correlated with D-dimer ($r = -0·38$, $P = 0·031$) and vWf ($r = -0·48$, $P = 0·006$). TIE-2 levels also negatively correlated with Ang-1 ($r = -0·36$, $P = 0·043$). In addition to Ang-1, levels of PDGF-AB correlated positively with PDGF-BB ($r = 0·67$, $P < 0·001$) and negatively with vWf ($r = -0·42$, $P = 0·017$). D-dimer, in turn, also demonstrated a negative correlation with fibrinogen levels ($r = -0·71$, $P < 0·001$), and finally, fibrinogen levels correlated positively with PAI-1 ($r = 0·41$, $P = 0·031$).

Discussion

The progression of vascular anomalies varies according to the type of abnormal vessel and the flow characteristics. The formation and progression of hemangiomas are known to be closely related to angiogenesis, but it has not yet been established whether it mediates the progression of vascular malformations. Marler *et al.*[12] found higher urine MMP and basic fibroblast growth factor levels in children with haemangiomas and vascular malformations than in control children. Meijer-Jorna *et al.*[13] identified areas of microvascular proliferation, interpreted as angiogenesis, in 30% of cases in a large series of 107 surgically removed vascular malformations, especially in arteriovenous malformations.

We report here for the first time that several angiogenic factors and endothelial damage/dysfunction markers are elevated in the circulation of selected patients with extensive slow-flow vascular malformations, probably playing a pathophysiological role. We found elevated Ang-2 levels, as also reported in some patients with arteriovenous malformations of the brain,[14,15]

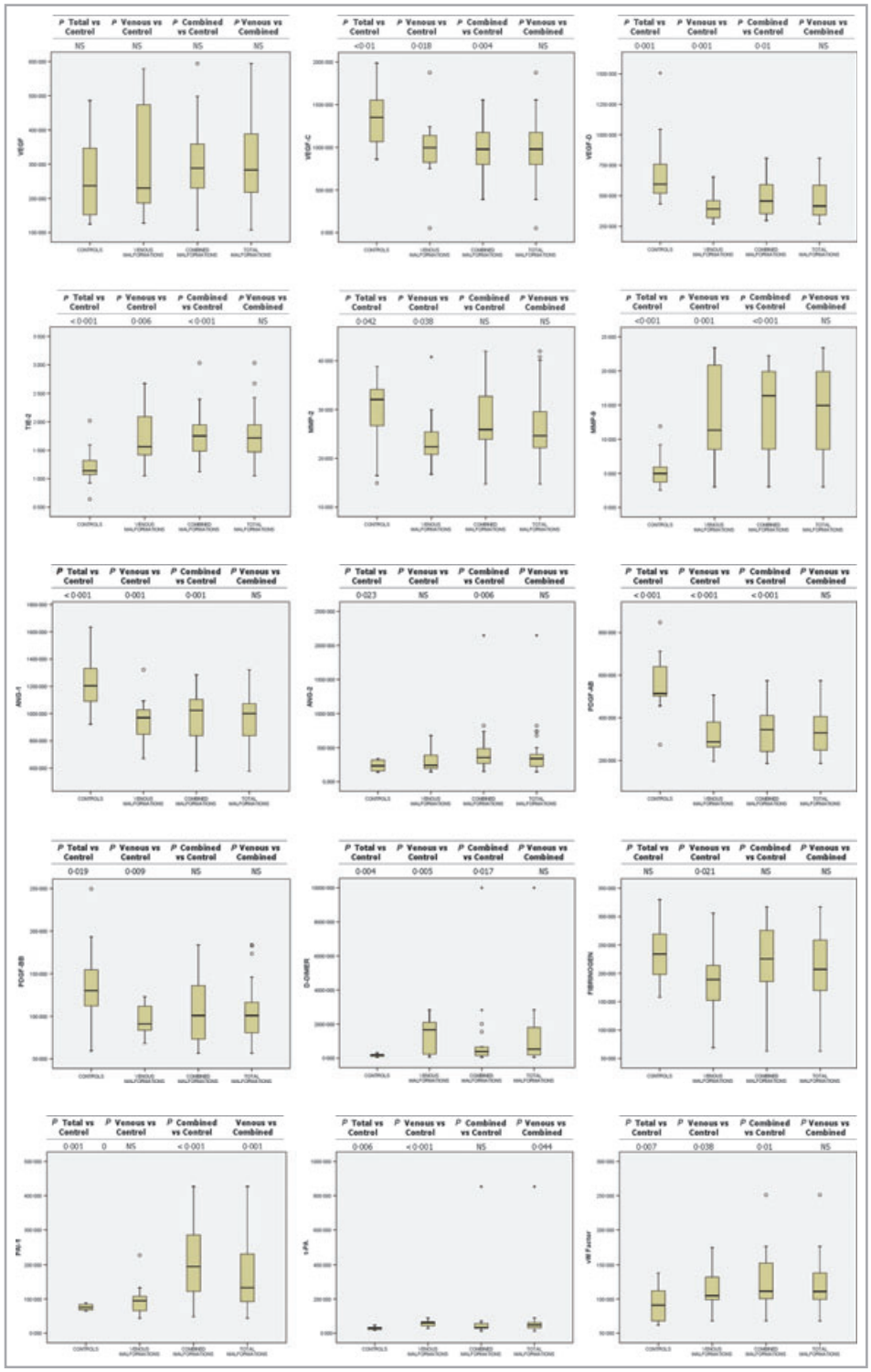

Fig 2. Box plots showing levels of angiogenic and prothrombotic markers in patients with vascular malformations. Results are shown as median and interquartile range. VEGF, vascular endothelial growth factor; TIE, tyrosine kinase with immunoglobulin and epidermal growth factor homology domain; MMP, matrix metalloproteinase; ANG, angiopoietin; PDGF, platelet-derived growth factor; PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue-type plasminogen activator; vW factor, von Willebrand factor.

and we also detected elevated levels of TIE-2 soluble receptor. Ang-1 and Ang-2 are ligands of the endothelial receptor tyrosine kinase TIE-2, which acts as critical regulator of vessel stabilization and maturation. Ang-2 is predominantly expressed in areas undergoing vascular remodelling.[16] These findings may suggest an abnormal disassembly level between endo-

thelial cells and mesenchymal cells due to an imbalance in the Ang–TIE-2 system, leading to dilated vessels with insufficient mural cell components.

Ang-2 is located in endothelial Weibel–Palade bodies (colocalized with vWf) and is rapidly released upon endothelial activation.[17] A recent study analysed Ang-2 expression in response to laminar shear stress in human endothelial cells. Long-term low laminar shear stress induced Ang-2 expression and release, whereas high laminar shear stress downregulated Ang-2 expression and release.[18] The current study confirms previous reports that Ang-2 can be identified and measured in the serum of patients with active angiogenesis, specifically tumour angiogenesis. We highlight the reduced Ang-1 levels in the present patients, in line with a previous report in human brain arteriovenous malformations.[15]

Transient Akt (v-akt murine thymoma viral oncogene homolog 1) signalling modulates physiological microvascular remodelling, vascular growth and homeostasis, whereas sustained Akt signalling causes microvascular malformations.[19] Akt signalling may contribute to vascular malformations when TIE-2 is constitutively activated.[20]

Ang-1-induced TIE-2 phosphorylation results in the activation of Akt.[21] Akt signalling leads to the phosphorylation and inactivation of the forkhead transcription factor, which targets Ang-2. Thus Ang-1/TIE-2 signalling directly drives a negative-feedback loop on endothelial cell Ang-2 production.[22,23] It had been proposed that in settings of decreased Akt activity, induction of Ang-2 is an adaptative mechanism that serves to promote endothelial cell survival and/or vascular maturation via TIE-2/Akt signalling. Akt activity might be reduced during vessel remodelling when endothelial cell contacts with other cells and with matrix are disrupted. In addition, pericyte-derived Akt activators such as PDGF and Ang-1 might be present in decreased levels during vessel remodelling. Thus, Ang-2 would be induced to compensate for the loss of Ang-1 signalling.[24] Our results (Ang-2 increased and Ang-1 reduced) suggest a model in which Ang-2 expression is induced in stressed endothelial cells, where it acts as an autocrine TIE-2/Akt agonist.[24]

A novel observation of this report is the significant increase in t-PA, PAI-1 and vWf in patients with extensive slow-flow vascular malformations. The levels of these endothelial damage/dysfunction markers have been related to various clinical conditions associated with thrombotic phenomena and to different angiogenic processes. Endothelial cells synthesize these proteins in situations of stress, venous stasis and inflammation.[25,26] Whereas deficiency of plasminogen delayed microvessel outgrowth, and t-PA deficiency alone did not dramatically affect microvessel formation,[27] a crucial role has been demonstrated for PAI-1 in tumour growth and angiogenesis. Clinical studies demonstrated that elevated PAI-1 levels are predictive of poor survival in patients with different types of cancer.[27] Nevertheless, this is a controversial issue, as PAI-1 is proangiogenic at physiological concentrations and antiangiogenic at higher levels.[28] Plasma vWf can be rapidly mobilized after endothelial cell activation, and it has been suggested that vWf, alongside fibrinogen, has a direct role in the promotion of thrombosis.[29,30] We show endothelial damage/dysfunction associated with vascular malformations, and propose that endothelial proliferation as a means of effecting endothelial repair may be a mechanism for attempting to preserve endothelial homeostasis. Hence, the development and slow proliferation of extensive slow-flow vascular malformations may be influenced by interaction between the fibrinolytic system and the angiogenesis.[31]

The development of a vascular system involves the assembly of endothelial cells and vascular smooth muscle cell/pericytes into many different types of blood vessels. Our patients had low serum levels of PDGF-AB and PDGF-BB. Analysis of the phenotypes of knockout mice has established critical roles for PDGF in vessel maturation via pericyte recruitment. The pericyte-deficient mutant microvessels of PDGF-deficient embryos show endothelial cell hyperplasia, hypervariable diameter, abundant microaneurysms, abnormal endothelial ultrastructure, and signs of increased permeability.[32] Pericytes express PDGF receptor-β and require PDGF-BB for their recruitment to new vessels in the course of angiogenesis.[33]

Elevated D-dimer levels were observed in 58% of our patients, similar or higher than previously reported,[34,35] possibly due to the larger size of their lesions. Action of plasmin on cross-linked fibrin produces fibrin degradation products that contain D-dimer fragments. Elevated D-dimers (> 500 ng mL^{-1}) are a hallmark of hypercoagulable/prothrombotic state. Several pieces of evidence indicate that angiogenesis and thrombosis may be closely linked. It has been shown that platelets contain and release VEGF, leading to the hypothesis that platelets and possibly local microthrombi promote angiogenesis.[36] *In vitro* data indicate that thrombin stimulates several critical pathways in angiogenesis, including MMPs, VEGF, VEGF receptors, Ang-2, and integrin αv/β3.[37] In addition, plasmin directly intervenes in the basal membrane rupture and extracellular matrix degradation of the angiogenic process, with the plasminogen activator system playing an essential role.[38]

The treatment of vascular malformations is limited, and we must often helplessly observe the natural progression of large lesions, unable to offer a definitive effective treatment. Administration of low molecular weight heparin (LMWH) reduces consumption of coagulation factors and therefore the state of LIC.[34,39] LIC is responsible for thrombotic painful events within the lesions, and when elevated D-dimer levels are associated with pain LMWH is the treatment of choice.[34] On the other hand, the observed high plasma levels of Ang-2, TIE-2 and MMP-9 in the series analysed suggest that these molecules may have a role in the pathogenesis of some vascular malformations. The Ang/TIE-2 system may be a therapeutic target for angiogenesis in vascular malformations.

The main limitation of this study is the relatively small sample size, due to the low incidence of extensive venous and combined vascular malformations. Studies with a larger number of patients are required to confirm these findings and to define therapeutic targets in subgroups of patients with active malformations who are candidates for antiangiogenic/antithrombotic therapies.

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