

REVIEW

Involvement of the TGF- β superfamily signalling pathway in hereditary haemorrhagic telangiectasia

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Summary

Hereditary haemorrhagic telangiectasia (HHT) is a vascular hereditary autosomic dominant disease associated with epistaxis, telangiectases, gastrointestinal haemorrhages and arteriovenous malformations in lung, liver and brain. It affects 1–2 in 10,000 people. There are at least three different genes mutated in HHT, *ENG*, *ACVRL1* and *MADH4* that encode endoglin, activin receptor-like kinase (ALK1) and Smad4 proteins, respectively. These proteins are involved in the transforming growth factor (TGF)- β superfamily signalling pathway of vascular endothelial cells. Mutations in *ENG* (HHT1) and *ACVRL1* (HHT2) account for more than 90% of all HHT mutations. In this article, we review the underlying molecular and cellular bases and the therapeutic approaches that have been addressed in our laboratory in recent years.

Key words: transforming growth factor; endothelial cells; hereditary haemorrhagic telangiectasia; endoglin; ALK1; Smad; anti-fibrinolytic agents; estrogens

HEREDITARY HAEMORRHAGIC TELANGIECTASIA: CLINICAL MANIFESTATIONS AND GENES INVOLVED

Hereditary haemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber syndrome is a vascular hereditary autosomic dominant disease associated with epistaxis, telangiectases, gastrointestinal haemorrhages and arteriovenous malformations in

lung, liver and brain. Prevalence is around 1–2 in 10,000 according to recent reviews (Abdalla and Letarte 2006, Govani and Shovlin 2009). The disease is included in the Online Mendelian Inheritance in Man (OMIM; #187300; #600376; #175050). The diagnosis is based on clinical criteria, known as the Curaçao criteria (Shovlin et al. 2000). A person is considered as an HHT patient if she/he has, at least 3 out of the following 4 criteria: (i) spontaneous and recurrent epistaxis; (ii) multiple telangiectases at characteristic locations (lips, oral cavity, fingers, nose); (iii) visceral lesions (gastrointestinal telangiectases, pulmonary, hepatic, cerebral or spinal arteriovenous malformations-AVMs); or (iv) a first degree relative with HHT. The penetrance of the disease increases with age and at 45 years, is about 90% (Plauchu et al. 1989). Since HHT patients may have lung and brain arteriovenous malformations before the onset of epistaxis and telangiectasia, the

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establishment of an early molecular diagnosis is necessary. These malformations may give rise to complications, such as brain ictus, brain infarction, brain abscesses, massive haemoptysis and paralysis. International guidelines for the diagnosis and management of HHT have been recently reported (Faughnan et al. 2010).

Two loci are involved by mutation in more than 90% of HHT cases. The first gene identified was *ENDOGLIN* (*ENG*) that maps to chromosome 9 (Fernández-Ruiz et al. 1993, McAllister et al. 1994), representing between 39–59% of the total HHT population. Next, *ACVRL1* (*activin receptor like kinase 1*, also known as *ALK1*) that maps to chromosome 12 was described (Johnson et al. 1996) as being involved in 25–57% of HHT cases. Mutations in *ENG* and *ACVRL1* give rise to HHT1 and HHT2 types, respectively. Up to date more than 700 different mutations have been described in *ENG* and *ALK1*. In around 2% of the total HHT population, the origin of the disease is a mutation in the *MADH4* gene leading to the combined syndrome of Juvenile Polyposis (JP) and HHT (JPHT), although an overlapping spectra of *MADH4* mutations in JP and JPHT has been found (Gallione et al. 2010). Two additional loci were described on chromosome 5 and chromosome 7, whose genes are still unidentified (Govani and Shovlin 2009). Haploinsufficiency is accepted as the cause of HHT1 and HHT2 pathogenicity (Abdalla et al. 2006).

REGULATED EXPRESSION OF HHT GENES

Endoglin and ALK1 are expressed in endothelial cells (ECs), which are the primary cell target in HHT. Endoglin is expressed at low levels in resting ECs, but at high levels in endothelial proliferating cells at sites of active angiogenesis and during embryogenesis (Bernabéu et al. 2009). Other cell types that express endoglin at their surface are macrophages, erythroid precursors in bone marrow, syncytiotrophoblasts and several cell types closely related to the cardiovascular system such as smooth muscle cells of atherosclerotic plaques and cardiac fibroblasts (Bernabéu et al. 2007). The human *ENG* promoter does not contain TATA or CAAT transcription initiation boxes but has GC-rich regions and consensus sites for Sp1, Ets, AP-2, NF κ B, GATA and Smad binding elements (SBE) (Rius et al., 1998). The basal activity of *ENG* transcription involves the proximal Sp1 motifs and an Ets site at –68 (Rius et al. 1998, Botella et al. 2001). Whereas the restricted expression to endothelium requires the presence of enhancers that bind Ets

family members, a negative regulation involves the presence of repressors that recruit Pu.1 and GATA-2 to inhibit *ENG* expression in blood stem/progenitors (Pimanda et al. 2008).

Upregulated expression of endoglin was found in inflamed or infected tissues, healing wounds, psoriatic skin, synovial arthritis, upon vascular injury and in tumoural vessels (Bernabéu et al. 2007, Fonsatti et al. 2010). There is a variety of stimuli responsible for the increased endoglin expression in activated vessels, including hypoxia, vascular injury and related cytokines. Indeed, endoglin expression is upregulated after ischemia in the heart, kidney and hind-limbs, as well as upon arterial injury (Botella et al. 2002, Bernabéu et al. 2007). Under hypoxic conditions, the hypoxia inducible factor-1 (HIF-1) complex binds a functional consensus hypoxia responsive element (HRE) in the *ENG* gene promoter (Sánchez-Elsner et al. 2002). TGF- β signalling, via Smad transcription factors, also potently stimulates endoglin expression (Rius et al. 1998, Botella et al. 2001). Whereas hypoxia alone moderately stimulates endoglin transcription, the addition of TGF- β 1 under hypoxic conditions results in a transcriptional cooperation between both signalling 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 (Sánchez-Elsner et al. 2002). Upon vascular injury, a transcriptional activation of endoglin mediated by the cooperative interaction between Sp1 and KLF6 transcription factors has been reported (Botella et al. 2002). Endoglin expression is also upregulated by synthetic agonists of the liver X receptor alpha, which binds to an LXR response element on the *ENG* promoter, suggesting the *in vivo* involvement of oxysterols, known as potent LXR activators (Henry-Berger et al. 2008). By contrast, tumour necrosis factor-alpha (TNF- α) decreases endoglin protein levels in ECs (Bernabéu et al. 2007).

ALK1 expression has been reported not only in highly vascularized tissues including lung, placenta, and heart, but also at specific sites of epithelial-mesenchymal interactions, and in other cell types such as monocytes, microglia, skin fibroblasts, stellate hepatic cells, chondrocytes, neural crest stem cells and more recently myoblasts (Bernabéu et al. 2007, Velasco et al. 2008). Nonetheless, most studies to date suggest that its major roles are related to the endothelial specific expression pattern. ALK1 is involved in angiogenesis and a regulatory region of *ACVRL1* gene is sufficient for endothelial expression in arteries feeding ischemic tissues (Li et al. 2009). The characterization of the *ACVRL1* promoter and the

study of its transcriptional regulation remain largely unknown. As in the case of *ENG*, the *ACVRL1* proximal promoter does not contain TATA or CAAT boxes, but has multiple GC-rich regions that recruit Sp1 to regulate basal transcription. ALK1 presents several transcriptional start sites and Sp1 is a key factor involved in its transcriptional regulation. Moreover, the methylation status of CpG islands markedly modulates the activity of the ALK1 promoter region.

STRUCTURE OF ENDOGLIN AND ALK1

Both endoglin and ALK1 are type I membrane proteins. Endoglin is expressed as a 180-kDa disulfide-linked homodimer (Gougos and Letarte 1990). It contains a large extracellular domain of 561 amino acids, highly glycosylated mainly in asparagine residues. Structurally, endoglin belongs to the Zona Pellucida (ZP) family of proteins that share a ZP domain of ~260 amino acid residues at their extracellular region (Jovine et al. 2005, Llorca et al. 2007). The three-dimensional structure of the extracellular domain of endoglin at 25Å resolution, using single-particle electron microscopy has been elucidated for the first time in our group (EMDB Entry: EMD-1559) (Llorca et al. 2007). Endoglin is arranged as a dome made of antiparallel orientated monomers enclosing a cavity at one end. Each subunit comprises three well-defined regions, two of them corresponding to the ZP domain. The third region does not show any significant homology to other protein family/domain and thereby has been named the “orphan” domain. A transmembrane region, spanning 25 hydrophobic residues, acts as a linker between the ectodomain and the cytosolic region. Two different alternatively spliced isoforms, the predominant long (L)-endoglin and the minor short (S)-endoglin, are expressed in human and mouse tissues (Gougos and Letarte 1990, Bellon et al. 2003, Pérez-Gómez et al. 2005). In humans, S-endoglin and L-endoglin proteins vary from each other in their cytoplasmic tails that contain 14 and 47 amino acids, respectively, with a sequence of only 7 residues being specific for S-endoglin. Both endoglin isoforms are constitutively phosphorylated and can be targeted by serine/threonine kinases, including the TGF- β type I (ALK1, ALK2 and ALK5) and II receptors (Guerrero-Esteo et al. 2002, Bernabéu et al. 2007). L-endoglin cytoplasmic domain contains a consensus PDZ binding motif (SerSerMetAla) at the carboxyl terminus that mediates endoglin interaction with several PDZ domain-containing proteins and endoglin

phosphorylation of distal threonine residues (Bernabéu et al. 2007, 2009).

ALK1 is a transmembrane protein of approximately 55 kDa with an N-glycosylated ectodomain of 97 amino acids carrying a cysteine-rich small sequence which probably confers the appropriate structural conformation to capture the ligand. The ALK1 cytoplasmic region of 362 amino acids contains (i) a GS domain, a conserved 30 amino acids glycine/serine-rich sequence involved in the regulation of the receptor activation and (ii) a serine/threonine kinase domain. Phosphorylation of serine/threonine residues of ALK1 in the GS domain by the type II receptor (T β R2) leads to a conformational change in ALK1 that allows phosphorylation of the downstream signalling molecules Smad1, Smad5 or Smad8 (Gordon and Blobbe 2008, Goumans et al. 2009). Although there are no data about the three dimensional structure of ALK1, it is possible to build a theoretical model of its cytosolic domain using homology modelling techniques based on the crystal structure of the type I receptor ALK5 (PDB: 1IAS) (Fontalba et al. 2008). The ALK1 structure of the cytosolic domain contains the L45 loop, a small region that interacts with Smads, which confers the signalling specificity among different type I receptors. In addition, the cytosolic region of ALK1 contains a consensus motif between residues 399-406 for the interaction with the scaffolding domain of caveolin-1, a major protein component of caveolae (Santibanez et al. 2008). As shown in Fig. 1, most of ALK1 mutations in HHT2 patients involve the cytoplasmic domain, at variance with HHT1 where endoglin mutations map to the extracellular domain (Fontalba et al. 2008).

FUNCTION OF PROTEINS ENCODED BY HHT GENES. THE TGF- β PATHWAY

The three identified genes mutated in HHT (*ACVRL1*, *ENG* and *MADH4*) encode for proteins involved in the TGF- β signalling pathway (Gordon and Blobbe 2008, Goumans et al. 2009, Govani and Shovlin 2009). Thus, ALK1 is a type I serine/threonine kinase receptor, endoglin is an auxiliary co-receptor without catalytic activity and Smad4 is a transcription factor that mediates the TGF- β signalling downstream of the type I receptors (Fig. 2). Endoglin forms a protein complex with the TGF- β types I and II receptors and the ligand. Several members of the TGF- β superfamily, including TGF- β 1, TGF- β 3, activin-A, BMP-2, BMP-7 and BMP-9 are able to bind endoglin and/or ALK1. This binding triggers the

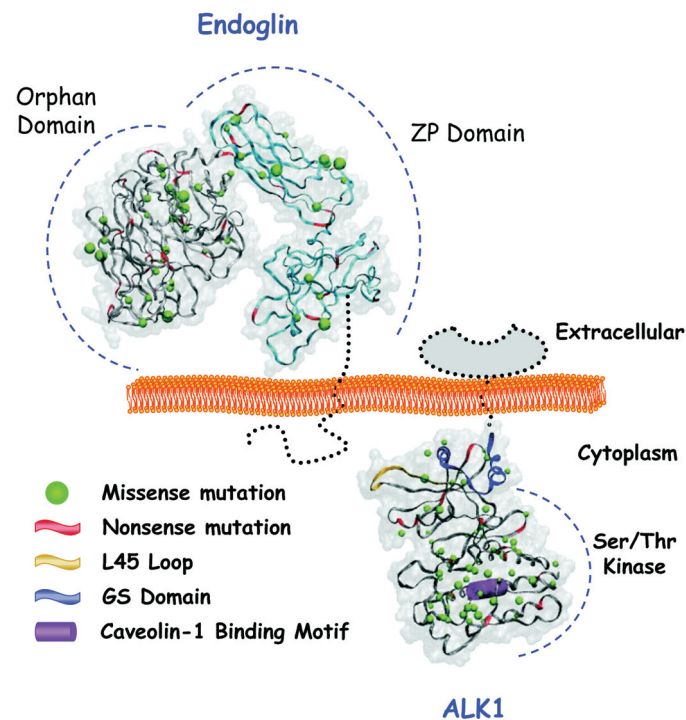


Fig. 1. Endoglin and ALK1 three dimensional structure and HHT mutations. The missense and nonsense mutations described for the extracellular part of endoglin and the intracellular part of ALK1 are shown as green spheres and red segments, respectively. The volume of the green spheres is exclusively related to the size of the mutated residue side chain. The L45 loop, the GS domain and the caveolin-1 binding motif of ALK1 are also indicated.

Smad-dependent downstream signalling (Guerrero-Esteo et al. 2002, Blanco et al. 2005, Gordon and Blobel 2008, David et al. 2009, Goumans et al. 2009). In ECs, endoglin modulates ligand binding and signalling by association with ALK1 and ALK5 (Guerrero-Esteo et al. 2002, Blanco et al. 2005, Santibanez et al. 2007, Velasco et al. 2008). Thus, endoglin inhibits the TGF- β /ALK5/Smad3-mediated cellular responses such as the increased expression of the plasminogen activator inhibitor 1 (PAI-1). By contrast, endoglin promotes the ALK5/Smad2-mediated upregulation of endothelial nitric oxide synthase (eNOS) as well as the TGF- β 1/ALK1-mediated increase of Id1. Interestingly, endoglin inhibits the BMP-9/ALK1 signalling in ECs. Overall, endoglin appears to be a critical modulator of the balance between ALK1 and ALK5 signalling. This balance plays a crucial role during vascular remodelling and angiogenesis, although the underlying molecular mechanisms remain to be elucidated (Bernabéu et al. 2007, Lebrin and Mummery 2008, David et al. 2009, Goumans et al. 2009).

Different studies support the view that endoglin and ALK1 participate in a common signalling pathway that is critical for EC responses to TGF- β family members (Bernabéu et al. 2007, 2009, Lebrin and Mummery 2008). This conclusion agrees with the fact that pathogenic mutations in *ENG* or *ACVRL1* genes result in HHT and that ALK1 and endoglin null mice have similar vascular phenotypes (Abdalla and Letarte 2006). Recently, it has been shown that S-endoglin is up-regulated during senescence of ECs and exerts an antagonistic role to that described above for L-endoglin. S-endoglin is able to interact with both endothelial type I receptors, but showing much more affinity for ALK5 than for ALK1. Consequently, S-endoglin inhibits cellular proliferation and promotes the expression of the ALK5 target gene PAI-1, whereas the ALK1 target Id1 is repressed (Blanco et al. 2008, Velasco et al. 2008).

Endoglin is involved in the control of vascular tone. In fact, endoglin deficient mice (*Eng*^{-/-}) show decreased levels of eNOS and elevated expression of cyclooxygenase-2 (COX-2), both of them key

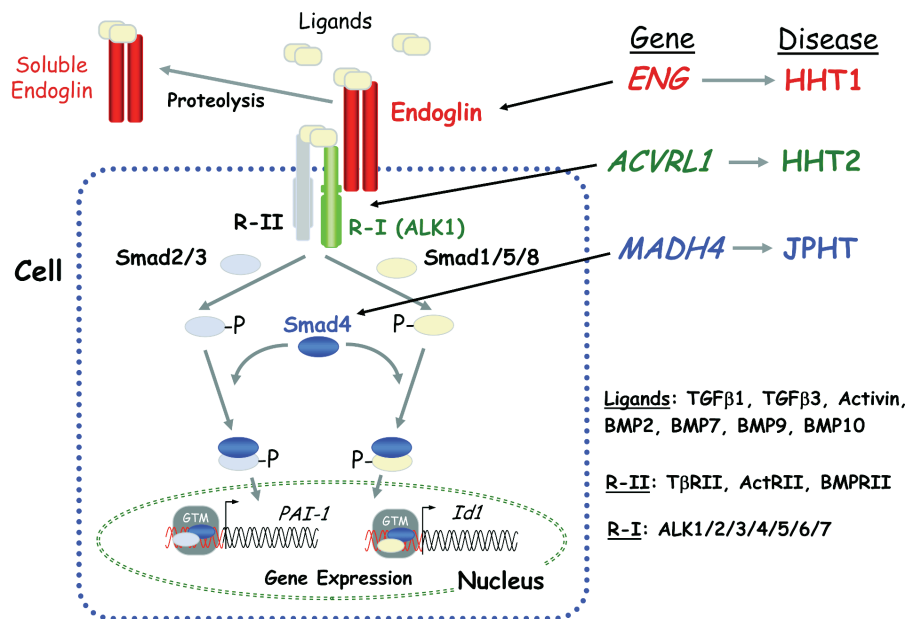


Fig. 2. HHT and the TGF- β signalling pathway. Members of the TGF- β family, which includes TGF- β s, activins and BMPs, bind to specific type I (R-I) and type II (R-II) cell surface receptors that exhibit serine/threonine kinase activity. Endoglin is an auxiliary receptor that associates with ligand, R-I and R-II and modulates signaling via R-I and R-II. A soluble form of endoglin can be generated by juxtamembrane proteolysis of the membrane bound receptor that can sequester ligands and thereby modulating their binding to R-I/R-II. The combinatorial heterodimeric association between R-I and R-II determines the specificity of the ligand signalling. Upon ligand binding, the R-II transphosphorylates R-I, which subsequently propagates the signal by phosphorylating the receptor-regulated Smad (R-Smad; Smad1, 2, 3, 5, 8) family of proteins. Once phosphorylated, R-Smads form heteromeric complexes with a cooperating homologue named Co-Smad (Smad4), and translocate into the nucleus where they regulate the transcriptional activity of target genes (Gordon and Blobel 2008, Goumans et al. 2009). In ECs the R-I, ALK1 and ALK5 activate signalling pathways via Smad1, 5, 8 (ALK1) or Smad2, 3 (ALK5), respectively. Endoglin, ALK1 and Smad4 proteins are encoded by *ENG*, *ACVRL1* and *MADH4* genes, whose pathogenic mutations give rise to HHT1, HHT2 and JPHT, respectively. ActR, activin receptor; BMP, bone morphogenetic protein; BMPR, BMP receptor; GTM, general transcription machinery.

enzymes in the control of the vasodilator responses (Jerkic et al. 2006, Santibanez et al. 2007). It is noteworthy that transgenic mice expressing human S-endoglin (*S-ENG*⁺) show a similar phenotype as *Eng*^{+/-} mice, in agreement with the opposing effects of L-endoglin versus S-endoglin (Blanco et al. 2008).

Endoglin is also implicated in the cytoskeletal organization. The cytoplasmic tail of L-endoglin interacts with members of the LIM domain-containing family of proteins, including zyxin and ZRP-1 (zyxin-related protein-1) (Sanz-Rodriguez et al. 2004). Both proteins serve as docking sites for the assembly of multimeric protein complexes involved in regulating cytoskeleton, assembly and cell motility. Accordingly, blood outgrowth ECs from HHT patients show an abnormal shape compared to controls, exhibiting poor organization of the actin cytoskeleton due to disorganized actin fibers and

depolymerization (Fernández-L et al. 2005). The organization of the capillary network during angiogenesis depends on the structure of ECs so that in the vasculature of HHT patients a disorganized cytoskeleton is prone to cell breaking with changes in shear stress and blood pressure. This might lead to vessel haemorrhages and eventual disappearance of the capillary network, as occurs in HHT.

Endoglin is emerging as a modulator of the TGF- β response with important roles in cancer. It is highly expressed in the tumour-associated vascular endothelium with prognostic significance in selected neoplasias and is a vascular target for antiangiogenic cancer therapy (Bernabéu et al. 2009, Fonsatti et al. 2010). On the other hand, expression of endoglin in the tumour cells appears to play an important role in the progression of cancer, influencing cell proliferation, motility, invasiveness and

tumorigenicity (Pérez-Gómez et al. 2005, Wong et al. 2008, Bernabéu et al. 2009). In addition, *in vitro* and *in vivo* experiments in which endoglin expression is modulated have provided evidence that it acts as a tumour suppressor (Pérez-Gómez et al. 2007).

Increased levels of soluble endoglin have been detected in plasma, serum and urine from patients with different pathologies, including pre-eclampsia and cancer (Bernabéu et al. 2009). Circulating soluble endoglin is a reliable marker of preeclampsia and is associated with poor prognosis in cancer. Whereas it has been postulated a pathogenic role for soluble endoglin in preeclampsia due to its anti-angiogenic activity, the role of soluble endoglin in tumour progression remains to be established (Pérez-Gómez et al. 2007).

HHT THERAPEUTIC APPROACHES. MOLECULAR MECHANISMS OF ACTION

So far, there is no cure for HHT and there is a need to find an effective drug for its treatment. The pharmacological therapeutic strategies should be ideally aimed at: (i) improving the coagulation process or preventing the haemorrhagic condition; (ii) increasing the amount of endoglin or ALK1 on the EC surface, since the pathogenesis of the HHT condition is due to haplo-insufficiency; and (iii) decreasing angiogenesis, because an excess of abnormal angiogenesis has been reported in the HHT condition. There are five different types of pharmacological drugs used for the treatment of HHT bleeding (Table 1). In this review, we will focus on the clinical and molecular data regarding antifibrinolytic drugs and raloxifene, therapeutic agents used in our research group.

The basis for the efficiency of antifibrinolytic agents, epsilon aminocaproic and tranexamic acid (TA) relies on the inhibition of the fibrinolytic activity, by binding the active center of plasmin in the tissues that leads to clot stabilization. TA is a derivative of lysine (4-aminomethyl cyclohexanecarboxylic acid), which binds reversibly to plasminogen, avoiding fibrin degradation by plasmin (Manucci 1998). TA is indicated in severe bleedings with hyperfibrinolysis as it is the case of HHT, showing hyperfibrinolysis secondary to intravascular coagulation. Previous studies have described the use of TA for the treatment of HHT patients with an improvement in epistaxis and the associated anaemia (Sabba et al. 2001).

Since 2003, more than 250 patients from more than 100 different families have been screened by the

HHT unit in Sierrallana Hospital (Cantabria, Spain). A pilot series of oral TA treatment was conducted with a total of 14 patients which had severe epistaxis interfering with their quality of life. In all these cases side-effect risks of thrombosis were absent. All patients showed a decrease in the intensity and frequency of nose bleeds after the first week of treatment. None of them have presented adverse side-effects until now (Fernández-L et al. 2007). However, there is a contraindication for TA in those patients prone to suffer thrombosis. In patients with high levels of coagulation factors, therapies that avoid bleeding may lead to deep venous thromboembolism, therefore alternative therapeutic sources to counteract HHT epistaxis are needed. Hormonal therapy, using estradiol/norethindrone for epistaxis and gastrointestinal management of HHT, has shown a variable degree of efficacy depending on the patient. A case based report with long-term cessation of epistaxis using tamoxifen in a postmenopausal woman was described (Zacharski et al. 2001). Based on these reports, the efficacy of raloxifene, another estrogen receptor modulator (SERM) as tamoxifen, was assessed in 19 postmenopausal HHT women (Albiñana et al. 2010). Raloxifene, a second generation SERM, exhibits an improved clinical profile versus that of tamoxifen and is currently used for the treatment and prevention of post-menopausal osteoporosis. These HHT women patients diagnosed with osteoporosis with ages ranging from 47 to 74 years, had no contraindication for the hormonal therapy, and were good candidates for a hormonal substitutive therapy. After the treatment, all of them showed an improvement of the HHT symptoms concerning epistaxis. The effects on epistaxis were evaluated after 6 months based on the Sadick-designed scale. This scale evaluates the amount and frequency of nose bleeds. In the nineteen patients treated with raloxifene, a decrease in the frequency and the quantity of epistaxis was observed in all patients with at least one grade in the Sadick scale: average of 2.36 versus 1.31 after treatment and 2.26 versus 1.42 after treatment, respectively (Albiñana et al. 2010).

Using *in vitro* cellular experiments, we have also addressed the possible molecular mechanism of action of TA and raloxifene. Both, TA and raloxifene upregulate endoglin and ALK1 protein and mRNA levels as well as their gene promoter activities, suggesting a positive effect on gene transcription. In this regard, we were able to show by chromatin immunoprecipitation experiments that estrogen receptors are involved in the raloxifene-induced transcription. In addition, tube formation in matrigel and wound healing experiments indicate that TA and

Table 1. Different types of pharmacological drugs used to treat bleeding in HHT Centers.

Therapeutic strategy	Observations
Antifibrinolytics	ϵ -aminocaproic acid (AC), tranexamic acid (TA). Not appropriate when patients have pro-thrombotic clinical history, or high level of coagulation factors V and VIII.
Hormonal estrogen therapy	Estrogen/progesterone, ethinyl, estradiol/norethindrone, danazol, phyto-estrogens, SERM (Selective Estrogen Receptor Modulator) like tamoxifene and raloxifene.
Anti-angiogenic drugs (anti-VEGF)	Thalidomide, bevacizumab (avastin). Currently used in trials. Only for severe cases.
Immunosuppressant agents	Sirolimus, tacrolimus, IFN γ . Only case reports available on kidney/liver HHT transplanted patients. Not enough information up to the moment.
Anti-inflammatory/antioxidant compounds	N-acetylcysteine

raloxifene promote functions dependent on the TGF- β /ALK1/endoglin pathway. The mechanism by which these drugs are able to stimulate this TGF- β pathway remains to be elucidated (Fernández-L et al. 2007, Albiñana et al. 2010). In summary, these experiments support the hypothesis that TA and raloxifene are counteracting, at least partially, endoglin or ALK1 haploinsufficiency in HHT patients.

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