Molecular Genetics of Vascular Malformations

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Advanced article

Article Contents

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Vascular anomalies are separated into vascular tumours and vascular malformations. Vascular malformations are named according to the affected type of vessels, that is venous, capillary, arteriovenous or lymphatic malformations. Up to now, sclerotherapy, embolisation and/or surgery are the treatments of choice, yet they do not often offer a curative treatment. Thus, there is an important need to develop novel disease-specific therapeutic approaches.

Inherited forms of vascular malformations led to the identification of several genes that are mutated encoding dysfunctional proteins. Demonstration that tissular second hits are commonly involved in inherited forms to explain development of lesions led to study somatic mutations in sporadically occurring forms. Since the primary discovery demonstrating that venous malformations are due to somatic mutations in TIE2/TEK, most types of vascular anomalies now have a known genetic cause. Thereby, the signalling pathways involved have been unravelled, leading to a better understanding of the aetiopathogenesis of vascular anomalies. As – like in cancers – the RAS/MAPK/ERK and the PI3K/AKT/mTOR signalling are enhanced in most vascular anomalies, treatment with cancer drugs interfering with these pathways could represent novel treatment options.

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Introduction

Vascular development is divided into vasculogenesis, angiogenesis and lymphangiogenesis. During these processes, arteries, capillaries, veins and lymphatic vessels arise, respectively. These vessels consist of a single intraluminal endothelial cell (EC) layer surrounded by a variable number of mural cells (i.e. smooth muscle cells and/or pericytes).

Defects during vascular development result in vascular malformations, the most common congenital and neonatal anomalies (Mulliken *et al.*, 2013). Mulliken and Glowacki provided the basis for a clear classification including physical findings, clinical behaviour and flow kinetics. They separated vascular anomalies into vascular tumours (mainly haemangiomas and other tumours) and vascular malformations that are named capillary, venous, lymphatic, arteriovenous or mixed malformations (Mulliken and Glowacki, 1982).

Commonly, vascular malformations are present at birth and grow proportionally with the child. However, in some cases, novel lesions appear with time. It is known that inherited and/or somatic mutations cause most vascular lesions. The identification of these mutations has given and will deepen our knowledge on the molecular and cellular bases of these lesions and thus the detailed underlying pathophysiology. This will hopefully help discover novel treatment options.

Vascular Tumours

Infantile haemangioma (IH)

Infantile haemangioma (IH) is the most frequent benign vascular tumour in childhood and with a female-to-male ratio of 2.4–4:1. IH seems to be associated with low birth weight (<1000 g), prematurity, multiple gestation and chorionic villus sampling (Mahady *et al.*, 2015). The majority of IHs are located in the face and usually appear a few weeks after birth (Mahady *et al.*, 2015) (**Figure 1a**). These tumours are characterised by a rapid proliferation phase, which lasts several months during young infancy.

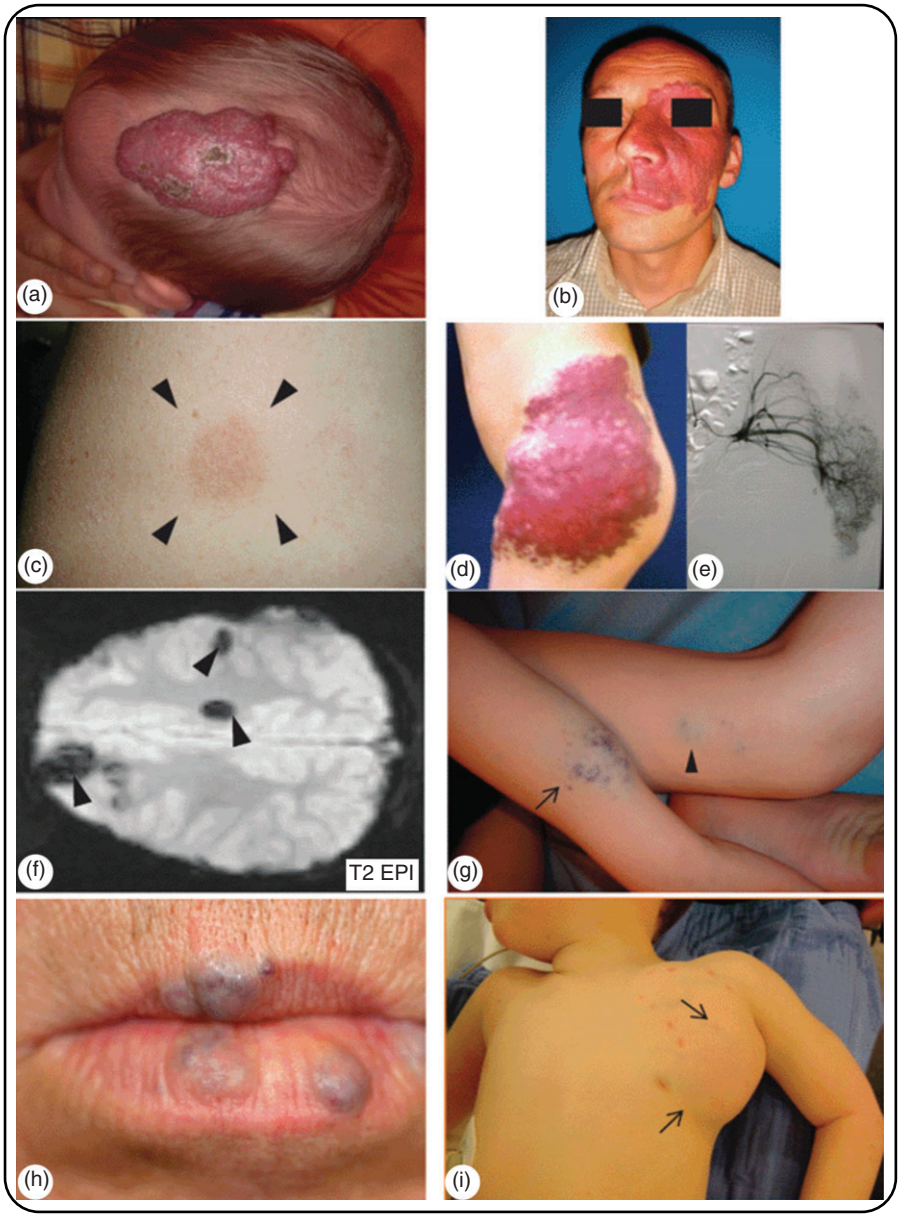

Figure 1 Images of vascular tumours and vascular malformations. (a) Voluminous haemangioma on the summit of the skull of a baby. (b) Hemifacial capillary malformation. (c) Characteristic capillary malformation of 'CM-AVM': oval and light-red appearance. Arrowheads indicate a pale halo around the malformation. (d) Arteriovenous malformation in an 8-year-old boy initially diagnosed as an infantile haemangioma. The lesion was warm and a thrill is felt on palpation. (e) Arteriography demonstrates abnormal vasculature. (f) Cerebral CT scan of a patient presenting cerebral cavernous malformation (arrowheads). (g) Characteristic glomuvenous malformations on the arm (arrow) and the leg (arrowhead) of a young boy. Note the nodular appearance and bluish-purple colouration. (h) Venous malformations of the lips. Note their homogenous aspect and bluish colouration. (i) Subcutaneous lymphatic malformation on the axilla (arrows).

Histologically, plump ECs can be detected as well as an elevated number of mast cells (Tucci *et al*., 2009). The proliferation phase is followed by a slow involution phase of several years, leading to complete regression and replacement of tumours with fibrous and/or adipocyte-rich tissue (Mahady *et al*., 2015).

Aberrant EC proliferation leads to EC hyperplasia, but the mechanism why this happens is unknown. Two or three theories are under debate: on the one hand, the involvement of embolic placental angioblasts based on the observation that these cells and IH-cells express same placental markers, such as GLUT1 (glucose transporter-1), Lewis Y antigen, merosin and Fcγ (Queisser *et al.*, 2017). On the other hand, there is the embryonic endothelial precursor theory, as CD133+/CD34+ circulating progenitor cells as well as stem cells can be detected in haemangiomas and

blood of haemangioma patients (Yu *et al*., 2004). This theory is also supported by the observation that nude mice developed haemangioma-like lesions after injection of 'haemangioma stem cells' (Khan *et al*., 2008).

Thirdly, genetic factors may play a role. Jinnin *et al*. sequenced 24 selected genes of 9 haemangioma EC cultures and identified germ-line 'risk-factor' variants in *integrin-like molecule tumour endothelial marker-8 (TEM8)* and in *VEGFR2* (Jinnin *et al*., 2008) (**Table 1**). Overexpression of mutant TEM8 reduced VEGFR1 expression and increased VEGFR2 phosphorylation, suggesting that VEGF-A signalling is a key mechanism (Jinnin *et al*., 2008). The identified TEM8 variant may act in a dominant-negative manner, whereas the VEGFR2 variants may act as a loss-of-function mutation (Jinnin *et al*., 2008). Mutated TEM8, as well as mutated VEGFR2, sequester ß-integrin and the complex negatively regulate ß-integrin activity and thereby nuclear factor of activated T-cells (NFAT) transcriptional activity, which leads to decreased VEGFR1 expression (Jinnin *et al*., 2008). Consequently, VEGFR2 can bind more VEGF, resulting in an increase in VEGFR2 signalling and HemEC proliferation (Jinnin *et al*., 2008) (**Figure 2**).

Besides VEGFR signalling, expressions of several other factors have been associated with the proliferating phase of IH: the basic fibroblast growth factor, insulin-like growth factor, matrix metalloproteinase 9 and the receptor tyrosine kinases TIE2 and TIE1 with the ligand angiopoietin-2 (Tucci *et al*., 2009; Uebelhoer *et al*., 2012). In cases of familial haemangiomas ($n = 6$), the transmission of IH seemed autosomal-dominant with incomplete penetrance. Three out of the six families showed linkage on chromosome 5q31-33 (Buckmiller *et al*., 2010), which includes *VEGFR3*, *PDGFR-ß* and *FGFR4* (**Table 1**). Genetic variants were detected in them, although without clear causality (Buckmiller *et al*., 2010).

Congenital haemangioma

Congenital haemangiomas are rare, and they are fully grown at birth as they arise and proliferate *in utero* (Boon *et al*., 1996; Mahady *et al*., 2015; Nasseri *et al*., 2014). Three types – which do not express the GLUT1 protein (Mahady *et al*., 2015) – are known: rapidly involuting congenital haemangioma (RICH), partially involuting congenital haemangioma (PICH) and noninvoluting congenital haemangioma (NICH) (Nasseri *et al*., 2014). In comparison to NICH, which remains unchanged throughout childhood with persistent fast-flow, RICH undergoes rapid regression within the first 6–14 months, whereas PICH involutes only partially (Ayturk *et al*., 2016; Nasseri *et al*., 2014).

As in more than 80% of uveal melanomas, mutually exclusive, mosaic missense mutations in *GNAQ* and *GNA11* at position glutamine 209 have been identified in congenital haemangiomas (Ayturk *et al*., 2016; Van Raamsdonk *et al*., 2009) (**Table 1**). *GNAQ* encodes the guanine nucleotide binding protein G(q) alpha subunit that hydrolyses GTP (guanosine triphosphate) to GDP (guanosine diphosphate) (Ayturk *et al*., 2016). In uveal melanomas, the above-described missense mutations are able to activate GTP-dependent signalling, which leads to constitutive activation of mitogen-activated protein kinase (MAPK) and/or YAP signalling pathways, suggesting that these pathways are involved in the development of congenital haemangiomas (Ayturk *et al*., 2016) (**Figure 3a**).

Pyogenic granuloma (PG)

Pyogenic granuloma (PG) is one of the most common benign vascular tumours, which appears as a rapidly growing angiomatous papule or polyp. It often occurs within a capillary malformation (CM), which results from an activating somatic GNAQ p.Arg183Gln mutation (secondary PG) (Groesser *et al*., 2016; Mulliken *et al*., 2013) (**Table 1**). This observation suggests that the origin of PG cells is in the underlying CM (Groesser *et al*., 2016). Furthermore, in secondary PGs, a somatic p.Val600Glu mutation in BRAF ($n = 8$) or p.Gln61Arg mutation in NRAS ($n = 1$) was detected (Groesser *et al*., 2016). In 25 isolated PGs (without underlying CM), the BRAF p.Val600Glu ($n = 3$), p.Gly464Glu mutation ($n = 1$) or KRAS p.Gly13Arg mutation ($n = 1$) was identified (Groesser *et al*., 2016) (**Table 1**). Thus, the BRAF p.Val600Glu mutation seems a 'driver' in isolated PGs, but only a 'second hit' on a GNAQ p.Arg183Gln mutated CM-background in secondary PGs (Groesser *et al*., 2016). *HRAS* mutations, which are commonly seen in colon cancers (p.Q61R, p.E49K, p.Q61R and p.G13S), seem to be important in PGs too (Lim *et al*., 2015) (**Table 1**). As BRAF, NRAS, KRAS and HRAS are involved in RAS/MAPK signalling, this pathway should play a major role in the development of PGs (**Figure 3a**).

Vascular Malformations

Capillary malformations

Capillary malformation (CM) or 'port-wine stain'

CMs or 'port-wine stains' are the most common cutaneous vascular malformations; about 0.3% of newborns are affected (Jacobs and Walton, 1976; Uebelhoer *et al*., 2012). CMs are flat, red-to-purple coloured lesions which appear sporadically (Uebelhoer *et al*., 2012) (**Figure 1b**). Abnormally dilated capillary-like vessels can be detected in the superficial dermis of the skin or mucosa (Tucci *et al*., 2009; Uebelhoer *et al*., 2012).

CMs as well as Sturge–Weber syndrome are caused by somatic activating p.Arg183Gln GNAQ mutations (Shirley *et al*., 2013) (**Table 1**). Expression of GNAQ p.Arg183Gln or p.Gln209Leu – which is also detected in congenital haemangiomas (Ayturk *et al*., 2016) – in HEK293T cells induced extracellular-signal regulated kinases (ERK) activation (**Figure 3a**), although with a more moderate effect for pArg183Gln (Shirley *et al*., 2013).

Capillary malformation – arteriovenous malformation 1 (CM-AVM1)

A distinct subentity of CMs, called CM-AVM, associates CMs with arteriovenous malformations (AVMs). The hallmarks of CM-AVM are small, multifocal, randomly distributed, red-to-brownish CMs, which are often surrounded by a pale

Table 1 Loci, genes and signalling pathways involved in vascular tumours and malformations

Malformation	Locus	Mutated gene	Type of mutation	Signalling pathway
Haemangioma				
Congenital haemangioma (NICH, RICH, PICH)	*9q21.2*	*GNAQ, GNA11*	Activating somatic missense mutations	MAPK and/or YAP signalling
Pyogenic granuloma (PG)	*9q21.2*	*GNAQ* (secondary PG)	Activating somatic missense mutations	RAS/MAPK/ERK signalling
	12p12.1	*KRAS*		
	7q34	*BRAF* (isolated PG)	Driver and second-hit mutation	
Infantile haemangioma (IH)	*5p31-33*	*VEGFR2* *TEM8*	Variants	Increased VEGFR2 signalling; reduced VEGFR1 signalling
	5q32	*PDGFR-ß*		
	5q35.3	*FLT4/VEGFR3*		
	5q35.2	*FGFR4*		
Capillary anomalies				
Capillary malformation (CM)/Sturge–Weber syndrome	*9q21.2*	*GNAQ*	Activating somatic missense mutations	RAS/MAPK/ERK signalling
Capillary malformation-arteriovenous malformation 1 (CM-AVM1)	*5q13-22*	*RASA1*	Loss-of-function mutations	RAS/MAPK/ERK signalling FAK signalling
Capillary malformation-arteriovenous malformation 2 (CM-AVM2)	*7q22.1*	*EPHB4*	Loss-of-function mutations	RAS/MAPK/ERK signalling
Arteriovenous anomalies				
Sporadic extracranial arteriovenous malformation	*15q22.31*	*MAP2K1*	Activating somatic missense mutations	RAS/MAPK/ERK signalling
Hereditary haemorrhagic telangiectasia (HHT)	*9q33-34*	*ENG*	All loss-of-function mutations	BMP9/10/ALK signalling
	12q11-14	*ALK1*		
	5q31.3-32	*HHT3*		PI3K/AKT/mTOR signalling
	7p14	*HHT4*		
	10q11.22	*GDF2* or *BMP9*		
Juvenile polyposis HHT	*18q21.1*	*SMAD4*		
PTEN hamartoma tumour syndrome (PHTS)	*10q23*	*PTEN*	Loss-of-function mutations	PI3K/AKT/mTOR signalling
Cerebral cavernous malformations				
Cerebral cavernous malformation (CCM)	*7q11-22*	*KRIT1*	Loss-of-function mutations	ß-Integrin signalling?
	7q13	*Malcavernin*		p38MAPK signalling
	3q26.1	*PDCD10*		Notch signalling
	3q26.3-27.2	*CCM4*		
Venous anomalies				
Venous malformation (VM)	*9p21*	*TEK* (common: L914F)	Somatic activating mutations	PI3K/AKT/mTOR signalling
	3q26.32	*PIK3CA*		RAS/MAPK/ERK signalling?
Multifocal venous malformation (MVM)	*9p21*	*TEK* (double mutations; common: Y897C-R915C)	Mosaic/somatic activating mutations	PI3K/AKT/mTOR signalling RAS/MAPK/ERK signalling?

Table 1 (*continued*)

Malformation	Locus	Mutated gene	Type of mutation	Signalling pathway
Inherited cutaneomucosal venous malformation (VMCM)	*9p21*	*TEK* (common: R849W)	Somatic activating mutations (weak TIE2 phosphorylation)	PI3K/AKT/mTOR signalling RAS/MAPK/ERK signalling?
Blue rubber bleb nevus syndrome (BRBN)	*9p21*	*TEK* (double mutations; common: T1105N-T1106P and Y897F-R915L)	Somatic activating mutations	PI3K/AKT/mTOR signalling
Hyperkeratotic cutaneous capillary-venous malformation (HCCVM)	*7q11-22*	*KRIT1*	Loss-of-function mutation	p38MAPK signalling ?
Verrucous venous malformation (VVM)	*17q23.3*	*MAP3K3*	Somatic activating mutations	MAP3K3/ERK signalling?
Glomuvenous malformation (GVM)	*1p21-22*	*Glomulin*	Loss-of-function mutations	HGF/c-Met signalling with PI3K downstream target p70S6K Role in ubiquitination of proteins TGF-ß signalling?
Lymphatic anomalies				
Lymphatic malformation (LM)	*3q26.32*	*PIK3CA*	Somatic activating mutation	PI3K/AKT/mTOR signalling
Hereditary primary congenital lymphedema (HPCL) or	*5q35.3*	*VEGFR3/FLT4* *VEGFC*	Missense mutations Missense mutations	Diminished VEGFR3 signalling
Nonne-Milroy disease	*1q42.13*	*GJC2*		Regulations of gap junctions
Late-onset lymphedema and lymphedema-distichiasis (LD)	*16q24.3*	*FOXC2*	Mutations outside of the forkhead domain cause gain-of-function Mutations in the forkhead domain cause loss-of-function	Transcription factor
Hypotrichosis-lymphedema-telangiectasia syndrome (HTLS)	*20q13.33*	*SOX18*	Missense mutation in HMG box or nonsense mutations	Transcription factor; target is PROX1
Hennekam syndrome	*18q21.32* *4q28.1* *4q13.3*	*CCBE1* *FAT4* *ADAMTS3*	Loss-of-function mutations	
Lymphedema-cholestasis syndrome (LCS)	*15q?*	*?*	?	?
Osteopetrosis lymphedema-anhydrotic ectodermal dysplasia-immunodeficiency syndrome (OL-EDA-ID syndrome)	*Xq28*	*IKBKG (NEMO)*	Hypomorphic mutations	Impaired NF-κB signalling

?, not known yet.

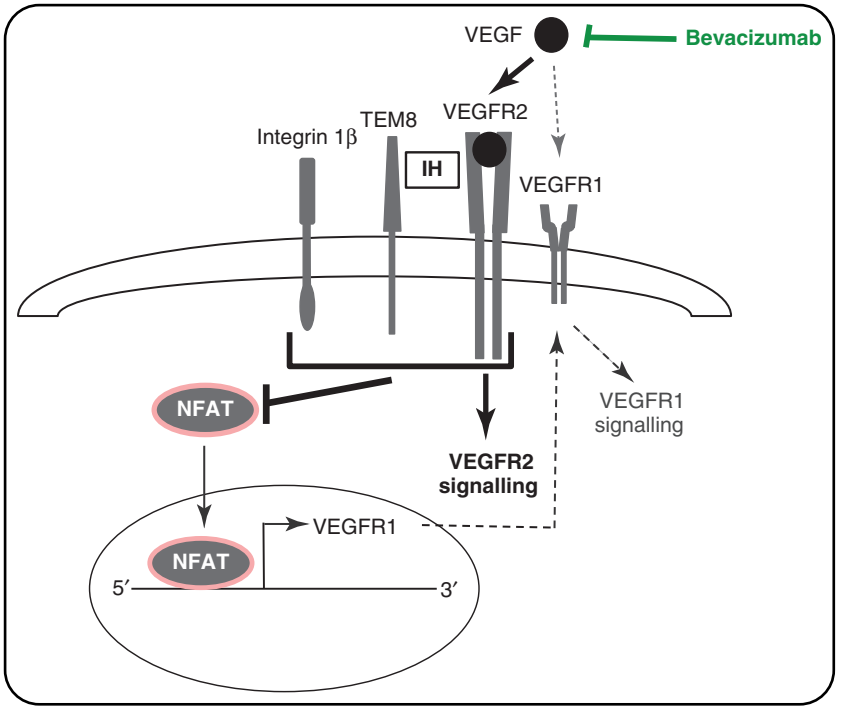

Figure 2 VEGFR2 signalling involved in IH. Variants in *TEM8* and *VEGFR2* lead to enhanced VEGFR2 and to reduced VEGFR1 signalling. A possible inhibitor is bevacizumab targeting VEGF. Grey, circled with light red: decreased signalling.

halo (Uebelhoer *et al*., 2012) (**Figure 1c**). **See also**: **Capillary Malformation-Arteriovenous Malformation and RASA1 Mutations**. They are often, but not always, combined with fast-flow lesions, such as arteriovenous fistula (AVF), AVM (**Figure 1d** and **e**) or Parkes Weber syndrome (Uebelhoer *et al*., 2012). Patients with CM-AVM usually have lesions already at birth, although additional lesions can appear with time. The lesion size varies from a few millimetres to several centimetres in diameter (Eerola *et al.*, 2003).

Autosomal-dominant inheritance of CM-AVM allowed to identify *RASA1* as the mutated gene (Uebelhoer *et al*., 2012; Eerola *et al.*, 2003) (**Table 1**). CM-AVMs due to a *RASA1* mutation are called CM-AVM1 (Queisser *et al.*, 2017). Until now, at least 100 CM-AVM families have been reported with more than 40 truncating RASA1 mutations (Eerola *et al*., 2003; Uebelhoer *et al*., 2012). Moreover, a somatic second-hit mutation on the second allele of RASA1 is leading to complete loss of *RASA1* has been shown twice (Macmurdo *et al*., 2016). This explains the phenotypic heterogeneity and reduced penetrance (98%) (Uebelhoer *et al*., 2012).

RASA1 codes for RAS p21 protein activator 1 (p120RasGAP), which negatively regulates the RAS/MAPK/ERK signalling pathway by enhancing the weak intrinsic GTPase activity of RAS. Following receptor tyrosine kinase activation, p120RasGAP is recruited to the membrane where it participates in the regulation of cellular processes such as cell growth, differentiation, proliferation and EC network organisation (Queisser *et al.*, 2017). Furthermore, p120RasGAP interacts with p190RhoGAP or FAK, both involved in EC movement (Uebelhoer *et al*., 2012). Hence, prolonged RAS/MAPK/ERK signalling underlies CM-AVM1 (**Figure 3a**).

Capillary malformation – arteriovenous malformation 2 (CM-AVM2)

CM-AVM2 is a second subentity, which slightly differs from CM-AVM1 (Amyere *et al*., 2017). In addition to the classic CM-AVM lesions, CM-AVM2 patients have small telangiectasias around the lips and upper thorax. They also encounter less frequently intracerebral fast-flow lesions. CM-AVM2 is caused by loss-of-function mutations in *EPHB4* (Amyere *et al*., 2017) (**Table 1**).

EPHB4 is a transmembrane receptor on the surface of venous ECs during vascular development (Wang *et al*., 1998) and its ligand EphrinB2 – also a transmembrane protein – is detectable on arterial ECs (Xiao *et al*., 2012). EPHB4 is involved in repressing the RAS/MAPK/ERK signalling pathway through interaction with p120RasGAP (Xiao *et al*., 2012) (**Figure 3a**). In summary, both loss-of-function of p120RasGAP (CM-AVM1) and EPHB4 (CM-AVM2) lead to activation of RAS and the MAPK/ERK pathway (Amyere *et al*., 2017).

Arteriovenous malformations (AVMs)

Arteriovenous malformation (AVM)

AVMs are the most devastating vascular anomalies, as they often destruct adjacent noble structures as they develop (**Figure 1d**

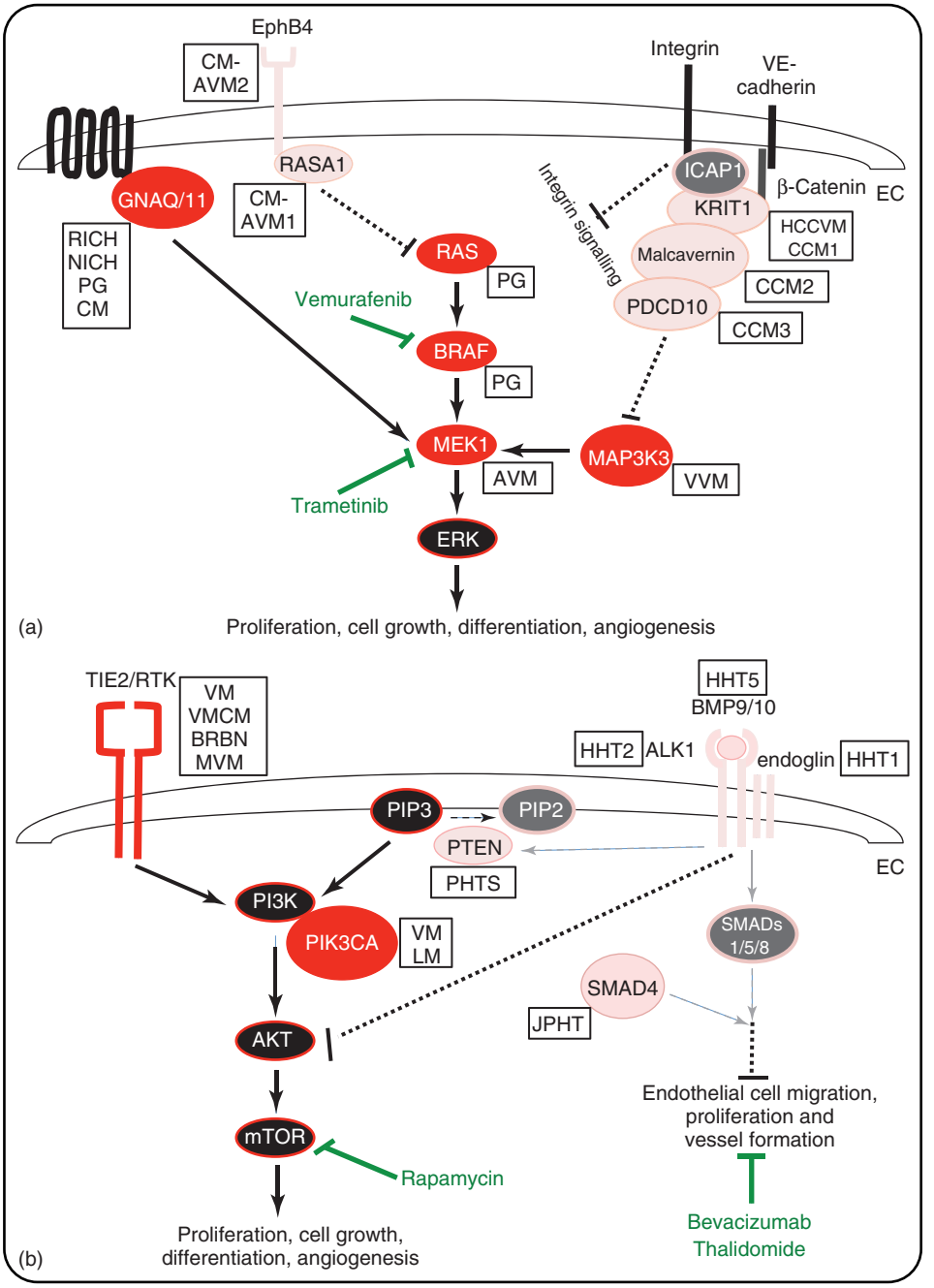

Figure 3 RAS/MAPK/ERK and PI3K/AKT/mTOR signalling involved in vascular tumours and vascular malformations. (a) Mutations in *GNAQ/11*, *RASA1*, *EPHB4*, *TIE2*, *MAP3K3*, *KRIT1*, *malcavernin*, *PDCD10*, *RAS*, *BRAF* and *MAP2K1* lead to the activation of RAS/MAPK/ERK signalling. Inhibitors Vemurafenib and Trametinib target BRAF and MEK, respectively. (b) Mutations in *TIE2*, *PIK3CA*, *PTEN*, *ALK1*, *BMP9/10* and *endoglin* activate PI3K/AKT/mTOR signalling. Inhibitor rapamycin targets mTOR. *ALK1*, *BMP9/10*, *endoglin* and *SMAD4* downregulate BMP9/10 signalling, leading to an enhanced vessel formation. Thalidomide could be utilised. Red: gain-of-function; light red: loss-of-function; black, circled with red: enhanced signalling; grey, circled with light red: decreased signalling; EC: endothelial cell.

and **e**). Treatment by embolisation associated or not with surgery should aim at removing the lesion, which oftentimes is not possible. Partial resection commonly leads to aggravation of the lesion. That is why cautiousness prevailed, whereas current thinking aims at earlier treatment before the lesions develop further.

Sporadically occurring AVMs can be found in any organ, for example the central nervous system, visceral organs or peripheral structures. Somatic activating MAP2K1 mutations – encoding the dual specificity mitogen-activated protein kinase MEK1 – were identified in a series of peripheral/extracranial AVMs (Couto

et al., 2017). These activate the RAS-MAPK signalling pathway and suggest proliferation of ECs to be an important aetiological factor (**Figure 3a**). Moreover, with other studies, this pinpoints RAS-MAPK pathway inhibitors as eventual molecular additions to the therapy of AVMs.

Capillary malformation-arteriovenous malformation (CM-AVM)

As discussed in the article on capillary malformations, arteriovenous malformations occur frequently in CM-AVM. Please see the earlier discussion.

Hereditary haemorrhagic telangiectasia (HHT)

Similar to CM-AVM (see the earlier discussion), HHT (hereditary haemorrhagic telangiectasia) or Osler–Weber–Rendu syndrome is characterised by multiple cutaneous lesions (telangiectasias) often associated with internal AVMs. HHT is an autosomal-dominant disorder in which the skin, mucosal surfaces and internal organs are involved (Chung, 2015). Patients have recurrent nosebleeds, which start in one-third of patients before the age of 10 years, commonly resulting in anaemia. At a later age, characteristic cutaneous telangiectasias occur, involving the face, oral mucosa and hands (Chung, 2015).

Five loci are linked to HHT (Queisser *et al.*, 2017; Uebelhoer *et al*., 2012). Loss-of-function mutations in *endoglin* (*ENG*) and in *activin receptor-like kinase 1* (*ALK1*) result in HHT1 and HHT2, respectively (Queisser *et al.*, 2017). Juvenile polyposis/HHT syndrome (JPHT) is caused by loss-of-function mutations in *MADH4,* which encodes SMAD4 (Gallione *et al*., 2004). Additional loci were identified on chromosomes *5q31.3-32* (HHT3) and on *7p14* (HHT4) (Queisser *et al.*, 2017). Some variants in the ALK-ligand growth/differentiation factor-2 (GDF2) or bone morphogenetic protein-9 (BMP9) have also been detected (Shovlin, 2010) (**Table 1**).

All the above-described genes encode proteins that are part of the bone morphogenetic protein (BMP) signalling pathway (Ola *et al*., 2016). On EC membranes, ALK1 and endoglin form a receptor complex after ligand binding, whereby SMAD1/5/8 is phosphorylated and makes a complex with the transcription factor SMAD4. The latter complex is able to suppress EC migration and proliferation, and thus to maintain the quiescent endothelial state (Tillet and Bailly, 2014) (**Figure 3b**). In *Alk* knockout mouse lung endothelial cells (mLECs), as well as in HUVECs with an *Alk1* knockdown or BMP9/10 ligand blockade, an enhanced VEGF and AKT (protein kinase B) signalling was detected owing to loss of PTEN (phosphatase and tensin homologue) activation. Increased carboxy-terminal PTEN phosphorylation and nuclear localisation (instead of cell membranous localisation) was observed (Ola *et al*., 2016) (**Figure 3b**).

PTEN hamartoma tumour syndrome (PHTS)

PHTS (PTEN hamartoma tumour syndrome) includes a spectrum of disorders linked to loss-of-function mutations in the tumour suppressor gene *PTEN* (Delannoy *et al*., 2017) (**Table 1**). **See also**: **Genetics of *PTEN* Hamartoma Tumour Syndrome**

PHTS is inherited in an autosomal-dominant manner (Delannoy *et al*., 2017). The Cowden and the Bannayan–Riley–Ruvalcaba syndrome are part of the clinical spectrum. The patients classically have macrocephaly, lipomas and papilledema, associated with nonpathognomic cutaneous vascular lesions and a predisposition to cancer. They develop intramuscular and multifocal vascular tumours. Histologically, lesions are often associated with ectopic fat and disruption of tissular architecture (Tan *et al*., 2007). Some of these are fast-flow lesions, similar to classic AVMs. PTEN inhibits phosphoinositide 3-kinase (PI3K) signalling. It converts phosphatidylinositol (3,4,5)-triphosphate (PIP3) into phosphatidylinositol (4,5)-bisphosphate (PIP2) (Delannoy *et al*., 2017). Therefore, loss of PTEN leads to constitutive activation of PI3K/AKT/mTOR signalling (**Figure 3b**), and mTOR (mammalian target of rapamycin) inhibitors are tested as a molecular therapy.

Cerebral cavernous malformations (CCM)

Cerebral cavernous malformations (CCMs) are mainly found in the brain (**Figure 1f**), but also occasionally in the spinal cord. They are comprised of slow-flow dilated capillaries surrounded by a defective support cell layer. This sometimes leads to vascular leakage (Uebelhoer *et al*., 2012). CCMs can either be inherited in an autosomal-dominant manner with incomplete penetrance or occur sporadically (Uebelhoer *et al*., 2012). **See also**: **Molecular Genetics of Familial Cerebral Cavernous Malformations**

Loss-of-function mutations in three genes lead to the formation of CCMs: *Krev interaction trapped 1* (*KRIT1* or *CCM1*), *cerebral cavernous malformation 2 (CCM2)* or *malcavernin* and *programed cell death protein 10* (*PDCD10* or *CCM3*) (Uebelhoer *et al*., 2012). A fourth locus has been suggested: *CCM4* on *3q26.3-27.2* (Uebelhoer *et al*., 2012) (**Table 1**).

The CCM proteins build a complex in which CCM2 acts as linker between KRIT1 and PDCD10 (Uebelhoer *et al*., 2012). In addition, KRIT1 interacts with the integrin cytoplasmic-associated protein-1 (ICAP1), which is a suppressor of integrin activation (Liu *et al*., 2013). Loss of KRIT1 or CCM2 leads to destabilisation of ICAP1 and increased integrin activation, disrupting normal tissular development (Faurobert *et al*., 2013) (**Figure 3a**). CCM1 is also involved in DLL4-NOTCH signalling, which leads to activation of AKT and diminution of ERK activity. This may explain why patients with loss-of-function mutations in CCM1 show elevated levels of phospho-ERK (Wustehube *et al*., 2010). Moreover, the CCM1/CCM2/CCM3 complex interacts with MAP3K3 (MEKK3) and the small GTPase RAC1 (Uhlik *et al*., 2003). Loss of the CCM complex leads to activated MAP3K3 signalling and the expression of its target genes *KLF2, KLF4, RHO* and *ADAMTS* (Zhou *et al*., 2016) (**Figure 3a**). These clues to pathogenesis have pinpointed targets for development of molecular therapeutics for CCM management.

Venous malformations (VMs)

VMs (venous malformation) are slow-flow lesions often located on the skin or mucosa. They are soft, compressible and blue

coloured (Natynki *et al*., 2015) (**Figure 1h**). Histologically, enlarged vein-like channels are detectable surrounded by a single layer of ECs with disorganised smooth muscle cells.

Sporadic venous malformation (sporadic VM) and multifocal venous malformation (MVM)

Somatic activating mutations in the endothelial receptor tyrosine kinase TIE2 – encoded by the *TEK* gene – are responsible for sporadic VM and MVM (multifocal venous malformation) (Limaye *et al*., 2009; Soblet *et al*., 2017; Uebelhoer *et al*., 2012) (**Table 1**). In sporadic VMs, the most frequent mutation is L914F, which is located in the intracellular tyrosine kinase (Limaye *et al*., 2009). Moreover, a series of double *cis*-mutations could be detected. *In vitro*, all of these mutations lead to ligand-independent hyperphosphorylation of TIE2. A stronger TIE2 phosphorylation could be seen in double mutations compared to single ones (Limaye *et al*., 2009). Some patients show multiple lesions (MVM) and they are prone to be mosaic for a first mutation. A second-hit mutation is detectable in lesional areas with the typical combination of Y897C-R915C (Soblet *et al*., 2017) (**Table 1**).

TIE2 has three known ligands: angiopoietin 1 (ANGPT1), angiopoietin 2 (ANGPT2) and angiopoietin 4 (ANGPT4) (Uebelhoer *et al*., 2012). ANGPT1 activates TIE2 and leads to receptor phosphorylation, whereas ANGPT2 modulates TIE2 activity context-dependently (Uebelhoer *et al*., 2012). Ligand binding results in multimerisation of TIE2 and their cross-phosphorylation. This leads to activation of the canonical PI3K/AKT/mTOR pathway (Uebelhoer *et al*., 2012) (**Figure 3b**). The importance of this pathway in VM-pathogenesis is underscored by the identification of mutations in *PIK3CA* in half of VM patients that lack a TIE2 mutation (**Table 1**). *PIK3CA* which encodes the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (p110alpha) is part of the PI3K complex (Boscolo *et al*., 2015a; Limaye *et al*., 2015; Natynki *et al*., 2015). Overexpression of mutant PIK3CA variants leads to activation of AKT and disruption of the EC monolayer as well as to loss of extracellular matrix fibronectin. Moreover, ANGPT2 and PDGF-ß are downregulated (Limaye *et al*., 2015).

Inherited cutaneomucosal venous malformations (VMCM)

Inherited cutaneomucosal venous malformations (VMCM) is transmitted through autosomal-dominant inheritance (Boon and Vikkula, 1993). As in sporadic VM and MVM, the causative mutations are found in TIE2. However, usually the positions are different, the most common being at position R849W (**Table 1**, **Figure 3b**). This mutation results in only weak ligand-independent TIE2 phosphorylation (**Figure 3b**). To develop the typical multifocal and small-sized VMCM lesions, a somatic second hit is needed (Limaye *et al*., 2009). The second hits vary between lesions even between lesions of a single patient.

Blue rubber bleb nevus syndrome (BRBN)

Blue rubber bleb nevus syndrome (BRBN) occurs sporadically and is characterised by a dominant venous lesion at birth and development of smaller gastrointestinal and palmoplantar lesions by age. Anaemia is also frequent, mostly thought to be due to GI-bleeding. In BRBN, *TEK* mutations also play an important role and lead to the activation of the receptor independently of the ligand (Soblet *et al*., 2017). Characteristic for BRBN is the occurrence of somatic *TEK* double mutations such as T1105N-T1106P and Y897F-R915L. Similar to other TIE2 mutations, they also activate the PI3K/AKT/mTOR pathway (Soblet *et al*., 2017) (**Table 1** and **Figure 3b**). Interestingly, BRBN patient are not mosaic (at least not at the level of their blood), and yet in distant lesions the same double mutations are identified. This has generated the hypothesis that temporarily restricted circulating cells could be the reason of novel lesions.

Hyperkeratotic cutaneous capillary-venous malformation (HCCVM)

Hyperkeratotic cutaneous capillary-venous malformations (HCCVMs) are crimson coloured and have an irregular shape. They can grow up to several centimetres and infiltrate dermis and hypodermis (Eerola *et al*., 2000). HCCVMs contain dilated capillaries and venous-like channels filled with blood. The epidermis that covers the lesion is often hyperkeratotic (Eerola *et al*., 2000). HCCVMs occur in association with inherited CCM (Eerola *et al*., 2000). These patients have germ-line loss-of-function mutations in *KRIT1* (Eerola *et al*., 2000) (see also section titled 'Cerebral Cavernous Malformations (CCM)) (**Table 1** and **Figure 3a**).

Verrucous venous malformation (VVM)

Verrucous venous malformation (VVM or verrucous haemangioma) is another nonhereditary, congenital disease (Couto *et al*., 2015). Lesions are raised, deep red and become hyperkeratotic with time (Couto *et al*., 2015). Histologically, lesions show verrucous hypoplasia of the epidermis with characteristic dilated capillaries and venules. Whole-exome sequencing identified somatic mutations in *MAP3K3* (Couto *et al*., 2015) (**Table 1** and **Figure 3a**). The mutations activate MAP3K3, which belongs to the MAP3K family of serine/threonine kinases. MAP3K3 seems to be downstream of angiopoietin-1/TIE2 signalling pathway and also involved in CCM signalling (Couto *et al*., 2015; Zhou *et al*., 2016).

Glomuvenous malformations (GVMs)

Glomuvenous malformation (GVM) links dysfunction of vascular smooth muscle cell (vSMC) development with vascular malformations (Uebelhoer *et al*., 2012). GVMs are autosomal-dominantly inherited cutaneous vascular lesions, which are bluish-purple in colour (**Figure 1g**) and mostly located on the extremities (Uebelhoer *et al*., 2012). Lesions are multifocal and painful on touching, and compression does not lead to emptying of lesions in contrast to venous malformations (Uebelhoer *et al*., 2012). Histologically, dilated veins are lined by normal looking ECs and abnormally differentiated vSMCs, called 'glomus cells' (Uebelhoer *et al*., 2012).

GVMs are caused by inherited loss-of-function mutations in *glomulin* (GLMN/FAP68) (Uebelhoer *et al*., 2012) (**Table 1**). Up to date, more than 40 different *glomulin* mutations have been

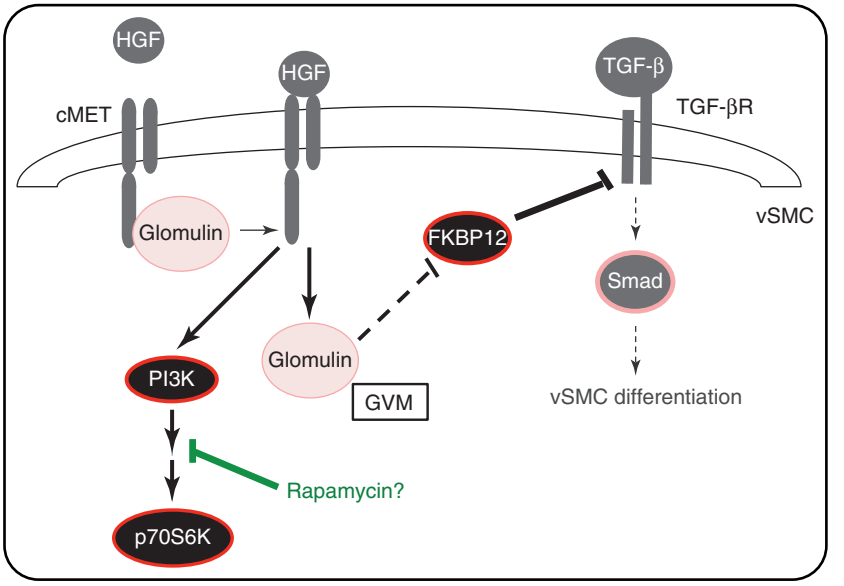

Figure 4 HGF and TGF-ß signalling in GVM. Mutations in *glomulin* may lead to enhanced PI3K signalling as well as to a downregulated TGF-ß/Smad signalling. Light red: loss-of-function; black, circled with red: enhanced signalling; grey, circled with light red: decreased signalling; vSMC: vascular smooth muscle cell.

described (Queisser *et al.*, 2017). As for VMCM, somatic second hits are needed for lesions to develop. The most common somatic second hit in GVMs is acquired uniparental isodisomy of the long arm of chromosome 1, where glomulin is located, rendering cells in the lesion homozygous for the inherited mutation (Amyere *et al*., 2013). This ensues complete localised loss of glomulin.

Glomulin seems to be expressed in both ECs and vSMCs. Glomulin seems to be involved in hepatocyte growth factor (HGF)/cMet signalling. When HGF binds to the unphosphorylated c-Met receptor, the subsequent cMet phosphorylation leads to release of glomulin. This results in activation of PI3K signalling and its downstream targets including p70S6K (Uebelhoer *et al*., 2012; Queisser *et al.*, 2017) (**Figure 4**). Furthermore, Glomulin seems to play a role in protein degradation via ubiquitination via Cul7 to form the Skp1-Cul7-Fbox-like complex. This complex is important for ubiquitination of various proteins (Queisser *et al.*, 2017).

Glomulin seems to be involved in vSMC differentiation as well (Uebelhoer *et al*., 2012). Inhibition of TGF-ß (transforming growth factor beta) signalling is mediated by binding of FK506 binding protein 12 (FKBP12) to the TGF-ß type I receptor (TßRI) (Uebelhoer *et al*., 2012). Glomulin interacts with FKBP12, and when glomulin is inactivated by a mutation, FKBP12 is able to bind TßRI, thus inhibiting TGF-ß signalling and differentiation of vSMCs. This may elevate mTOR activity (Uebelhoer *et al*., 2012) (**Figure 4**).

Lymphatic anomalies

The lymphatic system has two categories of pathologies considered under vascular anomalies: lymphatic malformations (LMs) and lymphedema. The latter are divided into primary and secondary lymphedemas. They are characterised by swelling, mostly commonly of the lower extremities (Brouillard *et al*., 2014). Overall, mutations in at least 23 genes are known to explain various syndromic and isolated forms of primary lymphedema (Brouillard *et al*., 2014). Only some forms are discussed here.

Lymphatic malformation (LM)

LMs are normally present at birth. They are focal lesions, just like venous malformations, but it is the lymphatic vessels that are dilated and filled with lymph (Uebelhoer *et al*., 2012) (**Figure 1i**). There is no evidence for inheritance supporting the theory that somatic mutations are the cause of these lesions. In fact, somatic activating mutations in *PIK3CA* – also seen in VMs – are a common cause of LMs (Boscolo *et al*., 2015a; Osborn *et al*., 2015) (**Table 1**). Mutated PIC3CA activates the AKT/mTOR pathway, which regulates proliferation, as well as cell growth and migration (Boscolo *et al*., 2015a; Osborn *et al*., 2015) (**Figure 3b**). As for VMs, rapamycin, an mTOR inhibitor, is in clinical trials as a novel molecular therapy.

Hereditary primary congenital lymphedema (HPCL)

Hereditary primary congenital lymphedema (HPCL) or Nonne-Milroy disease is an autosomal-dominantly transmitted disease with an incomplete penetrance. It is usually present at birth and sometimes even detected *in utero* (Uebelhoer *et al*., 2012). Unilateral or bilateral lymphedema occurs most commonly on toes and feet with extension towards the limbs. Patients often have prominent veins on legs, cellulites, upturned toenails

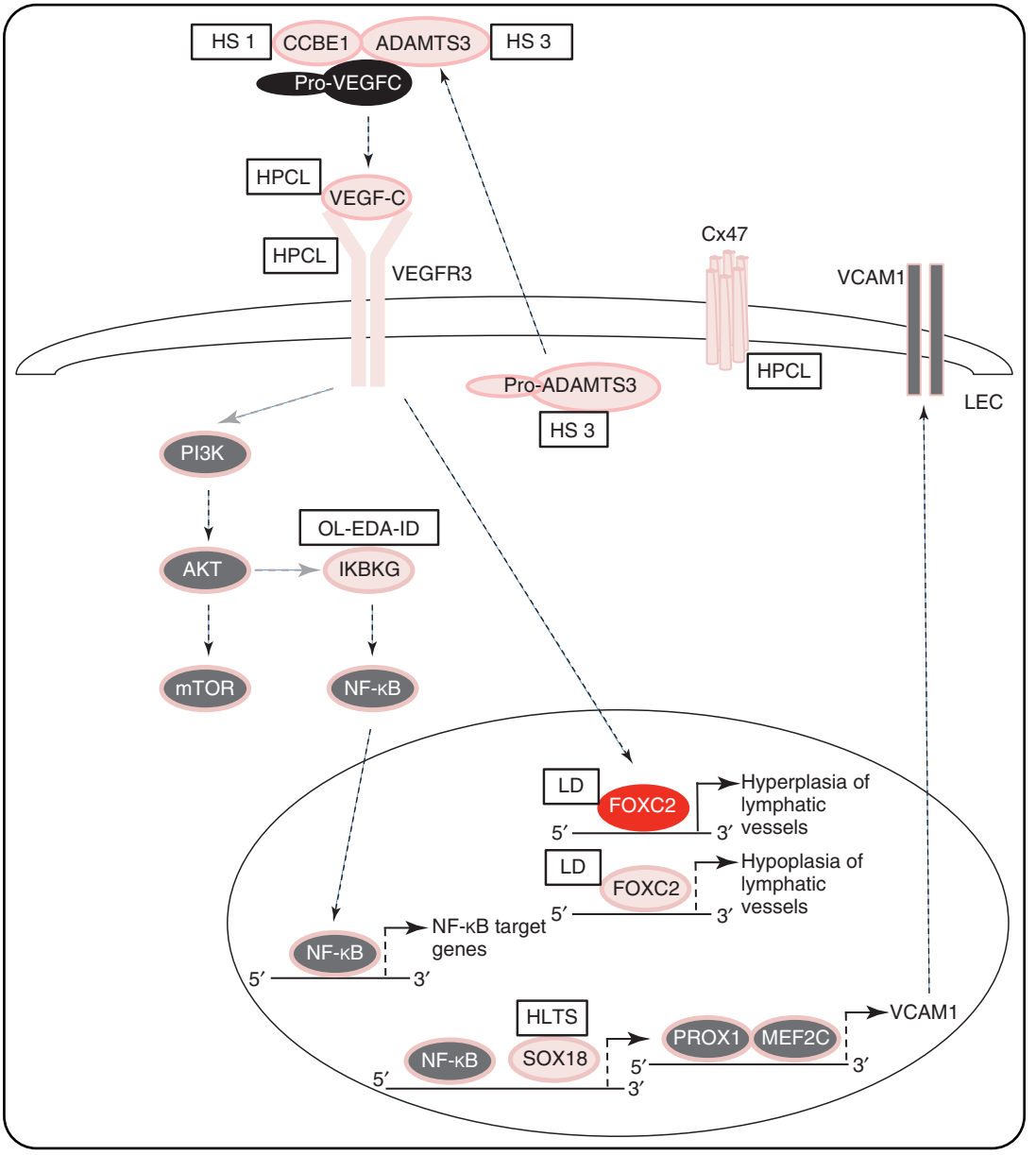

Figure 5 VEGFR3 signalling involved in different types of lymphedemas. Mutations in *VEGFR3*, *ADAMTS3*, *CCBE1*, *IKBKG*, *SOX18* and *FOXC2* lead to changes in signalling in LECs. Mutations in Cx47 may lead to changes in gap junctions and to disruption of lymphatic flow. Red: gain-of-function; light red: loss-of-function; grey, circled with light red: decreased signalling; LEC: lymphatic endothelial cell.

and papillomatosis. Male patients may have hydrocele, and sometimes hydrops fetalis is seen (Uebelhoer *et al*., 2012).

Lymphangiogenesis is controlled by vascular endothelial growth factor C (VEGFC)/vascular endothelial growth factor receptor 3 (VEGFR3) signalling (Brouillard *et al*., 2017). This pathway is also implicated in the aetiopathogenesis of primary lymphedema, as HPCL is caused by dominantly inherited missense mutations in *VEGFR3 (FLT4)* (**Table 1**). *De novo* mutations in *VEGFR3* cause sporadically occurring neonatal primary lymphedema (Uebelhoer *et al*., 2012). All mutations inhibit the phosphorylation of the receptor and downstream signalling (Uebelhoer *et al*., 2012) (**Figure 5**). Furthermore, in one family with congenital primary lymphedema, a homozygous recessive mutation in *VEGFR3* was identified (Brouillard *et al*., 2014).

Dominantly inherited mutations have also been seen in the ligand-encoding gene VEGFC in two families. Both were loss-of-function mutations (Gordon *et al*., 2013) (**Table 1** and **Figure 5**). In six other families, missense mutations in *GJC2* were identified (**Table 1**). *GJC2* encodes connexin 47 (Cx47), a

gap junction protein, important at least for the development of lymphatic valves (Uebelhoer *et al*., 2012) (**Figure 5**).

Late-onset lymphedema and lymphedema distichiasis (LD)

Late-onset lymphedema, type II lymphedema or Meige disease, as well as LD (lymphedema distichiasis) usually appear around puberty (Uebelhoer *et al*., 2012). This type of primary lymphedema associated with distichiasis is caused by dominant mutations in *FOXC2* (Brouillard *et al*., 2014; Uebelhoer *et al*., 2012) (**Table 1**). FOXC2 is a transcription factor, which belongs to the forkhead/winged-helix family involved in lymphatic EC differentiation and morphogenesis of lymphatic vessels. It is key for the formation of smooth muscle cell layers (Tavian *et al*., 2016). Loss of *FOXC2* results in overproduction of PDGF-ß and an increased recruitment of SMCs in lymphatic walls (Uebelhoer *et al*., 2012).

Up to date, dozens of different mutations in *FOXC2* have been described, the majority being small insertions, deletions or nonsense mutations that cause truncated proteins and likely haploinsufficiency (Brouillard *et al*., 2014). Moreover, missense mutations in the forkhead domain lead to an impaired binding of FOXC2 to DNA (deoxyribonucleic acid) and decreased transcription of target genes (Berry *et al*., 2005). Some missense mutations, which are located outside the forkhead domain, have been suggested to cause gain-of function effects (Tavian *et al*., 2016). The authors thus concluded that FOXC2 mutations may lead via gain-of-function effects to hyperplasia and loss-of-function effects to hypoplasia of lymphatic vessels, and that both lead to lymphedema (**Figure 5**).

Hypotrichosis-lymphedema-telangiectasia-renal defect syndrome (HLTRS)

Hallmark of hypotrichosis-lymphedema-telangiectasia-renal defect syndrome (HLTRS) is the combination of lymphedema with sparse hairs (hypotrichosis) and cutaneous telangiectasias (Uebelhoer *et al*., 2012). It can be inherited either as an autosomal-dominant or autosomal-recessive disorder (Irrthum *et al*., 2003). It is caused by mutations in the transcription factor SOX18 (Irrthum *et al*., 2003) (**Table 1**). Missense mutations in the high mobility group (HMG)-box are autosomal-recessive while nonsense mutations leading to truncated SOX18 are autosomal-dominant (Irrthum *et al*., 2003). This may be explained by the latter having dominant-negative effects. The dominant mutations that lead to the lack of the transactivation domain are associated with renal failure necessitating transplantation (Moalem *et al*., 2015). Interestingly, in comparison to humans, *Sox18*$^{-/-}$ ragged mice had only a mild coat defect (Moalem *et al*., 2015). However, in *Sox17/Sox18* double heterozygote mice renal alterations could be also observed. This underscores likely redundancy between Sox17 and Sox18, and the fact that the human mutations generate a situation in which such redundancy may not act. This could be due to the capability of the truncated SOX18 molecules binding to promoters without activation of transcription, and concurrent blocking of the sites from other factors, such as SOX17.

SOX18 expression can be detected in ECs, in hair and in the heart. One of its target genes is PROX1, which is an important regulator of lymphatic vessel differentiation. In ECs, PROX1 interacts with MEF2C – another transcription factor – and both are involved in the regulation of the adhesion molecule VCAM1, which has an important role in blood vascular and lymphatic endothelia (Uebelhoer *et al*., 2012) (**Figure 5**).

Hennekam lymphangiectasia-lymphedema syndrome

Hennekam lymphangiectasia-lymphedema syndrome is associated with lymphatic anomalies affecting various organs, such as the gastrointestinal tract and pericardium, with lymphedema of the limbs, facial dysmorphism and mental retardation (Crawford *et al*., 2016). There is important variability in expressivity, and, for example intellectual disability is not always present. The syndrome follows recessive inheritance.

Approximately 25% of the patients have biallelic loss-of-function mutations in collagen and calcium-binding EGF domain-containing protein 1 (*CCBE1*). This form was named Hennekam lymphangiectasia-lymphedema syndrome 1 (Crawford *et al*., 2016) (**Table 1** and **Figure 5**). CCBE1 is a secreted protein and involved in VEGFC processing and activation (Brouillard *et al*., 2014). Biallelic mutations in *FAT4* cause the Hennekam lymphangiectasia-lymphedema syndrome 2 in another 20% of patients (Crawford *et al*., 2016) (**Table 1**). The exact mechanisms of how these mutations lead to lymphedema are not known. The third gene that is mutated in Hennekam lymphangiectasia-lymphedema syndrome 3 is *ADAMTS3* (Brouillard *et al*., 2017). ADAMTS3 and CCBE1 interact functionally and are both required to convert pro-VEGFC into active VEGFC (Brouillard *et al*., 2014). The mutant ADAMTS3 is sequestered within the cells and thus pro-VEGFC cannot be converted into active VEGFC (Brouillard *et al*., 2017) (**Figure 5**).

Lymphedema-cholestasis syndrome (LCS)

Lymphedema-cholestasis syndrome (LCS) Type 1 or Aagenaes syndrome is characterised by severe neonatal cholestasis and chronic severe lymphedema. It is transmitted as an autosomal recessive disease. A linked locus was identified on 15q, yet the mutated gene is still unknown (Bull *et al*., 2000) (**Table 1**). However, in one family with a stillborn foetus and an affected child who died, a homozygous mutation in CCBE1 was identified (**Table 1**).

Osteopetrosis lymphedema-anhydrotic ectodermal dysplasia-immunodeficiency syndrome (OLEDAID syndrome)

Osteopetrosis lymphedema-anhydrotic ectodermal dysplasia-immunodeficiency (OLEDAID) syndrome is an X-linked disease with a broad spectrum of clinical phenotypes. It is caused by hypomorphic mutations in *IKBKG (NEMO)* (Doffinger *et al*., 2001) (**Table 1**). NEMO is the regulatory subunit of the IkappaB kinase complex (IKK) and essential for NF-κB (nuclear factor kappaB) pathway (Doffinger *et al*., 2001). The NEMO mutations

cause impaired, but not abolished, NF-κB signalling (Doffinger *et al*., 2001) (**Figure 5**).

Concluding Remarks

Vascular anomalies are treated by destruction or removal of abnormal vessels using laser, sclerotherapy, embolisation and/or surgery. No cause-specific treatments are available (Mulliken *et al.*, 2013). The careful investigation of the hereditary forms of vascular anomalies and the continuously improving sequencing technologies led to the discovery of mutations in various genes. This led to unravel that activation of EC signalling pathways is responsible for most vascular malformations. Two major pathways are involved in most of the lesions: the PI3K/AKT/mTOR and the RAS/RAF/MAPK/ERK pathways. Signalling via these pathways is also often enhanced in many types of cancers leading to the idea repurpose cancer drugs for the treatment of vascular anomalies. Thus, lesions with a constitutively active PI3K/AKT/mTOR pathway, such as VMs and LMs, may benefit from the mTOR inhibitor rapamycin (**Figure 3b**). Rapamycin is now tested in clinical trials as a novel molecular therapy for various venous malformations. The Phase II trial gave encouraging results, demonstrating increased quality of life and reduction of pain in six patients (Boscolo *et al*., 2015b). A similar study on mostly lymphatic and complex malformations also demonstrated benefits for those patients (Adams *et al*., 2016). Larger phase III trial is ongoing to confirm and refine the results (EudraCT Number: 2015-001703-32).

For lesions with an enhanced RAS/RAF/MAPK/ERK pathway, drugs that interfere with this pathway such as BRAF (vemurafinib) or MEK inhibitors (trametinib) are also conceivable (**Figure 3a**) (Queisser *et al.*, 2017). Both are also used to treat metastatic melanomas harbouring a *BRAF* mutation (Welsh and Corrie, 2015).

HHT patients that show a decrease in BMP signalling, and increased angiogenesis, may benefit form bevacizumab, an anti-VEGF antiangiogenic drug (Tillet and Bailly, 2014).

Although several key mutations leading to vascular anomalies have been discovered, and the main altered signalling pathways have been unravelled, further *in vitro* and *in vivo* studies are needed to better understand the exact molecular mechanism that lead to the development of vascular anomalies, and how these pathways are affected by the introduction of molecular therapies.

Glossary

Congenital Condition that is already present at birth and that can occur de novo, inherited or because of environmental factors

De novo mutation These are also called neo-mutations, i.e. mutations that occur during the division of the cell.

Mosaic Lesions, which are caused by somatic mutations, commonly contain both normal cells (nonmutated) and mutant cells. Similarly, an individual body can contain wild-type cells, and cells that contain de novo mutations. Thus, the individual is mosaic.

Mutation Permanent change in the nucleotide sequence of the DNA, which causes a pathological phenotype.

Rapamycin (also known as sirolimus) Molecule that functions as an immunosuppressant and which also inhibits mTOR, a protein involved in cellular proliferation. It is often used in renal transplantations.

Receptor tyrosine-kinase Proteins on cell membranes that after ligand binding get phosphorylated specifically on some of their tyrosine residues enabling downstream intracellular signalling.

Second-hit mutation Somatic second-hit occurs in the tissue on the same gene that already carries an inherited mutation. Knudson hypothesised this to explain the very high likelihood of bilateral occurrence of inherited retinoblastoma.

Somatic mutation Mutations that occur locally in tissue and are thus not inherited.

Vascular malformation Disease of the vascular system affecting locally veins, arteries, capillaries or lymphatics.

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