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Non-Hotspot *PIK3CA* Variants Have Higher Variant Allele Frequency and are More Common in Syndromic Vascular Malformations

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ABSTRACT

PIK3CA variants are known to cause vascular malformations. We were interested in studying the phenotypic spectrum, the location within the *PIK3CA* gene, and the variant allele frequency (VAF) of somatic *PI3KCA* variants in vascular malformations. Clinical data of consecutive patients with extracranial/extraspinal vascular malformations were collected in the context of the VASCOM cohort (2008–2022, $n = 558$). Starting October 2020, biopsy samples were tested with the TSO500 gene panel (*Illumina*). All consenting patients with *PIK3CA* variants were included in this study. Eighty-nine patients had available genetic results by June 2022. *PIK3CA* variants ($n = 25$) were found in 16 simple/combined (nonsyndromic) vascular malformations and in nine vascular malformations associated with other anomalies (syndromic). Four hotspot variants in exons 9 and 20 (c.1624G>A, c.1633G>A, c.3140A>G, c.3140A>T) were identified in 16/25 patients (VAF 0.9%–9.7%). Six non-hotspot variants (c.328_330del, c.323_337del, c.353G>A, c.1258T>C, c.3132T>A, c.3195_3203delinsT) were detected in nine patients (VAF 3.6%–31.7%). Non-hotspot variants were more frequent in syndromic than nonsyndromic vascular malformations ($p = 0.0034$) and exhibited a higher VAF than hotspot variants ($p = 0.0253$). Our study contributes to the growing body of knowledge of the genetic background in vascular malformations. Further studies will enrich the ever-growing list of pathogenic *PIK3CA* variants associated with vascular malformations.

1 | Introduction

Somatic gain-of-function variants in *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) cause activation of the PI3K/Akt/mTOR signaling pathway and were first described in cancer (Samuels et al. 2004). Their role in the pathogenesis of vascular malformations has been increasingly unveiled during the last decade (Queisser et al. 2021). *PIK3CA* variants were found in congenital lipomatous overgrowth with vascular anomalies, epidermal nevi and scoliosis (CLOVES) in 2012 (Kurek et al. 2012), in megalencephaly-capillary malformation syndrome (MCAP) in 2013 (Mirzaa, Rivière, and Dobyns 2013), and in lymphatic malformations (LM), venous malformations (VM), and Klippel-Trenauny syndrome (KTS) in 2015 (Luks et al. 2015; Limaye et al. 2015). Nowadays, it is known from the literature that the phenotypic spectrum of disorders associated with a *PIK3CA* variant is very broad; in fact, the term *PIK3CA*-related disorders was recently proposed, to include three subcategories of disorders: *PIK3CA*-related overgrowth spectrum (PROS), *PIK3CA*-related vascular malformations, and *PIK3CA*-related nonvascular lesions (Keppler-Noreuil et al. 2015; Canaud et al. 2021). Additionally, ongoing research keeps revealing novel *PIK3CA* variants (Mojarad et al. 2023).

According to the classification of the International Society for the Study of Vascular Anomalies (ISSVA), vascular malformations can be categorized in simple (when only one type of vessel is involved, that is, capillary, venous, lymphatic, or arteriovenous malformations), combined (when more than one types of vessels are involved in the same malformation), and vascular malformations associated with other anomalies (in the context of syndromes, such as KTS, CLOVES, Parkes Weber Syndrome etc., hence "syndromic") (ISSVA 2018). In this study, we aimed to characterize the *PIK3CA* variants identified in a series of patients with syndromic and nonsyndromic (simple or combined) vascular malformations, regarding their location within the *PIK3CA* gene and their variant allele frequency (VAF).

2 | Methods

2.1 | Editorial Policies and Ethical Considerations

The Bernese VAScular COngenital Malformation (VASCOM) cohort of the Inselspital—University Hospital of Bern, Switzerland was approved by the Ethics Committee of the Canton of Bern (ethics board number 2017-01960).

2.2 | Study Sample

Five hundred fifty-eight consecutively referred patients with congenital extra-cranial/extra-spinal vascular malformations were enrolled in the VASCOM cohort (2008–2022) (Tuleja et al. 2023). Genetic testing was implemented as standard of care in October 2020. All patients in the VASCOM cohort with available genetic testing results until June 2022 were reviewed for eligibility; patients with an identified *PIK3CA* variant were included in the present study. All patients (or their legal guardians) provided written informed consent forms for genetic testing and anonymized data analysis.

2.3 | Diagnostic Methods

The diagnostic procedures included physical examination and laboratory testing; duplex ultrasound and/or digital subtraction angiography were used for the evaluation of the anatomical location and the hemodynamic characterization of the vascular malformation; additional imaging studies (MRI, CT) were performed in some cases. D-dimer levels were routinely determined in venous blood samples using an immunoturbidimetric method with a cut-off value of > 500 mg/L.

Genetic testing was performed by next-generation sequencing (NGS) on frozen vascular malformation tissue available from diagnostic biopsies and stored in the Tissue Biobank Bern. The TruSight Oncology 500 gene panel (TSO500, *Illumina*) was used for the analysis; the TSO500 was originally designed to target exonic and splice site regions of 523 genes associated with solid tumors and covers most of the pathogenic variants that cause vascular malformations (Froyen et al. 2022). Paired-end sequencing was performed on a NovaSeq 6000 sequencing platform (*Illumina*) at the Clinical Genomics Lab of the Inselspital. The threshold for VAF detection was set at 0.5%; the recommended exon coverage for 0.5% level of detection following error correction was $>1000\times$. Copy number variations were not assessed, and the determined copy number was not corrected for malformation cell content. Clinically relevant variants (pathogenic or likely pathogenic) of *PIK3CA* (NM_006218.4; transcript ID: ENST00000263967.4) are being reported (Richards et al. 2015). All variants were submitted to a central variant database (ClinVar, https://www.ncbi.nlm.nih.gov).

2.4 | Data Collection and Analysis

Patient data were prospectively collected using caregiver-completed, electronic case-report forms. Following data extraction, patients' files were reviewed for missing data completion and retrospective application of the latest ISSVA classification (ISSVA 2018). A final decision was reached by consensus in cases with unclear diagnosis, after discussing the findings in our interdisciplinary boards (SINERGIA research group meetings and Inselspital Vascular Malformations Board).

The dataset was created using IBM SPSS Statistics (Version 28.0.0.0) and the statistical analysis was performed in RStudio (R version 4.3.1, http://www.r-project.org). Descriptive statistical methods were used to describe the data (median/standard deviation for quantitative variables i.e., VAF; numbers/percentages for categorical variables). The Shapiro–Wilk test was used to test for normality of the distribution of VAF. Parametric and nonparametric statistics were used for the comparisons of VAF in hotspot/non-hotspot variants and in syndromic/nonsyndromic vascular malformations; the Welch two sample *t*-test and the Mann–Whitney *U* test were used for the comparisons of mean VAF on the log-transformed scale and on the linear scale, respectively. For the comparison of categorical variables, the chi-square Fisher's exact test was used. The level of statistical significance was set at 5% ($p < 0.05$) and all calculated *p*-values were two-sided.

3 | Results

Biopsies of 59 slow-flow (without arterial component) and 30 fast-flow (with arterial component, i.e., AVM) vascular malformations were tested with the TSO500 panel ($n = 89$; 15.9% of the VASCOM cohort). In half of these cases ($n = 45$), we did not identify underlying pathogenic variants; in the remaining 44 cases (14/40 VM, 14/26 AVM, 1/3 LM, 4/6 combined malformations, and 11/14 malformations associated with other anomalies), we detected likely pathogenic variants in seven genes. Variants activating the PI3K/AKT/mTOR pathway were twice as common ($n = 30$) as variants activating the RAS/MAPK/ERK pathway ($n = 14$) (Figure 1).

Altogether, 25 patients with variants in *PIK3CA* were identified and included in this study; 16 patients with simple or combined vascular malformations (11 VM, 1 AVM, 1 LM, 2 LVM, 1 CVM) and nine patients with vascular malformations associated with other anomalies (6 KTS, 2 CLOVES, and 1 Parkes Weber Syndrome). Phenotypic and genotypic description are presented in Table 1.

In total, 10 different *PIK3CA* variants were identified, at allele frequencies ranging from 0.9% to 31.7% of total reads. Figure 2 depicts the location of the identified *PIK3CA* variants within the gene, and the clinical diagnoses associated with each variant in this study.

3.1 | Hotspot *PIK3CA* Variants

Four hotspot variants in the exons 9 and 20 of the *PIK3CA* gene dominated this case series, being present in almost two thirds of the samples ($n = 16$).

The p110 α-helical-domain substitutions c.1624G>A, p.(Glu542Lys) and c.1633G>A, p.(Glu545Lys) were identified in six and five patients, respectively. The kinase domain substitution c.3140A>G, p.(His1047Arg) was identified in four patients. The less common kinase domain substitution c.3140A>T, p.(His1047Leu), found in one patient, was also considered as a hotspot variant, being situated in a hotspot location, in line with previous publications (Brouillard et al. 2021).

Hotspot variants were detected at a mean VAF of 4.27% (SD = 2.51) in 13/16 simple or combined vascular malformations and in 3/9 malformations associated with other anomalies.

3.2 | Non-Hotspot *PIK3CA* Variants

The most common non-hotspot *PIK3CA* variant in our sample was the in-frame deletion c.328_330del, p.(Glu110del), which was identified in four patients. Another five non-hotspot *PIK3CA* variants were identified in one patient each (Table 1).

Non-hotspot variants were detected at a mean VAF of 9.4% (SD = 8.74) in 3/16 simple or combined vascular malformations and in 6/9 malformations associated with other anomalies.

3.3 | Statistical Analysis

The frequency of hotspot *PIK3CA* variants was significantly higher in simple/combined vascular malformations (81.25%) compared to those associated with other anomalies (33.33%) (Fisher's exact test = 5.74, $p = 0.0034$; Figure 3d). Moreover, the mean VAF was significantly higher in non-hotspot *PIK3CA* variants compared to hotspot variants, with values of 9.4% (SD = 8.74) and 4.27% (SD = 2.51), respectively (Welch two-samples *t*-test for log(VAF) $t = 2.586739$, $p = 0.01972378$; Mann–Whitney *U* test = 112, $p = 0.0253$; Figure 3b). Additionally, the mean VAF for vascular malformations associated with other anomalies was higher than that in simple/combined vascular malformations (9.03%, SD 8.89 vs. 4.48%, SD 2.65). However, this difference did not achieve statistical significance (Welch two-samples *t*-test for log(VAF) $t = -2.117618$, $p = 0.05021144$; Mann–Whitney *U* test = 45, $p = 0.133$; Figure 3a,c).

4 | Discussion

Despite our relatively small sample, this remains an interesting case series in the growing body of publications presenting the genetic background of vascular malformations.

Variants in *PIK3CA* emerged as the most prevalent genetic alterations in the tested samples, occurring in over a quarter of cases ($n = 25$) in the subset of 89 patients of the VASCOM cohort with available genetic results by June 2022 (Figure 1). This observation is not generalizable, as it is evidently the result of the overrepresentation of slow-flow malformations within our sample. Specifically, *PIK3CA* variants were found in 23/59 slow-flow malformations and 2/30 fast-flow malformations. Besides, the vast majority of patients with fast-flow vascular malformations (simple AVM or AVM in the context of Parkes Weber Syndrome) harbored variants in the RAS/MAPK/ERK pathway, as expected (Figure 1).

We detected likely pathogenic variants in 14/40 VM, out of which 11 were in *PIK3CA*. Variants in *PIK3CA* are reported to cause more than half of *TEK*-negative VM (Limaye et al. 2015). *TEK* were the first somatic variants to be associated with vascular malformations back in 2009, and are responsible for more than half of sporadic simple VM (Serio et al. 2022). The high percentage of negative genetic testing results within our VM group (26/40, 65%) was attributed to the inability of the TSO500 gene panel to detect variants in *TEK*. However, since the focus of this study is the phenotypic and genotypic description of patients with *PIK3CA*-related vascular malformations, we do not consider this to be a practical limitation.

A *PIK3CA* variant was found in 6/7 patients with KTS; failure to detect a *PIK3CA* variant in the remaining one patient with KTS may be attributed to a not representative biopsy sample. Both patients with CLOVES had a *PIK3CA* variant; although both patients were adults, it is worth mentioning that tumor surveillance with renal ultrasonography is recommended in pediatric patients every 3 months until the age of 7–8 years, since the risk of Wilm's tumor is increased in this population (Keppler-Noreuil et al. 2015; Palmieri et al. 2020). In other clinical entities of PROS, the risk of

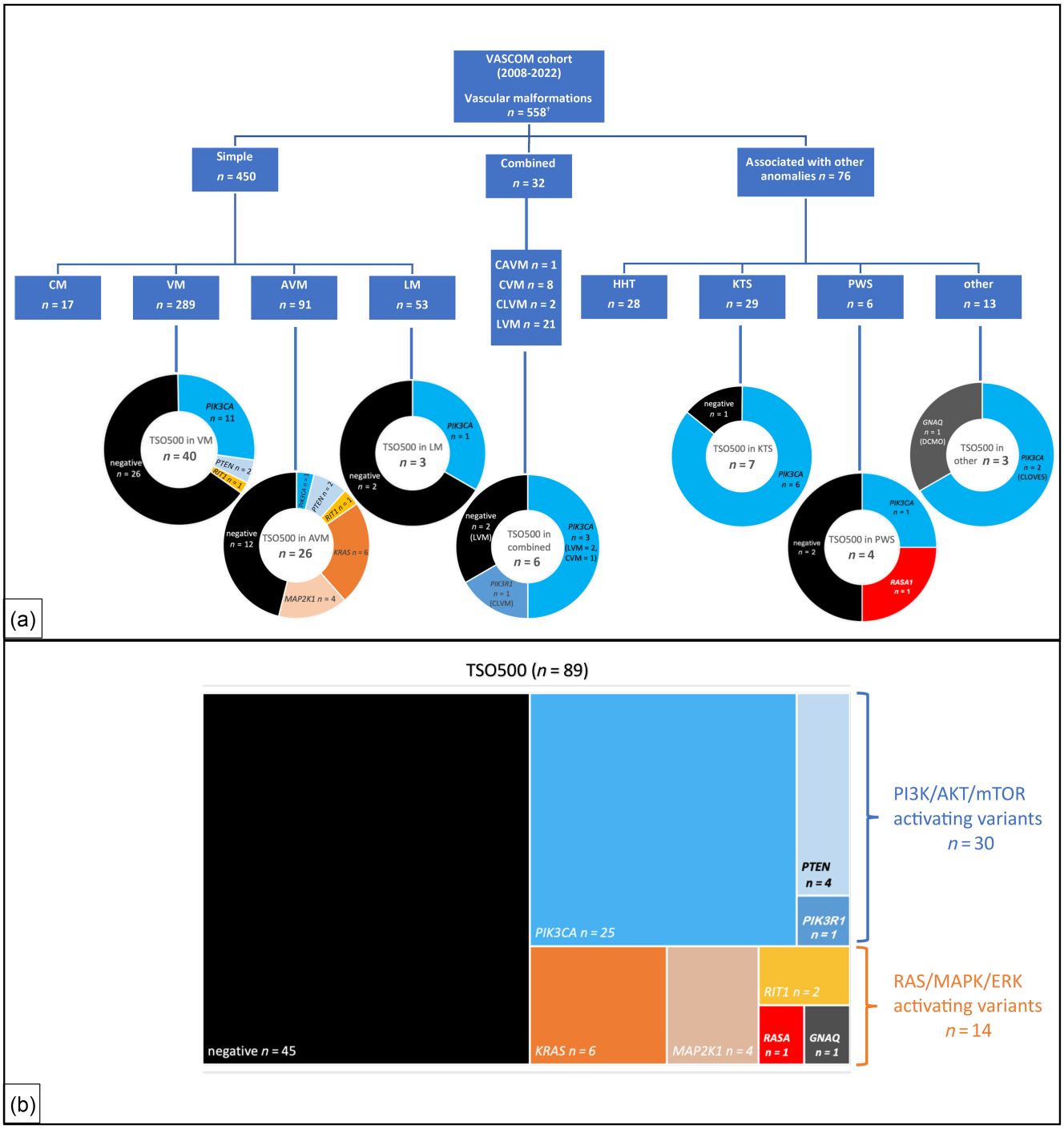

FIGURE 1 | (a) Flowchart of the VASCOM cohort of the Inselspital—University Hospital of Bern. (b) Overview of genetic results in the TSO500 subset. (a) Out of 558 patients with vascular malformations, genetic testing was performed on tissue biopsies in a subset of 89 patients (TSO500, Illumina). This subset included 69 patients with simple vascular malformations, 6 patients with combined vascular malformations, and 14 patients with vascular malformations associated with other anomalies. The pie charts summarize the genetic testing results of the TSO500 subset; for each diagnosis, the total number of patients with available genetic results is given in the center of the respective pie chart. Genetic testing results were not available for patients with CM or HHT (not tested). (b) Half of the tested patients had negative genetic results ($n = 45$). The other half had variants in seven different genes; two thirds had variants in (or related to) the PI3K/AKT/mTOR pathway ($n = 30$) and one third in (or related to) the RAS/MAPK/ERK pathway ($n = 14$). †Higher patient numbers (overall and per diagnostic category) are reported in this study as compared with the original publication presenting the VASCOM cohort (Tuleja et al. 2023), since this study corresponds to a later point in time and includes patients of all ages at presentation. Following a consent withdrawal, the sample size is reduced by one patient since our last publication (Andreoti et al. 2023). AVM, arteriovenous malformation; CAVM, capillary-arteriovenous malformation; CLOVES, congenital lipomatous overgrowth—epidermal nevi—skeletal anomalies syndrome; CLVM, capillary-lymphatic-venous malformation; CM, capillary malformation; CVM, capillary-venous malformation; DCMO, diffuse capillary malformation with overgrowth; HHT, hereditary hemorrhagic telangiectasia; KTS, Klippel-Trenaunay syndrome; LM, lymphatic malformation; LVM, lymphatic-venous malformation; PWS, Parkes Weber syndrome; VM, venous malformation.

TABLE 1 | Overview of the case series: Phenotypic and genotypic description of 25 patients with *PIK3CA*-related vascular malformations.

Patient characteristics			Clinical manifestations, complications, and treatments			Genetic testing results (TSO500 next-generation sequencing panel)[a]			
Age (y)/ case sex		Diagnosis	Localization (side; tissue compartments involved) and other clinical findings	Symptoms and complications	Treatment	*PIK3CA* domain	cDNA change, COSMIC ID, ClinVar ID	Amino acid change (single-letter code)	VAF%
Simple vascular malformations (nonsyndromic)									
1	9/f	Simple VM	• VM of the lower leg (left; M)	• Pain, swelling, thrombosis • D-dimers 156–3591 mg/L	• Alcohol embolizations • Alpelisib	Helical domain	**c.1624G>A** COSM760 31944	**p.(Glu542Lys)** (E542K)	5.10
2	26/f	Simple VM	• VM of the lower leg and foot (right; M)	• Pain, swelling, thrombosis • D-dimers 209–2045 mg/L	• Debulking surgery • Sclerotherapy and alcohol embolizations	Helical domain	**c.1624G>A** COSM760 31944	**p.(Glu542Lys)** (E542K)	1.20
3	49/f	Simple VM	• VM of the foot (left; M, B)	• Pain, swelling, thrombosis • D-dimers 231–583 mg/L	• Alcohol embolizations • Sirolimus	Helical domain	**c.1624G>A** COSM760 31944	**p.(Glu542Lys)** (E542K)	2.30
4	22/f	Simple VM	• VM of the lower leg (right; M)	• Pain, thrombosis	• Debulking surgery	Helical domain	**c.1624G>A** COSM760 31944	**p.(Glu542Lys)** (E542K)	3.80
5	14/m	Simple VM	• VM of the thigh (left; M)	• Pain, thrombosis	• Sclerotherapy, alcohol, and coil embolizations	Helical domain	**c.1624G>A** COSM760 31944	**p.(Glu542Lys)** (E542K)	9.70
6	42/f	Simple VM	• VM of the thigh (left; M)	• Pain • D-dimers 234–664 mg/L	• Alcohol embolizations	Helical domain	**c.1633G>A** COSM763 13655	**p.(Glu545Lys)** (E545K)	0.90
7	40/f	Simple VM	• Multifocal VM of the lower leg and big toe (right; SC, M) • Soft tissue hypertrophy	• Pain • D-dimers 196–724 mg/L	• Debulking surgery • Sclerotherapy and alcohol embolizations	Helical domain	**c.1633G>A** COSM763 13655	**p.(Glu545Lys)** (E545K)	2.00
8	20/m	Simple VM	• VM of the lower leg (left; M)	• Pain • D-dimers 155–185 mg/L	• Alcohol embolizations	Kinase domain	**c.3140A>G** COSM775 13652	**p.(His1047Arg)** (H1047R)	4.80
9	20/m	Simple VM	• VM of the thenar (right; M)	• Pain • D-dimers 155 mg/L	• Alcohol embolization	Kinase domain	**c.3140A>G** COSM775 13652	**p.(His1047Arg)** (H1047R)	6.10

(Continues)

TABLE 1 | (Continued)

Patient characteristics			Clinical manifestations, complications, and treatments			Genetic testing results (TSO500 next-generation sequencing panel)[a]			
Age (y)/ case sex		Diagnosis	Localization (side; tissue compartments involved) and other clinical findings	Symptoms and complications	Treatment	*PIK3CA* domain	cDNA change, COSMIC ID, ClinVar ID	Amino acid change (single-letter code)	VAF%
10	21/m	Simple VM	• VM of the thigh (left; SC, M) • Soft tissue hypertrophy, limb length difference	• Pain, thrombosis • D-dimers 259–1322 mg/L	• Debulking surgery • Sclerotherapy, alcohol, and coil embolizations • Laser treatment	Kinase domain	**c.3140A>G** COSM775 13652	**p.(His1047Arg)** (H1047R)	2.80
11[b]	15/f	Simple VM	• VM of the foot (right; M) • Soft tissue hypertrophy	• Pain, thrombosis • D-dimers 234–1315 mg/L	• Alcohol embolizations		c.328_330del COSM24710 995382	p.(Glu110del) (E110del)	4.10
12	0.15/f	Simple LM	• Macrocystic LM of the neck and axilla (left; SC)	• Functional impairment	• Sclerotherapy and alcohol embolizations	Kinase domain	**c.3140A>T** COSM776 13653	**p.(His1047Leu)** (H1047L)	5.00
13[b,d]	17/f	AVM	• AVM of the lower leg (left; M)	• Pain • D-dimers 272 mg/L	• Debulking surgery	C terminus	c.3195_3203delinsT COSM9358139 3233413	p.(His1065LeufsTer5) (H1065Lfs*5)	9.90
Combined vascular malformations (nonsyndromic)									
14	21/f	LVM	• LVM of the thigh (right; SC)	• Pain • D-dimers 194–338 mg/L	• Debulking surgery • Alcohol embolizations	Helical domain	**c.1633G>A** COSM763 13655	**p.(Glu545Lys)** (E545K)	4.60
15	32/m	LVM	• LVM of the left hemithorax (anterior and posterior mediastinum) and left hemiabdomen (peritoneal cavity) • Soft tissue hypertrophy	• Pain, disseminated intravascular coagulation, lymphorrhea • D-dimers 9156–32,641 mg/L	• Sclerotherapy	Kinase domain	**c.3140A>G** COSM775 13652	**p.(His1047Arg)** (H1047R)	2.80
16	16/f	CVM	• Skin CM of the left thigh and buttock • CVM of the thigh (left; SC, M) • Lateral marginal vein	• Pain • D-dimers 199–3912 mg/L	• Alcohol embolizations	C2-PIK3C-type domain	c.1258T>C COSM757 31945	p.(Cys420Arg) (C420R)	6.60

(Continues)

TABLE 1 | (Continued)

Patient characteristics			Clinical manifestations, complications, and treatments			Genetic testing results (TSO500 next-generation sequencing panel)[a]			
Age (y)/ case sex		**Diagnosis**	**Localization (side; tissue compartments involved) and other clinical findings**	**Symptoms and complications**	**Treatment**	***PIK3CA* domain**	**cDNA change, COSMIC ID, ClinVar ID**	**Amino acid change (single-letter code)**	**VAF%**
Vascular malformations associated with other anomalies (syndromic)									
17	49/f	KTS	• Skin CM of the right thigh • LVM of the lesser pelvis, anogenital area, buttock, and thigh (right; SC, M) • Lateral marginal vein • Soft tissue hypertrophy, limb length difference • Other: multiple spleen lesions	• Thrombosis, bleeding, recurrent infectious complications, lymphedema • D-dimers 1367–4415 mg/L	• Sclerotherapy and alcohol embolizations • Laser	Helical domain	**c.1633G>A** COSM763 13655	**p.(Glu545Lys)** (E545K)	3.50
18	32/m	KTS	• Skin CM of the right thigh and lower leg • LVM of the lesser pelvis, anogenital area, buttock, thigh, and lower leg (right; SC, M) • Lateral marginal vein • Limb length difference • Other: syndactyly of toes II–III	• Pain, thrombosis, bleeding, infection • D-dimers 1519–13,051 mg/L	• Debulking surgery • Alcohol and coil embolizations	Helical domain	**c.1633G>A** COSM763 13655	**p.(Glu545Lys)** (E545K)	4.50
19[c]	28/f	KTS	• Skin CM of the legs (bilateral) and the left side of the trunk, arm, and hand • LVM of the thigh, lower leg, and foot (left; SC) • Lateral marginal vein • Soft tissue hypertrophy, limb length difference • Other: syndactyly of toes III–IV	• Swelling, thrombosis	• Debulking surgery • Stripping of marginal vein • Correction of syndactyly • Alpelisib		c.353G>A COSM751 156446	p.(Gly118Asp) (G118D)	31.70
20[b]	41/f	KTS	• Skin CM of the left leg • LVM of the thigh, lower leg, and foot (left; SC, M) • Lateral marginal vein • Soft tissue hypertrophy, limb length difference	• Pain, swelling, bleeding, ulcer • D-dimers 45–856 mg/L	• Debulking surgery • Stripping of marginal vein and varices • Alcohol embolization • Sirolimus		c.328_330del COSM24710 995382	p.(Glu110del) (E110del)	4.00

(Continues)

TABLE 1 | (Continued)

Patient characteristics			Clinical manifestations, complications, and treatments			Genetic testing results (TSO500 next-generation sequencing panel)[a]			
Age (y)/ case sex		Diagnosis	Localization (side; tissue compartments involved) and other clinical findings	Symptoms and complications	Treatment	*PIK3CA* domain	cDNA change, COSMIC ID, ClinVar ID	Amino acid change (single-letter code)	VAF%
21	44/m	KTS	• Skin CM of the left buttock • VM of the lesser pelvis, anogenital area, thigh, and lower leg (left; SC, M) • Lateral marginal vein • Soft tissue hypertrophy	• Pain, swelling, thrombosis, and pulmonary embolism, bleeding, ulcer, infection • D-dimers 6571–41,839 mg/L	• Debulking surgery • Stripping of varices • Alcohol embolizations • Laser		c.328_330del COSM24710 995382	p.(Glu110del) (E110del)	3.60
22	27/m	KTS	• Skin CM of the right lateral abdominal wall • VM of the leg (right; SC, M) • Soft tissue hypertrophy, limb length difference	• Pain, thrombosis, infection • D-dimers 1034–1843 mg/L	• Debulking surgery • Sirolimus	Kinase domain	c.3132T>A COSM12592 663332	p.(Asn1044Lys) (N1044K)	8.40
23[d]	39/f	PWS	• Skin CM of the left leg and foot • AVM of the lower leg and foot (left; SC), no significant shunt • Soft tissue hypertrophy, macrodactyly toes II–III	• Pain, swelling, thrombosis, ulcer, infection • D-dimers 523–1091 mg/L	• Debulking surgery • Toe amputation • Sclerotherapy and alcohol embolizations		c.323_337del COSM7346032 3233414	p.(Arg108_Ile112del) (R108I112del)	10.50
24[b]	22/m	CLOVES	• Epidermal nevi • VM of the abdominal wall (right; SC, M) and the thoracic wall (bilateral; SC, M) • Intra- and retroperitoneal LVM • S-shaped scoliosis • Soft tissue hypertrophy	• Pain • D-dimers 1790–21,413 mg/L	• Alcohol and coil embolizations	Helical domain	**c.1624G>A** COSM760 31944	**p.(Glu542Lys)** (E542K)	9.30

(Continues)

TABLE 1 | (Continued)

Patient characteristics			Clinical manifestations, complications, and treatments			Genetic testing results (TSO500 next-generation sequencing panel)[a]			
Age (y)/ case sex		Diagnosis	Localization (side; tissue compartments involved) and other clinical findings	Symptoms and complications	Treatment	*PIK3CA* domain	cDNA change, COSMIC ID, ClinVar ID	Amino acid change (single-letter code)	VAF%
25[b]	40/f	CLOVES	• Linear epidermal nevus of the left axilla • Skin CM of the left arm • VM of the upper arm, thoracic and abdominal wall (left; SC, M) • Kyphoscoliosis • Soft tissue hypertrophy • Other: splenomegaly, splenic cysts	• Pain, thrombosis, and pulmonary embolism • D-dimers 3475–32,100 mg/L	• Debulking surgery • Toe amputation • Sclerotherapy		c.328_330del COSM24710 995382	p.(Glu110del) (E110del)	5.80

Note: Age at referral/enrollment is presented. No patients had pathogenic variants in the p85α-binding domain (amino acids 16–105) or the Ras-binding domain (amino acids 187–289). C2 PI3K-type domain (330–487); Helical domain (517–694); Kinase domain (765–1051) (*PIK3CA* transcript NM_006218.4, transcript ID: ENST00000263967.4; protein domains based on https://www.uniprot.org/uniprot/P42336).

Abbreviations: AVM, arterio-venous malformation; B, bones/joints; cDNA, complementary DNA; CLOVES, congenital lipomatous overgrowth with epidermal nevi and skeletal anomalies; CM, capillary malformation; COSMIC, catalogue of somatic mutations in cancer; KTS, Klippel-Trenaunay syndrome; LM, lymphatic malformation; LVM, combined lymphatic-venous malformation; M, muscle; m/f, male/female; PWS, Parkes Weber syndrome; SC, subcutaneous; VAF, variant allele frequency; VM, venous malformation.

[a]All *PIK3CA* variants were classified as likely pathogenic. Single-letter amino acid code in brackets, below the amino acid change. Hotspot variants in bold, variants of unknown significance not presented.

[b]The following additional likely pathogenic variants were identified in patients 11, 13, 20, 24, and 25: *AXIN2* (VAF 2.2%), *BCOR* (VAF 3.4%), *SDHD* (VAF 45.4%), *ARID5B* (VAF 1.1%), and *RAD51B* (VAF 47.3%), respectively.

[c]The clinical phenotype of patient 19 (non-hotspot *PIK3CA* variant with a VAF of 31.7%) was not compatible with a germline variant, thus no additional tests were performed to exclude this possibility.

[d]Patients 13 and 23 had a microfistular subtype of AVM, with post-capillary microfistular shunts (capillary-venule AVM) (Vuillemin et al. 2021). Patient 23 was previously presented as Patient 7 in a case series of patients with PWS (Andreoti et al. 2023).

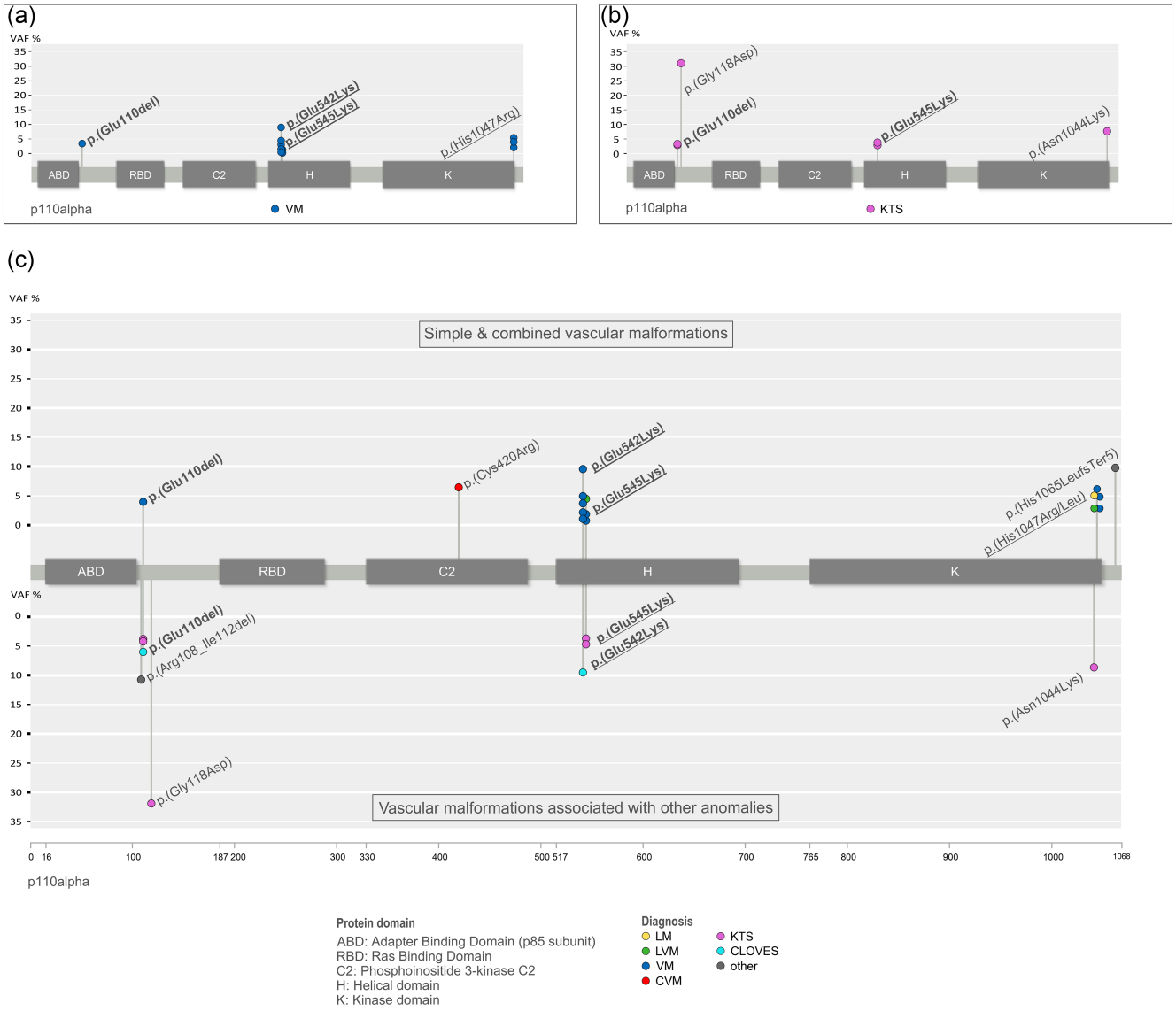

FIGURE 2 | Image of p110alpha, the protein encoded by *PIK3CA* (Human, 1068 aa), depicting the location of the detected *PIK3CA* variants: (a) in venous malformations, (b) in Klippel-Trenaunay syndrome, and (c) in the entire case series, with variants in nonsyndromic (simple or combined) and syndromic vascular malformations (associated with other anomalies) depicted above and below the p110alpha protein, respectively. Hotspot variants are underlined, variants detected both in syndromic and nonsyndromic malformations are in bold. The vertical axis represents the variant allele frequency (VAF%) of each detected variant. p85α-binding domain (amino acids 16–105); Ras-binding domain (amino acids 187–289); C2 PI3K-type domain (330–487); Helical domain (517–694); Kinase domain (765–1051) (*PIK3CA* transcript NM_006218.4, transcript ID: ENST00000263967.4; protein domains based on https://www.uniprot.org/uniprot/P42336). Simple vascular malformations: VM, venous malformation; LM, lymphatic malformation; AVM, arteriovenous malformation. Combined vascular malformations: CVM, capillary-venous malformation; LVM, lymphatic-venous malformation. Vascular malformations associated with other anomalies (syndromic): KTS, Klippel-Trenaunay syndrome; PWS, Parkes Weber syndrome; CLOVES, congenital lipomatous overgrowth—epidermal nevi—skeletal anomalies syndrome.

developing Wilm's tumor remains unclear and therefore routine screening is not recommended (Palmieri et al. 2020).

Apart from diagnostic reasons, genetic testing is also provided in order to identify individuals who could benefit from targeted treatment; indeed, a subgroup of our study sample ($n = 5$, 20%) was treated with molecular therapy with inhibitors of the PI3K/AKT/mTOR signaling pathway, either with sirolimus (mTOR inhibitor, $n = 3$) on a personalized, off-label basis, or with alpelisib (PI3K alpha inhibitor, $n = 2$) in the context of an ongoing trial (ClinicalTrials.gov ID: NCT04589650). Even though follow-up data are systematically collected in the context of the VASCOM cohort, this study was not designed to assess, and does not report on disease course or treatment outcomes.

4.1 | Limitations

Genetic testing on biopsy tissue was only recently implemented as part of standard clinical care in our center and, as far as the diagnostic procedure is concerned, many patients did not undergo biopsy for various reasons (e.g., otherwise established diagnosis, contraindication, refusal). The inherent risks of biopsies especially in LM, explain the underrepresentation of patients with

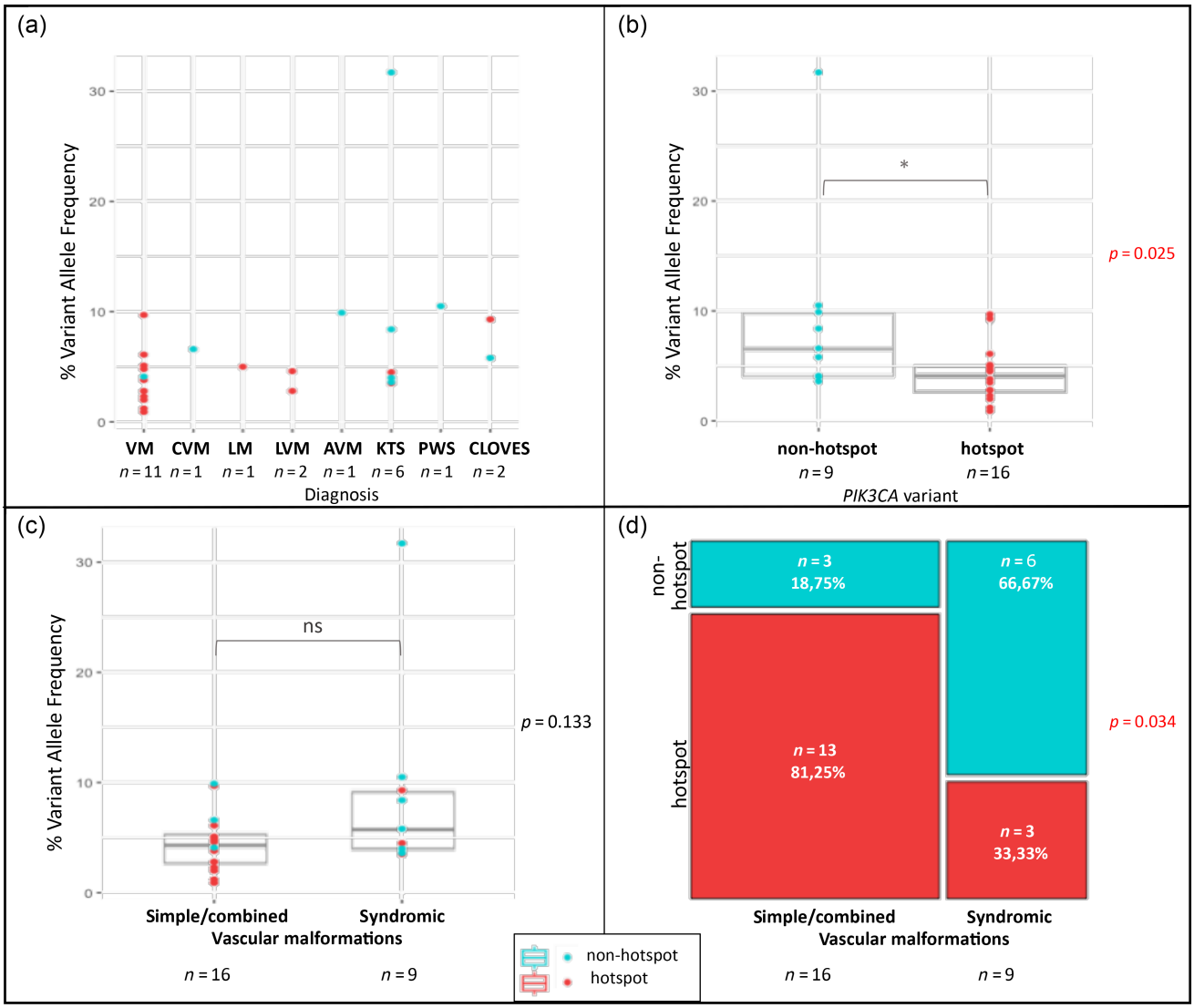

FIGURE 3 | (a–c) Scatterplots and/or boxplots depicting the variant allele frequency (%VAF) of *PIK3CA* variants: (a) by diagnosis, (b) by hotspot/non-hotspot PIK3CA variant, and (c) by category of vascular malformation (simple and combined/syndromic). (d) Mosaic plot of the distribution of hotspot PIK3CA variants by category of vascular malformation. We observed: A higher %VAF in non-hotspot compared with hotspot variants (Mann–Whitney U test = 112, $p = 0.0253$) (b); a higher %VAF of *PIK3CA* variants detected in syndromic vascular malformations compared with variants in simple/combined vascular malformations (Mann–Whitney U test = 45, $p = 0.133$) (c); a higher frequency of non-hotspot *PIK3CA* variants in syndromic vascular malformations compared to simple/combined vascular malformations (Fisher's exact test = 5.74, $p = 0.0034$) (d). ns: non-significant; *$p < 0.05$. Simple vascular malformations: VM, venous malformation; LM, lymphatic malformation; AVM, arteriovenous malformation. Combined vascular malformations: CVM, capillary-venous malformation; LVM, lymphatic-venous malformation. Vascular malformations associated with other anomalies (syndromic): KTS, Klippel-Trenaunay syndrome; PWS, Parkes Weber syndrome; CLOVES, congenital lipomatous overgrowth—epidermal nevi—skeletal anomalies syndrome.

LM in our sample, even though it is known from the literature that the majority of simple or combined LM are linked to somatic *PIK3CA* variants (Brouillard et al. 2021). In the future, emerging noninvasive tests, such as liquid biopsy (i.e., NGS in cell-free DNA extracted from blood samples taken as close to the vascular malformation as possible) or single-cell RNA transcriptome sequencing, may prove to be helpful diagnostic tools, allowing for more vascular malformations to be genotyped (Chavkin and Hirschi 2020; Limaye et al. 2008; Hughes, Hao, and Luu 2020). Furthermore, extensive genetic testing, such as exome sequencing, was not available to search for underlying pathogenic variants in patients who yielded negative results in TSO500. Besides, sequencing was not performed on other tissue samples (e.g., blood) to exclude the possibility of germline variants. However, low VAF observed in the tested samples most likely correspond to somatic variants.

Due to the retrospective nature of our study, a standardized procedure for obtaining tissue biopsies was not always followed, which could lead to variability in the composition of the tissue samples regarding gross cell distribution. The distribution of affected versus unaffected cells and the types of cells present (e.g., endothelial cells, fibroblasts) could vary significantly between samples. Consequently, while we report

higher VAF for non-hotspot *PIK3CA* variants in syndromic vascular malformations, these findings should be interpreted with caution. Further studies with standardized biopsy protocols and detailed cellular composition analyses are necessary to validate our observations and provide more definitive insights.

4.2 | Distribution of Hotspot *PIK3CA* Variants

We present a case series of 25 *PIK3CA*-associated vascular malformations. Hotspot variants were almost twice as common as non-hotspot variants. Hotspot variants were more common in simple or combined vascular malformations; inversely, non-hotspot variants were predominant in syndromic malformations.

Three hotspot variants (c.1624G>A, p.(Glu542Lys); c.1633G>A, p.(Glu545Lys), and c.3140A>G, p.(His1047Arg)), were detected in 15 out of 25 patients with *PIK3CA*-associated vascular malformations. These missense variants, frequently found in *PIK3CA*-associated cancers, overgrowth syndromes, and slow-flow vascular malformations, have been reported to account for >92% of individuals who carry *PIK3CA* variants (Limaye et al. 2015).

4.3 | Novel *PIK3CA* Variants

Two patients with fast-flow vascular malformations (microfistular AVM or CV-AVM (Vuillemin et al. 2021)) had non-hotspot *PIK3CA* variants, both of which were previously undescribed in vascular malformations.

One patient with a symptomatic intramuscular AVM of the foot had a variant near the C-terminal end of *PIK3CA* (c.3195_3203delinsT, p.(His1065LeufsTer5)); two similar variants leading to the same amino acid changes have been reported in one patient with congenital lipoma (c.3194_3202delinsT, p.(His1065LeufsTer5)) and one patient with breast cancer (c.3194_3203delinsTG, p.(His1065LeufsTer5); COSM9358139) (Mojarad et al. 2023; Razavi et al. 2018).

The second patient with AVM and phenotype of Parkes Weber Syndrome (Andreoti et al. 2023) had a *PIK3CA* variant that has only been reported in colorectal cancer (c.323_337del, p.(Arg108_Ile112del)) (Hampel et al. 2018). A nearby amino acid deletion had been previously reported to be an activating mutation (Ng et al. 2018); we thereby presume this to be an activating mutation as well.

Both variants being categorized as likely pathogenic, we assumed causality between the genotype and the observed phenotype in these two patients.

4.4 | Variant Allele Frequency: Higher in Non-Hotspot *PIK3CA* Variants

A higher VAF was found in syndromic versus simple or combined malformations and a significantly higher VAF in non-hotspot versus hotspot *PIK3CA* variants. This finding aligns with our hypothesis that *PIK3CA* variants at higher VAF, including possible germline variants, are only expected to be tolerated in the context of non-hotspot variants.

In this case series, the only case with a high VAF (31.7%) was indeed a non-hotspot variant detected in a syndromic vascular malformation (Patient 19). The clinical phenotype of this patient is compatible with a somatic variant; thus, no additional tests were performed to exclude the possibility of a germline variant. Somatic variants at higher VAF possibly lead to widely spread vascular malformations; germline *PIK3CA* variants lead to vascular anomalies and systemic clinical presentations (generalized overgrowth, macrocephaly, dysmorphic traits), that were absent in patient 19. Afterall, germline *PIK3CA* variants are considerably rare, with only 20 cases reported in the literature, reflecting the important role of *PIK3CA* for survival (Rivière et al. 2012; Orloff et al. 2013; Yeung et al. 2017; Zollino et al. 2019; Di Donato et al. 2016).

Brouillard et al. detected non-hotspot *PIK3CA* variants more frequently in syndromic LM than in simple or combined LM (Brouillard et al. 2021), which aligns with the findings of our broader comparison between syndromic and nonsyndromic (simple or combined) vascular malformations. The same study presented higher VAF in CLOVES than in simple or combined LM (Brouillard et al. 2021). Although we could not confirm this finding per se (due to our low number of LM), we were able to perform a more general comparison between syndromic and nonsyndromic vascular malformations, which yielded results that support their findings. We interpret the higher VAF of *PIK3CA* variants in syndromic malformations as likely attributable to the higher prevalence of non-hotspot variants in this subgroup of patients, since non-hotspot variants were found to have significantly higher VAF.

5 | Conclusions

PIK3CA variants were the most common genetic cause for slow-flow vascular malformations in the subset of the VASCOM cohort of non-CNS vascular malformations tested with the TSO500 panel. The high prevalence of negative results (even though partially explained by the limitations of TSO500 in detecting *TEK* variants) indicates the possibility of undiscovered variants within the genetic landscape of vascular malformations. As the body of evidence in the field of underlying genetics of vascular malformations is increasing, a new classification system based on pathogenetic mechanisms is anticipated.

In this cross-sectional study, patient data from a monocentric cohort of vascular malformations were analyzed and presented as a case series, demonstrating the wide spectrum of clinical phenotypes associated with *PIK3CA* variants. We present two patients with variants previously undescribed in vascular malformations; as novel *PIK3CA* variants continue to arise, genetic testing focusing on hotspot variants is not recommended. We found that hotspot *PIK3CA* variants at lower VAF were more common in simple or combined vascular malformations, while non-hotspot variants at higher VAF

were more common in syndromic vascular malformations. Whether treatment response to targeted therapies is different in patients with hotspot versus non-hotspot *PIK3CA* variants, or in patients with *PIK3CA* variants at lower or higher VAF, is yet to be explored.

Author Contributions

Themis-Areti A. Andreoti: conceptualization, data extraction, methodology, statistical analysis, writing (original draft, lead). **Massimo Maiolo:** writing (original draft, supporting). **Aleksandra Tuleja:** data curation, investigation. **Yvonne Döring:** investigation. **André Schaller:** investigation. **Erik Vassella:** investigation. **Laurence M. Boon:** writing (review and editing, supporting). **Iris Baumgartner:** project supervision, funding acquisition. **Sarah M. Bernhard:** investigation. **Christiane Zweier:** writing (review and editing, supporting). **Miikka Vikkula:** writing (review and editing, supporting). **Jochen Rössler:** project supervision, funding acquisition, writing (review and editing, lead).

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Disclosure

This manuscript is an original work that has neither been published, nor is it under consideration for publication elsewhere. The abstract was presented at the ISSVA World Congress 2024, May 7–10, Madrid, Spain, https://www.issva.org/2024/ (poster presentation).

Ethics Statement

The study was approved by the Ethics Committee of the Canton of Bern (ethics board number 2017–01960). All patients (or their legal guardians) provided written informed consent forms for genetic testing and anonymized data analysis.

Conflicts of Interest

Themis-Areti Andreoti, Sarah M. Bernhard, Aleksandra Tuleja, and Iris Baumgartner are investigators in the EPIK-P2 study (https://clinicaltrials.gov/ct2/show/NCT04589650), sponsored by Novartis. Jochen Rössler is currently an employee of Novartis Pharma Basel. Massimo Maiolo, Yvonne Döring, André Schaller, Erik Vassella, Christiane Zweier, Laurence M. Boon, and Miikka Vikkula have no actual or potential conflicts of interest to disclose in relation to this publication.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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