Hereditary haemorrhagic telangiectasia: from physiopathology to future treatments

Thalgott, J.H.

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

Thalgott JH, Galaris G, Dos-Santos D and Lebrin F

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General introduction

Clinical overview of Hereditary Haemorrhagic Telangiectasia

Hereditary Haemorrhagic Telangiectasia (HHT), also known as Osler-Weber-Rendu syndrome, is an autosomal dominant inherited disease characterized by systemic angiodysplasia. Vascular malformations range from small telangiectasias, which are characterized by dilated weak-walled blood vessels that easily bleed and are located in the nasal spectrum, the oral mucosa and gastrointestinal tract, to large Arteriovenous Venous Malformations (AVMs) in the lungs, brain and liver. AVMs consist of direct connections between arterioles and venules without an intervening capillary bed (**Fig. 1**). The disease prevalence is estimated to be 1 in 5,000–8,000 affecting theoretically between 950,000 and 1,500,000 persons worldwide[1-3]. However, HHT is certainly undiagnosed since many people, as well as doctors, are not familiar with its wide range of symptoms and only around 500,000 individuals around the world are officially diagnosed with the disease. HHT can be diagnosed through DNA screening or by the Curacao Criteria, which includes four criteria[4]:

1. Spontaneous or recurrent epistaxis. It affects 95% of HHT patients and appears around the age of six and intensifying with age[5]. Epistaxis episodes occur spontaneously, irregularly, and repeatedly. Recurrent bleeding can cause asthenia, dyspnoea and anaemia, thereby impairing the quality of life[6] .
2. The presence of telangiectasias located on the lips, oral cavity, nose or fingers. Their number and size increase with age. They are present in 30% of cases before the age of 20, and in 75% of patients afterwards[5]. The lesions are described as small, ruby-coloured, non-pulsatile, and blanch under pressure. They are mainly visible on the face, lips, buccal mucosa, ears, and fingers, especially on the fingertips. In addition to causing aesthetic damage, these lesions can also bleed.
3. The presence of AVMs in gastrointestinal tract, lungs, liver, brain or spine. Visceral lesions such **as gastrointestinal telangiectasias** affect approximately 80-90% of patients with HHT. Primarily in the stomach and small intestine, and less commonly in the colon. Nevertheless, symptoms manifest in only 25-30% of these patients[7]. Symptoms can range from external haemorrhage to acute or chronic anaemia that cannot be easily

explained by epistaxis or simple iron deficiency. The symptoms typically arise after the age of fifty and tend to deteriorate as the patient gets older. **Pulmonary AVMs** (PAVMs) are also found in patients with HHT. It corresponds to a direct connection between an artery and a vein without an intermediate capillary (fistula) responsible for a right-to-left shunt. They affect almost half of all patients[8] and can cause paradoxical embolisms (14% of the cases)[9], septic embolisms responsible for brain abscesses (5 to 17% of the patients around 50 years of age)[8], ischemic embolisms (9 to 37% of the patients)[8-10]. **Hepatic AVM** are more frequently diagnosed in women and depending on the diagnostic technique used, 42% to 74% of patients are diagnosed with a hepatic AVM (hepatic ultrasound, abdominal CT scan)[11,12]. Only 8% of patients with hepatic AVM, generally from the age of 50, are symptomatic (dyspnoea, right hypochondrium pain, pruritus, jaundice)[12]. These AVMs give rise three types of shunt, which may coexist: hepatic artery (HA)-hepatic vein (HV), HA-portal trunk (PT), PT-HV. HA-HV and PT-HV shunts, which increase cardiac output and can lead to high-flow heart failure, is one of the most frequent complications with pulmonary hypertension[13]. **Brain AVM** have a prevalence of approximately 14%[14] and have been described as AVMs, arteriovenous fistulas (no nidus), telangiectasias, developmental venous anomalies and carvernomas[15]. The incidence of bleeding is low, at 0.5% per year, but the consequences are serious in terms of morbidity and mortality. The risk of bleeding also varies with the type of cerebral anomaly: it is higher in the case of malformations and fistulas than in the case of cerebral telangiectasias[15]. **Spinal AVM** prevalence is less than 1%[16] and the vast majority are found in children. Although they may be rare, these conditions can lead to severe and possibly life-threatening bleeding complications[17,18].

4. Family member matching the above-mentioned criteria. A diagnosis of HHT is considered definite if presented with three criteria, possible or suspected if presented with two criteria, and unlikely if presented with fewer than two criteria.

Genetic basis of the disease

Mutations in the ENG gene (Endoglin, 9q34.11)[19] or in the ACVRL1 gene (activin receptor-like kinase 1 or ALK1, 12q13.13)[20] are responsible for HHT1 or HHT2, respectively and account for more than 90% of cases of HHT. Both ENG and ACVRL1 encode for receptors of Transforming Growth Factor-b (TGF-β)/Bone Morphogenetic Protein (BMP) (**Fig. 1**) that are expressed in endothelial cells and share functions in signalling[21]. All classical features of HHT can be seen in both HHT1 and HHT2, but the prevalence of specific vascular anomalies varies

according to the genotype. Pulmonary and cerebral AVMs are more common in HHT1 than HHT2, 85% versus 35%[22] and 20% versus 2%[23], respectively. While HHT2 individuals have a higher incidence of hepatic AVMs and gastrointestinal hemorrhages[22,23]. A rare form of HHT disease in which vascular lesions are combined with Juvenile Polyposis is associated with mutations in the gene MADH4 (Mothers Against Decapentaplegic Homolog 4). MADH4 encodes for SMAD4, a downstream effector of TGF-β/BMP family ligands[24]. While HHT3[25] and HHT4[26] have only been linked to a particular locus and no specific genes have been identified yet, HHT5 is due to mutations in the Growth Differentiation Factor 2 (GDF2) gene[27]. GDF2 gene encodes for BMP9, a high-affinity ligand for ALK1 that controls endothelial cell quiescence[28,29]. Known gene mutations include deletion, insertion, and missense mutations as well as splice site changes and represent null allele indicating that haploinsufficiency is the underlying mechanism of HHT. As consequence, the remaining wild-type allele is unable to contribute sufficient protein for normal TGF-β/BMP signalling in endothelial cells leading to blood vessel dysfunctions[30].

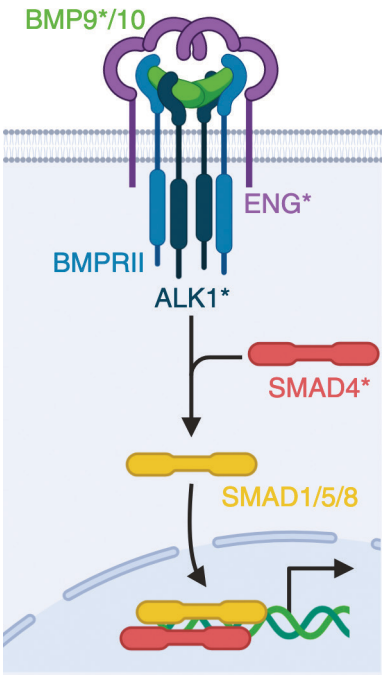

Fig. 1 | BMP9/10 signalling pathway in endothelial cells.
** Indicates HHT gene mutations*

TGF-β/BMP signalling in vascular development and homeostasis

TGF-β/BMP signalling in endothelial cells

Classically, TGF-β and BMP signalling pathways are each initiated by ligand-mediated activation of distinct type I and type II serine/threonine kinase transmembrane receptors. Within the ligand-induced heteromeric receptor complex, the constitutively active type II receptor phosphorylates the type I receptor on specific serine/threonine residues in the intracellular juxtamembrane region named GS-domain leading to the phosphorylation of TGF-β or BMP-specific-receptor-regulated Smad proteins (R-Smad). R-Smads then associate with the common mediator (co)-Smad (Smad4) and translocate to the nucleus to regulate the transcription of specific target genes in association with other partner proteins. R-Smads are divided in two groups. The first group consists of Smad1/5/8 and are preferentially activated by BMP type I receptors that include ALK1, 2, 3 and 6. The second group contains Smad2 and 3 and is activated by TGF-β type I receptor ALK5[31,32]. TGF-β and BMPs can also activate Mitogen Activated Protein (MAP)-Kinase signalling pathways, Rho-like GTPase and PI3K/Akt cascades independently of Smad signalling pathways[33]. Genetic studies in mice and humans have clearly demonstrated the importance of TGF-β/BMP signalling pathways in vascular morphogenesis and angiogenesis. Information gathered the various loss-of-function mouse models of TGF-β signalling components have been reviewed in detail[34]. In all cases, targeted deletions of tgfb1[35], genes encoding TGF-β receptors, *acvrl1*[36,37], *Alk5*[38], *TβrII*[39] or *Eng*[40-43] as well as the downstream target Smad5[44,45] lead to embryonic lethality at mid-gestation with severe cardiovascular defects including impaired angiogenesis and differentiation of mural cells (**Table 1**). The primary target cells for TGF-β/BMP are endothelial cells since mice deficient in endothelial TβRII[46], ALK1[47-51], ALK5[46,52] or endoglin[49,53-55] show various vascular defects ranging from vessel hyper-branching, enlarged blood vessels to AVM formation.

TGF-β has been proposed to regulate the activation state of endothelial cells by differentially activating two TGF-β type I receptors, ALK5 and ALK1. ALK5 is broadly expressed in almost all tissues whereas ALK1 is restricted to the endothelium. Upon TGF-β stimulation, ALK5 phosphorylates Smad2/3 leading to inhibition of endothelial cell proliferation and migration, whereas ALK1 phosphorylates Smad1/5 to induce opposite effects[56]. The existence of two type I receptors activated by one ligand, raises the question of how their activation is controlled and why these two cascades coincide. Although not experimentally

proven, one explanation is that ALK1 signalling first may dominate, leading to the activation phase of angiogenesis triggered by VEGF, whereas ALK5 may induce later vessel stabilization and extracellular matrix production. However, ALK5 kinase activity seems to be required for optimal ALK1 signalling whereas ALK1/Smad1/5 signalling directly antagonizes ALK5/Smad2/3 signalling cascade[52,57]. The net effect of TGF-β may therefore depend on the relative levels of ALK1/ALK5 expression and also on the different levels of TGF-β[56]. The type III TGF-β co-receptor endoglin is highly expressed on activated endothelial cells. It is required for efficient ALK1 signalling. Interestingly, endothelial cells lacking endoglin do not proliferate due to enhanced ALK5 signalling cascade. Endoglin may therefore regulate fine-tuning between ALK1 and ALK5 activated cascades[21,58,59].

BMP9 and BMP10 have been shown to bind to ALK1 with a much higher affinity than TGF-β[28,29,60]. Since then, accumulative evidence indicates that BMP9/BMP10 through the ALK1-Smad1/5 signalling pathway play essential functions in vascular development and in the maintenance of the vascular quiescence[28,29,61-63] (**Fig. 2**). Although *BMP10*$^{-/-}$ mice exhibit a cardiac phenotype[64], its role on the vasculature should not be disregarded. BMP10 through ALK1 has been shown to induce flow-arterial quiescence in zebrafish[65] and has recently been reported to function independently of BMP9 for the development of the arteriovenous network[66]. *BMP9*$^{-/-}$ mice do not display defective vasculature[61,64], unless BMP10 is removed from the circulation leading to impairment of the retinal vasculature and defective closure of the ductus arteriosus[61,62]. BMP9/ALK1 signalling has been shown to regulate target genes important for blood vessel maturation and stabilization. These genes included Notch targets (Hes1, Jag1, Hey1, Hey2), inhibitors of VEGF signalling (VEGFR1), Angiopoietin-2 (Angpt2) and suppression of endothelial tip cell markers (Unc5b)[48,67,68]. Their role in the context of HHT has also been elucidated for some of them[69,70].

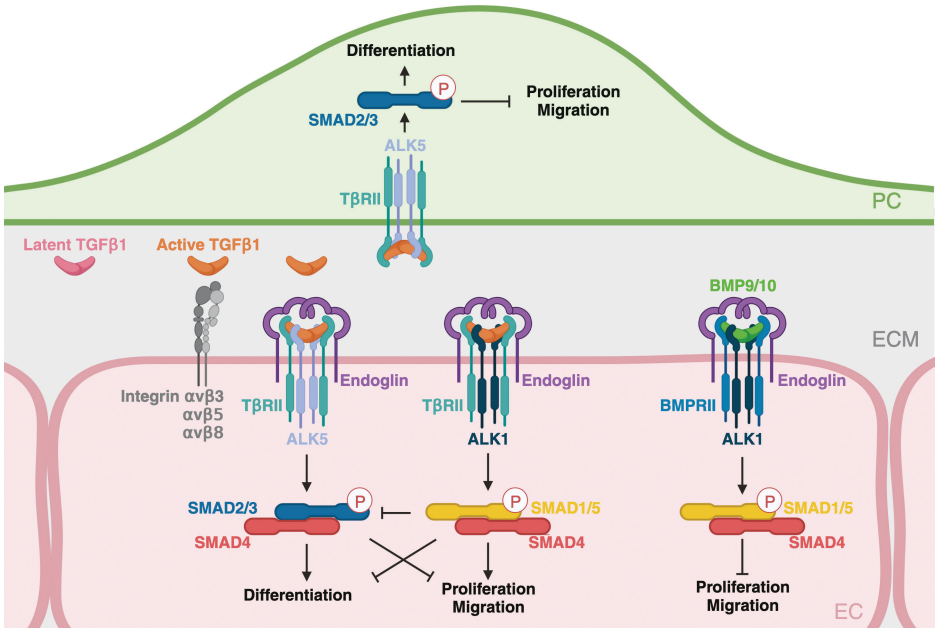

Fig.2 | Close physical endothelial cell-mural cell contacts promote paracrine TGF-β/ALK5 signalling in mural cells and differentiation. In endothelial cells, TGF-β signals through ALK5-Smad2/3 and ALK1-Smad1/5 to mediate opposite responses on proliferation and migration. In contrast, BMP9 that binds with much higher affinity to ALK5 promotes endothelial cell quiescence. TGF-β is secreted by both endothelial cells and mural cells in an inactive form. Close physical contacts between endothelial cells and mural cells are necessary to activate TGF-β. The mechanisms leading to its activation remain poorly understood, depends on the cell types and on different mediators that include connexins, integrins, and tissue factor. Upon TGF-β binding to ALK5 on mural cells, it stimulates the production of contractile proteins, mural cell quiescence and differentiation.

The involvement and activity of these TGF-β/BMP signalling components are strictly linked to the development stage[34] (**Table 1**). Interestingly, impaired TGF-β/BMP signalling pathways not only affect endothelial cells but they are also important for proper recruitment and differentiation of mural cells (**Table 1**). Moreover, mural cell specific deletion of TGF-β/BMP components are linked to vascular defects but at later stages of development indicating that TGF-β/BMP signalling pathways regulate vessel remodelling[46] (**Table 1**). One important issue over the past decade has been to identify whether the mural cell defects observed in the TGF-β mutants reflected the primary effects of TGF-β signalling in mural cells or occurred secondarily to the impairment of endothelial cell functions (**Table 1**). Both constitutive and conditional deletion of *Acvrl1* or *Eng* in the endothelial cells lead to impaired angiogenesis and the development

Table 1: Major endothelial-mural cell defects in TGF-β/BMP receptors and Smad deficiency mice.

	Knockout mouse models				
	Genotype	**Tissue specific deletion**	**Lethality**	**Phenotype**	**Ref.**
Receptor Type I	*Acvrl1*$^{-/-}$	Germline deletion	E10.5 - E11.5	Excessive fusion of capillary plexes into cavernous vessels and hyper dilation of large vessels. Deficient differentiation and recruitment of VSMCs	Oh et al., 2000
	Acvrl$^{+/-}$	Germline deletion	Viable	Multiorgan vascular defects with bleeding, similar to those seen in HHT individuals. Dilated vessels with thin walls	Srinivasan et al., 2003
	Acvrl1$^{f/f}$	Ad-Cre, Ad-VEGF	Viable	Reduction of mural cell coverage after VEGF stimulation is a potential mechanism for the impairment of vessel wall integrity in HHT2-associated brain AVM	Chen et al., 2013
	TβRI$^{-/-}$	Germline deletion	E10 - E11	Defective angiogenensis. Yolk sacs show malformed vessels with few blood cells. Mutant ECs exhibit impaired migration and fibronectin synthesis Some vessels lack VSMCs due to EC defects	Larsson et al., 2001
	TβRI$^{LACZ/LACZ}$	Germline deletion	E10 - E11	Defects in yolk sac and placenta vascular development Defects in the vascular smooth muscle development	Seki et al., 2006
	TβRI$^{KI/KI}$	Asp266 in the L45 Loop remplaced by Ala	E10.5 - E11.5	Angiogenic defects similar to *TβRI*$^{-/-}$. Mural cells are unable to contribute to vascular formation because of the decreased motility	Itoh et al., 2009
Receptor Type II	*Bmpr2*	Sh-mediated Knock Down	Viable	BMP receptor signaling regulates vascular remodeling during angiogenesis by maintaining the expression of EC guidance molecules that promote vessel maturation. Incomplete mural cell coverage on vessel walls	Liu et al., 2007

	Knockout mouse models				
	Genotype	**Tissue specific deletion**	**Lethality**	**Phenotype**	**Ref.**
Receptor Type III	*Eng*$^{-/-}$	Germline deletion	E10.5 - E11.5	Defective yolk sac vasculogenesis, embryonic angiogenesis; Defective VSMC developement precedes disruption in endothelial remodeling	Li et al., 1999 Bourdeau et al., 1999 Arthur et al., 2000
	Eng$^{+/-}$	Germline deletion	Viable	Multiorgan vascular defects with bleeding, similar to those seen in HHT individuals. Dilated vessels with thin walls	Bourdeau et al., 2001 Torsney et al., 2003
	Eng$^{-/-}$	Germline deletion	E10.5	ALK5 signaling from ECs to adjacents mesothelial cells is defective. Reduced availability of TGFβ1 protein to promote recruitment and differentiation of mural cells	Carvalho et al., 2004
	Eng$^{+/-}$	Germline deletion	Viable	Irregular VSMC coverage of skin arteries First demontration that strategies targeting mural cells are beneficial to treat bleeding from HHT indivituals	Lebrin et al., 2010
	Eng$^{f/f}$	*PGK-Cre*, ubiquitous deletion	E10.5	Defective yolk sac vasculogenesis, embryonic angiogenesis. Defective VSMC developement	Allinson et al., 2007
TFs	*Smad5*$^{-/-}$	Germline deletion	E10.5 - E11.5	Defective angiogenesis and enlarged blood vessels. Decreased numbers of VSMCs	Yang et al., 1999
	Smad5f/f	*PGK-Cre*, ubiquitous deletion	E10.5 - E11.5	Defective angiogenesis and enlarged blood vessels. Decreased numbers of VSMCs	Umans et al., 2003

Endothelial cell specific deletion mouse models

	Genotype	Tissue specific deletion	Lethality	Phenotype	Ref.
Receptor Type I	*Acvrl1*$^{f/f}$	Scl-CreER	Viable	Deletion of *Acvrl1* in ECs results in wound-induced skin AVMs.	Garrido-Martin et al., 2014
	Acvrl1$^{f/f}$	*Cdh5-CreERT2, Acvrl1-iKOe*	10 days post Acvrl1 deletion	Loss of endothelial *Acvrl1* leads to venous enlargement, vascular hyperbranching and AVMs. Reduced pericyte coverage in retinal capillaries	Tual-Chalot et al., 2014
	TβRI$^{f/f}$	*Tie1-Cre*	E10.5	Defective angiogenesis. Yolk sacs lack networks of vessels at all stages. Impaired VSMC differentiation is a direct consequence of functional defects in ECs	Carvalho et al., 2007
	Bmpr1a$^{flox/flox}$	Flk1$^{+/Cre}$	E10.5-E11.5	ALK3-mediated Id gene activation in ECs is crucial for vessel remodeling in yolk sac	Park et al., 2006
Receptor Type II	*Tgfbr2*$^{f/f}$	*Cdh5-CreERT2, Tgfbr2-iKOe*	E10.5	Defective angiogenesis. Yolk sacs lack networks of vessels at all stages. Impaired VSMC differentiation is a direct consequence of functional defects in ECs	Carvalho et al., 2007
	Bmpr2$^{f/f}$	Tg(ALK1)-*Cre*, pulmonary EC deletion	Viable	Right ventricular hypertrophy and an increase in the number and wall thickness of muscularized distal pulmonary arteries	Hong et al., 2008
Receptor Type III	*Eng*$^{f/f}$	wScl-Cre^{ER}	Viable	Deletion of *Eng* in ECs results in wound-induced skin AVMs	Garrido-Martin et al., 2014
	Eng$^{f/f}$	Cdh5-Cre^{ERT2}, Eng-iKO^{e}	Viable	Retinas exhibit delayed remodeling of the capillary plexus, increased EC proliferation and localized AVMs. Increased VSMC coverage following AVM formation appears to be secondary response to increased blood flow	Mahmoud et al., 2010
TFs	*Smad5*$^{f/f}$	Tie-2-Cre	Viable	No obvious vascular phenotype	Umans et al., 2007
	Smad2$^{f/f}$; *Smad3*$^{f/f}$	Tie-2-Cre	E12.5	Defective angiogenesis in the yolk sac and in the proper embryo. Inadequate assembly of mural cells in the vasculature. Reduction of N-cadherin expression	Itoh et al., 2012
	Smad4$^{f/f}$	*SP-A-Cre*, Brain vascular EC deletion	perinatal lethality	Intracranial hemorrhagies. Defective mural cell coverage. Smad4 stabilizes cerebrovascular EC-pericyte interaction by regulating the transcription of N-cadherin through associating with Notch	Li et al., 2011

VSMC specific deletion mouse models

	Genotype	Tissue specific deletion	Lethality	Phenotype	Ref.
Receptor Type I	*Acvrl1*$^{f/f}$	Myh11-Cre^{ER}	Viable	*Acvrl1* in VSMCs is dispensable for maintaining normal vasculature and for the formation of a normal vascular network during wound healing in adult stages	Garrido-Martin et al., 2014
	TβRI$^{f/f}$	*SM22-Cre*	E12.5	Embryos at E9.5 do not exhibit yolk sac defects and resemble wild-type embryos. However at E12.5 embyos are pale and anaemic with obvious vasculature defects	Carvalho et al., 2007
	TβRIf/f	*GATA5-Cre, epicardium*	Viable	Defective formation of a smooth muscle cell layer around coronary arteries	Sridurongrit et al., 2008
	Bmpr1a$^{flox/flox}$	*SM22α-Cre*	E11 - E11.5	Severe dilatation of the aorta and large vessels with impaired investment of VSMCs that are also related to reduced proliferation	El-Bizri et al., 2008
	Bmpr1a$^{flox/flox}$	*Flk1*$^{+/Cre}$	E10.5 - E11.5	Vessel integrity is defective. These mutants display dilated vessels in the brain and abnormal branching in the trunk and show reduced number of VSMCs	Park et al., 2006
Receptor Type II	Tgfbr2f/f	*SM22-Cre, Tgfbr2-iKO*^{e}	E12.5	E12.5 embyos are pale and anaemic with obvious vasculature defects	Carvalho et al., 2007
Receptor Type III	*Eng*$^{f/f}$	Myh11-Cre^{ER}	Viable	Eng in VSMCs is dispensable for maintaining normal vasculature and for the formation of a normal vascular network during wound healing in adult stages	Garrido-Martin et al., 2014
TFs	Smad5$^{f/f}$	SM22-Cre	Viable	No obvious vascular phenotype	Umans et al., 2007
	Msx1f/f; Msx2f/f	SM22-Cre	perinatal lethality	"Defects in head vascularization, aneurysms and hemorrhages are observed BMP signaling is affected, VSMC coverage is reduced and endothelium maturation is impaired"	Lopes et al., 2011

of vascular malformations indicating that these receptors share functions in signalling[47-51,53-55]. The situation is yet more complex for ALK5 since conflicting data exist on its expression pattern in the endothelium. Using an *acvrl1* (ALK1)-Cre to delete *TβRII* and *ALK5* specifically in the endothelium, Park et al., have suggested that the effects of these receptors on vessel morphogenesis and angiogenesis were not due to their functions in endothelial cells[71] supporting that ALK5 expression preferentially occurs in mural cells as suggested by Seki et al.[72]. However, floxed *TβRII* and floxed *Alk5* mice crossed with transgenic mice expressing the Cre-recombinase under control of the vascular endothelial specific Tie1 promoter resulted in embryonic lethality at E10.5 because of aberrant angiogenesis as for the conventional *TβRII* and *Alk5* knockout mice[46]. Another Cre-driver where EGFP-Cre was knocked into the acvrl1 (active at E9.5 in the endothelium) to delete TβRII and Alk5 also led to severe blood vessel anomalies and intracranial hemorrhages[73]. The temporal regulation of the promoters used indicate that ALK5 expression in endothelial cells may be required for angiogenesis only at certain developmental stages and may be dispensable for the maintenance of the mature vasculature. Whilst this hypothesis awaits further experiments for support, ALK1 is known to trigger Smad1/5 pathway upon BMP9 or BMP10 stimulation to induce blood vessel quiescence[28,29,61,74]. *Bmp9*$^{-/-}$ and *BMP10*$^{-/-}$ mice do not display lethal defects in yolk sac development[61,64], but they seem to be important in vascular remodelling post-natally[61,63]. The primary cause of HHT may thus be considered as dysfunctions of BMP9/10 in endothelial cells. However, how defects in the delicate balance between TGF-β/ALK5 and BMP9/10-ALK1-endoglin signalling in endothelial cells lead to disease pathology remains to be clarified. Crosstalks in cellular signalling between BMP9/10-ALK1-endoglin, VEGF, Notch and Angiopoietin signalling pathways have also been reported [48,69] [51,55,70] and will be discussed in more detail in the concluding chapter of this thesis.

TGF-β signalling between endothelial cells and mural cells

The exact molecular changes leading to HHT are not clear yet. However, following the recent identification of targets implicated in blood capillary stabilization, this would suggest that the baseline situation in HHT is likely to be an abnormal activation of the endothelium that may affect mural cell attachment. Indeed, one of the best-understood roles of TGF-β signalling in vascular development is that of promoting mural cell differentiation[75,76]. Muscularization is achieved when endothelial cells promote paracrine TGF-β signalling to the neighbouring mural cells to promote their differentiation. Upon TGF-β binding to ALK5 expressed on mural cells, ALK5 phosphorylates Smad2/3 to promote the

production of contractile proteins inducing cell quiescence and differentiation to mature VSMCs or pericytes[77,78] (**Fig. 2**). Importantly, the establishment of close physical endothelial cell-mural cell contacts is absolutely required for TGF-β activation and mural cell differentiation[79]. The mechanism by which latent TGF-β is converted into the active form to promote mural cell differentiation is not fully understood, but Connexin-43 and Connexin-45, Tissue Factor, and integrins such as αvβ8 have been suggested to play a role[75]. How defective TGF-β/BMP signalling in endothelial cells affect mural cell differentiation has also been studied. Carvalho et al. have analysed TGF-β signalling in yolk sacs from endoglin knockout embryos. They revealed that endothelial disrupted TGF-β signalling in endothelial cells also affected TGF-β/ALK5 signalling in the adjacent mesenchymal cells. Interestingly, they also demonstrated that application of exogenous TGF-β in cultured yolk sacs was sufficient to induce VSMC differentiation[80].

Concept of AVM and telangiectasia formation

Animal models have confirmed that *Gdf2*, *Eng*, *Acvrl1*, or *Smad4* mutations causes HHT and have brought important insights into the mechanisms by which HHT mutations lead to the development of vascular malformations. These models have employed classical null and heterozygous mice for either *Eng* or *Acvrl1*[40,41,43,70,81,82], mice bearing conditional loxP knockout alleles for *Eng*, *Acvrl1*, or *Smad4* crossed with endothelial or mural tissue specific Cre-recombinase mouse lines[54,67,83], mice injected with blocking antibodies targeting BMP9 and BMP10[84] or *zebrafish* embryos harbouring a mutation in *alk1* or *eng* gene[85]. From these studies, it appears that HHT mutations are deleterious predominantly during some forms of angiogenesis and that additional triggers to the gene mutations are required for AVMs to form (**Fig. 3**). The heterozygous for *Eng* or *Acvrl1* mice that are the closest genetic models of HHT patients in terms of genotype exhibit a very mild phenotype with HHT-like features appearing only at low frequency and in an unpredictable manner. Blood vessels develop and function normally in these mice, although they have a widespread abnormality of the vascular walls due to defective mural cell recruitment and attachment[70,75,82,86,87]. Defective blood vessel stability represents the baseline situation in HHT and is caused by decreased TGF-β bioavailability[80] and increased VEGF signalling in endothelial cells[51,54,55,70,88-90] (**Fig. 3**). This is thought to favour inadequate responses of the endothelial cells to angiogenic triggers leading to excessive angiogenesis and the development of vascular malformations. Others and we have reported that a second event such as inflammation, infection, wound healing and/or angiogenesis is indeed required to initiate the formation of AV shunts[70,91-93] (**Fig.**

3). An additional somatic mutation in the remaining wild-type allele has also been proposed to explain the clinical observation that vascular lesions occur focally[94]. This concept is supported by the recent generation of conditional knockout mice for *Eng*, *Acvrl1*, or *Smad4*, which develop robust AVMs resembling those seen in HHT individuals[53,67,95]. However, the local loss of heterozygosity concept may be somehow simplistic to explain HHT pathogenesis. It seems unlikely that many endothelial cells over the entire body could acquire somatic mutations in the *Eng* or *Acvrl1* gene to explain the multiplicity of vascular malformations found in patients with HHT. Moreover, it has been reported that AVMs in HHT1 patients express the same level of endoglin as unaffected blood vessels, which is approximately half of the normal level[42,96]. Alternatively, inflammatory cytokines such as Tumour Necrosis Factor-α (TNF-α) that regulate receptor release from the cell surface have been proposed to result in a transient and local null-endoglin phenotype during inflammation, although this hypothesis needs to be experimentally confirmed[53]. Advanced real-time imaging technologies using skinfold window chamber systems have revealed that the initial AV shunts are able to remodel due to hemodynamic changes with veins and arteries that dilate and adjacent blood capillaries that regress resulting in the maturation of the AV shunts[47,49,97]. Interestingly, recent studies point in the direction of a specific synergy between blood flow and Endoglin-Alk1 signalling pathway for the regulation of vessel calibre supporting the key role of shear stress in AVM formation and maturation[55,85,93,98,99]. Finally, several genetic modifiers have also been described to play a role in susceptibility to HHT disease[93,100-103] (**Fig. 3**).

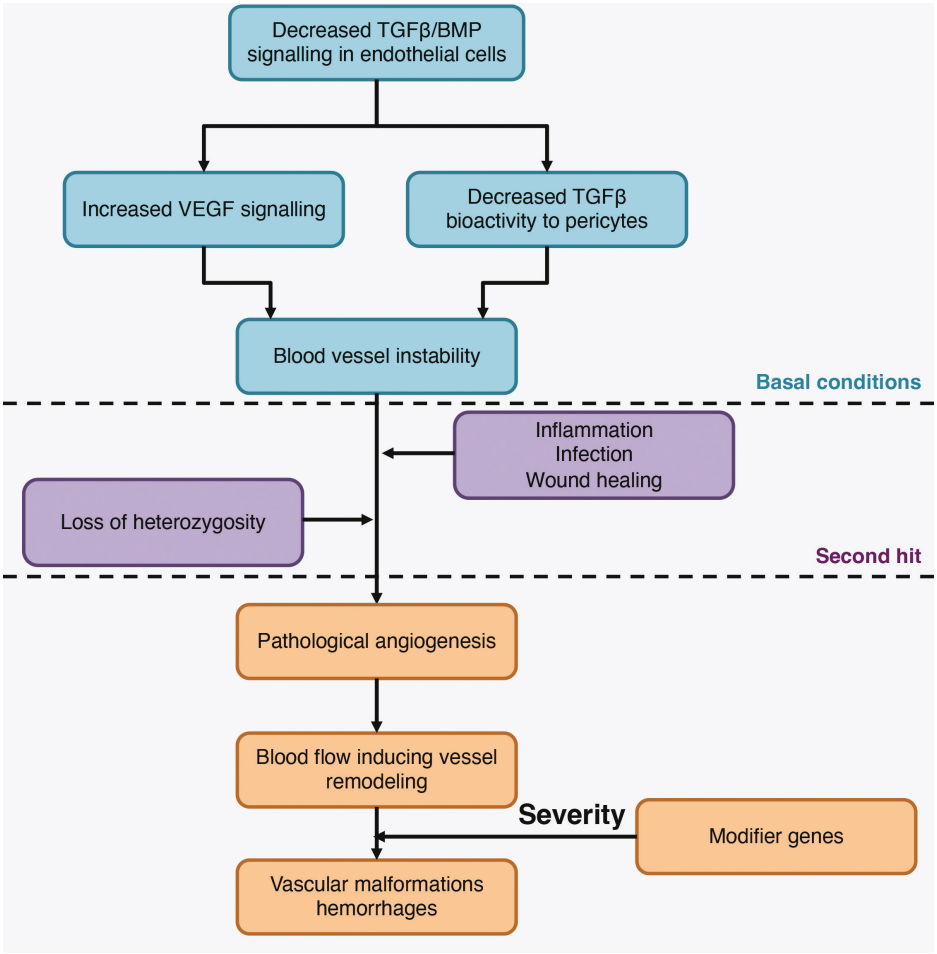

Fig. 3 | How gene mutations in HHT lead to the development of AVMs. Decreased TGF-β/BMP signalling in endothelial cells is combined with increased VEGF signalling leading to blood capillary destabilization and poor mural cell attachment. A second hit such as inflammation, infection, wound healing or loss of heterozygosity is required to induce pathological angiogenesis and the formation of vessel abnormalities that subsequently remodel due to blood flow changes to form stable and mature AVMs. In addition, modifier genes are contribution to the severity of the disease.

Therapeutic options for patients with HHT

Anti-VEGF

After discovering severe angiogenesis defects and increased expression of angiogenic factors, including VEGF, in the ALK1$^{-/-}$ mouse model[36], elevated levels of VEGF were found in the serum of HHT patients[104]. This led to consideration of the potential of anti-angiogenic drugs targeting VEGF. In 2006, Bevacizumab,

one of the most prominent anti-VEGF drugs still in use today, was tested on a severely affected patient. The treatment resulted in a significant decrease in transfusion frequency, improvement in haemoglobin concentration, and physical condition immediately after treatment[105,106]. From this date, clinicians have tested bevacizumab and pazopanib, a kinase inhibitor targeting VEGFR2 on patients with HHT and have demonstrated their efficacy in preventing nosebleeding[107-111]. Bevacizumab delivered by nasal spray has also been tested but did not provide positive outcomes[112]. Several studies including ours, have deciphered the mechanisms by which HHT mutations lead to high VEGF activity in blood vessels and have demonstrated that strategies targeting VEGF signalling can prevent the formation of AVMs in mice depleted of either *Eng* or *Acvrl1*[51,54,55,70,88-90,113,114]. Also, not fully proven, one explanation of the effects of the anti-VEGF therapies in HHT might be a reduction of VEGFR2 signalling activity in endothelial cells, VEGFR2 activity levels that may become normalized promoting blood vessel stabilization[70]. This will be extensively discussed in the concluding paragraph of this thesis. Bevacizumab is an expensive option. However, small chemical inhibitors such as nintedanib and pazopanib, which block VEGFR2 activity, have been tested in animal models and patient case reports showing positive results, including improvements in epistaxis and normalization of haemoglobin and iron levels[110,111,114,115]. While showing promise, there remains a necessity to thoroughly assess long term safety and efficacy of employing anti-VEGF therapies. Primary and late resistance to anti-VEGF therapies are quite common, and adverse effects including hypertension, proteinuria, gastrointestinal perforation, endocrine dysfunction and cardiac impairment have been reported.

Immunomodulatory Imide drugs (IMiDs)

We have revealed a novel mechanism of action of thalidomide, namely stimulation of vessel stabilization and have reported that oral administration of thalidomide reduced both the frequency and duration of nosebleeds with significant decreases of blood transfusion requirement and improvement of quality of life[86]. Few other cases have been reported so far, but the published literature is concordant regarding the potential benefit of thalidomide in HHT individuals (**Table 2**).

Table 2: IMIDs drugs prevents bleeding from HHT individuals. Case reports from the literature.

No. of patients	Follow-up period	Sex /Age	IMIDs dose	Haemoglobin level (mmol/ml)		number of bleedings (per week)		Duration of bleeding		Number of transfusions		Side effects	Reference
	(months)	(years)	(mg/daily)	Before	After	Before	After	Before	After	Before	After (during follow-up)		
1	13	M/77	Thalidomide 150–250	NA	NA	21 - 28	3 - 4	45 -90	1 - 2	NA	NA	Mild peripheral neuropathy in the fingers and toes Mild sedative effects	Kurstin, 2002
8	6	NR/NR (57–69)	Thalidomide 100 - 300	4.8 - 7.7	6.2 - 12.2	NA	NA	NA	NA	2- 10 blood units / months	0 - 5 blood units / months	Minor side effects (constipation and vertigo) except for one patient who stopped after 4 months because of poor responses and side effects	Buscarini et al., 2009
4	6	NR/NR	Thalidomide 50–200	NA	NA	NA	decrease 54 - 89%	NA	NA	2 units of blood / year	19 - 57% decrease	No serious complications (drowsiness)	Gossage et al., 2009
1	13	1F (69)	Lenalidomide 10 - 15	97	132	NA	NA	NA	NA	3 - 4 per months	0	Marrow suppression (mild)	Bowcock et al., 2009
7	6–60	6 M/1 F/60 (43–75)[a]	Thalidomide 100	4.3 - 7.2	6.2 - 8.7	18 - 59	1 - 35	10 - 90	<5 - 20	0 - 8	0 - NA	Mild constipation, loss of libido, drowsiness, lethargy. One individual stopped treatment after 19 months because of peripheral neuropathy	Lebrin et al., 2010
1	1	F/59	Thalidomide 100	NA	NA	NA	NA	NA	NA	NA	NA	Deep vein trombosis	Penaloza et al., 2011
1	16	F/77	Thalidomide 100	5.8	13.0	NA	NA	NA	NA	24 - 36 blood units / year	< 1 blood unit / year	NA	Alam et al., 2011
31	median 15.9	20M/11F mean (SD) 62.6 (11.1)	Thalidomide 50–200	≈8.2 ± 0.5	≈10.4±2	NA	NA	NA	NA	NA	NA	No serious complications (constipation and drowsiness)	Invernizzi et al., 2015

5	6	5F (45-56)	Thalidomide 100		NA	3 - 5	0	1 - 4	0	NA	0	NA	Peng et al., 2015
7	5	1M/6F (30-76)	Thalidomide 50 - 100	NA	NA	NA	NA	NA	NA	NA	NA	drowsiness, dizziness, constipation, nausea, and peripheral neuropathy	Fang et al., 2017
6	12	3M/3F (44-74)	Thalidomide 50 - 100	5.9 - 11.6	8.8 - 14.0	NA	NA	NA	NA	0 - 3 per months	NA	dizziness and nausea	Baysal et al., 2019
144	6	74M/70F (mean 58.8)	Placebo and Pomalidomide 4									mild to moderate neutropenia (45% vs. 10%), constipation/diarrhea (60% vs. 37%), and rash (36% vs. 10%)	Al-Samkari et al., 2023

All subjects treated with thalidomide had severe and recurrent epistaxis and they were refractory individuals to standard medical and local surgical treatments. Thalidomide was administrated orally and the doses given were comparable to that prescribed in the 1960s to treat nausea in pregnancy, ranging from 50 mg to 300 mg of thalidomide daily. In most cases, only minor side effects have been reported and include mild constipation, loss of libido, drowsiness and lethargy. However, three individuals stopped treatment due to peripheral neuropathies in two individuals and deep vein thrombosis in one subject (**Table 2**). Therefore, thalidomide appears to be a potential candidate for the treatment of severe bleeding in HHT individuals unresponsive to conventional therapies. However, these studies have not yet been supported by data from randomized controlled trials and future research should be directed toward identifying the minimum dose of thalidomide effective to prevent bleeding from HHT vascular anomalies without inducing side effects.

Thalidomide was first introduced as a sedative used to prevent nausea during pregnancy in the late 1950s. In 1961, it was withdrawn from the market due to teratogenicity and neuropathy[116]. The use of thalidomide resulted in one of the biggest tragedies in the history of drug development. As a result of using thalidomide, it caused an estimated 10,000 children in 46 countries to be born with birth defects, marked by limb malformations and congenital defects affecting ears, eyes, heart and kidney. These defects occurred when drug exposure took place within a short, time-sensitive window between day 20 and day 36 of gestation. Just one 100 mg tablet of thalidomide was enough to cause limb defects[117,118]. This drug was abandoned but has recently undergone a renaissance. Emerging insight into thalidomide's anti-inflammatory, immunomodulatory and anti-angiogenic activity led to clinical trials in AIDS-related aphthous ulceration, Behcet's syndrome, Crohn's disease cutaneous lupus and various malignancies[119]. In 1999, effectiveness against multiple myeloma (MM) was reported[120]. In respect to Erythema Nodosum Leprosum (ENL) and MM, the US FDA approved thalidomide for use under strict guidelines and carefully controlled inclusion criteria in 1998 and 2006, respectively. Decades of investigation have identified a multitude of biological effects that are regulated by thalidomide. In addition to suppression of Tumour Necrosis Factor-α (TNF-α), thalidomide affects the generation and elaboration of a cascade of pro-inflammatory cytokines that activate cytotoxic T-cells even in absence of co-stimulatory signals. Furthermore, VEGF and basic Fibroblast Growth Factor (bFGF) secretion and cellular response are suppressed by thalidomide, thus antagonizing angiogenesis and altering the bone marrow

stromal microenvironment in hematologic malignancies[119,121]. More recently, it has been discovered that cereblon (CRBN) is the main target through which thalidomide exerts its anti-cancer and teratogenic effects. CRBN, along with DDB1, CUL4A, and ROC1 forms the CRBN-CRL4 E3 ubiquitin ligase ($CRL4^{CRBN}$) and regulates the enzyme's substrate specificity. This leads to the ubiquitination of proteasomal degradation of specific neo-substrates. Thalidomide has been reported to act as a molecular bridge that links selected neo-substrates to CRBN and ubiquitin-proteasome system[122-125]. The neo-substrates identified include the Ikaros family zinc finger transcription factors 1 and 3 (IKZF1 and IKZF3), casein kinase 1α and ARID2 that all, contribute to the efficacy in the treatment of MM. Conversely, spalt-like transcription factor 4 (SALL4), PLZF and p63 have been implicated in the teratogenic effects induced by IMiD's treatments[125-132] (**Fig. 4**). The mechanisms by which thalidomide inhibits angiogenesis, promotes blood vessel stability and integrity are much less understood.

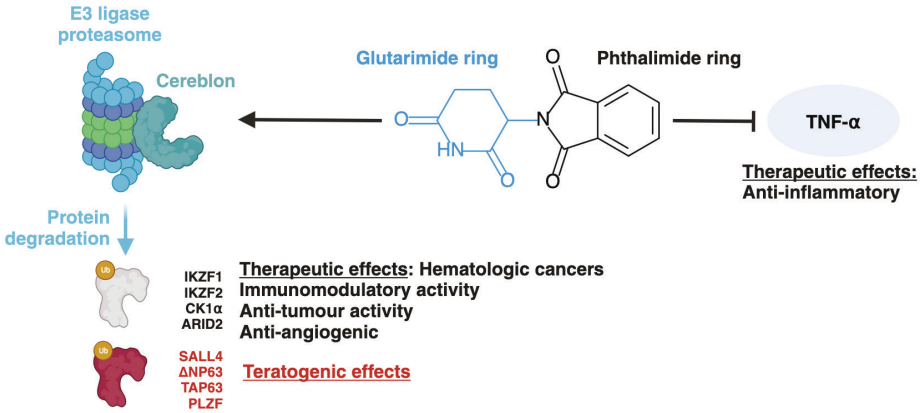

Fig. 4 | Mechanism of action of thalidomide

Previously, my lab has reported that the anti-haemorrhagic property of thalidomide is not the result of direct inhibition of endothelial cell proliferation and migration but is rather due to increased mural cell coverage of the vasculature. Thalidomide increased the number of pericytes and their recruitment to blood vessels, enhancing the apposition between the inner endothelial and supportive pericyte layers and resulting in vessel stabilization in *Eng*$^{+/-}$ mutant mice, a well-characterized model of HHT. Moreover, high doses of thalidomide (150 mg/kg body weight) stimulated the number of pericytes that expressed α-SMA, an established marker of the pericyte contractile phenotype. At the molecular level, thalidomide treated mouse retinas unexpectedly showed

only marginally reduced VEGF mRNA levels compared to untreated controls. However, we observed a marked and rapid increase of PDGF-B mRNA levels in endothelial cells in response to thalidomide. PDGF-B is a key molecule in pericyte chemotaxis that promotes endothelial-pericyte cell-cell contact. The observation that the anti-angiogenic effects of thalidomide were prevented by concurrent administration of STI-571, a kinase inhibitor that blocks PDGFR-β but not VEGF signalling suggests a functional role for PDGF-B in this thalidomide-stimulated reduction in angiogenesis. Moreover, we took advantage of the *Pdgf*$^{ret/ret}$ mouse model, in which PDGF-B is secreted but is not retained by the extracellular matrix and so does not form the gradient required to stimulate tight adhesion of pericytes to the abluminal surface of microvessels and showed that thalidomide did not rescue the pericyte recruitment defect in post-natal *Pdgf*$^{ret/ret}$ mice. Finally, we revealed that thalidomide might target mural cells directly to stimulate their proliferation and ability to form protrusions independently of effects on PDGF-B signalling. The exact mechanisms underlying this effect need further investigation. These data provide to our knowledge, the first evidence that a therapy targeting pericytes to stimulate vessel maturation can have beneficial effects on bleeding from vascular malformations[86]. Recently, it has been reported that thalidomide treatment prevented Blood Brain Barrier leakage and cognitive decline induced whole brain ionizing radiation in both humans and mice. This effect was partly mediated through PDGFr-β signalling confirming that thalidomide might be beneficial in treating diseases associated with vascular dysfunction[133].

Lenalidomide and pomalidomide are IMiD analogue of thalidomide. They have been approved by the US Food and Drug Administration (FDA) for the treatment of MM in June 2006 and February 2013, respectively. They share a common glutarimide ring that binds to CRBN, linked to a phthaloyl ring with minor chemical variations dictating their interactions with a limited number of neo-substrate proteins. Therefore, their mechanisms of action are similar in some aspects, but lenalidomide and pomalidomide are more potent compared to thalidomide. A clinical trial using pomalidomide to treat patients with HHT has recently been completed (NCT03910244) and preliminary data show a reduction in epistaxis severity score (tool used to evaluate the current severity of HHT patient nosebleeds) and improvement in quality of life in treated patients compared with placebo[134]. The use of pomalidomide is not without risk and neutropenia, thrombocytopenia and an increased risk of second solid tumours and haematopoietic malignancies, primarily MDS and AML[135,136] have been reported. Moreover, pomalidomide is a potent teratogen[137]. The clinical

implementation of thalidomide's analogues will require the identification of the mechanisms of action and protein targets as well as the generation of safer drugs to treat chronic diseases such as HHT.

Scope of the thesis

This thesis provides insights into the mechanism underlying HHT. Novel and promising treatments are also introduced, along with an innovative imaging technique designed to assess drug efficacy.

Chapter 2 examines tissue-specific characteristics in HHT1, revealing a correlation between low endoglin levels and vascular malformations in organs.

Chapter 3 demonstrated the significant role of VEGFR1 in HHT2 pathogenesis and provided mechanisms explaining why HHT2 blood vessels respond abnormally to angiogenic signals. This supports the use of anti-VEGF therapy in HHT2.

In **Chapter 4**, we focused on pericyte-endothelial cell interactions in endoglin-deficient mice. Additionally, we used revolutionary ultrasound imaging techniques to reveal cerebral blood flow deregulation. In addition, our study highlights the important role of TGF-β1 in this phenotype.

Chapter 5 explains the mechanism of action of Thalidomide and introduces new analogues that could potentially treat HHT and other cerebral microvascular disorders.

Chapter 6 critically discusses the main findings of the studies included in this thesis in the context of recent literature and presents promising prospects for future research and treatment.

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