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REVIEW



## Endoglin and *alk1* as therapeutic targets for hereditary hemorrhagic telangiectasia

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### ABSTRACT

**Introduction:** Hereditary Haemorrhagic Telangiectasia (HHT) is as an autosomal dominant trait characterized by frequent nose bleeds, mucocutaneous telangiectases, arteriovenous malformations (AVMs) of the lung, liver and brain, and gastrointestinal bleedings due to telangiectases. HHT is originated by mutations in genes whose encoded proteins are involved in the transforming growth factor  $\beta$  (TGF- $\beta$ ) family signalling of vascular endothelial cells. In spite of the great advances in the diagnosis as well as in the molecular, cellular and animal models of HHT, the current treatments remain just at the palliative level.

**Areas covered:** Pathogenic mutations in genes coding for the TGF- $\beta$  receptors endoglin (*ENG*) (HHT1) or the activin receptor-like kinase-1 (*ACVRL1* or *ALK1*) (HHT2), are responsible for more than 80% of patients with HHT. Therefore, *ENG* and *ALK1* are the main potential therapeutic targets for HHT and the focus of this review. The current status of the preclinical and clinical studies, including the anti-angiogenic strategy, have been addressed.

**Expert opinion:** Endoglin and *ALK1* are attractive therapeutic targets in HHT. Because haploinsufficiency is the pathogenic mechanism in HHT, several therapeutic approaches able to enhance protein expression and/or function of endoglin and *ALK1* are keys to find novel and efficient treatments for the disease.

### ARTICLE HISTORY

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### KEYWORDS

HHT; endoglin; *ALK1*; haploinsufficiency; bleeding; angiogenesis; *AVM*; bevacizumab; thalidomide

### 1. Clinical symptoms and histological findings in hereditary hemorrhagic telangiectasia

Hereditary hemorrhagic telangiectasia (HHT) is an extremely challenging disorder both to understand and manage and spans a vast range of clinical and scientific disciplines. It is an autosomal-dominant vascular disease characterized by frequent nose bleeds, mucocutaneous telangiectases, arteriovenous malformations (AVMs) of the lung, liver and brain, as well as involvement of the gastrointestinal (GI) tract associated with GI bleedings due to telangiectases. Both AVMs and telangiectases have in common the lack of intervening capillaries leading to a direct connection between arteries and veins. The cutaneous telangiectases derive from a focal dilatation of the post-capillary venule in the upper horizontal plexus [1]. An in silico reconstruction of serial sections suggests that the dilated post-capillary venules enlarge, connect with dilated arterioles with loss of the intervening capillary bed, and form arteriovenous shunts. Microscopic telangiectasia is observed not only in the skin but also in other vascular beds such as the pulmonary circulation where they may cause low grade intrapulmonary right-left shunting detectable by contrast echocardiography in the absence of macroscopic vascular abnormalities [2,3]. HHT is a highly penetrant disorder where most patients present age-

dependent symptoms. Although infants are occasionally severely affected, the proper diagnosis is often not reached until adolescence or later. Telangiectases close to the surface of the skin and mucous membranes are fragile and frequently rupture and bleed upon slight trauma. The most common clinical manifestation in more than 90% of patients is spontaneous and recurrent nosebleeds (epistaxis) beginning on average at age 12 years. In addition, approximately 25% of HHT patients show GI bleeding, which usually presents after the age of 50 years. Large AVMs often cause symptoms when they occur in the brain, liver, or lungs, whereas complications from bleeding or shunting may be sudden and catastrophic. The phenotype of each HHT patient develops differently depending on their repertoire of susceptibility genes and/or environmental triggers to which each individual is exposed. Diagnosis of HHT follows the Curaçao Criteria, a widely accepted international consensus diagnostic criteria [4,5]. It is based on the presence of (1) nose bleeds, (2) cutaneous or mucosal telangiectases, (3) visceral AVMs, and (4) family history. Thus, an individual has a diagnosis of 'definite HHT' if three criteria are present, 'suspected HHT' if two are present, and 'unlikely HHT' if only one is present [2,3]. Once an index case has been characterized within a family, a shortcut to the suspected diagnosis of HHT

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### Article highlights

- Hereditary hemorrhagic telangiectasia (HHT) presents frequent nose bleeds, mucocutaneous telangiectases, arteriovenous malformations of the lung, liver and brain, and gastrointestinal bleedings. The current treatments of this disease remain just at the palliative level.
- Heterozygous mutations in several genes whose encoded proteins (endoglin, ALK1, BMP9, Smad4) are involved in the same TGF- $\beta$  signaling pathway of endothelial cells, can lead to classical HHT or HHT-like phenotypes through a pathogenic mechanism of haploinsufficiency.
- The majority of patients with HHT have mutations in endoglin (HHT1) or ALK1 (HHT2) genes; therefore, endoglin or ALK1 represent potential and preferential therapeutic targets to find novel treatments for the disease.
- Many of the current pharmacological strategies appear to target the expression or function of endoglin/ALK1 in endothelial cells, including their involvement in angiogenesis, pericyte recruitment, cell proliferation/apoptosis, and expression and function of endothelial nitric oxide synthase, among others.
- Overall, the current pharmacological treatments are not always effective and some of them display adverse side effects. Consequently, there is a need to find novel and better drug treatments.
- The recent generation of new HHT1 and HHT2 animal models are a valuable tool to screen for novel candidate drugs previously identified as promising modulators of endoglin/ALK1 expression and function.
- Whole exome and genome testing and multi-gene next generation sequencing (NGS) of HHT families with phenotypic variability, as well as those families with compelling evidence of an HHT disorder but without identifiable mutations in the known HHT genes, should be useful to identify new genes with pathobiological, diagnostics and therapeutic implications in HHT.

This box summarizes key points contained in the article.

can also be made by identifying the familial pathogenic variant in an individual.

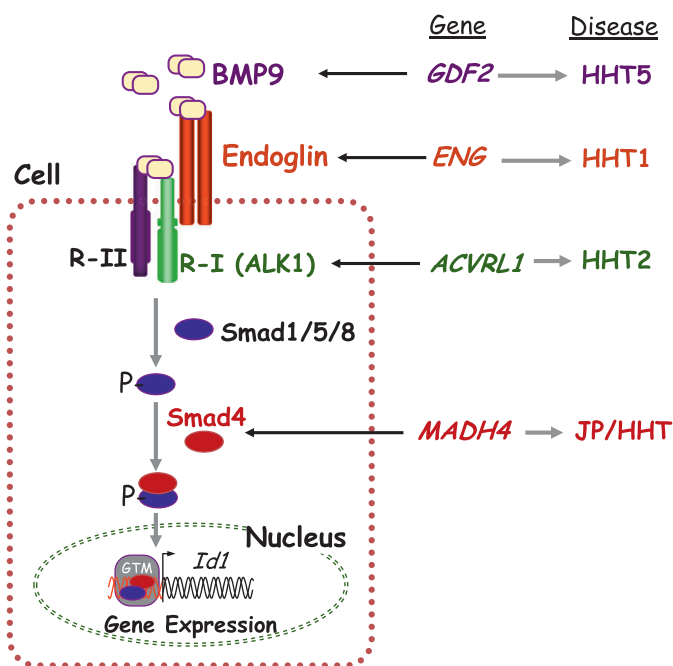
## 2. Genetics of HHT and the TGF- $\beta$ -signaling pathway

So far, all known genes whose mutations cause HHT are found within the transforming growth factor- $\beta$  (TGF- $\beta$ )-signaling pathway. Mutations in the membrane TGF- $\beta$  receptors endoglin (*ENG*) and activin A receptor type II-like 1 (*ACVRL1*, also known as *ALK1*) cause HHT1 (MIM 131195) and HHT2 (MIM 601284) variants, respectively. Interestingly, these two genes, *ENG* or *ACVRL1*, are mutated in over 80% of patients with HHT [6–10]. In addition, a combined syndrome of juvenile polyposis (JP) and HHT was reported to be caused by mutations in *MADH4* (also as *SMAD4*) that encodes a transcription factor (Smad4) of the TGF- $\beta$ -signaling pathway [11]. This combined syndrome (JP-HHT; MIM 600993) occurs only in approximately 2% of HHT patients. Recently, mutations in BMP9 (*GDF2*), a member of the TGF- $\beta$  family able to interact with endoglin and ALK1, have also been shown to cause an HHT-like phenotype [12], which has been named as the HHT5 variant of the disease (MIM 615506). Overall, all the HHT variants share common symptoms, but they differ from each other in the frequency of the specific vascular lesions. For example, the frequency of pulmonary AVMs in HHT2 is much lower than in HHT1 patients, whereas liver AVMs are more frequent in HHT2 than in HHT1 patients. Because of the high penetrance of the disease, given an index case within the family,

the molecular genetic testing of *ENG*, *ACVRL1*, *SMAD4*, and *GDF2* can be used as a highly predictive molecular diagnosis of the other family members. Interestingly, several studies have concluded that a genetic screening strategy with targeted clinical screening is cheaper than conventional clinical screening, resulting in a reduction of the number of clinical tests for family members who do not have HHT [13].

Intrafamilial phenotypic variability has been reported in HHT, suggesting that clinical penetrance and severity of the disease are modulated by additional genetic factors, also called genetic modifiers [7]. These determinants may include gene mutations/polymorphisms that are not able to fully disrupt the function of the encoded protein and are not sufficient to cause disease by themselves. For example, the *ACVRL1* c.314-35A>G polymorphism is associated with pulmonary, liver, and brain AVMs in HHT1, but not in HHT2 patients [14], whereas the *ENG* rs10987746 intronic polymorphism correlates with pulmonary AVMs in HHT1 patients [15]. In addition, variants of novel modifier genes such as protein tyrosine phosphatase non-receptor type 14 (PTPN4) and a disintegrin and metalloprotease 17 (ADAM17) appear to influence the development of AVMs in HHT and can potentiate a TGF- $\beta$ -regulated vascular disease [16,17]. Further genomic analysis of HHT families with phenotypic variability, as well as those families with compelling evidence of an HHT disorder but without identifiable mutations in the known HHT genes, should be useful to identify new genes with pathobiological, diagnostics, and therapeutic implications in HHT. In this context, the use of whole exome and genome testing and multi-gene next-generation sequencing may contribute to explain a heterogeneous spectrum of the HHT phenotype [7].

Because genes mutated in HHT encode protein components of the TGF- $\beta$ -signaling pathway, its perturbation appears to be at the basis of HHT pathogenesis. Members of the TGF- $\beta$  family such as TGF- $\beta$ s, BMPs, activins, nodals, GDFs, and inhibins acting as ligands of specific receptors regulate diverse cellular functions, such as cellular proliferation, differentiation, and apoptosis. These ligands of the TGF- $\beta$  family exert their action through binding to a heteromeric complex of type I and type II TGF- $\beta$  serine/threonine kinase receptors [18]. Signaling can be propagated through a cascade of protein phosphorylations via the canonical Smad-dependent pathway, in which the gene products of the three classical HHT genes (*ENG*, *ACVRL1*, *SMAD4*) and *GDF2* function. As endoglin and ALK1 are predominantly expressed in endothelial cells, these are widely accepted as the HHT target cells, where mutations in HHT genes may lead vascular lesions. Overall, ligand binding activates a TGF- $\beta$  type II receptor, which in turn phosphorylates a type I receptor (T $\beta$ RI). Once activated, the type I receptor phosphorylates and activates the receptor-associated (R)-Smads including Smads 1/2/3/5 and 8. Then, phosphorylated R-Smads bind to Smad4 and translocate to the nucleus, to regulate the transcriptional activity with coactivators and corepressors. This TGF- $\beta$ -signaling pathway is regulated by a negative feedback loop via the inhibitory Smads (Smad6 and Smad7) which target R-Smads for degradation. In most cell types, the TGF- $\beta$  type II receptor signals through ALK5 (T $\beta$ RI) via Smad2/3, but in endothelial cells, it can also signal through ALK1 (T $\beta$ RI) via Smad1/5/8. Remarkably, BMP9 is able to specifically bind ALK1 and endoglin [19–21]. A schematic representation of the HHT-



**Figure 1. HHT and the TGF- $\beta$  signalling pathway in endothelial cells.** BMP9, and probably other members of the TGF- $\beta$  family, bind to an endothelial cell surface receptor complex composed by the type I (R-I) receptor named ALK1 and the type II (R-II) receptor, both exhibiting serine/threonine kinase activity, as well as the auxiliary receptor endoglin. The combinatorial heterodimeric association between different R-I and R-II determines the specificity of the ligand signalling. Upon ligand binding, the R-II transphosphorylates ALK1, which subsequently propagates the signal by phosphorylating the receptor-regulated Smad (R-Smad) family of proteins Smad1/5/8. Once phosphorylated, R-Smads form heteromeric complexes with a cooperating homologue named Smad4, and translocate into the nucleus where they regulate the transcriptional activity of different target genes [18]. BMP9, Endoglin, ALK1 and Smad4 proteins are encoded by GDF2, ENG, ACVRL1 and MADH4 genes, whose pathogenic mutations give rise to HHT5, HHT1, HHT2 and JPHT, respectively. BMP, bone morphogenetic protein; GTM, general transcription machinery. Adapted from McDonald et al. [7].

signaling pathway within the TGF- $\beta$  system of endothelial cells is shown in Figure 1. Identifying new components on the roadmap to this pathway may be useful in developing diagnostics and therapeutics to target the pathogenesis of HHT.

### 3. Pathogenic mechanisms in HHT

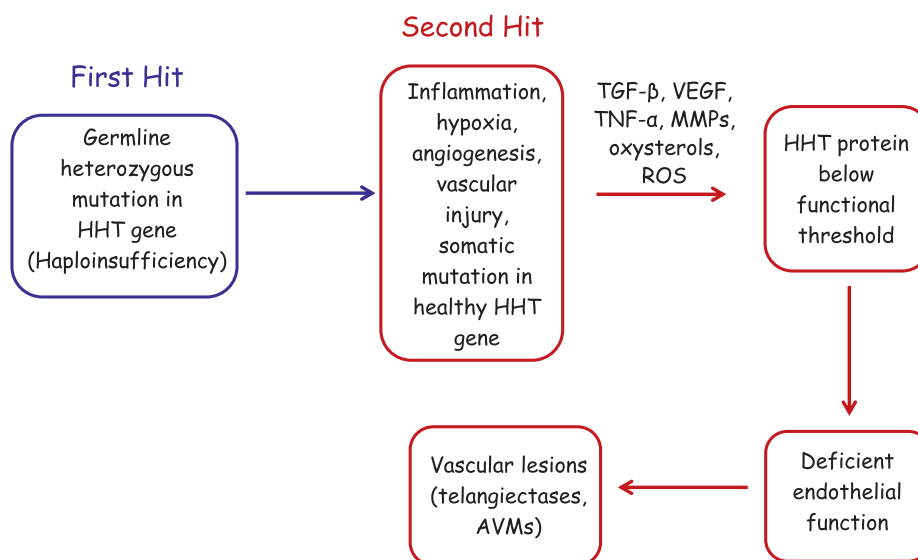
Expression analysis of mutant endoglin and ALK1 proteins, the clinical diagnosis associated with these pathogenic mutations, and HHT-like phenotypes in heterozygous mice strongly suggest haploinsufficiency of the respective protein as the cause of HHT1 and HHT2 [6]. Over 1000 human ENG and ALK1 gene variants linked to HHT have been reported ([http://www.arup.utah.edu/database/ENG/ENG\\_welcome.php](http://www.arup.utah.edu/database/ENG/ENG_welcome.php)). The majority of HHT mutations analyzed so far lead to a mutant protein that is either not expressed at all or retained intracellularly, supporting a model of haploinsufficiency. However, *in vitro* studies have shown that some missense endoglin mutants can heterodimerize with wild-type endoglin, interfering with its expression at the cell surface, likely through a dominant negative effect [22,23]. Most of HHT1 missense mutations affect buried hydrophobic residues and analysis of the 3D structure of endoglin suggests that the majority of HHT1 mutations

impair its folding resulting in a reduced amount of functional endoglin at the cell surface [24]. In addition, some missense mutations in the ALK1 extracellular domain abrogate ligand-dependent signaling *in vitro* [25], whereas functional analysis in zebrafish of HHT2 mutations in the kinase domain of ALK1 has revealed different pathogenic mechanisms, including a null phenotype via loss of protein expression or receptor activity, as well as a dominant negative effect [26]. Overall, haploinsufficiency is widely accepted as the cause of HHT1 and HHT2 pathogenicity and most of HHT mouse models are haploinsufficient [6,27]. Nonetheless, haploinsufficiency by itself does not account for the localized generation of vascular in HHT patients. In this sense, it is intriguing why the vascular HHT lesions appear only at distinct sites within certain organs, rather than being present throughout the body and in all organs/tissues. This paradox has been explained, as in many other genetic diseases, postulating the need for an external trigger, or second hit, such as vascular injury, inflammation, infection, ischemia, angiogenic stimuli, or a second somatic mutation in the healthy HHT gene that synergizes with endoglin haploinsufficiency to generate the lesion [28] (Figure 2). Interestingly, some of these potential hits upregulate the expression of endoglin and ALK1 (see item below), suggesting that the function of ALK1 and endoglin is required under those stressful settings. Experimental support for the second hit hypothesis has been reported using a mouse with a conditional mutation in *Eng*, demonstrating that AVMs develop when an angiogenic stimulus is combined with endoglin depletion in the vasculature of the neonatal retina [27,29]. Similarly, wounding or stimulation with the angiogenic vascular endothelial growth factor (VEGF) can induce vessels to develop skin AVMs in Alk1-deficient mice [30–32]. Conversely, VEGF neutralization can prevent and normalize AVMs in a mouse model for HHT2 [33]. Thus, in the haploinsufficient HHT setting subjected to the second hit, protein levels of endoglin or ALK1 may not reach the minimum threshold to exert their optimal function and this may be critical to generate the vascular lesion (Figure 2). Of note, a somatic mutation in the healthy HHT allele may also lead to a critical drop in the endothelial function, as suggested by the inducible HHT gene knockout animal models [27]. However, the existence of a second somatic mutation in the vascular lesions of HHT patients has not been demonstrated yet. In summary, targeting gene expression and function of endoglin and ALK1 appears essential to counteract HHT haploinsufficiency.

### 4. Targeting gene expression of endoglin and ALK1

Endoglin and ALK1 are expressed in endothelial cells, which are the primary cell target in HHT [27,28,34,35]. In resting endothelial cells, endoglin is expressed at low levels, but it is highly upregulated when these cells are actively proliferating at sites of active angiogenesis and during embryogenesis [28,34,36]. Comparative expression studies of the endothelium in lung vessels have shown that endoglin and ALK1 have distinct expression patterns in the pulmonary vasculature and are only coexpressed in the distal (precapillary) arteries,

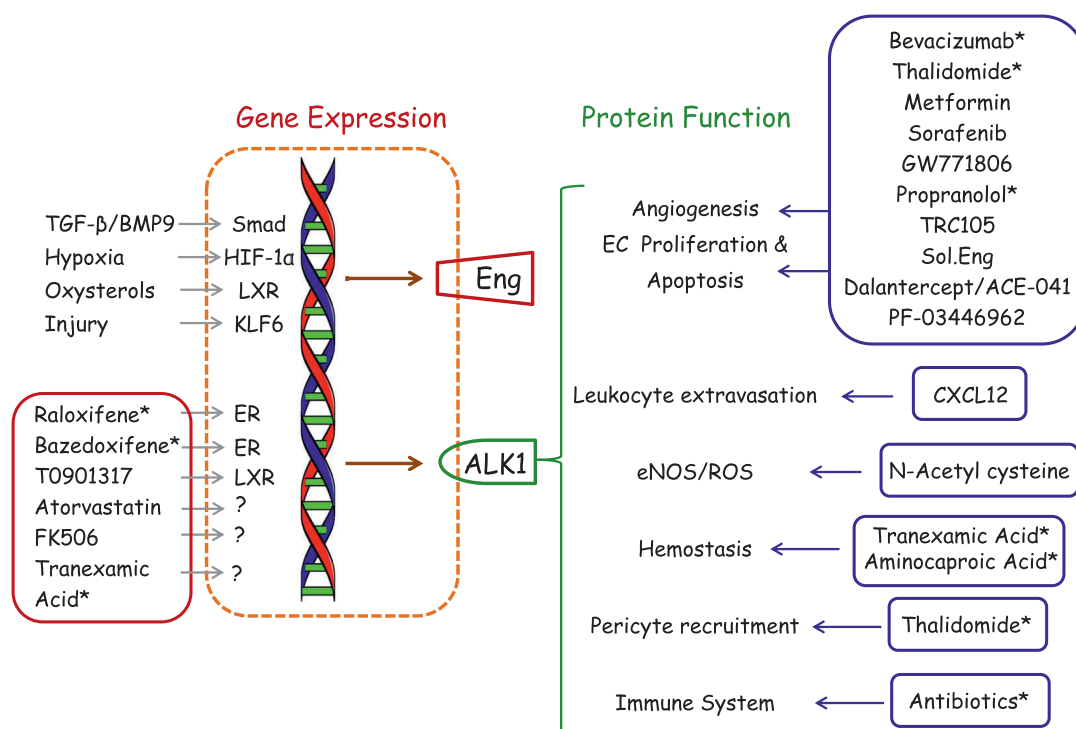




**Figure 2. Hypothetical second hit model in HHT.** The germline heterozygous mutation in the HHT gene leads to haploinsufficiency (First hit). A subsequent event (Second hit) involves inflammation, hypoxia, neoangiogenesis, vascular injury, or a somatic mutation in the healthy HHT allele. Some of these events induce the expression/activation of mediators, which generate a microenvironment where HHT protein levels are below the needed functional threshold. This drop in the HHT functional protein can also be directly obtained after a second somatic mutation of the healthy HHT allele. The resulting deficiency in endothelial cells function leads to the generation of vascular lesions.

distal veins, and capillaries, consistent with the tendency for pulmonary AVMs to form in the distal pulmonary vessels in HHT [37]. A regulatory region of the *ALK1* is sufficient for endothelial expression in arteries feeding ischemic tissues [38]. In addition to endothelial cells, other cell lineages such as macrophages and cells closely related to the vascular system, such as smooth muscle cells of atherosclerotic plaques and cardiac fibroblasts, can express at lower levels endoglin and/or *ALK1* at their surface [28,39–42]. Several studies have analyzed the specific regulated expression of *ENG* and *ALK1* at the transcriptional level. The human endoglin and *ALK1* promoters do not contain TATA or CAAT transcription initiation boxes, but guanine-cytosine (GC)-rich regions with consensus sites for Sp1 which drive their basal transcriptional activity [43–45]. Upregulated expression of endoglin was found in inflamed or infected tissues, healing wounds, psoriatic skin, synovial arthritis, upon vascular injury, and in tumoral vessels [28,46,47]. Moreover, *ALK1* is highly expressed in the vascular structures of the embryo and markedly upregulated in response to several angiogenic stimuli [35,37,48,49]. A variety of stimuli have been reported to increase endoglin or *ALK1* expression in activated vessels, including hypoxia, shear stress, vascular injury, inflammation, and some related cytokines (Figure 3). For example, endoglin expression is upregulated after ischemia in the heart, kidney, and hind-limbs, as well as upon arterial injury [47,50]. Under hypoxic conditions, the hypoxia-inducible factor-1 (HIF-1) complex binds a functional consensus hypoxia-responsive element in the endoglin gene promoter [51]. TGF- $\beta$ 1 signaling, via Smad transcription factors, also potently stimulates endoglin expression [43,44]. Interestingly, BMP9, another member of the TGF- $\beta$  family mutated in HHT5, can also enhance the expression of endoglin [52]. Whereas the hypoxic environment moderately enhances endoglin transcription, addition of TGF- $\beta$ 1 under hypoxic conditions results in a transcriptional cooperation between both

signaling pathways, leading to a marked stimulation of endoglin expression. This synergic stimulation involves the formation of a transcriptional multicomplex containing Smad3/Smad4, Sp1, and HIF-1 [51]. In addition, endoglin expression can be enhanced by the hypoxia-induced pathway involving reactive oxygen species (ROS) which, in turn, oxidize cholesterol yielding oxysterols. Then, oxysterols associate with the nuclear receptor liver X receptor alpha (LXR), which binds to LXR response elements on the endoglin promoter [53]. As a member of the nuclear hormone receptors (NHRs) family, LXR is a transcription factor that works in concert with other family members to regulate gene expression. Some examples of NHRs are the retinoid acid receptor, the retinoid X receptor, or the vitamin D receptor. Among the ligands for NHRs are bile acids, retinoids, steroid hormones, thyroid hormone, and vitamin D. Of note, consensus vitamin D- and estrogen-responsive elements have been identified in the endoglin gene promoter [43]. The increased expression of endoglin can also be obtained upon *in vitro* culture of endothelial cells using the LXR synthetic agonist T0901317, suggesting the potential use of this agonist *in vivo* to counteract endoglin haploinsufficiency [53,54]. Other stress conditions that may act as putative second hits in HHT are vascular injury and shear stress. On vascular injury, the transcription factor Krüppel-like factor 6 translocates to the nucleus of endothelial cells activating endoglin, *ALK1*, and MMP-14 gene transcription, in synergy with Sp1 [39,50,55] (Figure 4). In addition, endothelial cells exposed to shear stress *in vitro* induce increased levels of endoglin mRNA, but not *ALK1* [58]. Of note, fluid shear stress potentiates BMPs to activate *ALK1* signaling, which correlates with enhanced association of *ALK1* and endoglin [59]. The same authors also showed that this pathway mediates both inhibition of endothelial proliferation and recruitment of vascular mural cells, suggesting that a deficient endoglin/*ALK1* pathway blocks flow-induced vascular stabilization and this



**Figure 3. Modulators of gene expression and protein function of endoglin and ALK1.** Left, gene expression of endoglin (Eng) and/or ALK1 can be upregulated by different physiological stimuli including TGF- $\beta$ 1/BMP9, hypoxia, oxysterols or vascular injury through the activation of the transcription factors Smad, HIF-1 $\alpha$ , LXR or KLF6, respectively. Several drugs have shown to be able to stimulate endoglin and ALK1 expression (red rectangle), including raloxifene, bazedoxifene, the synthetic agonist of oxysterols T0901317, the immunosuppressor FK506, atorvastatin and tranexamic acid. Some of these drugs stimulate HHT gene expression via estrogen receptors (ER) or Liver X Receptors (LXR), as indicated. Right, endoglin and ALK1 proteins are involved in several functions and biological processes, including angiogenesis, proliferation/apoptosis, leukocyte extravasation, cytoskeletal organization, vasodilatation, caveolae-dependent signaling, hemostasis, immune system and pericyte recruitment, among others. Several drugs have been shown to modulate some of the endoglin/ALK1-dependent functions (blue rectangles). Among these, the angiogenesis process appears as a preferential target where bevacizumab, thalidomide, metformin, sorafenib, GW771806, propranolol, TRC105, Sol.Eng, dalantercept/ACE-041, and PF-03446962 may act displaying an anti-angiogenic effect. The procoagulant drugs tranexamic and aminocaproic acids can act on the hemostasis process, whereas thalidomide stimulates the pericyte recruitment to the endothelium. The asterisks indicate those drugs which have been used in the treatment of HHT patients. Full color available online.

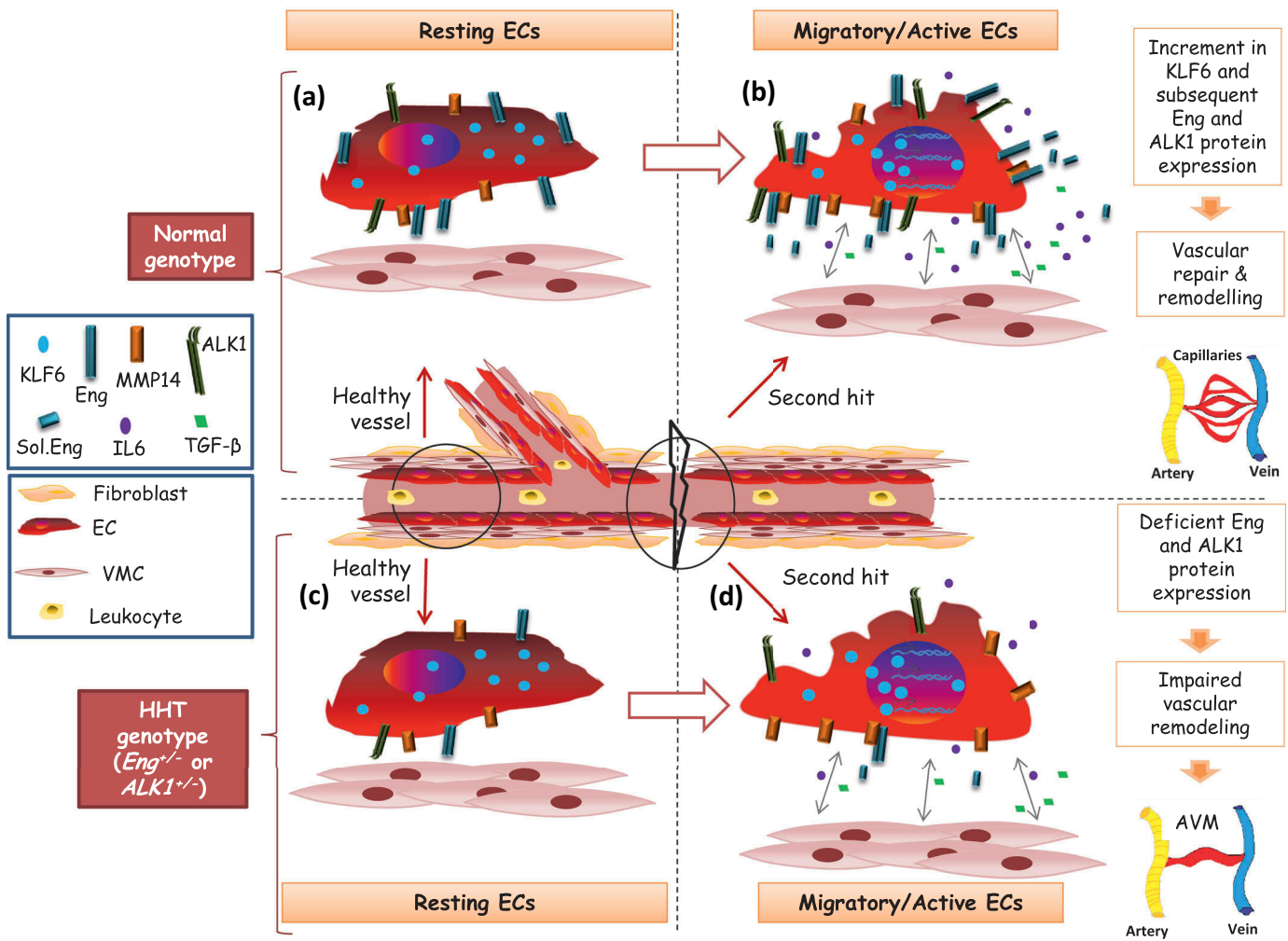
may be a molecular mechanism for development of HHT lesions. Indeed, endoglin prevents AVMs by regulating flow-induced cell migration and specification through VEGFR2 signaling [60].

At variance with the upregulators of HHT genes, certain stimuli may induce their decreased expression. For example, in endothelial cells, the inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) decreases endoglin protein levels [56,61]. It is likely that this effect is mediated by proteases, which acting on membrane endoglin can release a soluble form of the protein. In this regard, the membrane-bound metalloprotease MMP-14 cleaves cell surface endoglin at a Gly-Leu bond in the juxtamembrane region, releasing most of its extracellular domain [54,57]. This catalytic activity can be enhanced when using antibodies to endoglin, including TRC105, which enhance endoglin shedding through direct coupling of endoglin and MMP-14 at the cell surface to release soluble endoglin [62]. By decreasing the surface expression of endoglin, these putative second hit factors may enhance endoglin haploinsufficiency, thus favoring the loss of endoglin function and, in turn, leading to vascular lesions.

### 5. Targeting endoglin and ALK1 protein function

Endoglin and ALK1 play a critical role in angiogenesis, and this function is the target of many therapeutic approaches used in

HHT. Angiogenesis is a physiological homeostatic process by which the body supplies oxygen and metabolites to the different organs or tissues. This process involves the formation of new vessels through two consecutive steps of activation and maturation, both driven by endothelial cells, the target cells in HHT. Thus, upon angiogenic stimulation, endothelial cells undergo proliferation, migration, budding, tube formation, maturation, and maintenance of quiescent endothelium. The maturation phase involves a progressive decrease in endothelial cell proliferation and the recruitment of vascular mural cells, namely pericytes or vascular smooth muscle cells (VSMCs). While pericytes serve to stabilize capillaries, VSMCs are essential for arterial structure and function. These processes are driven by a complex interaction of different growth factors such as VEGF, fibroblast growth factor, and TGF- $\beta$  and their specific receptors, including endoglin and ALK1. Indeed, the signaling pathway driven by these two receptors has been shown to modulate *in vitro* the TGF- $\beta$  responses of endothelial cell to proliferation and apoptosis [63,64] and its deficient expression in HHT1 or HHT2 animal models leads to a markedly reduced level of angiogenesis [27]. The protective role of the endothelial endoglin/ALK1 route in response to TGF- $\beta$ -induced inhibition of cell proliferation and apoptosis has been proposed to explain the disappearance of the capillary bed in the haploinsufficient HHT context [28]. Pericyte recruitment is a promising therapeutic target in HHT [65]. Indeed, the involvement of endoglin [40–42] and

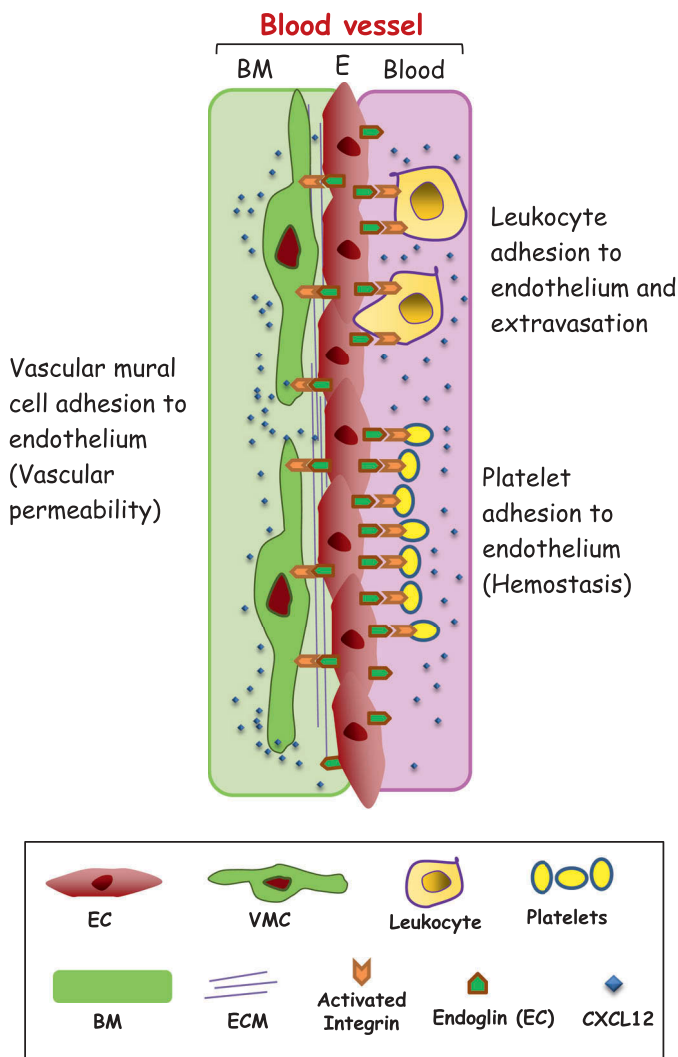


**Figure 4. Hypothetical model of vascular injury as a second hit in HHT.** In a healthy vessel with normal genotype ( $Eng^{+/+}$  or  $Acv11^{+/+}$ ) there is basal protein expression of Eng and ALK1 and a crosstalk between vascular mural cells (VMCs), namely pericytes and vascular smooth muscle cells (vSMCs), and endothelial cells (ECs) which maintain a well balanced angiogenesis (a). Upon vascular injury (second hit), KLF6 translocates from the cytoplasm to the nucleus and activates endothelial genes such as endoglin [50], ALK1 [39], IL6 [39] and MMP14 [55]. MMP14 co-localizes with endoglin at the cell surface, triggering the release of soluble endoglin. Soluble proteins such as IL6, endoglin and TGF- $\beta$  are potential players in the cross-talk between ECs and VMCs during vascular repair. Under these conditions, the injury-induced increased expression of endoglin or ALK1 allows for a proper vascular repair and remodeling (b). In a quiescent vessel with an HHT1 or HHT2 genotype ( $Eng^{+/-}$  or  $Acv11^{+/-}$ ), although there is a decreased basal expression level of Eng and ALK1 proteins, this does not affect the vessel stability (c). However, upon vascular injury (second hit), KLF6 is able to upregulate the expression of the healthy HHT allele, but the final expression levels of endoglin or ALK1 are not able to reach the functional threshold required for the optimal vascular repair and remodeling, leading to the generation of AVMs. In addition, the HHT haploinsufficiency status may be enhanced by a second somatic mutation in the healthy HHT allele [27] or by the proinflammatory TNF- $\alpha$  [56] or the metalloproteinase MMP-14 [55,57], which may trigger the shedding of soluble endoglin and decrease the levels of membrane bound endoglin (d). This hypothetical model can also apply when the second hit is inflammation, trauma, hypoxia, second somatic mutation, etc. Adapted from Gallardo-Vara et al. [55].

ALK1 [66] in the recruitment of vascular mural cells to the endothelium has been reported. In the case of endoglin, this process is due, at least in part, to the role of endoglin as an integrin counter receptor in cell–cell adhesion [41] (Figure 5). Interestingly, thalidomide, an antiangiogenic drug used in HHT therapy, has been shown to act by enhancing pericyte recruitment to the endothelium [67]. The adhesive activity of endothelial endoglin has also been described to engage leukocyte [56] and platelet [68] integrins, in turn regulating leukocyte extravasation and endothelial-dependent hemostasis, respectively (Figure 5). In this regard, the leukocyte infiltration has been postulated to be involved in the vascular remodeling associated with the generation of HHT vascular lesions [69–71]. Noteworthy, the adhesion of endoglin to integrins is markedly enhanced under inflammatory conditions, as well as in the presence of the chemokine CXCL12, which is able to activate integrins to bind their ligands [41,56], suggesting that this

chemokine is a potential therapeutic target to activate the endoglin adhesive function. Moreover, several procoagulant therapies, using tranexamic and aminocaproic acids, aim at regulating the hemostasis process in HHT [2,72].

The relevance of angiogenesis as a therapeutic target in HHT [73] is emphasized by the fact that several antiangiogenic drugs have been used in the clinic, as well as in animal and *in vitro* models of HHT (Figure 3). Among them, bevacizumab, a humanized antibody to VEGF, and thalidomide have been used for HHT patients [67,74]. Using an HHT2 animal model, the oral antiangiogenic tyrosine-kinase inhibitors (TKIs) sorafenib and GW771806 significantly improved anemia and GI bleeding but were not effective for inhibiting the development of wound-induced skin AVMs [75]. Based on its pleiotropic effects that include antiangiogenic properties, metformin has been recently proposed as a prophylactic or



**Figure 5. Schematic diagram showing the role of endoglin in integrin-mediated cell adhesion between ECs and vascular or circulatory cells.** Left, the endothelium (E) of the blood vessel is surrounded by vascular mural cells (VMCs) and ECM proteins. Endothelial cells (ECs) and VMCs share a common basal membrane (BM). During vascular development and stabilization, binding of the homeostatic chemokine CXCL12 can activate VMCs integrins, leading to binding of endothelial endoglin to integrins on VMCs. This endoglin-dependent cellular adhesion may be involved in vascular development, stabilization and permeability. Right, on inflammatory stimuli, different soluble factors are released, including the chemokine CXCL12, leading to activation of integrins present on leukocytes and platelets. Once activated, integrins bind to endoglin present on the endothelial cell (EC) surface, allowing the extravasation and migration of leukocytes to the inflammatory site and contributing to the complex process of endothelium dependent hemostasis. In the HHT1 vascular context subjected to inflammation/injury, endoglin haploinsufficiency leads to a decreased binding of endothelial endoglin to integrins in VMCs, leukocytes and platelets, leading to an impaired VSMC recruitment, leukocyte extravasation, and endothelial-dependent hemostasis. The presence of other adhesion molecules in endothelial cells has been omitted for simplification.

curative therapy in HHT patients with lung involvement [76]. Moreover, the  $\beta$ -blocker propranolol, originally designed for cardiological indications, is nowadays considered as an antiangiogenic drug for HHT therapy [77]. In fact, topical application of timolol, a propranolol related compound with nonselective beta blocker activity, decreases the severity and frequency of epistaxis in HHT patients [78].

Because endoglin and ALK1 play a crucial role in angiogenesis, several related reagents have been generated aimed to

inhibit angiogenesis in pathologies associated with abnormal angiogenesis. Among these, TRC105 is a chimeric monoclonal antibody to endoglin that inhibits angiogenesis and causes antibody-dependent cellular cytotoxicity and apoptosis of proliferating endothelium [79]. TRC105 is currently used, alone or in combination with other antineoplastic agents, in antiangiogenic therapies of several types of cancer [79–81], including more than 20 clinical trials involving solid tumors (<https://clinicaltrials.gov/>). A soluble form of endoglin, encompassing the extracellular domain of the membrane bound protein, also displays antiangiogenic activity *in vivo* [19,41], an effect likely mediated by sequestering endoglin-specific ligands as BMP9, and thus antagonizing the function of membrane-bound endoglin. Two ALK1-related pharmacological inhibitors, an ALK1-Fc fusion protein (Dalantercept/ACE-041) and a human antibody against the extracellular domain of ALK1 (PF-03446962), have been used as an antiangiogenic strategy in preclinical studies and are currently under clinical development [35]. All these antiangiogenic reagents related to endoglin and ALK1 somehow antagonize the function of membrane endoglin and ALK1, and in line with the antiangiogenic strategy in HHT [73], it would be interesting and intriguing to test their potential therapeutic effects in this disease.

Endoglin and ALK1 interact with endothelial nitric oxide synthase (eNOS) and regulate its activation [82–84]. Also, mice heterozygous for *Eng* and *Acvrl1* cause eNOS uncoupling and generation of ROS, leading to impaired NO-mediated vasodilatation. The mice showed age signs of pulmonary arterial hypertension attributable to eNOS-derived ROS, which was preventable by antioxidant treatment. In line with these findings, the antioxidant *N*-acetyl-cysteine (NAC) has been used to treat epistaxis in HHT patients with beneficial effects [85].

Recently, it has been reported that mice lacking endoglin in macrophages show an impaired immune response [86], which could account for the higher rate of infectious diseases seen in HHT [87]. Indeed, antibiotic prophylaxis to prevent infectious outcomes is highly recommended when dental and invasive procedures are required in HHT patients, primarily if pulmonary AVMs are present or suspected [2,3,5].

Endoglin and ALK1 have been shown to regulate other functions, widely discussed in previous reviews [28,34,35,47,88,89], but apparently not targeted by HHT therapeutic approaches.

## 6. Current and prospective therapies

### 6.1. Surgical therapies

Embolizations are used to close the AVMs, direct shunt connecting arterial blood, often present in lung up to 30–50% of the cases, being more frequent in HHT1. Pulmonary AVMs with feeding vessels 2–3 mm or greater in diameter require catheter-based occlusion. The devices used for embolization are balloons, coils, and more recently Amplatzer® (St. Jude Medical). Brain arteriovenous fistulae are less frequent than pulmonary AVMs, up to 5–10% in HHT patients. Although treatment of asymptomatic brain AVMs is controversial, in some specific cases where the lesions are accessible for



embolization, they can be closed by onyx glue. Liver AVMs are present in 30–70% of patients and are most frequent in HHT2. Fortunately in most cases, liver AVMs remain asymptomatic. However, liver transplantation is the only alternative when the AVMs lead to serious impairment of the hepatic functionality [2,5,90]. GI bleeding is treated with iron replacement therapy and if needed with endoscopic ablation or surgical resection of bleeding sites. When treating epistaxis, minor surgeries are often used such as low penetrance laser closure by NYag or Argon laser, and sclerotherapy near the telangiectases with local injections of polydocanol or ethoxysclerol, which act as a plug in the vessel [91]. Major surgeries include septodermoplasty or substitution of the nasal mucosa by skin of the upper thighs, and the modified Young's technique, which involves the complete nose closure by sewing the internal nasal mucosa.

## 6.2. Pharmacological therapies

Different options to control nose and gastric bleeding are used [2,92], according to different strategies where endoglin and ALK1 can be direct or indirect targets.

### 6.2.1. Antifibrinolytics

Although HHT is not a disease derived from a coagulation failure, reinforcing coagulation versus fibrinolysis is useful. The decrease of hemorrhages stabilizing the fibrin network with antifibrinolytics is at the first line of epistaxis treatment. Tranexamic acid is an antifibrinolytic agent 10-fold more potent than  $\epsilon$ -aminocaproic acid, and with longer half-life. An increase of fibrinolytic activity on the wall of telangiectases has been postulated [93], and  $\epsilon$ -aminocaproic and tranexamic acids may act by inhibiting fibrinolysis associated with telangiectases, in addition to increase the amount of endoglin and ALK1 [72]. The only contraindication of antifibrinolytics would be in those patients prone to thrombosis [94]. The first results encouraged specialized centers to design multidisciplinary clinical trials to assess the benefits of tranexamic acid in HHT patients. Thus, the ATERO study demonstrated a significant decrease in the duration of epistaxis in HHT patients taking tranexamic acid in a multinational center study [95]. Another report found that tranexamic acid reduces epistaxis in patients with HHT in a double-blind crossover clinical trial phase IIIB (clinical trial registration no. BfArM 141 CHC 9008-001 and NCT01031992) [96].

### 6.2.2. Hormonal therapy: specific estrogen receptor modulators (SERMs)

The observation that epistaxis often tend to increase after the menopause in women led to the idea that estrogens might exert a kind of protective effect concerning HHT-derived bleeding in women. Next, the option of using specific estrogen receptor modulators (SERMs) to regulate the corresponding NHRs, and not directly estrogens or progesterone, was considered. Tamoxifen, well tolerated in postmenopausal women, was successfully used in two clinical trials, significantly decreasing epistaxis [97]. In the context of SERMs, the efficacy and safety of raloxifene hydrochloride (a drug of the same group as tamoxifen, and with advantages on bone

mineralization, cardiovascular and gynecological cancer prevention) was assayed in a pilot study including 19 postmenopausal women diagnosed with osteoporosis. The results showed a significant decrease in frequency and quantity of epistaxis, as well as a concomitant increase in hemoglobin levels, after 6 months of raloxifene oral treatment (60 mg/day) [98]. Studies on the biochemical basis of raloxifene action on endothelial cells showed that estrogens, and raloxifene in particular, are transcriptional activators of ENG and ACVRL1/ALK1 gene promoters. Thus, the effective amount of endoglin and ALK1 proteins, haploinsufficient products in patients from HHT, is actually increased compensating, at least partially, for the haploinsufficiency. Raloxifene hydrochloride was designated as the first orphan drug for the treatment of HHT-derived bleeding in 2010 by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) (EU/3/10/730; FDA 21 U.S.C 360 bb). A more recent study with the third-generation SERM bazedoxifene acetate yielded similar results to those obtained with raloxifene [99]. Significant decreases in the frequency and quantity of epistaxis, with an improvement in the hemoglobin levels, were detected already after 1 month of treatment, with a dose of 20 mg/day. Both SERMs, raloxifene and bazedoxifene, are effective in upregulating endoglin and ALK1 transcription. Bazedoxifene is more cost-effective than raloxifene (dose of 20 vs. 60 mg/day, respectively) [99] and has obtained the orphan drug designation for HHT in 2014 by the EMA (EU/3/14/1367).

### 6.2.3. Immunosuppressor (FK506)

The efficacy of tacrolimus (FK506) in increasing ENG and ALK1 expression was previously reported [100]. The rationale to consider FK506 came from a case report of a patient with HHT who received a liver transplantation after hepatic failure due to liver AVMs. The liver was transplanted, and the immunosuppressor FK506 was used to prevent the rejection. After the first month of FK506 treatment, the internal and external telangiectases, epistaxes, and anemia disappeared [101]. It should be noted that continuous use of FK506 is not recommended due to its suppressor effect on the immune system. *In vitro* studies showed that FK506 increases the protein and mRNA expression of ENG and ALK1 in cultured endothelial cells and enhances the TGF- $\beta$ 1/ALK1-signaling pathway and endothelial cell functions like tubulogenesis and migration [100]. These results suggest that the mechanism of action of FK506 involves a partial compensation of endoglin and ALK1 haploinsufficiency and might therefore be an interesting drug for use in patients with HHT who undergo transplantation.

### 6.2.4. Atorvastatin

It has been published that atorvastatin (ATV) is able to increase endoglin and eNOS expression and reduce plaque size beyond its lipid-lowering effects by unknown mechanisms [102]. After treatment of human umbilical vein endothelial cells (HUVECs) with TNF- $\alpha$ , endoglin and eNOS protein expression was reduced. By contrast, ATV treatment increased endoglin and eNOS protein expression, while preventing TNF- $\alpha$ -mediated downregulation of endoglin and eNOS protein levels [102]. These results suggest that ATV treatment prevents inflammation-reduced endoglin and eNOS expression in

endothelial cells. On the other hand, ATV-induced eNOS expression strongly depends on the proper expression of endoglin in HUVECs. These findings might have implications in pathological conditions characterized by reduced expression of endoglin and eNOS as for example in HHT. Thus, it would be interesting to analyze whether hypercholesterolemic HHT patients treated with statins show an epistaxis improvement respect to untreated patients of the same age and condition. Nonetheless, secondary effects of statins, like muscle pain, increased risk of diabetes mellitus, and abnormalities in liver enzyme tests should be considered.

### 6.2.5. Antiangiogenic approach

Decreasing the excess of abnormal vasculature present on the nasal mucosa by anti-angiogenesis is a current strategy to decrease bleeding in HHT. Protein levels of VEGF appear to be elevated in HHT patients [103], a finding compatible with the fact that partial ALK1 deficiency *in vitro* and *in vivo* results in elevated VEGF expression [104]. Since VEGF is the main angiogenic factor, these results suggest that the VEGF-dependent angiogenic stimulus may be involved as a second hit in the generation of HHT AVMs. In agreement with this hypothesis, it has been shown that VEGF neutralization can prevent and normalize AVMs in an HHT2 animal model [33]. This experimental model represents an invaluable system to analyze the precise molecular mechanism of action of VEGF blockades, as well as for preclinical screening of drug candidates for epistaxis and GI bleedings. Neutralization of VEGF with bevacizumab has been shown to be beneficial when treating HHT patients with severe hepatic AVMs [74] or severe GI bleeding [105,106]. However, in two independent studies, a bevacizumab nasal spray treatment, compared with a placebo, did not reduce epistaxis duration in HHT patients [107,108]. Of note, certain undesired side effects on the use of bevacizumab have been reported, including wounds that don't heal, nausea, vomiting, GI perforation, or constipation.

VEGF and PI3K/AKT signaling are increased on Alk1 deletion and BMP9/10 ligand blockade [109], whereas genetic deletion of the signal-transducing Vegfr2 receptor prevents excessive angiogenesis but does not fully revert AVM formation. Conversely, pharmacological PI3K inhibition efficiently prevents AVM formation and reverts established AVMs. Thus, Alk1 deletion leads to increased endothelial PI3K pathway activation, a route that may be a putative target for the treatment of vascular lesions in HHT2. More recently, Kim et al. [75] have evaluated the response of an adult Alk1-inducible knockout (iKO) model to oral administration of the different TKIs: sorafenib, sunitinib, erlotinib, and a pazopanib analog (GW771806) on hemoglobin level, GI hemorrhages, and formation of wound-induced skin AVMs. While sorafenib and GW771806 significantly improved, yet erlotinib worsened, anemia and GI bleeding in the Alk1-iKO model. However, none of these TKIs were effective for inhibiting the development of wound-induced skin AVMs. These results suggest that oral delivery of antiangiogenic TKIs is selectively more effective for GI bleeding than for mucocutaneous AVMs.

Thalidomide is another antiangiogenic drug, reported to suppress levels of angiogenic and growth factors, including VEGF, TNF- $\alpha$ , and bFGF. Lebrin et al. [67] have shown that

thalidomide reduces epistaxis by stimulating vessel maturation through pericyte recruitment on the vasculature walls. A pilot study on the clinical use of thalidomide in HHT has been reported [110], indicating that thalidomide may have a positive effect on the number and severity of epistaxis. However, adverse side effects of this drug, including severe malformations in infants born from mothers who had taken the drug during pregnancy, depression, muscle weakness, constipation, fainting, or dullness, should be taken into account.

The beta-blocker propranolol displays antiangiogenic properties and has been recently designed as an orphan drug for the von Hippel Lindau disease (EU/3/17/1841). This drug, initially prescribed as antihypertensive and antiarrhythmic, is currently the choice treatment for infantile hemangioma. An *in vitro* study demonstrated the antiangiogenic and pro-apoptotic properties of propranolol on endothelial cells, suggesting the use of propranolol for topical use in HHT [77]. Since then, several reports have shown that topical application of nonselective beta blockers, timolol and propranolol, decreases the severity and frequency of epistaxis in HHT [78,111]. The potential systemic administration of this drug family of beta blockers should consider its hypotensive activity.

### 6.2.6. Antioxidants

The effect of the antioxidant NAC was tested in large series of 43 HHT patients taking oral NAC 600 mg three times a day for 12 weeks, demonstrating a significant decrease in frequency and severity of nose bleeds [85]. The improvement was most evident in male patients and HHT1 patients with an *ENG* mutation. In women and HHT2 patients with an ALK1 mutation, only a trend for improvement was observed. Because of the favorable side effects and the positive results with NAC, these results may somehow justify a future randomized clinical trial. Antioxidants are expected to counteract the harmful effects of ROS and consequently may prevent or treat oxidative stress-related diseases. Because oxidative stress contributes to endothelial dysfunction in mouse models of HHT1 and HHT2 [112], it is possible that ROS contributes to precapillary sphincter abnormalities, resulting in epistaxis. In this regard, eNOS is coupled to the endoglin/ALK1/Hsp70 protein complex for its proper enzymatic activity and the decrease of endoglin or ALK1 uncouples eNOS, leading to less NO production and an increase of O<sub>2</sub> radicals [84]. In addition to a direct effect of NAC neutralizing ROS, it has been described that NAC can modulate the TGF- $\beta$ -signaling pathway, in part, by driving the monomerization of the disulfide-linked dimer of native endoglin [113].

### 6.3. Gene therapy

Despite great progress in drug development, effective therapies for genetic diseases like HHT are still needed. In this regard, gene therapy holds a promising future and can be used to prevent, treat, or cure different disorders. It deals with the replacement of the defective genes with their correct copies to produce functional proteins, the inactivation of a mutated gene or the introduction of a new gene into the patient. In the two last decades, gene therapy has been driven by improvements in viral and nonviral delivery strategies and many are

now in clinical trials ([www.genetherapynet.com](http://www.genetherapynet.com)). Currently, gene therapy is a promising alternative in inherited disorders, such as severe combined immune deficiency (ADA-SCID) [114], chronic granulomatous disorder [115], and hemophilia [116]. Given the lack of effective treatments for HHT, gene therapy may be considered a promising alternative as it is in other hereditary pathologies. The first *in vivo* nonviral gene delivery approach to treat HHT by gene therapy was performed by inserting endoglin and ICAM-2 promoters upstream of human endoglin cDNA in an expression plasmid. *In vivo* expression of this transgene was clearly detected in several organs from adult transgenic mouse [117]. After systemic delivery, high levels of endoglin were detected in lung, spleen, and liver and upon local gene delivery, endoglin was expressed in the endothelium of vessels present in the dermis and subcutaneous tissue. These results suggested that endoglin and ICAM-2 promoters can be considered excellent tools to specifically deliver HHT genes *in vivo* to the endothelium [117].

Several types of viruses have been engineered to deliver genetic material therapeutically, including the classical retrovirus, lentivirus, adenovirus, and adeno-associated viruses (AAVs). Retroviruses were the first class of viruses to be harnessed for gene transfer, although this type of viral therapy is full of serious safety concerns. CRISPR–Cas9 system is an alternative technology that allows the edition of genome using programmable DNA nucleases packed in an AAV vector. This novel gene editing tool offers a precise method to correct DNA changes but implies a personalized gene therapy as each family's mutation would need to be managed individually [118]. Lentiviral vectors targeting endoglin have also been used for endothelial cell-targeted therapy. This approach allowed targeted gene transfer to endothelial cells with a high degree of specificity *in vitro* [119]. Upon systemic injection, endoglin lentiviral vector specifically transduced liver sinusoidal endothelial cells, but not other liver cells, and a strong gene expression was detected [120]. Recently, it has tested an innovative gene therapy strategy injecting an adeno-associated viral vector serotype-9 expressing soluble FLT1 (AAV9-sFLT1) into the brain to alleviate AVMs. sFlt1 binds to VEGF and therefore neutralizes VEGF pathogenic effects in HHT-associated brain AVMs [121]. This viral vector can potentially be developed into a safer therapy to reduce not only brain AVMs but also those that appear in other organs. One potential setback to the use of AAV vectors for gene therapy is the presence of neutralizing antibodies generated upon natural exposure. In fact, patients with elevated titers of these neutralizing antibodies are usually excluded from the treatment with AAV vectors. In addition, patients who have received AAV-based gene therapy may develop antibodies to the viruses, thus making these individuals poorly suited for retreatment with AAV vectors [122]. Much further research is required to consider gene therapy a plausible option for HHT patients.

#### 6.4. Genetic modulators

Many of the identified HHT mutations in ENG and ACVRL1 are premature termination codons or missense variants, which prevent the proper synthesis of the functional protein.

Interestingly, the FDA and the EMA have approved several innovative drugs, termed genetic modulators, which target some genetic diseases by modulating the efficiency of the mutant protein translation, yielding a more functional protein. Among these drugs are selected antibiotics of the aminoglycoside family such as geneticin (G418), as well as multiple novel synthetic molecules like ataluren [123,124]. The promising results of ataluren in Duchenne muscular dystrophy and cystic fibrosis [125] have prompted preclinical studies aimed to evaluate whether ataluren is able to read through the stop codons in HHT1 and HHT2 mutations [126]; if successful, these studies may lead to new and more effective therapies for HHT.

## 7. Conclusions

In HHT, clinical manifestations range from mild to life-threatening and all patients require a coordinated multidisciplinary approach. In recent years, there has been much progress in the discovery of the genetic basis of HHT and the role of the angiogenesis process in its pathogenesis. HHT spans a vast range of scientific and clinical disciplines and is a challenging disorder both to understand and to manage. Pathogenic mutations in endoglin (HHT1) or ALK1 (HHT2) genes represent a majority of HHT patients, and cellular and molecular mechanisms underlying the generation of HHT vascular lesions are being unraveled in HHT1 and HHT2 experimental models. A wide range of pharmacological strategies has been used to treat nose and GI bleedings, including antifibrinolytics, estrogen receptor modulators, immunosuppressors, and antioxidants. In recent years, there has been much excitement about the use of the antiangiogenic strategy with the VEGF antagonist bevacizumab, whether administered intranasally or systematically. However, these drugs are not always effective, relapses occur after withdrawal, and some of the drugs display undesired secondary effects. Therefore, future research on the identification of novel treatments using the available HHT animal models should yield better therapies to improve the work ability and quality of life of HHT patients. Also, exploring the administration of the current drugs, not only as monotherapy but also in combination with other strategic compounds or stimuli, is needed.

## 8. Expert opinion

To our current knowledge, HHT is caused by a deregulation of the balance between pro-angiogenic and antiangiogenic factors that leads to the generation of mucocutaneous telangiectases and AVMs in internal organs. There is ample and strong evidence from *in vitro* and *in vivo* animal models, supporting the involvement of endoglin and ALK1 in the angiogenic process. Because pathogenic mutations in endoglin (HHT1) or ALK1 (HHT2) genes involve approximately 80% of HHT patients, studies to counteract endoglin or ALK1 haploinsufficiency are critical to discover new and better therapies. The cellular and molecular mechanisms underlying the generation of HHT vascular lesions are quickly being unraveled in HHT1 and HHT2 experimental models, with some data focusing on a defective response to angiogenic stimuli in particular settings where the VEGF system plays a critical role. In fact,

neutralization of VEGF with bevacizumab, a humanized anti-VEGF monoclonal antibody previously used in cancer therapy, can prevent and normalize AMVs in an HHT mouse model. In this line, treatment with bevacizumab of severe GI bleeding in HHT has been achieved with beneficial effects. There are also some HHT case reports on temporal reversal of liver transplantation with bevacizumab administration, suggesting that this drug may be useful in liver failure due to hepatic AVMs. Nonetheless, since HHT patients who have undergone liver transplantation have a long time survival, bevacizumab does not appear as replacement of liver transplantation but may probably serve to delay it. Also, nasal spray treatment with bevacizumab of spontaneous epistaxis has yielded poor results. Blood pressure is a well known on-target effect for anti-VEGF drugs, and adverse events and complications associated with systemic bevacizumab treatments include hypertension, headache, nausea or vomiting, asthenia, diarrhea, and rash. Normalization of the HHT vasculature can also be achieved with thalidomide, another antiangiogenic drug, which has been shown to reduce nose bleeds, likely by promoting pericyte recruitment to the endothelium and vessel maturation. Adverse side effects of thalidomide treatment include nausea, drowsiness, dizziness, and peripheral neuropathy. Although current results show that thalidomide may be a treatment choice for recurrent nosebleeds in HHT patients, the side effects should be considered. A novel derivative of thalidomide, known as pamolidomide (3-amino-thalidomide), was recently approved by the US FDA as a treatment for relapsed and refractory multiple myeloma [127]. It is therefore tempting to investigate the therapeutic effect of pamolidomide in HHT. Other pharmacological strategies include antifibrinolytics (tranexamic and aminocaproic acids), estrogen receptor modulators (raloxifene and bazedoxifene), the immunosuppressor tacrolimus, and the antioxidant NAC, which can counteract haploinsufficiency by upregulating endoglin and ALK1 expression or function.

This review focuses on the new evidence and concepts of this complex condition, placing these in context for both clinicians and scientists. Unfortunately, the current pharmacological treatments remain at the palliative level and some of them display adverse side effects. Overall, there is no fully effective registered drug for HHT. Therefore, novel therapeutic targets need to be explored. To this end, currently available HHT1 and HHT2 animal models are a valuable tool to screen for and identify novel drugs. While the pharmacological treatments for epistaxis and GI bleeding, or embolotherapy in pulmonary AVMs, alleviate the symptoms in HHT patients, achieving the reversion of the vascular lesions remains as an important challenge. It can be postulated that this reversion may be triggered by an angiogenic cue to facilitate a proper vascular remodeling leading to the disappearance of telangiectasia and AMVs. This process would be somehow opposite to the generation of vascular lesions that occurs in an HHT haploinsufficient background, where a second hit is involved. In this case, however, in order to make sure that the second hit induces a proper remodeling, HHT haploinsufficiency should be minimized by increasing the expression or functional activity of the corresponding HHT protein. As described above, several stimuli can enhance

the expression of endoglin or ALK1, including TGF- $\beta$ , BMP9, vascular injury, hypoxia, oxysterols, raloxifene, bazedoxifene, and tranexamic acid, and may serve to this purpose. Experiments with HHT cellular and animal models have yielded additional putative modulators of endoglin/ALK1 function. Among these are (1) PI3K inhibitors which inhibit AVM formation; (2) TKIs, which show beneficial effects on anemia and GI bleeding; (3) integrin activators as CXCL12, which allows binding of endothelial endoglin to the corresponding integrins on leukocytes, pericytes, or platelets; (4) BMP9 which acts upstream of endoglin and ALK1 in the HHT-signaling pathway and its blockade aggravates HHT symptoms; (5) oxysterols and other agonists of nuclear LXR, which can upregulate ENG expression; and (6) compounds such as propranolol, soluble endoglin, TRC105, and Dalantercept/ACE-041, all with antiangiogenic properties. Gene therapy is also a good option to target endoglin or ALK1 expression to the vascular endothelium, although this strategy has been poorly explored and deserves further investigation. Overall, we conclude that the accumulated knowledge about the expression and function of endoglin and ALK1, as well as the currently available HHT1 and HHT2 animal models, offer promising opportunities to screen for and identify better treatments able to normalize the vascular lesions in HHT.

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### Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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