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Thrombosis risk factors in PIK3CA-related overgrowth spectrum and Proteus syndrome

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Abstract

Increased risk of thromboembolism has been recognized in individuals with mosaic overgrowth disorders, Proteus syndrome (PS) and *PIK3CA*-related overgrowth spectrum (PROS), including Klippel–Trenaunay syndrome and CLOVES syndrome. PS and PROS have distinct, yet overlapping clinical findings and are caused by somatic pathogenic variants in the PI3K/AKT gene signaling pathway. PS is caused by a single somatic activating *AKT1* c.49G > A p.E17K variant while PROS can be caused one of multiple variants in *PIK3CA*. The role of prothrombotic factors, endothelial cell adhesion molecules, and vascular malformations in both PS and PROS have not been previously investigated. A pilot study of prospective clinical and laboratory evaluations with the purposes of identifying potential risk factors for thrombosis was conducted. Doppler ultrasounds and magnetic resonance angiogram/ venography (MRA/MRV) scans identified vascular malformations in PS and PROS that were not appreciated on physical examination. Abnormal D-dimers (0.60–2.0 mcg/ml) occurred in half of individuals, many having vascular malformations, but no thromboses. Soluble vascular endothelial markers, including thrombomodulin, soluble vascular adhesion molecule (sVCAM), soluble intercellular adhesion molecule (sICAM), E-selectin, and P-selectin were significantly higher in PS and PROS compared to controls. However, no single attribute was identified that explained the risk of thrombosis. Predisposition to thrombosis is likely multifactorial with risk factors including chronic stasis within vascular malformations, stasis from impaired mobility (e.g., following surgery), decreased anticoagulant proteins, and effects of *AKT1* and *PIK3CA* variants on vascular endothelium. Based on our findings, we propose clinical recommendations for surveillance of thrombosis in PS and PROS.

KEYWORDS
deep vein thrombosis (DVT), PIK3CA-related overgrowth spectrum (PROS), Proteus syndrome, pulmonary embolism (PE), thrombosis

1 | INTRODUCTION

Proteus syndrome is characterized by asymmetric and disproportionate overgrowth, connective tissue nevi, epidermal nevi, dysregulated adipose tissue, and vascular malformations (Biesecker, 2006; Biesecker et al., 1999) caused by a somatic activating variant in *AKT1* (Lindhurst et al., 2011). Those with Proteus syndrome have an increased risk for deep vein thrombosis (DVT) and pulmonary embolism (PE) (Eberhard, 1994; Keppler-Noreuil, Lozier, Sapp, & Biesecker, 2017; Skovby, Graham Jr, Sonne-Holm, & Cohen Jr, 1993; Slavotinek,

Vacha, Peters, & Biesecker, 2000; Staub, Schmid, & Huber, 2006). Pulmonary embolism is one of the most common causes of premature death in Proteus syndrome, which contributes to the 25% mortality by 22 years of age (Cohen, 2005; Sapp et al., 2007). Vascular anomalies are common and have included prominent, enlarged, and/or ectatic/dilated veins in the abdomen, lower extremities, spine, and chest (Takyar et al., 2018). Superficial and varicose veins may be prominent over affected areas of overgrowth and occur at a young age (Keppler-Noreuil et al., 2017). Absence of the left saphenous vein and right peroneal vein has also been reported (Slavotinek et al., 2000). In our retrospective cohort study of thrombosis in Proteus syndrome, of 57 individuals evaluated at the National Institutes of Health (NIH), 6 of 10 deaths (60%) were caused by a DVT and bilateral PE, and among 47 living individuals, six had DVTs (16%), three had PE, and seven (15%) had thrombophlebitis (Keppler-Noreuil et al., 2017). DVT occurred in overgrown affected limbs with vascular malformations and often in the postoperative period with a median age of 17 years.

PIK3CA-related overgrowth spectrum (PROS) has phenotypic overlap with Proteus syndrome, but is caused by somatic variants in *PIK3CA*, which is in the same signaling pathway as *AKT1* (Keppler-Noreuil et al., 2014; Kurek et al., 2012; Lindhurst et al., 2012). Disorders now recognized to be part of the PROS, include Klippel-Trenaunay syndrome (KTS) and CLOVES syndrome. These disorders also have an increased risk for DVT and PE (Alomari et al., 2010; Douma et al., 2012; Gianlupi, Harper, Dwyre, & Marelich, 1999; Huiras, Barnes, Eichenfield, Pelech, & Drolet, 2005; Keppler-Noreuil et al., 2015; Sapp et al., 2007; Reis III et al., 2018). In KTS and CLOVES syndrome, individuals have combined capillary-lymphatic-venous malformations and are at higher risk for thromboembolism than are those with less extensive, isolated vascular malformations (Alomari et al., 2010; Huiras et al., 2005). The incidence of DVT and PE in those with KTS has been reported to be 17% and 14–22%, respectively (Baskerville et al., 1985). Similarly, other studies have noted chronic thromboembolism in 4% and PE in 12% individuals (64% occurring after surgery or sclerotherapy) (Douma et al., 2012; Reis III et al., 2018). CLOVES syndrome also has capillary, lymphatic, venous and combined vascular malformations. Two of 11 individuals with CLOVES syndrome with central and thoracic phlebectasia developed perioperative PE, one of which caused death (Alomari et al., 2010). Another study reported PE in 9% (10/110) of individuals with CLOVES syndrome (Reis III et al., 2018). Spinal thromboses and neonatal cerebral infarcts have been described (Keppler-Noreuil et al., 2014). Vascular malformations are frequent in PROS, occurring in 59% of one recently described cohort (Parker et al., 2018). Vascular malformations may manifest in some individuals with PROS as spinal myelopathy due to fast-flow spinal-paraspinal vascular lesions (Alomari et al., 2010). Megalencephaly-capillary malformation (MCAP or M-CM) syndrome, one of the phenotypes caused by *PIK3CA* somatic and germline pathogenic variants (Kurek et al., 2012; Mirzaa, Conway, Graham Jr, & Dobyns, 2013), has vascular abnormalities as a component feature, although they occur less frequently. These vascular abnormalities include: infantile hemangiomas, dilated and prominent cutaneous and intracranial venous networks, aberrant vascular in various locations, venous malformations, and vascular rings (Clayton-Smith et al., 1997; Gripp et al., 2009; Mirzaa et al., 2012; Wright et al., 2009). In addition, there are reports of venous thrombosis, dural sinus stasis, and Kasabach–Merritt syndrome in MCAP or M-CM (Wright et al., 2009).

There are multiple reports of individuals with isolated congenital vascular and lymphatic malformations not associated with an underlying syndrome that have increased risk of DVT and PE (Dompmartin et al., 2008; Enjolras, Ciabrini, Mazoyer, Laurian, & Herbreteau, 1997; Mason et al., 2001; Mazoyer et al., 2008; Mazoyer, Enjolras, Laurian, Houdart, & Drouet, 2002; Merli, 2005; Oduber, Gerdes, van der Horst, & Bresser, 2009). Many of these vascular and lymphatic malformations are caused by somatic *PIK3CA* variants. Therefore, the mechanisms contributing to an increased risk of thrombosis may be attributed, at least in part, to dysfunction of the PI3K/AKT gene pathway.

Vascular endothelium is considered to be a highly specialized metabolically active tissue (Behrendt & Ganz, 2002; Celermajer, 1997). Activation of vascular endothelial cells by various factors consists of an increased expression of cellular adhesion molecules (CAMs) and selectins, whose soluble forms can be detected in the blood (Glowinska, Urban, Peczynska, & Florys, 2005). Although CAMs are necessary for normal development and function of blood vessels, they also have been implicated in pathogenesis of cardiovascular disease. In atherosclerosis, attachment of monocytes and lymphocytes to endothelial cells is under the influence of CAMs, including intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule (VCAM-1), and selectins on the endothelium (Hillis & Flapan, 1998; Ross, 1999). Elevated levels of sICAM, sVCAM, E-selectin, and P-selectin have been found in obese, hypertensive, and diabetic children and adolescents with evidence of vascular abnormalities (Glowinska et al., 2005). The activation of these vascular endothelial cellular adhesion molecules has not been evaluated, but may be a risk factor for thrombosis in Proteus syndrome or PROS.

The mechanism of hypercoagulability and risk for thrombosis in Proteus syndrome and PROS remains to be identified. We conducted a pilot study of prospective clinical and laboratory evaluations to identify potential risk factors for thrombosis. Based on these findings, we propose clinical recommendations for surveillance of thrombosis in Proteus syndrome and PROS.

2 | METHODS

2.1 | Clinical

Twenty-two individuals with Proteus syndrome and ten individuals with PROS were evaluated at the NIH Clinical Center, as part of a natural history study approved by the National Human Genome Research Institute (NHGRI) IRB (study #04-HG-0132). Written informed consent from the participant or their guardians was obtained. All individuals with Proteus syndrome and PROS met clinical diagnostic criteria (Biesecker, 2006; Biesecker et al., 1999; Keppler-Noreuil et al., 2015). Twenty-one of 22 individuals with a Proteus syndrome clinical

diagnosis had the somatic activating variant in *AKT1* confirmed from affected tissue samples. All ten of the individuals with PROS had somatic activating variants in *PIK3CA* in affected tissue samples. The individuals' medical history and physical examination findings were recorded, including their history of superficial and deep venous thromboembolic events, and use of anticoagulant medications. Retrospective data on 17 of the 22 individuals with Proteus syndrome were described in our previous study (Keppler-Noreuil et al., 2017). This pilot study protocol included a prospective evaluation algorithm of clinical and research laboratory and radiological studies. The clinical coagulation studies included: fibrinogen levels, thrombin clotting time (TCT), prothrombin time (PT), partial thromboplastin time (PTT), antithrombin activity, protein C and S activities, factor V Leiden variant, prothrombin 20210 variant, lupus anticoagulant (LA), plasminogen activator inhibitor (PAI), platelet function analyzer (PFA-100), and homocysteine. The D-dimer assay used at the NIH Clinical Center (STA Liatest, Stago Diagnostica, Parsippany, NJ) has been validated for DVT screening in the general population with a threshold of 0.5 mcg/ml to rule out thrombosis (Aguilar et al., 2002; Kulstad, Kulstad, & Lovell, 2004; Rathbun, Whitsett, & Raskob, 2004). Doppler ultrasounds of the arms and legs were performed on all individuals. MRA/MRV scans of the abdomen and pelvis with contrast material (Ablavar) were performed on all individuals who did not require sedation.

Research tests included a global in vitro assessment of blood coagulation and fibrinolysis, as well as markers of endothelial damage or activation. These included assays for prothrombin activation (prothrombin fragment 1.2), thrombin inactivation (thrombin-antithrombin complex, TAT), initiator of coagulation (tissue factor, TF), and markers of endothelial activation and damage (thrombomodulin, sVCAM and sICAM), and platelet activation (P-selectin, E-selectin). The thromboelastogram (TEG) is a test of overall coagulation function and fibrinolysis. TEG parameters analyzed included indicators of the time to activate thrombin and generate fibrin (the R time, in minutes), the rate of initial clot formation (alpha angle in degrees), and the maximum clot strength (maximum amplitude, MA, in millimeters). All the research studies were performed on a normal control group of 32 individuals, who had similar age and gender distribution to those with Proteus syndrome and PROS.

2.2 | Statistics

The analyses compared the results of the research laboratory tests in the individuals with Proteus syndrome and PROS versus the control group using univariate *t*-tests (chi-squared tests for gender). Normal controls were compared to PROS individuals, and separately to Proteus individuals, with respect to the variables (P-selectin, E-selectin, sICAM, sVCAM, thrombomodulin, tissue factor, TAT, Prothrombin F1 -F2, and TEG components: MA, A, R, K by means of student's two-sample t-tests, and, for gender, by chi-squared tests. When the Folded F test for equality of variances was significant at level 0.05, the t-test was adjusted for unequal variances by the Satterthwaite method. We used the Bonferroni technique to control the overall type I error rate at 0.05 by nominating only those tests as Bonferroni-significant for which the nominal *p*-value was less than .05/K = 0.002, where K = 24 is the total number of tests we performed. In these results, we discuss only results that are Bonferroni-significant. We also show Box-and-Whisker diagrams of selected comparisons, to give a clearer idea of how the control, PROS, and Proteus groups differed with respect to variables of interest. In these Box and Whisker plots, the bottom and top edges of the box indicate the 25th and 75th centiles, (the interquartile range—IQR), while the middle line indicates the median. The marker inside the box indicates the mean. Whiskers indicate values outside IQR but too close to be outliers. Open circles indicate outliers, more than 1.5*IQR away. All calculations were carried out in SAS 9.4.

3 | RESULTS

Of the 22 individuals with Proteus syndrome, the male-to-female ratio was 1.44 to 1, and the median age was 13.5 years (range 1.5--55 years). All but one had a somatic activating variant, in *AKT1* confirmed from the affected tissue samples. Key clinical features of these individuals are summarized in Table 1. Eighteen of 22 (82%) had one

TABLE 1 Summary of clinical findings in 22 individuals with Proteus syndrome

Summary of clinical findings in Proteus syndrome (*N* = 22)		
Gender		
Male	13	
Female	9	
Median age (range)	13.5 y (1.5–55 y)	
AKT1 pathogenic variant	21/22	
Diagnostic features		
	Present (#)	# present/Total (percent)
Vascular malformations	18	18/22 (82%)
Superficial venous	18	18/22 (82%)
Deep venous/ visceral	11	11/22 (50%)
History of DVT	4	4/22 (18%)
History of superficial thrombophlebitis	5	5/22 (23%)
Skeletal overgrowth	22	22/22 (100%)
Legs	19	19/22 (86%)
Feet	20	20/22 (91%)
Hands/arms	14	14/22 (64%)
Craniofacial	8	8/22 (36%)
Vertebral/scoliosis	11	11/22 (50%)
Cerebriform connective tissue nevus (CCTN)	19	19/22 (86%)
Epidermal nevus	19	19/22 (86%)
Tumors		
Ovarian/Paraovarian	3	3/9 (33%)
Testicular/Paratesticular	5	5/13 (38%)
Other (labia/cervix)	1	1/22 (4.5%)

or more venous malformations comprised of superficial venous abnormalities in all, and deep venous and/or visceral malformations present in 50%. Of these, 4/18 (18%) had a history of DVT in an affected limb, and one of these individuals had a history of PE. Five of 22 (23%) had a history of superficial thrombophlebitis. None had an acute or newly identified DVT or thrombophlebitis; however, two had evidence of an "old" thrombus by imaging, at the time of obtaining the clinical and laboratory studies. All had Doppler ultrasounds of the arms and legs, which were negative for thrombi. Doppler ultrasound did identify venous malformations in 10/22 (45%) of individuals. MRA/MRV scans of the abdomen and pelvis with Ablavar were performed on 14 of 22 (64%); 10 of 14 (71%) had multiple deep venous and visceral venous malformations, which were not evident from physical examination (Supplemental Table 1). Abnormalities included dilation, enlargement, tortuosity and ectasia of portal, splenic, renal, mesenteric, superior mesenteric vein, inferior vena cava, and iliac veins (common external and internal), saphenous, and popliteal veins, and involving multiple veins in various body parts, especially in thoracic, paraspinal and pelvic regions. There were also venous plexuses, and development of collateral veins (Figure 1a,b). There was enlargement of superficial veins in all patients.

Clinical laboratory results showed that most individuals (16/22, 73%) had one or more heterogenous abnormalities on the clinical coagulation test panel. The most common abnormality was a positive lupus anticoagulant in 11/22 (50%), and of these individuals 8/11 (73%) had additional abnormalities. Six had abnormal PFA, four had abnormal PAI, one had Factor V Leiden heterozygous variant, and one had anti-Beta$_2$ glycoprotein 1 antibody. There were a total of four individuals that had a history of DVT; there were two individuals with protein C and S deficiencies, and elevated PT, PTT levels, and one of them also had hyperhomocystinemia (these two individuals had residual chronic, non-obstructing clot, one had combined abnormalities of positive lupus anticoagulation, high PFA, anti-β_2 glycoprotein 1 antibody and low PAI, and the fourth had positive lupus anticoagulant and low PAI)(Supplemental Table I).

Almost half (10/22, 45%) had abnormal D-dimer levels (>0.50 mcg/ml FEU) ranging from 0.60–3.77 mcg/ml, and none of these individuals had evidence of DVT on imaging. Of the six individuals with abnormal D-dimer levels who underwent MRA/MRV scans, five underlying deep venous malformations. Of the nine with normal D-dimer levels who had MRA/MRV scans, five had deep venous malformations. A CT of the chest was not done if there were no symptoms or signs of PE, except in the individual with the D-dimer level of 3.77 mcg/ml, who had a normal CT scan of the chest.

The 10 individuals with PROS had a male-to-female ratio of 1 to 1, and median age was 10 years (range 11 months to 48 years). All had somatic activating variants in *PIK3CA*: Six with c.3140A>G, p.(His1047Arg), two with c.16246G>A, p.(Glu542Lys), one with c.3140A>T, p.(His1047Leu), and one with c.328_330delGAA p.(Glu110del). The clinical findings are summarized in Table 2, and Supplemental Table II. Vascular malformations were identified in half of the individuals; however, complete ascertainment of deep venous/visceral malformations was not achieved because MRA/MRV scans of the abdomen and pelvis were only done in four individuals. Three of these studies showed abnormal vasculature, primarily venous abnormalities not identified by physical examination, (Supplemental

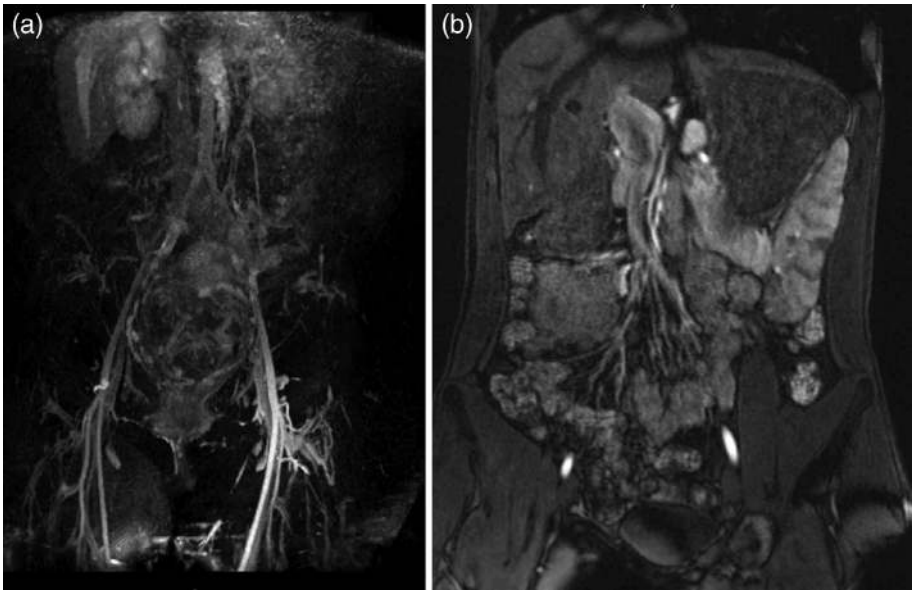

FIGURE 1 MRA/MRV scans on two individuals with Proteus syndrome (a) PS47 (42 year old woman): Multiple findings including 1) A large varicosity in the right gluteal region, 2) multiple dilated superficial vein in the left subcutaneous tissue posteriorly in the upper thigh, 3) A prominent large anterior vein in the left thigh, draining to the left iliac vessels, 4) prominent hepatic vein varicosity in the liver below the level of the portal vein, 5) large cervical mass—No high flow arterial involvement, just evidence of low-flow state. (b) PS38 (29-year-old man) multiple findings including: 1) diffusely enlarged inferior vena cava, 2) portal vein focally dilated at confluence with superior mesenteric vein and splenic vein, and 3) left renal vein also slightly enlarged. MRA/MRV, magnetic resonance angiogram/ venography

TABLE 2 Clinical findings in 10 individuals with *PIK3CA*-related overgrowth spectrum

Summary of clinical findings in PROS (*N* = 10)		
Gender		
Male	5	
Female	5	
Median age (range)	10 y (11 m–48 y)	
PIK3CA pathogenic variants	c.3140A > G, p.(His1047Arg) (*N* = 6); c.16246G > A, p.(Glu542Lys) (*N* = 2); c.3140A > T, p.(His1047Leu) (*N* = 1); c.328_330delGAA p.(Glu110del) (*N* = 1)	
Diagnostic features		
	Present (#)	# Present/Total (percent)
Bone overgrowth	10	10/10 (100%)
Fibroadipose overgrowth	9	9/10 (90%)
Regional lipohypoplasia (affected areas)	2	2/10 (20%)
Vascular malformations	5	5/10 (50%)
Superficial venous	5	5/10 (40%)
Deep venous/ visceral	3	[a]3/4 (75%)
History of deep venous thrombosis (DVT)	0	0
History of neonatal infarct	1	1/10 (10%)
History of Paraspinal vascular malformations/ infiltrate in epidural space	2	2/10 (20%)
History of superficial thrombophlebitis	1	1/10 (10%)
Polydactyly	2	2/10 (20%)
Syndactyly	2	2/10 (20%)
Tumors (unilateral cystic mass of testis, bilateral Nephroblastomatosis)	2	2/10 (20%)
Epidermal nevus	4	4/10 (40%)
Cerebriform connective tissue nevus (CCTN)	1	1/10 (10%)

Abbreviations: MRA/MRV, magnetic resonance angiogram/ venography; PROS, *PIK3CA*-related overgrowth spectrum.
[a]Ascertainment by MRA/MRV scan done in 4/10.

Table II; Figure 2a,b). All Doppler ultrasounds on the arms and legs were negative for thromboses. Only one patient had a venous malformation identified on Doppler ultrasound, which was a small vein in the right femur.

Clinical laboratory studies showed five individuals with abnormal D-dimer levels (0.56, 0.79, 1.38, 1.81, and 5.93 mcg/ml). As described earlier, Doppler ultrasounds of the arms and legs did not identify thrombosis in any of these. In the three individuals with D-dimer level above 1.0 mcg/ml, these individuals were all identified to have venous malformations on MRA/MRV scans. There were three other individuals with normal D-dimer and Doppler ultrasound studies, but they did not have an MRA/MRV scan. Four individuals with PROS had a positive lupus anticoagulant, one of which also had a positive PFA. One other person had an elevated PFA, and one had a positive anticardiolipin antibody (Supplemental Table II).

Thrombomodulin levels were significantly higher in individuals with Proteus syndrome and PROS compared to normal controls (*p*-values .0071 and .0129, respectively) (Figure 3a). The endothelial cellular adhesion molecules, sVCAM, and sICAM (*p*-value .0011 PROS, *p*-value .3150 Proteus syndrome) were also elevated in individuals with Proteus syndrome and PROS compared to controls with sVCAM having highly significant increased levels in both conditions (*p*-value .0029 Proteus syndrome, and *p*-value 0.0 PROS) (Figure 3b,c). E-selectin was significantly higher for those with PROS (*p*-value .0039) (Figure 3d), and P-selectin was significantly higher for those with Proteus syndrome (*p*-value .00207) (Figure 3e). Tissue factor, prothrombin fragment 1.2, and thrombin-antithrombin complexes were not significantly different from normal controls for individuals with Proteus syndrome or PROS, nor were TEG results significantly different.

4 | DISCUSSION

We evaluated 32 individuals with Proteus syndrome or PROS to explore the potential association of vascular malformations in these disorders with various factors with known or suggested roles in vascular structure and function, and disease. We confirmed that individuals with both Proteus syndrome and PROS had frequent vascular malformations including deep venous/visceral venous malformations. In particular, vascular malformations occurred in 80% of those with Proteus syndrome, a greater frequency than the ~60% previously reported (Slavotinek et al., 2000). Notably, the majority of these were only identifiable by MRA/MRV scans; this suggests that ultrasound is not a sensitive diagnostic technique for these lesions. It has been suggested that the higher incidence of DVT and PE in Proteus syndrome and PROS is primarily related to blood stasis in underlying vascular malformations (Nielsen, 1991; Slavotinek et al., 2000). Blood stasis leading to activation of coagulation within the distorted and enlarged venous blood vessels is assumed to be in part responsible for thromboembolism (Dompmartin et al., 2008; Enjolras et al., 1997; Mason et al., 2001; Mazoyer et al., 2002; Mazoyer et al., 2008; Merli, 2005; Oduber et al., 2009). However, plethysmography in one study could not differentiate the venous anatomic abnormalities of individuals with KTS with DVT or PE from those without DVT or PE. Fibrinopeptide A and thrombin activity were abnormal in those individuals with KTS and thrombosis suggesting increased fibrinogen conversion to fibrin by activated thrombin as the etiology for the high incidence of DVT (Baskerville et al., 1985).

In our previous retrospective study, we found that the occurrence of thrombosis in individuals with Proteus syndrome was not primarily attributable to an increase in any one of the well-known prothrombotic conditions (such as the factor V Leiden variant, prothrombin 20210 variant, deficiency of antithrombin, protein C, or protein S)

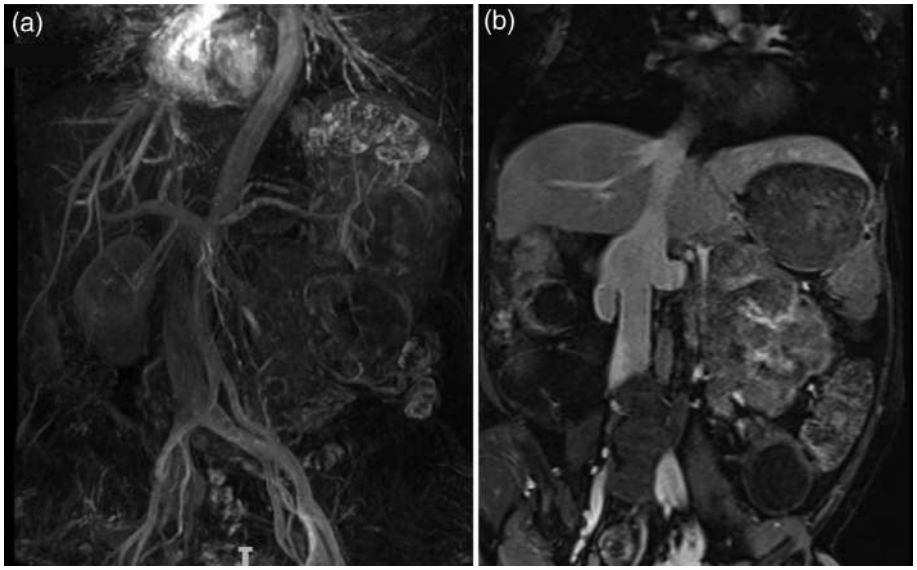

FIGURE 2 MRA/MRV scan on two individuals with PROS (a) PS113, 48-year-old woman. Abnormalities include a varix of the right iliac vein and abnormal superficial vessels in the posterior thoracic and abdominal wall. (b) PS138, 16-year-old girl. Abnormalities include a dilated IVC and left renal vein. IVC, inferior vena cavae; MRA/MRV, magnetic resonance angiogram/ venography; PROS, *PIK3CA*-related overgrowth spectrum

that increase the rate of thrombin production and fibrin clot generation (Keppler-Noreuil et al., 2017). However, here, over 70% of the individuals with PS had one or more prothrombotic factors, including 50% with a positive lupus anticoagulant. There were also additional prothrombotic factors identified in multiple individuals including PAI and PFA. The presence of these factors may contribute to susceptibility for developing DVT and/or PE and may eventually provide a better estimation of risk. However, these results are individually uncommon, and it is difficult to assign a causative role to any one of these individual factors. Elevated D-dimer levels were found in about half of the individuals with Proteus syndrome and PROS, and although these individuals did not have evidence of a DVT or PE, they were more likely to be associated with evidence of an underlying vascular malformation on MRA/MRV scan.

In a previous report of 17 individuals with Proteus syndrome, eight had abnormal D-dimer levels. Three of these were greater than 0.5mcg/ml but below 1.0 mcg/ml; none of these individuals had evidence of DVT/PE on Doppler ultrasound of legs and CT scan of the chest. Four of the five remaining individuals with levels ≥1.0 mcg/ml had DVT/PE, and one had recurrent superficial thrombi (Keppler-Noreuil et al., 2017). Based on these results, we suggested considering upward adjustment of the screening threshold for thrombosis in the Proteus syndrome population from 0.5 mcg/ml (the accepted threshold in the general population for the STA Liatest D-dimer assay) to >1.0 mcg/ml to improve the specificity of an abnormal D-dimer test result. None of the patients with Proteus syndrome in the present analysis had DVT/PE and 40% had D-dimer levels >1.0, but less than 2.0 mcg/ml. It may thus be reasonable to adjust the threshold for high suspicion for an underlying DVT/PE to >2.0 mcg/ml. Notably, applying this threshold to the individuals we report here and previously would result in one instance of missed DVT and concurrent PE; notably, this individual also had a prothrombin 20210 mutation (Keppler-Noreuil et al., 2017).

Thromboelastography (a global measurement of blood coagulation), prothrombin fragment 1.2 (an indicator of prothrombin activation), thrombin-antithrombin complex (an indicator of thrombin turnover), and tissue factor (the key initiator of coagulation) did not show significant differences between individuals with Proteus syndrome or PROS and normal controls. These results suggest that abnormalities in the components of the coagulation cascade and fibrinolysis may not be the major cause of increased risk of thrombosis.

There is strong evidence for disturbances in the vascular endothelial lining in both Proteus syndrome and PROS. We show herein that elevations in soluble forms of thrombomodulin, sVCAM, and sICAM, E-selectin and P-selectin in plasma are present in patients with PROS and Proteus syndrome. Activation of these soluble endothelial cellular adhesion molecules has been associated with multiple vascular disorders, and have been used as markers for DVT risk, in the case of P-selectin (Blann, Noteboom, & Rosendaal, 2000; Rectenwald et al., 2005; Shi et al., 2014; Smith, Quarmby, Collin, Lockhart, & Burnand, 1999). Thrombomodulin was significantly increased in individuals with Proteus syndrome and PROS compared to normal controls. Thrombomodulin is a membrane-bound protein that is part of the protein C receptor complex on endothelial cells and serves as a cofactor for the conversion of protein C to activated protein C (an anticoagulant protein) by thrombin and also mediates activation of procarboxypeptidase B, a thrombin activatable fibrinolysis inhibitor that inactivates complement factors C3a and C5a (Campbell, Lazoura, Okada, & Okada, 2002; Van de Wouwer, Collen, & Conway, 2004). Soluble thrombomodulin circulates in plasma at levels of 3,000–5,000 pg/ml and its levels are increased by endothelial damage from sepsis, infection, or inflammation, presumably by enzymatic cleavage from neutrophils (Boehme et al., 1996; Van de Wouwer et al., 2004). The level of thrombomodulin in the circulation correlates with disease activity in vasculitides such as Wegeners granulomatosis and is a marker of endothelial damage associated with these diseases (Zycinska, Wardyn, Zielonka, Krupa, & Lukas, 2009).

Similar to thrombomodulin, the endothelial associated vascular adhesion molecules sVCAM and sICAM were significantly elevated in the Proteus and PROS individuals compared to normal controls. sVCAM and sICAM have been studied as markers of disease activity

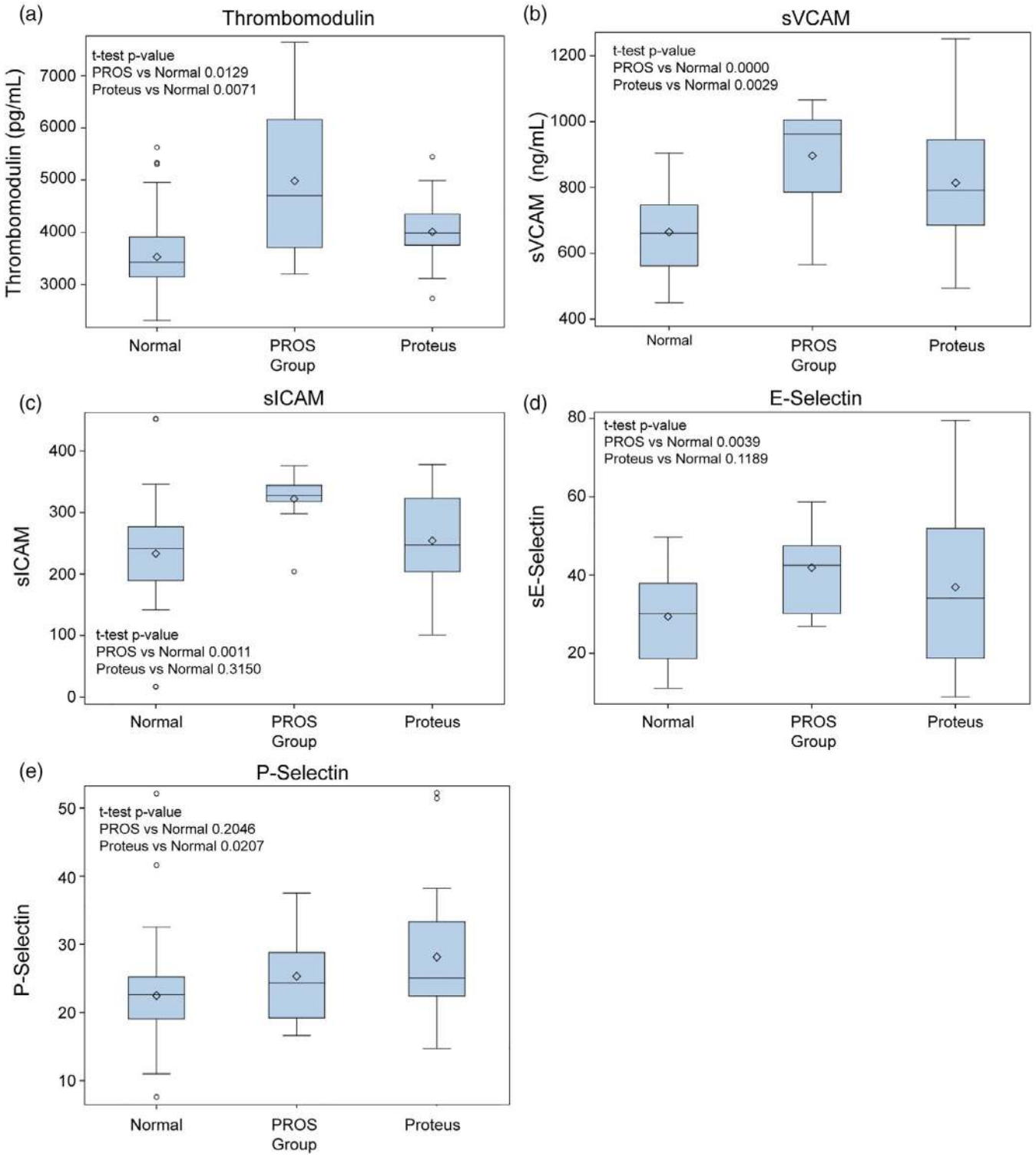

FIGURE 3 Box and whisker plot of soluble cellular endothelial marker levels with *t*-test *p*-values. (a) Thrombomodulin levels were significantly increased in Proteus syndrome and PROS compared to normal controls. (b) sVCAM levels were significantly increased in Proteus syndrome and PROS compared to normal controls. (c) sICAM levels were significantly increased in PROS compared to normal controls. (d) E-selectin levels were significantly increased in PROS compared to normal controls. (e) P-selectin levels were significantly increased in Proteus syndrome compared to normal controls. PROS, *PIK3CA*-related overgrowth spectrum; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular adhesion molecule

in coronary artery and peripheral vascular disease, diabetes, and inflammatory conditions (Fasching et al., 1996; Hulok et al., 2014; Kado and Nagata, 1999). In addition, both of the selectins were higher in Proteus syndrome and PROS than in normal controls. All are implicated as markers of endothelial cell activation, injury, or dysfunction (Glowinska et al., 2005; Hillis & Flapan, 1998; Ross, 1999). In

particular, P-selectin is involved in activation of platelets, adhesion of leukocytes and platelets to the endothelium, and has been associated with blood coagulation and venous thrombosis (Celi, Pellegrini, & Lorenzet, 1994; Furie & Furie, 2004; Merten & Thiagarajan, 2000; Theoret, Yacoub, Hachem, Gillis, & Merhi, 2011). The significant elevation of endothelium-associated proteins such as thrombomodulin, sVCAM, sICAM, E-selectin and P-selectin in Proteus syndrome and PROS suggests a disturbance of vascular endothelial metabolism, which we hypothesize, may be a marker or point to a possible contributing mechanism for risk of thrombosis in these conditions.

The role of the p110alpha catalytic subunit of PI3K is essential for endothelial cell migration and angiogenesis and sustained endothelial activation of *AKT1* has been shown to induce the formation of structurally and functionally abnormal blood vessels (Graupera et al., 2008; Phung et al., 2006). RAS activation in endothelial cells resulted in abnormal vascular morphogenesis, which is regulated by PI3K signaling (Bajaj, Zheng, Adam, Vincent, & Pumiglia, 2010).

Based on these findings, we suggest that the biology of Proteus syndrome and PROS may have adverse effects on hemostasis by mechanisms such as endothelial abnormalities or platelet dysfunction, beyond the well-recognized effects of stasis associated with vascular malformations. The increased risk of thrombosis in these disorders may be attributable to multiple factors, which may be additive, including specific vascular endothelial dysfunction, particular endothelial adhesion molecules, caused by the somatic activating *AKT1* p.E17K and *PIK3CA* variants, known prothrombotic factors, and acquired prothrombotic risk factors (e.g., surgery, immobilization, and dehydration) (Figure 4).

4.1 | Surveillance & Management Recommendations

We suggest that the presence of these vascular malformations, in addition to our findings herein of elevated specific vascular endothelial molecular factors in both Proteus syndrome and PROS supports heightened surveillance for development of DVT/ PE, and intervention based on the results of the evaluations. We have previously found that while most DVT and PE in individuals with Proteus syndrome developed perioperatively, some did not. Treatment for individuals with Proteus syndrome and PROS will be dependent on the findings in each individual, which are variable in onset and course, tissue affected and location of the affected lesions. A complete history re. DVT and PE and assessment of the extent of the vascular findings with follow-up of abnormal findings is appropriate. Individuals with both Proteus syndrome and PROS may benefit from an MRI of the chest, abdomen, pelvis and lower extremities at the time of initial diagnosis. This helps define deeper components of the syndrome that may require intervention in early childhood (e.g., lymphatic and venous malformations, gastrointestinal, and genitourinary involvement); and characterize overgrowth and extension into the retroperitoneum, peritoneum, superior and posterior mediastinum, pelvis, pleural spaces and paraspinal muscles, tethered spinal cord, neural tube defects. Observation versus interventions (e.g., sclerotherapy, or surgical repair) will depend on the findings from these studies, in addition to clinical symptoms. Optimal timing of imaging is best balanced by the risks of anesthesia with the information to be gained. Because the overgrowth is progressive, especially in Proteus syndrome, this study

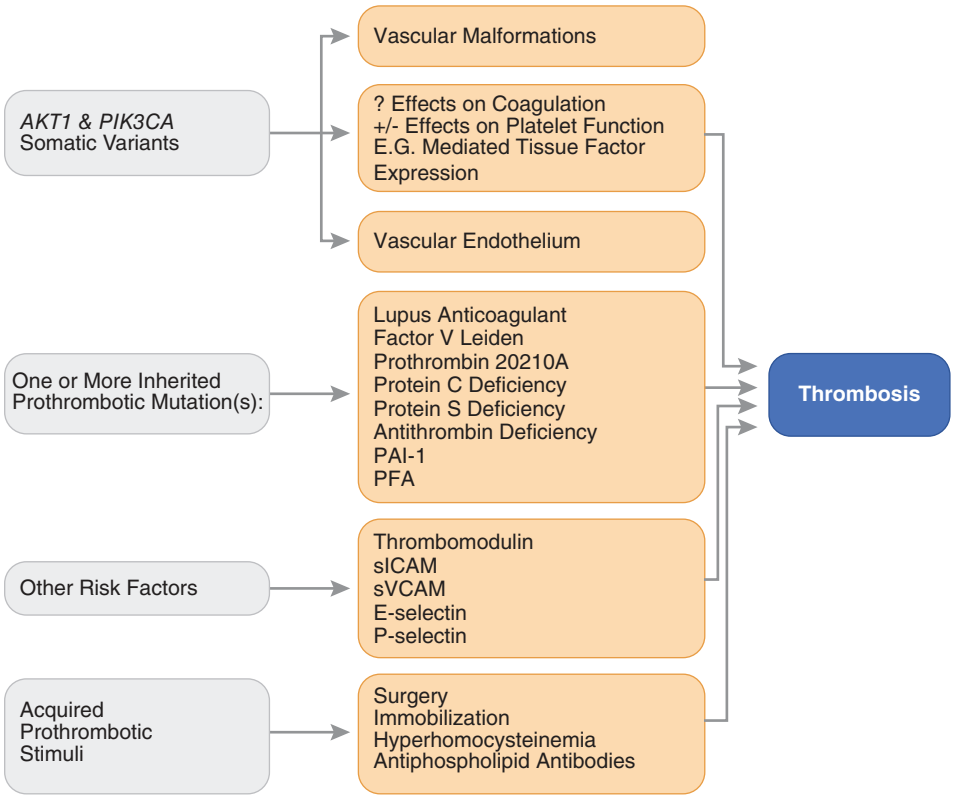

FIGURE 4 Contributing risk factors for thrombosis in Proteus syndrome and *PIK3CA*-related overgrowth spectrum

may need to be repeated based on clinical history and physical examination findings.

Individuals with PROS, who are identified to have venous malformations, including phlebectasia or dilatation of veins often seen in the lower extremities and truncal wall, may need close monitoring for risk of developing thromboembolism and painful thrombophlebitis, especially in situations where there is increased risk of thromboembolism, for example, surgery, immobilization, dehydration. These individuals with PROS having these findings are at increased risk (Alomari et al., 2010). Routine imaging such as Doppler ultrasounds of the limbs or MRI of the spine for surveillance or if the patient is symptomatic may be warranted depending on the location and characterization of associated vascular malformations. Doppler ultrasound may demonstrate certain veins including subclavian, axillary, saphenous and marginal veins in the lower extremity and the lateral truncal wall. Doppler ultrasound helps to characterize the venous malformations, and screen for the presence of blood clots. Deep veins (innominate, azygous, and sciatic veins as well as the superior and inferior vena cavae [SVC, IVC]) can be visualized by MRI. Vascular malformations may occur into the retroperitoneum, peritoneum, superior and posterior mediastinum, pelvis, pleural spaces and paraspinal muscles. Those patients with PROS having a prior history of DVT and/or vascular malformations may be considered for anticoagulant therapy during and after surgical procedures in situations with increased risk of thrombosis, for example with immobility, until they are ambulatory, similar to management for DVT/PE in Proteus syndrome. The clinical behavior of paraspinal-posterior mediastinal infiltrative tissue is distinct from the larger fatty masses. Paraspinal lesions can be hypervascular, aggressive and infiltrate into the epidural space and compromise the spinal cord and adjacent vessels.

Individuals with Proteus syndrome or PROS with vascular malformations may benefit from a hematology evaluation, and basic clinical coagulation laboratory studies, including baseline D-dimer test, which is a useful screen for DVT and PE (Keppler-Noreuil et al., 2017; Pulivarthi & Gurram, 2014), and is informative as a baseline for comparison when evaluating possible venous thromboembolic (VTE) events, or during heightened VTE monitoring at the time of invasive procedures. Prior to invasive surgical procedures, preoperative imaging with Doppler ultrasounds to evaluate the venous anatomy, in addition to a routine hematology/hypercoagulable workup is recommended. Patients with Proteus syndrome, who had orthopedic surgeries for leg overgrowth at the NIH all were managed with DVT prophylaxis of 0.5 mg/kg enoxaparin (Sanofi Aventis US, Bridgewater, NJ) q12h IM, sequential compression devices until the patient was reasonably ambulatory, and all were mobilized quickly postoperatively to mitigate the risk of DVT and PE (Crenshaw et al., 2018; Tosi, Sapp, Allen, O'Keefe, & Biesecker, 2011). Although no patients in this series developed a perioperative DVT or PE, this is a common problem in patients with PS and the absence of this complication in this case series was noteworthy. We hypothesized that this trend was attributable to consistent prophylaxis and aggressive postoperative ambulation. For those patients with PROS having vascular malformations, we would suggest DVT prophylaxis and management similar to that recommended for Proteus syndrome with 0.5 mg/kg enoxaparin, sequential compression devices until the patient is ambulatory, and mobilization postoperatively to reduce the risk of thrombosis. Those individuals with Proteus syndrome and PROS who are positive for DVT or PE on imaging should undergo acute anticoagulation according to the standard American College of Chest Physicians guideline (Antithrombotic Therapy for VTE Disease: CHEST Guideline and Expert Panel Report) (Kearon et al., 2016).

The question of whether these patients should be routinely anticoagulated is not addressed by the data presented here. Although we are convinced that perioperative prophylactic anticoagulation is essential, it is important to recognize that this is a delimited situation where the risks of DVT/PE are higher than baseline and the risks of treatment are lower than baseline. The latter is because individuals are less likely to fall or if they do, are proximate to expert medical care. In normal daily life, patients with these disorders may not have quite as high a risk of DVT/PE as they do perioperatively, but the risks of treatment are higher from common trauma (bicycle or workplace injuries) and they may not have access to immediate healthcare for a hemorrhage. We can only recommend that hematology consultation is undertaken and the risks and benefits of chronic anticoagulation are evaluated on an individualized basis. In summary, in the absence of risk factors for DVT or PE, such as vascular malformations and surgical procedures in individuals with Proteus syndrome and PROS, there is no data for use of prophylactic primary anticoagulation treatment to improve survival. We do not recommend prophylactic anticoagulation therapy with warfarin due to the inherent long-term risk of risk of bleeding, and the burden of frequent laboratory monitoring of INR levels. We have no experience with direct oral anticoagulants (DOACs) such as dabigatran, rivaroxaban, apixaban or edoxaban in patients with Proteus syndrome and PROS. As experience with long-term anticoagulation with DOACs in other prothrombotic conditions is gained, primary prophylaxis with these agents should be studied in Proteus syndrome and PROS patients with risk factors for VTE.

Further longitudinal data of larger cohorts are needed to advance our understanding of the use of markers of vascular endothelium in management of thrombosis for both Proteus syndrome and PROS. We propose establishing a registry to collect data on D-dimer screening and subsequent evaluations for all patients with Proteus syndrome or PROS. With these approaches, we are optimistic that the morbidity and mortality associated with thrombosis can be reduced in these disorders.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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