# Development of Molecular Therapies for Venous Malformations

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*Abstract:* Vascular anomalies are localized defects of morphogenesis that can affect lymphatic and blood vessels. They are generally called birthmarks, typically observed soon after birth and occurring in up to 10% of children. Based on their clinical and histological characteristics, they are classified into vascular tumours and vascular malformations. The most common malformations are venous malformations (VMs) resulting in chronic vascular diseases that can be associated with significant morbidity necessitating often demanding and repeating clinical management. The current treatment is based on surgical resection and sclerotherapy, which can be impossible due to the size or location of lesions or ineffective due to the regrowth of malformed vessels. Therefore, medical therapies for VMs are highly desired. Recent studies have identified genetic defects that result in the constantly active endothelial cell receptor tyrosine kinase TIE2 / phosphoinositide 3 -kinase PI3K signalling pathway as a frequent cause for VMs. The first treatment to inhibit this pathway with sirolimus indicated that molecular treatment can be effective against VMs. In addition, certain VM "hotspot" mutations have been previously found in tumours, providing the rationale for the exploration and repurposing of existing and investigational cancer drugs for VMs. Finally, discoveries of molecular and cellular abnormalities that characterize a large proportion of VMs and the generation of pre-clinical VM mouse models provide the

necessary basis for the development of the targeted molecular treatment strategies we discuss in this MiniReview.

Altered functions of the vasculature are involved in many common human diseases, such as growth of malignant tumours and metastasis, cardiovascular diseases, diabetes and retinopathies. Studies focusing on the principles of vascular development have revealed growth factor families and cellular signalling mechanisms that are critical for morphogenesis and homeostasis of blood and lymphatic vessels. These studies have identified the Ang/Tie system as a major endothelial cell (EC) signalling pathway consisting of angiopoietin ligands (Ang 1–4) and the Tie (Tie1 and Tie2) receptor tyrosine kinases [1,2]. Gene-targeting experiments have indicated that the Ang/Tie signalling system is needed for physiological and pathological remodelling of both lymphatic and blood vessels in embryonic, postnatal and adult mice [1]. In humans, an altered expression of angiopoietins is implicated in many vascular diseases [2], and loss-of-function mutations in genes encoding TIE2 and its activating ANG1 ligand have been shown to result in the defective growth of Schlemm's canal (a specific type of hybrid blood/lymphatic vessel for aqueous humour outflow in the eye), causing increased intraocular pressure and primary congenital glaucoma in children [3,4]. Clearly, identification of effective therapeutic means to modulate Ang/Tie signalling has a high clinical value, and drugs targeting this pathway are in clinical trials for cancer and neovascular eye diseases [2].

Venous malformations (VMs) are the most common vascular malformations, with an estimated incidence of 1–2 per 10,000 [5]. VMs can cause significant morbidity due to pain, disfigurement and organ dysfunction. Due to poor understanding of pathological mechanisms underlying the development of VMs, therapies have long been limited to compression therapy and ablation of malformed veins by sclerotherapy and surgery. Recent human genetic and molecular biology studies have made breakthroughs in unravelling the genetic, molecular and cellular alterations that characterize a large proportion of VMs; more than half of VMs are found to be positive for gain-of-function mutations in TIE2 [6–8]. In addition, activating mutations in PIK3CA – encoding the p110α catalytic subunit of the phosphoinositide 3-kinase PI3K – are found in about half of the TIE2 mutation-negative VMs [6,7,9,10]. These data have explained the genetic cause for most VMs and have identified the endothelial TIE2-PI3K axis as a major VM-causing signalling pathway that has been further investigated in cell culture and mouse models [6,9–13]. In this MiniReview, we summarize the recent findings on VM genetics and molecular pathology and discuss the advancements made in *in vitro* and VM pre-clinical models. These have yielded insightful data on the pathological

mechanism of VMs and have also led to the development of the first evidence-based molecular therapies which have already shown preliminary efficacy in pre-clinical and clinical studies [9,10,13,14].

**Clinical presentation and current treatment of venous malformations**

VMs are localized, congenital lesions of distorted slow flow venous-like vessels. They are predominantly located in the skin and mucosa, but can also be found in deeper structures such as muscles and internal organs [15]. Macroscopically, VMs appear as a bluish/purple, soft, compressible vascular mass [16]. In histology, VMs are characterized by venous-like vessels with enlarged, irregular lumens, flat EC lining, thick, poorly organized fibrotic extracellular matrix (ECM) and uneven smooth muscle cell (SMC) layering [6,17,18]. VMs are not considered to be proliferative lesions, but they do grow in proportion to the individual and never regress spontaneously [19]. Typically, the patient has a single lesion present at birth, but in a subset of patients, multiple separate lesions are observed [7,20]. Nevertheless, malignant transformation has not been reported. VMs are also observed as a part of vascular anomaly syndromes (e.g. Klippel-Trenaunay syndrome) and in combination with lymphatic and/or capillary malformations [21].

Symptoms of VMs vary depending on the anatomical location of the lesion. Common complaints are pain, swelling and disfigurement [16]. Diagnosis is usually made in early childhood, based on symptoms, clinical findings and imaging data [22]. Since the clinical presentation is highly variable, the treatment strategy should be carefully planned by an experienced multidisciplinary team on a case-by-case basis [23,24]. Sclerotherapy is usually the first-line treatment for symptomatic VM. A sclerosing agent such as ethanol is percutaneously injected into the lumen of the affected vessel in order to cause EC destruction and shrinkage of the lesion, also resulting in inflammation and subsequent fibrosis [25]. Usually, multiple treatment sessions are needed to achieve adequate symptom relief [26]. In the case of superficial VM with clear margins, surgical resection may be the primary treatment, but more often surgery is performed after sclerotherapy if symptoms persist [23]. As limitations, surgical resection is often impossible due to the size or location of lesions, and current treatments are only rarely curative since malformed veins can seldom be removed or destroyed completely and they tend to recanalize and recur [26]. Medical therapies are thus needed to improve the treatment of VM patients.

**Genetic cause of VMs and subtypes**

Genetic studies have been pivotal in elucidating the pathogenic mechanisms of VMs. More than two decades ago, cutaneomucosal venous malformation (VMCM), a rare autosomal dominantly inherited form of VM, was found to be associated with a TIE2 missense mutation (from arginine to tryptophan at residue 849 in the kinase domain) that increased TIE2 kinase activity indicating a gain-of-function effect [17]. The first observation was followed by the identification of somatic TIE2 mutations in more common sporadic VMs some ten years later [7]. It is noteworthy that while inherited mutations are found from blood DNA, somatic mutations are only detected in affected tissue with a relatively low allele frequency [6,7]. More than 20 different TIE2 mutations (Supplemental Table I) have been described in the literature occurring alone or as double mutations in cis (in the same allele) in exons 15, 17, 22 and 23 encoding intracellular kinase, kinase insert and C-terminal tail domains (Fig. 1). Collectively, the genetic data indicate that most TIE2 mutations are somatic and explain more than half of all VMs.

Recently, PIK3CA was identified as a second major gene mutated in VMs [6,9,10]. Physiological angiopoietin ligand-induced TIE2 activation triggers PI3K signalling, which in ECs is mainly mediated by PIK3CA [27]. *In vitro* studies have confirmed that mutations in these genes reside in a common signalling pathway [6] (Fig. 1). Based on genetic data from studies where both genes were extensively sequenced, TIE2 mutations account for 56% and PIK3CA 22% of all VM cases (Table I and Supplemental Table I). The remaining 23% TIE2/PIK3CA mutation negative VMs are likely caused by infrequent mutations in several different genes connected to PI3K and MAPK signalling pathways, as suggested by Castel *et al.* [9]. This study, using targeted exome sequencing of 341 cancer-associated genes, found solitary VM cases with mutations in GNAQ, NF1, MAP2K1, MAP3K1, AKT2, AKT3 and IRS2 alone or in combination with a TIE2 or PIK3CA mutation. It is noteworthy that in only one case, a mutation in both TIE2 and PIK3CA has been reported in combination [9].

Comprehensive clinical and genetic characterization has revealed distinct subtypes of VMs with differential clinical presentation and pattern of genetic mutations [6–8,17,20,28]. Among the subtypes, sporadic unifocal VM where the patient has a single VM lesion without a family history is by far the most common form caused by either TIE2 or PIK3CA mutations. In a large cohort of 130 unifocal VM patients, it was reported that 59 out of 80 (73.8%) TIE2 mutation positive patients harboured the c.2740C>T (L914F) mutation, the most frequent TIE2 mutation reported in the literature overall and found in ~28% of all VMs

[6] (Supplemental Table I). 25 out of 27 (92.6%) PIK3CA-positive VMs had mutations causing amino acid changes E542K, E545K or H1047R, which are also frequently found in cancer and other PIK3CA-associated malformation/overgrowth syndromes [29,30]. Although TIE2 and PIK3CA are in the same pathway, some gene-specific effects may be present in unifocal VMs. For example, PIK3CA mutation positive VMs were noted as more deeper lesions and not extending into the skin, in contrast to common TIE2 mutation positive VMs, TIE2 and PIK3CA mutation positive VM patients who had significantly higher D-dimer levels compared with patients having no identified mutations in these two genes, and certain mutations were found to be enriched in histological VM subtypes [6]. When expressed in cultured ECs, both TIE2 and PIK3CA mutations activate Akt, but only TIE2 mutations cause phosphorylation of Erk1/2 and Stat1, suggesting some differences in the activation of downstream signalling [6,12]. However, further studies are needed to reveal the significance of these observations.

Rare familial VMCM patients have multifocal variable-sized VMs predominantly in the skin and mucosa [17,28]. The most common inherited mutation in TIE2 is R849W (11 of 18 cases reported), with other mutations reported as a single case each [17,28,31,32] (Table II). Based on the current view, inherited TIE2 mutations are likely not able to cause VM lesions alone but need another genetic change. This is supported by the fact that somatic "second-hits" in TIE2 have been found from lesions of VMCM patients, as a loss-of-function deletion mutation *in trans* (deleting a possibly protecting WT allele) or as a second missense mutation *in cis* [7,20], likely potentiating the gain-of-function effect of relatively weaker inherited mutations when compared to mutations found alone in sporadic VMs [11,12]. On the other hand, TIE2-L914F, TIE2 double mutations or PIK3CA mutations have never been found as inherited, suggesting that they all are lethal in germline, as already shown for PIK3CA-H1049R using genetic mouse models [9,33].

Multifocal VMs can also occur sporadically, and they frequently associate with TIE2 double mutations. Soblet *et al.* described 23 patients with non-familial multifocal VMs [20]. In the study, 17 out of 23 patients were diagnosed with Blue Rubber Bleb Nevus syndrome (BRBN). BRBN patients usually had a single large dominant lesion present at birth, but in contrast to unifocal VMs, the number of lesions increased with age, resulting in multiple smaller VMs, preferentially in the palms and soles. Importantly, BRBN patients also had gastrointestinal lesions and frequently needed iron supplementation due to bleeding complications. In the same study, 6 out of 23 patients also had multiple VM lesions but lacked gastrointestinal VMs and did not present with dominant lesions or anatomical

preference [20]. These individuals were classified under a diagnosis of multifocal sporadic VM (MSVM). While both groups had TIE2 double mutations, they differed in terms of mutational spectrum and acquisition of the mutations. BRBN patients typically had somatic T1105N-T1106P double mutation, which was not detected in blood DNA. In contrast, MSVM patients most commonly had R915C combined with Y897C or L914F *in cis*. Interestingly, in 3 out of 6 patients, R951C was also detected from blood DNA with a low allele frequency, suggesting that these patients are a mosaic for R915C, and a second mutation would be a somatic second hit needed for lesion formation reminiscent of inherited VMCM [7]. In contrast to TIE2, PIK3CA mutations have not been reported in VMCM, BRBN or MSVM. With clinical and genetic data taken together, distinct presentations of VM diseases may exist with a differential mutation spectrum and acquisition sequence of mutations. Whether specific mutations enriched in distinct clinical and genetic entities can cause specific cellular or molecular effects that may explain different phenotype presentations has been explored to a limited extent [20].

**Molecular and cellular pathology of VMs**

The majority of VMs are due to gain-of-function somatic mutations in the genes encoding TIE2 or PIK3CA, resulting in chronic activation of the TIE2/PI3K/Akt signalling pathway (Fig. 1) [6,7,9,10]. In addition, both VM EC transplantation studies and use of EC-specific Cre drivers together with inducible VM mutations have indicated that TIE2 or PIK3CA mutation in an EC compartment is sufficient for VM lesion formation [9,10,12,13,33]. Disease mechanisms downstream from a mutated TIE2/PIK3CA are not completely understood. However, several molecular and cellular dysfunctions have been identified that may have a role in the initiation, growth and maintenance of VM lesions (Fig. 2). These may affect EC intrinsic functions, EC–SMC interactions, perivascular ECM remodelling and venous specification [10–12]. Not all *in vitro* alterations have been confirmed in VM tissues, and cellular abnormalities observed on plastic dishes or in 3D fibrin gels are not necessarily directly comparable to the VM lesions *in vivo*. For translational studies, however, identification of quantifiable *in vitro* phenotypes due to increased TIE2/PIK3CA signalling should provide feasible readouts for identification of novel molecular inhibitors able to normalize VM signalling. Common to all TIE2 mutations is an increase in ligand-independent TIE2 tyrosine phosphorylation (i.e. kinase activation). In cell culture models, the extent of TIE2 autophosphorylation is highly variable, however, and no correlation with severity of clinical phenotype can be made [7,12,20,28]. Another common feature is altered

trafficking and low plasma membrane translocation of TIE2 VM protein, which may hamper normal, ligand-regulated TIE2 functions and thus potentiate VM pathogenesis [7,12] (Fig. 2C).

In addition to Akt, studies focusing on signalling pathway(s) downstream from mutations have revealed increased Erk1/2 phosphorylation in cell lysates. The MAPK inhibitor (PD98059) restored major features of VM ECs [12], supporting the importance for MAPK signalling. Additional evidence for involvement of MAPK comes from genetic analysis of TIE2 and PIK3CA mutation negative VMs that have indicated some sporadic mutations in the MAPK pathway [9]. On the other hand, in cultured ECs PIK3CA mutations seem to mainly activate Akt and not MAPK but nevertheless lead to highly similar cellular phenotype compared with TIE2-VMs [6]. Currently, the exact importance of MAPK in VM pathogenesis and possible cooperation/crosstalk of the MAPK and PI3K signalling pathways in VMs is unclear.

Akt activation provides a strong cell survival signal that is also induced by ANG1-activated TIE2-WT [34]. Consistently, TIE2-VM mutations increase the viability of non-adhered ECs [20] and dominant-negative Akt inhibited pro-survival phenotype of TIE2-R849W in cultured ECs [18]. In addition, less apoptotic p53 staining was histologically observed in mutant ECs in VM lesions compared to TIE2-WT in a mouse transplantation model [12]. It has been proposed that an increase in Akt may support EC survival in SMC-deficient malformed veins [18], but the importance of increased resistance to apoptosis for VMs is not established.

Vascular anomalies are classified into two main groups based on cellular proliferation: highly proliferating vascular neoplasms including hemangiomas and malformations, in which EC proliferation is not considered pathological [21]. On the other hand, as VMs are characterized by abnormally enlarged vascular channels, it is reasonable to assume that increased EC proliferation has a role. However, in clinical VM biopsies, proliferation is not a prominent feature [35–38]. In addition, TIE2-L914F increases the number of human umbilical vein ECs (HUVECs) by extending their life span rather than proliferation *in vitro,* and in the mouse transplantation model no increase in EC proliferation was observed [11,12]. On the other hand, VEGF-A stimulated PIK3CA-VM transduced ECs proliferated more than PIK3CA-WT ECs [33] and *Pik3ca*[H1047R] increased EC proliferation but not the rate of apoptosis in genetic mouse models [9,10]. These discrepancies could be due to the different model systems and time points studied. It could be that VMs have an initial proliferative phase followed by slow growth in an established lesion where resistance

to apoptosis maintaining ECs would be more important than proliferation. As VMs are typically present at birth, a developmental stage when ECs are more sensitive to respond to proliferative signalling may exist, which may depend on the presence of additional growth factors. Cell migration is another EC function predicted to be influenced by TIE2 mutations based on microarray transcriptome screening of TIE2-L914F transduced HUVECs [11]. However, migration velocities are not increased based on an *in vitro* assay [20].

Histologically, VMs are characterized by defective SMC coverage around malformed veins. In lesions, SMCs are focally either absent or present as irregularly distributed clumps indicating that the EC-SMC interplay is impaired in VMs [6,17]. The forkhead box protein O1 (FOXO1) transcription factor is negatively regulated by activated Akt [39]. As a consequence, FOXO1 target genes, implicated in vascular morphogenesis and remodelling, are down-regulated in ECs expressing either TIE2 or PIK3CA mutations [6,10,11]. These include reduction in a potent vascular SMC attractant PDGF-B in VM mutant gene transduced HUVECs, retinas of *Pik3ca*[H1047R] mice, biopsies from TIE2 mutation positive VM lesions [10,11], and also serum samples from non-genotyped VMs [40]. Although not mechanistically yet proved, the decrease in PDGF-B could explain the defective SMC coverage characterizing the VM lesions. Another factor essential for SMC migration, differentiation and vascular stabilization is the transforming growth factor beta (TGF-β) [41]. Immunohistochemistry of VM tissue shows reduced expression of TGF-β mRNAs compared to normal human skin [42,43]. In another study, Xia *et al.* observed that levels of miR-145 – a microRNA regulator of SMC differentiation and motility – correlate with TGF-β expression and low levels of αSMA$^+$ cell coverage in VM tissue [44]. Interestingly, their levels were restored in human VM samples after sclerotherapy using bleomycin A5 and in HUVECs treated with bleomycin *in vitro* [42–44]. As TGF-β seemingly also composes a balancing partner for TIE2 activity [43,45] more thorough knowledge of its regulation and downstream signalling might offer a route to a better understanding of VM pathogenesis.

At the cellular level, over-expression of TIE2 or PIK3CA VM mutations in HUVECs causes perturbation of the organized EC monolayer typical for TIE-WT ECs [6,12]. Instead of a cuboidal, cobblestone-like phenotype, VM mutant cells are elongated and cross over each other with a fibroblast-like appearance. In TIE2-VM transduced ECs, this results from the loss of ECM fibronectin (FN), which can be rescued with Erk1/2 inhibition (PD98059) [12]. Loss of ECM FN and EC monolayer organization is common to all TIE2 and PIK3CA VM mutations analysed [6,12]. Interestingly, in PIK3CA-VM HUVECs, FN is lost without evident Erk1/2 activation [6]. On the other hand, in both TIE2-VM and PIK3CA-VM ECs,

the PIK3CA inhibitor (alpelisib) could restore the EC monolayer organization and ECM FN, while Akt inhibition (LY294002 or sirolimus) had no clear effect [6,12]. Although altered morphology of mutant ECs observed *in vitro* does not have a directly comparable counterpart *in vivo*, transmission electron microscopy (TEM) analysis of VM tissues reveals uneven and discontinued endothelium, EC elongations and disorganized alignment of cytoskeletal filaments [12,43]. Collectively, these observations suggest that altered ECM and cytoskeleton signalling may have a key role in VM pathogenesis and importantly serve as an easily observable parameter for EC normalization for *in vitro* studies. Abnormalities in EC–EC interactions may relate to reduced expression of adhesion proteins VE-cadherin and N-cadherin seen in VM tissue biopsies [43].

As a prominent and consistent change in VM biopsies from patients and mouse transplantations, TEM has revealed abnormalities in perivascular ECM. These include structural changes in the basement membranes (BMs) and fibrillar collagen matrix, suggesting continuous remodelling [12,43]. Interestingly, structurally aberrant ECM is accompanied by strong induction of pericellular proteolytic pathways *in vitro*: up-regulated gene expression of the ADAMTS family of peptidases and plasminogen activation system (increase in tissue and urokinase type plasminogen activators, tPA and uPA, decrease in plasminogen activator inhibitor PAI-1). These proteases can degrade many ECM and BM constituents and activate matrix metalloproteinases for ECM remodelling [6,11,12]. Finally, downstream from TGF-β, connective tissue growth factor 2 (CCN2), a matricellular protein regulating cell–ECM interplay, is down-regulated. Low TGF-β/CCN2 has shown to be associated with a reduction in ECM and BM components such as FN, collagen I and laminins α3 and α5 [42].

Taken together, SMC coverage and structural alterations in perivascular ECM may alter ECM to EC signalling and cell cytoskeleton, weaken the perivascular support in the vascular wall, and contribute to lesion formation. The exact roles of increased EC's resistance to apoptosis and proliferation are currently not clear. Since VMs are usually present congenitally, it could be that VMs are preferentially induced during a state in vascular development where the vessel structure and constituent cells are permissive to venous remodelling. On the other hand, in some cases, this hypothesis does not hold. For example, in BRBN, new lesions seem to develop in adulthood and VM-like lesions can also be induced postnatally in a genetic mouse model [20,33].

## VM biomarkers

The recent characterization of TIE2 and PIK3CA mutation positive *in vitro* EC models, VM mice and patients have increased the knowledge about VM pathophysiology and unravelled altered molecules that can potentially be used as biomarkers for VMs [6,9–13]. A gene tests for TIE2 and PIK3CA mutations is the most definite biomarker for VMs. The recurrent mutations cover a significant proportion (about 80%) of all VMs; however, sequencing currently requires a tissue biopsy for mutation detection. Whether the mutational status is clinically useful for predicting the response to a specific treatment modality remains to be studied. Of note, the gain-of-function mutation in TIE2 is thus far the only VM-specific molecular abnormality, while PIK3CA substitutions are also detected in cancers and over-growth syndromes [29,30,46].

In a blood coagulation reaction, thrombin converts fibrinogen to fibrin that is cleaved by plasmin in fibrinolysis, resulting in the formation of D-dimers as a fibrin degradation product. Unlike other vascular malformations, VMs are associated with localized intravascular coagulopathy, and patients often have elevated D-dimers and in severe cases low fibrinogen serum levels [6,13,40,47–51]. D-dimer testing has shown to be useful to separate VMs from other vascular or lymphatic malformations which usually present with normal D-dimers [48,50]. Interestingly, VM patients with identified TIE2 or PIK3CA mutations had high D-dimers when compared to patients with no detectable mutation in these genes [6]. The utilization of D-dimers and fibrinogen as serum biomarkers for VMs is further strengthened by the observation of strongly up-regulated plasminogen/plasmin proteolytic activity in TIE2 and PIK3CA VM mutation transduced HUVECs [6,12]. Interestingly, these *in vitro* studies suggest that a high serum level of D-dimers is not solely due to static blood flow in the lesions, but also to an intrinsic signalling defect in ECs due to constantly high TIE2/PIK3CA activity.

Another potential biomarker that can be measured from the blood or serum is PDGF-B, which is consistently down-regulated in different models and clinical samples, including VM mutant transduced ECs, genetic VM mouse model, tissue biopsies and serum from VM patients [10,11,40]. It has also been recently shown that the peripheral blood of VM patients contains more cell-derived microparticles than similar samples of healthy controls [52]. The number of microparticles is higher if the sample is taken directly from the lesional fluid and correlates with the size of the lesion. In addition, the mRNA and microRNA content of these microparticles differs significantly from that of the healthy controls. The exact function or importance of microparticles is unknown, but they could represent the parent cell they are

budded from and therefore provide a molecular readout of VM ECs [52]. ANG2, a context-dependent antagonistic ligand of TIE2 induced in normal vascular remodelling, is another Akt/FOXO1-dependent gene strongly down-regulated in VM HUVECs [6,11]. In addition, other biomarkers whose use may be limited to *in vitro* tests include decreased ECM FN and activation status of Akt and Erk1/2 [12]. Further studies are needed to evaluate the utility of potential VM biomarkers, if and how they could be potentially used in experimental and clinical settings to follow responses to treatment. As many observed changes are not specific to VMs, their use in diagnosis is likely limited.

**Preclinical development of molecular treatment for VMs in mouse models**

Both genetic and EC transplantation based mouse models for VMs have been developed [9,10,12,13,33]. Models have proven useful for the study of VM pathogenesis and molecular therapies (Fig. 3). In the xenotransplantation model, HUVECs retrovirally transduced to over-express mutated TIE2 forms were injected subcutaneously into immunocompromised mice, either as a suspension in matrigel or as EC spheroids in a matrigel/fibrin matrix [12,13]. In the transplanted plugs, ECs form tubular structures that can connect to the host mouse vasculature to form VMs. VMs observed in the transplantation model are abnormally enlarged, slow flow, blood-filled channels with a continuous EC layer, patchy pericyte/SMC coverage and aberrant ECM, closely resembling the histopathology and defective blood flow of human VMs [12,13]. The effects of the mechanistic target of rapamycin (mTOR) inhibitor sirolimus (also known as rapamycin) and TIE2 tyrosine kinase inhibitor (TIE2-TKI) were tested in the transplantation model using TIE2-L914F expressing HUVECs [13]. *In vitro* sirolimus inhibited TIE2-L914F induced Akt phosphorylation in HUVECs, and when injected intraperitoneally, sirolimus was effective in reducing VM formation and stopping the growth of established lesions, but was not able to diminish established VMs. Surprisingly, TIE2-TKI was far less potent in treating VMs in this model, which is likely explained by the weak inhibition of TIE2-L914F by this inhibitor. In addition to the human EC based transplantation model, PIK3CA mutations have been tested using allografting of vascular lesions isolated from *Pik3ca*[H1047R]-CAG-CreER mice [9]. In this model, another mTOR inhibitor everolimus and PIK3CA inhibitor alpelisib (BYL719) were able to prevent VM growth with marked response observed with alpelisib and partial reduction with everolimus. The importance of high Akt signalling for vascular malformations has also been previously noticed when immortalized murine ECs overexpressing constitutively active myristoylated Akt formed VM-like lesions when transplanted into nude mice [53].

Genetic mouse models [9,10,33] for VMs are based on inducible *Pik3ca* gain-of-function mutation H1047R (either as allelic knock-in or in Rosa26 locus). Of note, H1047R is frequently found in VMs, but also in lymphatic malformations [29] and overgrowth syndromes [46]. Castillo *et al*. used T promoter driven CreER and a suboptimal dose of 4-hydroxytamoxifen to induce a mosaic expression of *Pik3ca*[H1047R] in embryonic mesoderm (*MosMes-Pik3ca*[H1047R]), which gives rise to an EC lineage [10]. This resulted in cutaneous and internal VMs, without apparent defects in other tissues. *Pik3ca*[H1047R] was further analysed in a postnatal retina model of angiogenesis using inducible, EC-specific Pdgfb-Cre [10]. This resulted in abnormally enlarged vascular structures, EC hyperplasia, impaired pericyte coverage and low expression of arteriovenous specification marker genes, but not increased angiogenic sprouting. The other genetic models were also based on inducible PIK3CA-H1047R mutation, but using a Rosa26 locus targeted allele [9,33]. In this model, expression of mutant *Pik3ca*[H1047R] was induced either in endometrial epithelial cells and in some ECs in spine vasculature (by crossing with Sprr2f-Cre driver mouse line) or ubiquitously in 6–8-week-old mice (CAG-CreER) and in developing vasculature (Tie2-Cre) [9]. Di Blasio *et al*. similarly induced expression of *Pik3ca*[H1047R] using Tie2-Cre or in posterior leg vasculature using EC-specific inducible Cre (Cdh5-CreERT2) and local injection of 4-hydroxytamoxifen [33]. These genetic models indicated that, depending on the extent of expression and age of induction, *Pik3ca*[H1047R] results in vascular dysmorphogenesis and formation of VMs that well phenocopied characteristics of VMs observed in patients [9,10,33].

Collectively, both genetic and transplantation-based models offer versatile tools to study the pathology of VMs, as well as the efficacy and safety of potential molecular therapies. Transplantation models allow analysis of localized lesions, which mimic somatic VMs in patients, are flexible for additional genetic modifications and feasible for screening of different VM mutations. The disadvantages of HUVEC-based models are that they use immunocompromised mice and retrovirally transduced ECs that overexpress mutant proteins and are anatomically artificial compared to genetic mouse models where lesions are present in native tissues.

**Pharmacotherapy of venous malformations**

Like many other rare diseases, investments needed for development of drugs designed against VMs are higher than the possible profit from the markets, and therefore it is unlikely that VMs will attract sufficient interest from pharmaceutical companies. Pathways up-regulated in

VMs, especially PI3K/Akt and MAPK, are extensively studied major culprits in cancer and many experimental and clinically approved drugs targeting these pathways exist [54,55], thus making drug repurposing a logical strategy to develop pharmacotherapy for VMs (Table III). Direct targeting of the chronically activated TIE2 or PIK3CA kinases using specific inhibitors to stop the pathological signalling should yield the best response. Unfortunately, drug compounds specifically inhibiting TIE2 or PIK3CA have not yet reached the clinic in any indication [2,56].

TIE2 inhibition has been tested in a mouse transplantation model of VM, but likely due to poor inhibition efficiency of the TIE2-TKI used, the results were only modest [13]. In postnatal and adult mice, TIE2 seems to have only a limited role in overall vascular homeostasis, since conditional knockout after birth leads to defects that seem to be mainly limited to Schlemm's canal of the eye, lymphatic capillaries of the corneal limbus [57] and postnatal mouse retina [58]. Inhibition of the mutated TIE2 in VMs could thus be possible without disrupting normal vascular functions. TIE2 inhibitors currently in preclinical and clinical trials are multi-kinase inhibitors targeting additional kinases such as PDGFRβ, VEGFR and MAPKs [2]. Even though the inhibition of multiple targets in general would appear undesirable, the targeting of TIE2 and MAPKs signalling in combination could be beneficial in VMs, as Erk1/2 mediated signalling causes part of the TIE2 mutation induced cellular dysfunctions in *in vitro* studies [12]. Perhaps the most significant limitation of targeting TIE2 is that it would likely be of no use in VMs caused by mutations in PIK3CA or other genes downstream of TIE2. In addition, TIE2 inhibition cannot be generalized to other vascular anomalies since TIE2 mutations have thus far only been found in VMs.

As TIE2 and its downstream target PIK3CA activate the same major pathway to cause VMs and mutations in these two genes to account for three quarters of all VM cases, targeting PIK3CA should cover for the majority of patients [6] (Table I). One potent drug for repurposing is alpelisib, a highly specific PIK3CA inhibitor currently in phase I–III clinical trials for treatment of various types of cancer as combination therapy [56,59]. Alpelisib was able to efficiently inhibit Akt phosphorylation and normalize the phenotype of both TIE2-VM and PIK3CA-VM transduced HUVECs *in vitro* [6] and drastically diminish VM lesion growth in mice [9]. Importantly, alpelisib was also efficient in treating cutaneous VMs in mouse allotransplantation model when administered topically as a cream, a method which could be useful in limiting adverse effects of systemic PI3K-inhibition [9]. Alpelisib has not yet been tested clinically in VM, but a case report describes remarkably beneficial effects in

two patients with rare, life-threatening overgrowth syndrome (CLOVES) caused by somatic PIK3CA mutations and affecting multiple tissue types including vasculature [60].

By far the most advanced pharmacotherapy for VMs currently is sirolimus [13,14]. Sirolimus is an mTOR inhibitor indicated for the prevention of organ rejection in patients older than 13 receiving a renal transplant [61]. The exact mechanism of action of sirolimus in VMs is not completely understood. The main target of sirolimus is mTOR complex 1 (mTORC1), which is a multiprotein signalling hub integrating signalling inputs from various sources such as growth factors, nutritional status and energy metabolism state [62]. However, on chronic exposure, sirolimus can also inhibit mTORC2, which is known to directly phosphorylate Ser473 residue of Akt, needed for its full activation [62,63]. Sirolimus inhibited TIE2-VM and PIK3CA-VM induced Akt activation in transduced ECs *in vitro* and *in vivo* [6,13], and in mouse models it diminished lesion growth, normalized SMC coverage and decreased EC proliferation [10,13]. It is noteworthy that compared with alpelisib, sirolimus was not able to normalize all TIE2-VM and PIK3CA-VM EC phenotypic features in retrovirally transduced ECs, suggesting that not all pathogenic signalling pathways are blocked by sirolimus in VMs [6].

Encouraged by the accumulated knowledge of pathogenic mechanisms of vascular malformations as outlined above, success in other PI3K/Akt/mTOR-signalling related diseases [64,65] and positive experiences published as case reports [66], large-scale prospective clinical trials testing sirolimus in the treatment of vascular malformations have been launched (Supplemental Table II). In the largest trial published so far, Adams *et al*. enrolled 61 patients with heterogeneous complicated vascular malformations not adequately manageable with standard care and treated them with twice daily oral sirolimus [14]. More than 80% of the patients who completed 6 or 12 cycles of continuous therapy (28 days per cycle) had a partial response defined by an improvement in radiological evaluation, quality of life and functional assessment. None of the patients had a complete response. Six patients who chose to pause sirolimus experienced a worsening of symptoms, and most patients continued sirolimus after the study. Similarly, in a prospective pilot cohort of six TIE2 and PIK3CA mutation positive vascular malformation patients, improvement in quality of life, diminishment in pain, bleeding and oozing and a decrease in lesion volumes were reported [6,13]. Although the data are limited, Adams *et al*. suggested that sirolimus might be more effective if started in early childhood [14]. This idea is supported by preclinical findings in mice where sirolimus was efficient in preventing growth of new lesions but less potent to diminish them once established [13]. It is noteworthy that in two other mTOR-related

diseases – lymphangioleiomyomatosis and tuberous sclerosis – sirolimus was also able to halt the disease progression, but after ending the treatment, the disease progressed similarly to the untreated group [64,65]. Two additional large clinical trials enrolling patients with extensive vascular malformations are ongoing (Supplemental Table II) [67,68].

Based on the published clinical data thus far, it seems evident that sirolimus can significantly ameliorate symptoms and diminish lesion volumes in patients with complicated vascular anomalies. Although reports on long-term efficacy are still lacking, sirolimus is unlikely to be completely curative in most patients [13,14,66,69]. One intriguing yet unexplored possibility to boost treatment results could be the combination of sirolimus with MAPK inhibition. Sirolimus was not able to normalize all VM cellular phenotypes *in vitro* and in fact increased Erk1/2 phosphorylation, which could potentiate some of the cellular abnormalities [6,12]. mTORC1 inhibition with sirolimus has been shown to induce negative feedback regulation leading to increased activation of PI3K, Akt, S6K1 and Erk pathways, likely limiting its efficacy in cancer and perhaps also in vascular anomalies [70]. Clinically approved Mek inhibitors (trametinib, cobimetinib) exist, which could be considered for repurposing to vascular anomalies as a combination therapy with sirolimus if first shown beneficial in pre-clinical models [71].

Long-term or even lifelong medical treatment might be needed for VMs, meaning that the therapy must be well-tolerated. It should also be applicable to paediatric patients. Targeting either PIK3CA or mTOR is expected to cause similar adverse effects (e.g. hyperglycaemia, hyperlipidaemia) since they predominantly inhibit the same PI3K/Akt/mTOR pathway [70]. Due to immunosuppression, sirolimus also increases susceptibility to infections and risk of malignancies [61]. In vascular anomaly patients, sirolimus has been generally relatively well-tolerated from adults to infants, even neonates [13,14,66,69,72,73] (Supplemental Table II). Most commonly observed adverse effects have been bone marrow toxicity, hypertriglyceridaemia and infections. Most of the reported adverse effects have been mild and manageable with dose reduction. But occasionally persistent adverse effects, such as chronic mucositis, have led to cessation of treatment. In general, adverse effects seem as expected from previous sirolimus studies in its primary indication, and no new safety concerns have been raised. In contrast to sirolimus, only limited safety data exist on alpelisib, which has been used only as an investigational cancer drug in a limited number of adult patients [74–76]. A frequent adverse effect observed has been hyperglycaemia but usually as mild and requiring no action. Other common adverse effects include nausea, diarrhoea and skin reactions. In general, the safety profile has been deemed

acceptable to continue further trials [75,76]. In general, PI3K inhibitors have been found to cause significant adverse effects such as liver toxicity, rash, stomatitis and gastrointestinal symptoms, which have led to the termination of the development of many drug compounds of this class and especially the ones targeting multiple PI3K isoforms unselectively [56,77]. Larger clinical trials are needed to better determine the safety profile of alpelisib. The crucial open question is whether paediatric patients tolerate it. It is possible that in VM patients, a lower dosage than in cancer could be used, since VMs are caused by the specific activation of the TIE2/PIK3CA pathway, in contrast to cancer where multiple oncogenic pathways are simultaneously activated. For superficial VMs, topical administration of the drugs might be an option for limiting systemic adverse effects [9]. Ideally, medical treatment would lead to sustained response without rebound growth, which would allow short-term or intermittent medication with limited adverse effects. Whether this can be achieved with PIK3CA inhibition remains to be tested.

**Conclusion and future directions**

Recent molecular, cellular and genetic studies have unravelled the cause of most VMs resulting from activating mutations in the TIE2/PI3K signalling pathway, and the first clinical studies indicate that molecular therapy of VMs can be effective. Although many candidate genes and proteins are found to be dysregulated, the exact pathogenic mechanisms downstream from mutated proteins are not clear however, and the first molecular treatment in the clinic using sirolimus has limitations. The genetic model for TIE2 gain-of-function mutation induced VMs has not been reported, but it is plausible that similarly to mosaic expression of $Pik3ca^{H1047R}$, targeted TIE2 mutations would lead to VM formation in mice. This would be a valuable model to test possible differences in pathology and treatment response in PIK3CA- and TIE2-positive VMs, which may not be identical. Since alpelisib but not sirolimus was able to completely restore phenotypes of mutant TIE2 and PIK3CA transfected HUVECs [6], it would be intriguing to directly compare the effects of these two compounds in an *in vivo* model of TIE2 and PIK3CA mutation positive VMs. As VMs are due to somatic gain-of-function mutations in one allele, they are suitable candidates for gene therapy. The approach could be based on shRNAs for the silencing of expression of the mutated allele or silencing/correction of the mutated gene using genome editing tools. Development of such approaches for VM therapies, however, have not yet been published. The management of difficult-to-treat VMs has already been revolutionized by sirolimus. However, long-term sirolimus treatment may cause significant side effects, and in clinical

studies thus far it does not always reduce the volume of existing VM lesions. Mechanistic molecular data supports the idea that PIK3CA inhibition would lead to a significantly better response if a suitable and safe molecule is found. Furthermore, in cultured HUVECs, sirolimus increases Erk1/2 activation, which potentiates cellular abnormalities in VM ECs [6,12], thus making further search for more specific inhibitors attractive and necessary for the development of VM‑targeted molecular therapies. Currently, it seems unrealistic to expect that even most potent kinase inhibitors would lead to complete recovery of malformed tissue. However, they may prove to be important to stabilize difficult‑to‑treat VMs, and if combined with sclerotherapy or surgery they may inhibit regrowth of malformed vasculature.

**Conflict of interest**

The authors have no conflicts of interest to disclose.

**References**

1       Eklund L, Kangas J, Saharinen P. Angiopoietin-Tie signalling in the cardiovascular and lymphatic systems. Clin Sci (Lond) 2017;131:87–103.

2       Saharinen P, Eklund L, Alitalo K. Therapeutic targeting of the angiopoietin-TIE pathway. Nat Rev Drug Discov 2017;16:635–61.

3       Souma T, Tompson SW, Thomson BR, Siggs OM, Kizhatil K, Yamaguchi S, et al. Angiopoietin receptor TEK mutations underlie primary congenital glaucoma with variable expressivity. J Clin Invest 2016;126:2575–87.

4       Thomson BR, Souma T, Tompson SW, Onay T, Kizhatil K, Siggs OM, et al. Angiopoietin-1 is required for Schlemm's canal development in mice and humans. J Clin Invest 2017;127:4421–36.

5       Uebelhoer M, Boon LM, Vikkula M. Vascular Anomalies: From Genetics toward Models for Therapeutic Trials. Cold Spring Harb Perspect Med 2012;2:a009688– a009688.

6       Limaye N, Kangas J, Mendola A, Godfraind C, Schlögel MJ, Helaers R, et al. Somatic Activating PIK3CA Mutations Cause Venous Malformation. Am J Hum Genet 2015;97:914–21.

7       Limaye N, Wouters V, Uebelhoer M, Tuominen M, Wirkkala R, Mulliken JB, et al. Somatic mutations in angiopoietin receptor gene TEK cause solitary and multiple sporadic venous malformations. Nat Genet 2009;41:118–24.

8       Soblet J, Limaye N, Uebelhoer M, Boon LM, Vikkula M. Variable Somatic TIE2 Mutations in Half of Sporadic Venous Malformations. Mol Syndromol 2013;4:179–83.

9       Castel P, Carmona FJ, Grego-Bessa J, Berger MF, Viale A, Anderson K V., et al. Somatic PIK3CA mutations as a driver of sporadic venous malformations. Sci Transl Med 2016;8:332ra42-332ra42.

10      Castillo SD, Tzouanacou E, Zaw-Thin M, Berenjeno IM, Parker VER, Chivite I, et al. Somatic activating mutations in Pik3ca cause sporadic venous malformations in mice and humans. Sci Transl Med 2016;8:332ra43.

11      Uebelhoer M, Nätynki M, Kangas J, Mendola A, Nguyen H-L, Soblet J, et al. Venous malformation-causative TIE2 mutations mediate an AKT-dependent decrease in PDGFB. Hum Mol Genet 2013;22:3438–48.

12      Nätynki M, Kangas J, Miinalainen I, Sormunen R, Pietilä R, Soblet J, et al. Common and specific effects of TIE2 mutations causing venous malformations. Hum Mol Genet 2015;24:6374–89.

13      Boscolo E, Limaye N, Huang L, Kang K-T, Soblet J, Uebelhoer M, et al. Rapamycin improves TIE2-mutated venous malformation in murine model and human subjects. J Clin Invest 2015;125:3491–504.

14      Adams DM, Trenor CC, Hammill AM, Vinks AA, Patel MN, Chaudry G, et al. Efficacy and Safety of Sirolimus in the Treatment of Complicated Vascular Anomalies. Pediatrics 2016;137:e20153257.

15      Mulliken JB, Burrows PE, Fishman SJ. Mulliken and Young's Vascular Anomalies Hemangiomas and Malformations. 2nd ed. Oxford University Press; 2013.

16      Vogel SA, Hess CP, Dowd CF, Hoffman WY, Kane AJ, Rajaii R, et al. Early versus later presentations of venous malformations: Where and why? Pediatr Dermatol 2013;30:534–40.

17  Vikkula M, Boon LM, Carraway KL, Calvert JT, Diamonti a. J, Goumnerov B, et al. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. Cell 1996;87:1181–90.

18  Morris PN, Dunmore BJ, Tadros A, Marchuk DA, Darland DC, D'Amore PA, et al. Functional analysis of a mutant form of the receptor tyrosine kinase Tie2 causing venous malformations. J Mol Med (Berl) 2005;83:58–63.

19  Boon LM, Ballieux F, Vikkula M. Pathogenesis of vascular anomalies. Clin Plast Surg 2011;38:7–19.

20  Soblet J, Kangas J, Nätynki M, Mendola A, Helaers R, Uebelhoer M, et al. Blue Rubber Bleb Nevus (BRBN) Syndrome Is Caused by Somatic TEK (TIE2) Mutations. J Invest Dermatol 2017;137:207–16.

21  Wassef M, Blei F, Adams D, Alomari A, Baselga E, Berenstein A, et al. Vascular Anomalies Classification: Recommendations From the International Society for the Study of Vascular Anomalies. Pediatrics 2015;136:e203-14.

22  Behravesh S, Yakes W, Gupta N, Naidu S, Chong BW, Khademhosseini A, et al. Venous malformations: clinical diagnosis and treatment. Cardiovasc Diagn Ther 2016;6:557–69.

23  Dompmartin A, Vikkula M, Boon LM. Venous malformation: update on aetiopathogenesis, diagnosis and management. Phlebology 2010;25:224–35.

24  Mattila KA, Kervinen K, Kalajoki-Helmiö T, Lappalainen K, Vuola P, Lohi J, et al. An interdisciplinary specialist team leads to improved diagnostics and treatment for paediatric patients with vascular anomalies. Acta Paediatr 2015;104:1109–16.

25  Aronniemi J, Castrén E, Lappalainen K, Vuola P, Salminen P, Pitkäranta A, et al. Sclerotherapy complications of peripheral venous malformations. Phlebology 2016;31:712–22.

26  Ali S, Mitchell S. Outcomes of Venous Malformation Sclerotherapy: A Review of Study Methodology and Long-Term Results. Semin Intervent Radiol 2017;34:288–93.

27  Graupera M, Guillermet-Guibert J, Foukas LC, Phng L-K, Cain RJ, Salpekar A, et al.

Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. Nature 2008;453:662–6.

28 Wouters V, Limaye N, Uebelhoer M, Irrthum A, Boon LM, Mulliken JB, et al. Hereditary cutaneomucosal venous malformations are caused by TIE2 mutations with widely variable hyper-phosphorylating effects. Eur J Hum Genet 2010;18:414–20.

29 Luks VL, Kamitaki N, Vivero MP, Uller W, Rab R, Bovée JVMG, et al. Lymphatic and Other Vascular Malformative/Overgrowth Disorders Are Caused by Somatic Mutations in PIK3CA. J Pediatr 2015;166:1048–1054.e5.

30 Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004;304:554.

31 Calvert JT, Riney TJ, Kontos CD, Cha EH, Prieto VG, Shea CR, et al. Allelic and locus heterogeneity in inherited venous malformations. Hum Mol Genet 1999;8:1279–89.

32 Shu W, Lin Y, Hua R, Luo Y, He N, Fang L, et al. Cutaneomucosal venous malformations are linked to the TIE2 mutation in a large Chinese family. Exp Dermatol 2012;21:456–7.

33 di Blasio L, Puliafito A, Gagliardi PA, Comunanza V, Somale D, Chiaverina G, et al. PI3K/mTOR inhibition promotes the regression of experimental vascular malformations driven by PIK3CA-activating mutations. Cell Death Dis 2018;9:45.

34 Kim I, Kim HG, So JN, Kim JH, Kwak HJ, Koh GY. Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway. Circ Res 2000;86:24–9.

35 Rössler J, Haubold M, Gilsbach R, Jüttner E, Schmitt D, Niemeyer CM, et al. β1-Adrenoceptor mRNA level reveals distinctions between infantile hemangioma and vascular malformations. Pediatr Res 2013;73:409–13.

36 Kräling BM, Razon MJ, Boon LM, Zurakowski D, Seachord C, Darveau RP, et al. E-selectin is present in proliferating endothelial cells in human hemangiomas. Am J Pathol 1996;148:1181–91.

37    Takahashi K, Mulliken JB, Kozakewich HPW, Rogers RA, Folkman J, Ezekowitz RAB. Cellular markers that distinguish the phases of hemangioma during infancy and childhood. J Clin Invest 1994;93:2357–64.

38    Meijer-Jorna LB, Van Der Loos CM, De Boer OJ, Van Der Horst CMAM, Van Der Wal AC. Microvascular proliferation in congenital vascular malformations of skin and soft tissue. J Clin Pathol 2007;60:798–803.

39    Daly C. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes Dev 2004;18:1060–71.

40    Redondo P, Aguado L, Marquina M, Paramo JA, Sierra A, Sánchez-Ibarrola A, et al. Angiogenic and prothrombotic markers in extensive slow-flow vascular malformations: implications for antiangiogenic/antithrombotic strategies. Br J Dermatol 2010;162:350–6.

41    Gaengel K, Genové G, Armulik A, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler Thromb Vasc Biol 2009;29:630–8.

42    Ren J-G, Chen G, Zhu J-Y, Zhang W, Sun Y-F, Jia J, et al. Downregulation of the transforming growth factor-β/connective tissue growth factor 2 signalling pathway in venous malformations: its target potential for sclerotherapy. Br J Dermatol 2014;171:242–51.

43    Chen G, Ren J-G, Zhang W, Sun Y-F, Wang F-Q, Li R-F, et al. Disorganized vascular structures in sporadic venous malformations: a possible correlation with balancing effect between Tie2 and TGF-β. Sci Rep 2014;4:5457.

44    Xia H-F, Ren J-G, Zhu J-Y, Yu Z-L, Zhang W, Sun Y-F, et al. Downregulation of miR-145 in venous malformations: Its association with disorganized vessels and sclerotherapy. Eur J Pharm Sci 2017;100:126–31.

45    Jeansson M, Gawlik A, Anderson G, Li C, Kerjaschki D, Henkelman M, et al. Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. J Clin Invest 2011;121:2278–89.

46    Kang H-C, Baek ST, Song S, Gleeson JG. Clinical and Genetic Aspects of the Segmental Overgrowth Spectrum Due to Somatic Mutations in PIK3CA. J Pediatr 2015;167:957–62.

47    Dompmartin A, Acher A, Thibon P, Tourbach S, Hermans C, Deneys V, et al. Association of localized intravascular coagulopathy with venous malformations. Arch Dermatol 2008;144:873–7.

48    Dompmartin A, Ballieux F, Thibon P, Lequerrec A, Hermans C, Clapuyt P, et al. Elevated D-dimer level in the differential diagnosis of venous malformations. Arch Dermatol 2009;145:1239–44.

49    Mazoyer E, Enjolras O, Laurian C, Houdart E, Drouet L. Coagulation abnormalities associated with extensive venous malformations of the limbs: differentiation from Kasabach-Merritt syndrome. Clin Lab Haematol 2002;24:243–51.

50    Mazoyer E, Enjolras O, Bisdorff A, Perdu J, Wassef M, Drouet L. Coagulation disorders in patients with venous malformation of the limbs and trunk: a case series of 118 patients. Arch Dermatol 2008;144:861–7.

51    Leung YC-L, Leung MW-Y, Yam S-D, Hung JW-S, Liu CS-W, Chung L-Y, et al. D-dimer level correlation with treatment response in children with venous malformations. J Pediatr Surg 2017;53:737–41.

52    Zhu J-Y, Ren J-G, Zhang W, Wang F-Q, Cai Y, Zhao J-H, et al. Characterization of microparticles in patients with venous malformations of the head and neck. Oral Dis 2017;23:110–9.

53    Perry B, Banyard J, McLaughlin ER, Watnick R, Sohn A, Brindley DN, et al. AKT1 overexpression in endothelial cells leads to the development of cutaneous vascular malformations in vivo. Arch Dermatol 2007;143:504–6.

54    Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. Nat Rev Clin Oncol 2013;10:143–53.

55    Asati V, Mahapatra DK, Bharti SK. PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological

perspectives. Eur J Med Chem 2016;109:314–41.

56    Rodon J, Tabernero J. Improving the Armamentarium of PI3K Inhibitors with
      Isoform-Selective Agents: A New Light in the Darkness. Cancer Discov 2017;7:666–
      9.

57    Thomson BR, Heinen S, Jeansson M, Ghosh AK, Fatima A, Sung H-K, et al. A
      lymphatic defect causes ocular hypertension and glaucoma in mice. J Clin Invest
      2014;124:4320–4.

58    Chu M, Li T, Shen B, Cao X, Zhong H, Zhang L, et al. Angiopoietin receptor Tie2 is
      required for vein specification and maintenance via regulating COUP-TFII. Elife
      2016;5:1–16.

59    Furet P, Guagnano V, Fairhurst R a., Imbach-Weese P, Bruce I, Knapp M, et al.
      Discovery of NVP-BYL719 a potent and selective phosphatidylinositol-3 kinase alpha
      inhibitor selected for clinical evaluation. Bioorganic Med Chem Lett 2013;23:3741–8.

60    International Patent Application WO/2017/140828, BYL719 (Alpelisib) for Use in the
      Treatment of PIK3CA-Related Overgrowth Spectrum (PROS—CLOVES Syndrome),
      Inventor: Canaud G; applicants: INSERM, France; CNRS, France; Universite, Paris
      Descartes, France; published: August 24, 2017.

61    Pfizer [Internet]. Rapamune package insert 2017.
      http://labeling.pfizer.com/showlabeling.aspx?id=139 (accessed December 25, 2017).

62    Li J, Kim SG, Blenis J. Rapamycin: One drug, many effects. Cell Metab 2014;19:373–
      9.

63    Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell
      2012;149:274–93.

64    Bissler JJ, McCormack FX, Young LR, Elwing JM, Chuck G, Leonard JM, et al.
      Sirolimus for Angiomyolipoma in Tuberous Sclerosis Complex or
      Lymphangioleiomyomatosis. N Engl J Med 2008;358:140–51.

65    McCormack FX, Inoue Y, Moss J, Singer LG, Strange C, Nakata K, et al. Efficacy and
      safety of sirolimus in lymphangioleiomyomatosis. N Engl J Med 2011;364:1595–606.

66   Nadal M, Giraudeau B, Tavernier E, Jonville-Bera A-P, Lorette G, Maruani A. Efficacy and Safety of Mammalian Target of Rapamycin Inhibitors in Vascular Anomalies: A Systematic Review. Acta Derm Venereol 2016;96:448–52.

67   ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000 Feb 29 -. suPERficial Slow-flow Vascular malFORMations Treated With sirolimUS. Identifier NCT02509468 2015. https://clinicaltrials.gov/ct2/show/NCT02509468 (accessed December 27, 2017).

68   ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000 Feb 29 -. Efficacy and Safety of Sirolimus in Vascular Anomalies That Are Refractory to Standard Care. Identifier NCT02638389, 2015. https://clinicaltrials.gov/ct2/show/NCT02638389 (accessed December 27, 2017).

69   Triana P, Dore M, Cerezo V, Cervantes M, Sánchez A, Ferrero M, et al. Sirolimus in the Treatment of Vascular Anomalies. Eur J Pediatr Surg 2016;27:86–90.

70   Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT. The PI3K Pathway in Human Disease. Cell 2017;170:605–35.

71   Tran KA, Cheng MY, Mitra A, Ogawa H, Shi VY, Olney LP, et al. MEK inhibitors and their potential in the treatment of advanced melanoma: the advantages of combination therapy. Drug Des Devel Ther 2016;10:43–52.

72   Czechowicz JA, Long-Boyle JR, Rosbe KW, Mathes E, Frieden IJ, Shimano KA. Sirolimus for management of complex vascular anomalies – A proposed dosing regimen for very young infants. Int J Pediatr Otorhinolaryngol 2018;105:48–51.

73   Lackner H, Karastaneva A, Schwinger W, Benesch M, Sovinz P, Seidel M, et al. Sirolimus for the treatment of children with various complicated vascular anomalies. Eur J Pediatr 2015;174:1579–84.

74   Juric D, Gonzalez-Angulo A, Burris H, Schuler M, Schellens J, Berlin J, et al. Abstract P2-16-14: Preliminary safety, pharmacokinetics and anti-tumor activity of BYL719, an alpha-specific PI3K inhibitor in combination with fulvestrant: Results from a phase I study. Cancer Res 2013;73:P2-16-14-P2-16–14.

75    van Geel RMJM, Tabernero J, Elez E, Bendell JC, Spreafico A, Schuler M, et al. A Phase Ib Dose-Escalation Study of Encorafenib and Cetuximab with or without Alpelisib in Metastatic BRAF-Mutant Colorectal Cancer. Cancer Discov 2017;7:610–9.

76    Mayer IA, Abramson VG, Formisano L, Balko JM, Estrada M V., Sanders ME, et al. A Phase Ib Study of Alpelisib (BYL719), a PI3Kα-Specific Inhibitor, with Letrozole in ER+/HER2- Metastatic Breast Cancer. Clin Cancer Res 2017;23:26–34.

77    Janku F, Yap TA, Meric-Bernstam F. Targeting the PI3K pathway in cancer: are we making headway? Nat Rev Clin Oncol 2018. Doi: 10.1038/nrclinonc.2018.28

78    Lee J, Kim KE, Choi D-K, Jang JY, Jung J-J, Kiyonari H, et al. Angiopoietin-1 guides directional angiogenesis through integrin αvβ5 signaling for recovery of ischemic retinopathy. Sci Transl Med 2013;5:203ra127.

79    Cho C-H, Kim KE, Byun J, Jang H-S, Kim D-K, Baluk P, et al. Long-term and sustained COMP-Ang1 induces long-lasting vascular enlargement and enhanced blood flow. Circ Res 2005;97:86–94.

80    Thurston G, Wang Q, Baffert F, Rudge J, Papadopoulos N, Jean-Guillaume D, et al. Angiopoietin 1 causes vessel enlargement, without angiogenic sprouting, during a critical developmental period. Development 2005;132:3317–26.

81    Wang G, Jacquet L, Karamariti E, Xu Q. Origin and differentiation of vascular smooth muscle cells. J Physiol 2015;593:3013–30.

82    Saharinen P, Eklund L, Miettinen J, Wirkkala R, Anisimov A, Winderlich M, et al. Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol 2008;10:527–37.

83    Fukuhara S, Sako K, Minami T, Noda K, Kim HZ, Kodama T, et al. Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. Nat Cell Biol 2008;10:513–26.

84    Pietilä R, Nätynki M, Tammela T, Kangas J, Pulkki KH, Limaye N, et al. Ligand oligomerization state controls Tie2 receptor trafficking and angiopoietin-2-specific

responses. J Cell Sci 2012;125:2212–23.

85    Ye C, Pan L, Huang Y, Ye R, Han A, Li S, et al. Somatic mutations in exon 17 of the
      TEK gene in vascular tumors and vascular malformations. J Vasc Surg 2011;54:1760–
      8.

86    Zhou M, Jiang R, Zhao G, Wang L, Wang H, Li W, et al. Classification and Tie2
      mutations in spinal and soft tissue vascular anomalies. Gene 2015;571:91–6.

**FIGURE LEGENDS**

**Figure 1. VM signalling pathway and potential targets for molecular therapies.** More
than 20 gain-of-function mutations have been found in TIE2 in VM tissues. (See a detailed
list and frequencies in the Supplemental Table I.) Mutations induce ligand independent
tyrosine phosphorylation (P) and downstream activation of the PI3K/AKT signalling
pathway. In TIE2 mutation negative VMs, activating mutations are frequently found in
PIK3CA encoding the catalytic subunit of PI3K [6,9,10] (Supplemental Table I).
Biochemical and cell biological data shows that TIE2 and PIK3CA participate in the same
VM causative signalling pathway [6]. Some gene-specific effects may also exist. In addition
to PI3K signalling, both *in vitro* and genetic data suggest the involvement of activation of
MAPK, which may be important for some cellular abnormalities in VMs [9,12]. Most potent
inhibitors for VMs (sirolimus and alpelisib) are indicated.

**Figure 2. (A) Endothelial angiopoietin/TIE/PI3K pathway regulates vascular
morphogenesis and remodelling in normal development.** EC-specific deletion of *Pik3ca*
(encoding p110 catalytic subunit of PI3K) results in defective angiogenic sprouting and
vascular remodelling [27]. Ang/Tie signalling is required for vascular remodelling in
different vascular beds [1]. In the postnatal mouse retina model of angiogenesis, Tie2 is
expressed more in venous than in arterial endothelial cells (ECs) to support newly formed
veins via COUP transcription factor 2 (COUP-TFII) [58]. In addition, an excess of Tie2
activating ligand Ang1 stimulates capillary-to-venous remodelling [78–80]. EC to smooth
muscle cell (SMC) signalling is also regulated by other signalling systems, including platelet-
derived (PDGF) and transforming (TGF) growth factor families [81]. During vascular
morphogenesis ECs, SMCs and interstitial cells secrete structural and matricellular proteins

to form a basement membrane and fibrillar collagen matrix for signalling functions and structural support. The perivascular extracellular matrix (ECM) is remodelled as a response to different stimuli and involves proteolytic degradation, ECM deposition and regulation of gene expression in ECs, SMCs and fibroblasts.

**(B) Activating TIE2 and PIK3CA VM mutations cause molecular and cellular dysfunctions that may disturb normal vascular development.** In retrovirally transduced HUVECs, TIE2‑VM forms activate MAPK and Akt signalling pathways. Inhibition of Erk1/2 restores loss of ECM fibronectin (FN) and EC monolayer organization, and normalizes the plasminogen (PA) activation system [12]. Increase in Akt signalling reduces EC secretion of SMC attractant PDGF‑B in HUVECs and increases EC survival *in vitro* [11,18]. In *Pik3ca*[H1047R] mouse models, Akt also increased EC proliferation [9,10] and decreased expression of arteriovenous specification markers (COUP‑TFII, Eph‑B4 and Ephrin‑B2) [10]. qPCR and immunostaining analysis of human VM tissues have revealed down‑regulated TGF‑β, CCN2 and cadherins regulating ECM composition, SMC recruitment, and EC–EC adhesion [42,43]. Ultrastructural analyses of VM lesions in the VM mouse model [12] and patient biopsies [12,43] showed a disorganized fibrillar collagen matrix and basement membrane (BM) abnormalities that may reflect increased *in vitro* expression of ADAMTS peptidases [11] and plasminogen activators (tPA, uPA) [6,12] that degrade ECM proteins and activate matrix metalloproteinases for ECM remodelling. Fluid from VM lesions [52] and cultures of retrovirally transduced VM‑mutation HUVECs (J.K., L.E, unpublished) contain an increased amount of cell shed microparticles, whose importance for VM pathogenesis is currently unknown.

**(C) Schematic representation of TIE2**-**VM (left) and normal TIE2/ANG signalling (right) in ECs**. In normal ECs, extracellular angiopoietin ligands induce TIE2 translocation and activation in specific subcellular domains in EC–EC and EC–ECM contact sites to regulate vascular stability and motility [82–84]. Several mechanisms have been identified in TIE2‑VM protein trafficking that may hamper normal ligand‑regulated TIE2 signalling in specific cell compartments. These include TIE2 intracellular retention, increased turnover rate, extracellular domain shedding, and incomplete translocation and activation in EC–EC junctions [12]. In contrast to angiopoietin ligand regulated activation of TIE2‑WT, TIE2-VM receptors are constantly phosphorylated (P) ligand‑independently in abnormal subcellular compartments. Another mechanism that silences the normal TIE2 function in VMs is a

somatic second‑hit deletion mutation in a TIE2‑WT allele *in trans* resulting in intracellular retention [7].

**Figure 3. *In vivo* models for venous malformations**

In the VM transplantation model [12,13], retrovirally transduced human ECs stably expressing TIE2-VM mutations are transplanted into immunocompromised mice where they give rise to a network of vessels that connects to the host vasculature. The model recapitulates well VM phenotypes and mimics local subcutaneous somatic VM lesions. The disadvantage is that it uses immunocompromised mice and TIE2 over-expression. Also allograft studies, using matrigel‑embedded minced VM lesion material from a genetic PIK3CA‑VM mouse has been used in pharmacological studies in an immunodeficient host [9]. Genetic models are based on floxed *Pik3ca*[H1047R] alleles induced at different development stages, either ubiquitously or conditionally in non‑ECs and ECs, resulting in vascular dysmorphogenesis and development of VMs closely resembling human lesions [9,10,33]. No gene‑targeted VM model based on TIE2 mutation has been published. (B) Example of clinical appearance of VM lesions in a tongue and (C) in a mouse EC transplantation assay that closely resemble each other. Panels (B) and (C) reprinted from [12] with permission of Oxford University Press.

**Table I. Frequencies of TIE2 and PIK3CA mutations from studies where both genes were sequenced.**

| Amino acid change | [7] | [8] | [6] | [9] | [10] | Total number | % of all screened |
|---|---|---|---|---|---|---|---|
| **PIK3CA[a]** | | | | | | | |
| Hot-spot mutation[b] | | | 25 | 6 | 3 | 34 | 19.3 % |
| Other | | | 2 | 2 | | 4 | 2.3 % |
| **TIE2[c]** | | | | | | | |
| L914F | 24 | 9 | 26 | 7 | 3 | 69 | 39.2 % |
| Double mutation | 4 | 5 | 5 | 4 | 2 | 20 | 11.4 % |
| Single mutation[d] | | 3 | 5 | | 1 | 9 | 5.1 % |
| **TIE2/PIK3CA negative** | | | | | | | |
| Negative | | | 23 | 14 | 4 | 41 | 23.3 % |
| **Total screened** | **28** | **17** | **86** | **32** | **13** | **176** | |

(a) 21.6% of screened VMs are PIK3CA mutation positive. (b) Residues E542, E545 and H1047.
(c) 55.7% of screened VMs are TEK mutation positive. (d) Other TIE2 single amino acid substitution than most common L914F.


**Table II. Inherited TIE2 mutations found in VMCM and comparison to sporadic mutations.**

| Amino acid change | Number of cases[a] | Found alone in sporadic VMs[b] | Found as part of double mutation in sporadic VMs[a] |
|---|---|---|---|
| R849W | 11 | 1 or 2/320[c] | 0/320 |
| Y897S | 1 | 0/320 | 2/320 |
| K1100N | 1 | 0/320 | 0/320 |
| Y897C | 1 | 4/320[d] | 10/320 |
| R915H | 1 | 0/320 | 0/320 |
| R918C | 1 | 1/320[d] | 6/320 |
| A925S | 1 | 0/320 | 0/320 |
| V919L | 1 | 0/320 | 0/320 |
| Total | 18 | 6/320 | 18/320 |

(a) Based on [17,28,31,32]. Mutation found from blood DNA and most patients had confirmed family history. (b) cases/screened. (c) Information ambiguous in [8] (d) [85,86] sequenced only exon 17 of TIE2, so the presence of other TIE2 mutation cannot be excluded.

**Table III. Potential pharmacotherapies for venous malformations.**

| Drug | Target | Mechanism | Advantages | Limitations | *In vitro* EC | Pre-clinical | Clinical | Ref. |
|---|---|---|---|---|---|---|---|---|
| Sirolimus | mTOR | Inhibits Akt activation via mTORC2. | Clinically approved and used in other indications. | Does not target MAPK signalling in VMs. | Inhibited VM mutation induced Akt but didn't normalize all VM phenotypes. | Diminished growth in mouse VMs. | Phase II/III in VMs. | [6,13,14,67,68] |
| Alpelisib | PIK3CA isoform specific | Inhibits PI3K activation. | Targets TIE2 VM downstream signalling and directly PIK3CA VM mutations. | Not yet clinically approved, nor studied in paediatric patients. | Inhibited TIE2 and PIK3CA VM mutation induced Akt, normalized VM phenotypes. | Diminished VM lesions when administered systemically or topically. | Phase I/II in cancers. | [6,9] |
| TIE2 kinase inhibitor (CAS 948557-43-5) | TIE2 | TIE2 tyrosine kinase inhibitor. | Designed to target TIE2. | Not beneficial for PIK3CA VMs. Potent and specific TIE2 inhibitor is currently lacking. | Inhibited phosphorylation of some but not all TIE2-VM mutations. | Did not inhibit TIE2-L914F VMs in mice. | Not tested | [13] |
| Dactolisib (BEZ-235) | PI3K/mTOR | Inhibits PI3K/Akt activation. | Targets both PI3K and mTOR for more efficient inhibition. | Development discontinued as a cancer drug. | Inhibited Akt activation and normalized PIK3CA VM phenotypes. | Reduced and normalized vessels and bleeding in VM mouse. | Not in use | [33,77] |
| MEK inhibitors (Trametinib, Cobimetinib) | MEK1 | Inhibits TIE2-VM MAPK signalling. | Clinically approved. Provides possible combination treatment with Akt inhibitors. | No effect on PI3K/Akt activation. | MAPK inhibitor (PD98059) inhibited TIE2-VM induced ERK1/2 and VM phenotype. | Clinically approved MEK inhibitors have not been tested in VM models. | Not tested in VMs | [12] |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| MK-2206 | Akt | Inhibits VM mutation induced Akt activation. | Potent inhibitor of Akt in VM pathway. | Does not target pathogenic MAPK signaling in VMs. | Inhibited Akt activation and normalized some PIK3CA VM phenotypes. | Not tested | Not tested | [11,33] |
| Non-isoform specific PI3K inhibitors (Copanlisib) | PI3K | Inhibits VM mutations induced PI3K activation. | Alternative to isoform specific PIK3CA inhibitors. | More adverse effects than PIK3CA isoform specific inhibition. Intravenous administration. | Not tested | Not tested | FDA approved for lymphoma. | [77] |
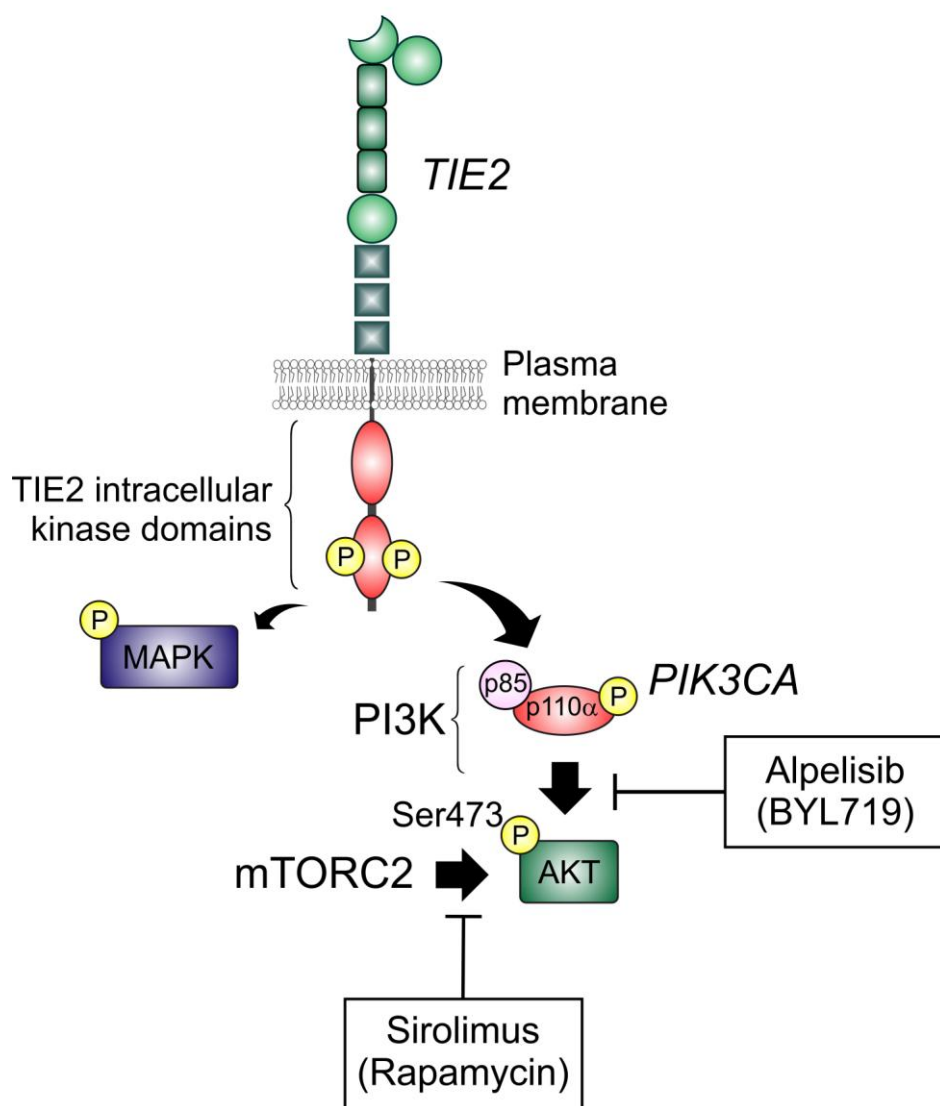
**FIGURE 1.**

**FIGURE 2.**



A. Normal vein morphogenesis
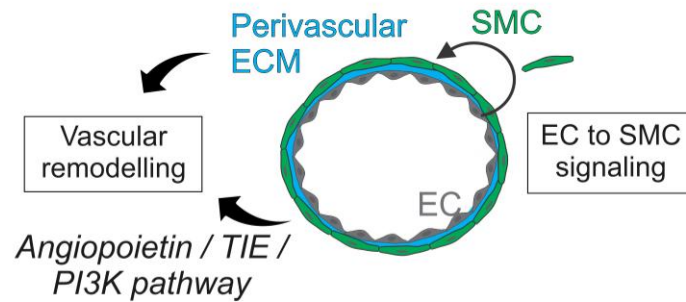
B. Venous malformation
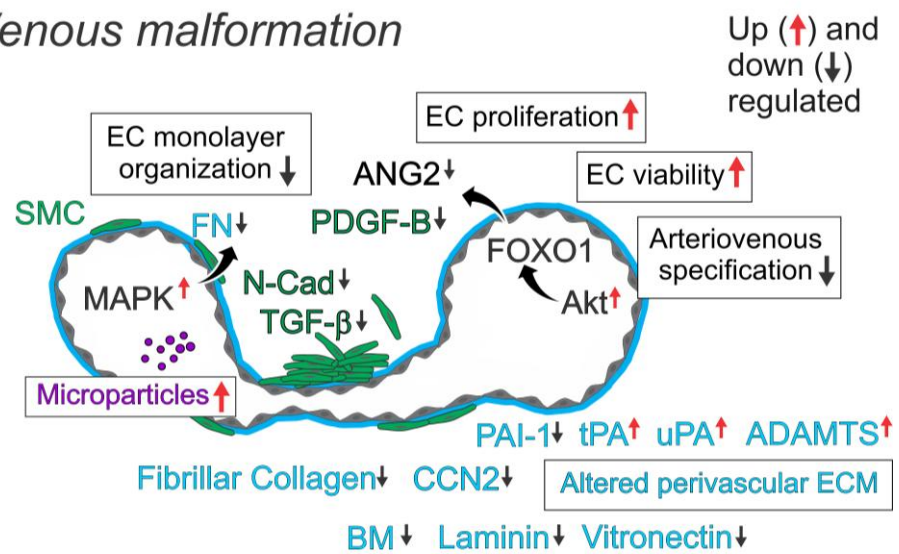
C. Dysregulated TIE2 signaling in VMs

**FIGURE 3.**