UNIVERSITÀ DEGLI STUDI DI PAVIA

Dipartimento di Medicina Molecolare
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Hereditary Haemorrhagic Telangiectasia: mutation analysis, new variant characterization and study of circulating microRNAs in a rare disease

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Dottorato di Ricerca in
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Abstract

The topic of my PhD project is the Hereditary Haemorrhagic Telangiectasia (HHT), also known as Rendu-Osler-Weber syndrome, a rare genetic disease with an incidence of about 1:5000, inherited as an autosomal dominant trait. Up to date, mutations in four genes (*ENG*, *ACVRL1*, *MADH4* and *GDF2*) have been described in HHT, however, *ENG* and *ACVRL1* mutations account for the 85% of patients. All these genes encode for proteins belonging to the TGF-β/BMPs signalling pathway and are involved in the regulation of angiogenesis. HHT is clinically diagnosed if a patient presents at least three out of four criteria, known as "Curaçao criteria": (i) spontaneous and recurrent epistaxis; (ii) telangiectases at characteristic sites, as lips, oral cavity, nose, fingertips and gastrointestinal mucosa; (iii) arteriovenous malformations at characteristic sites, as liver, lungs and central nervous system, and (iv) family history, i.e. a first degree relative with a diagnosis of HHT according to the same criteria.

The aim of this thesis is to further investigate the genetic background of HHT in order to better understand the underlying molecular mechanisms and the genotype-phenotype correlations.

In particular, my work involved the mutation analyses of *ENG* and *ACVRL1* coding exons and the study of circulating miRNAs in a group of HHT patients.

Mutation analyses were performed in new index cases with a HHT clinical diagnosis and their relatives, to identify the disease-causing mutation. Then, the results collected were added to data previously obtained by the Medical Genetics Laboratory (headed by Prof. C. Danesino) and used to perform a descriptive study of the HHT Italian Population, carried out in collaboration with Prof. C. Sabbà, from the University of Bari. Moreover, during my PhD I attended a three-months period in the laboratory of Prof. C. Bernabeu, in Madrid, in order to study the putative pathogenic effect of a particular *ENG* variant (c.1852+42 C>T) found in a HHT family.

The study of circulating miRNAs was performed in collaboration with Prof. M. Denti (University of Trento). We collected plasma samples from 15 people (5 controls; 5 patients with *ENG* mutation and 5 patients with *ACVRL1* mutation) and evaluated the expression level of 752 human miRNAs to identify those misregulated in patients compared to controls. Firstly, we analysed results grouping

the patients according to the mutated gene and we identified about 20 misregulated miRNAs. Then, we evaluated if some miRNAs were differentially expressed in patients according to their clinical symptoms.
In conclusion, the obtained results increased the knowledge on the molecular pathogenesis, and shed a light on the role exploited by miRNAs in this disorder, thus suggesting new genotype-phenotype correlations, although a validation of the results in a bigger population is mandatory.

Acknowledgements

I would use this opportunity to express my gratitude to all the people who sustained me during the last three years.
First of all, I am thankful to Professor Cesare Danesino, who lets me work in his laboratory, and to my scientific supervisor, Dr. Carla Olivieri, for her help and her advices during the whole project work.
I would express my thanks to the other persons of the Medical Genetics laboratory, Dr. Antonella Minelli, Dr. Paola De Filippi, Dr. Federica Ornati, Dr. Lucia Nacci and Dr. Alice Spano, for their suggestions and precious support.
I would also like to thank Dr. Jacopo Morini and Dr. Gabriele Babini, of the Radiobiology and Radiation Biophysics laboratory, for their active support and for their constructive comments.
I would express my gratitude also to Dr. Giuseppe Spinozzi, of the Fondazione IRCSS Policlinico San Matteo, for his availability and kindness.
I thank Prof. Carlo Sabbà and Dr. Gennaro Lenato, of the University of Bari, for their important collaboration.
I would like to extend my warm thanks to Prof. Carmelo Bernabeu for welcoming me in his laboratory, in Madrid, and to Dr. Carmen Langa Poza and Dr. Lidia Ruiz Llorente for all the teachings and for their extremely availability.
Also, I am thankful to Prof. Michela Denti and her team, specially Dr. Valerio Del Vescovo and Dr. Margherita Grasso, for giving me the opportunity to attend the laboratory of RNA Biology and Biotechnology and for teaching me so much.

Abbreviations

ACE: Angiotensin Converting Enzyme
ACVR2A/B: Activin A Receptor Type 2A/B
ACVRL1: Activin A Receptor, Type II-Like Kinase 1
ALK1-7: Activin receptor-Like Kinase 1-7
AMHR2: Anti-Mullerian Hormone Receptor Type 2
APC: Argon Plasma Coagulation
ASF/SF2: Alternative Splicing Factor/Splicing Factor 2
AVM: Arteriovenous Malformation
BMP9: Bone Morphogenetic Protein 9
BMPR1A: Bone morphogenetic protein receptor type 1A
BMPR2: Bone morphogenetic protein receptor type 2
BMPs: Bone Morphogenetic Proteins
CAV1: Caveolin 1
CAVMs: Cerebral Arteriovenous Malformations
Co-SMAD: Common-mediator Small Mother Against Decapentaplegic
CVMs: Cerebral Vascular Malformations
DMEM: Dulbecco's Modified Eagle's medium
ECs: Endothelial Cells
EGD: Esophago-Gastro-Duodenoscopy
EIF2AK4: Eukaryotic translation Initiation Factor 2 alpha Kinase 4
EMSA: Electrophoretic Mobility Shift Assay
ENG: Endoglin
ERK: Extracellular signal–Regulated Kinases
ESS: Epistaxis Severity Score
FISH: Fluorescent In Situ Hybridization
FNH: Focal Nodular Hyperplasia
GDF2: Growth Differentiation Factor 2
GI: GastroIntestinal
GTPase: Guanosine Triphosphatase
HAVMs: Hepatic Arteriovenous Malformation

HES1: Hes family bHLH transcription factor 1
HEY1-2: Hes related family bHLH transcription factor with YRPW motif 1-2
HHT: Hereditary Haemorrhagic Telangiectasia
HOHF: High-Output Heart Failure
HPAH: Hereditable Pulmonary Arterial Hypertension
IAs: Intracranial Aneurysms
ID1: Inhibitor of DNA binding 1
IPAH: Idiopathic Pulmonary Arterial Hypertension
ISMAD: Inhibitory Small Mother Against Decapentaplegic
JNK: Jun N-terminal Kinase
JP: Juvenile Polyposis
KCNA5: potassium voltage-gated channel subfamily A member 5
KCNK3: potassium two pore domain channel subfamily K member 3
MADH4: Mothers Against Decapentaplegic Homolog 4
MH1-2: Mad Homology 1-2
miRNA: micro RNA
MRI: Magnetic Resonance Imaging
MT1-MMP:Membrane Type 1-Matrix Metalloproteinase
NE: Nuclear Extract
NHR: Nodular Regenerative Hyperplasia
OD: Orphan Domain
ORF: Open Reading Frame
PAH: Pulmonary Arterial Hypertension
PAP: Pulmonary Artery Pressure
PAVMs: Pulmonary Arteriovenous Malformations
PC: Proprotein Convertases
PH: Pulmonary Hypertension
PI3K-AKT: Phosphatidylinositide 3-kinase – Protein Kinase B
PVR: Pulmonary Vascular Resistance
qRT-PCR: quantitative Real Time – Polymerase Chain Reaction
rER: rough Endoplasmic Reticulum
ROW: Rendu-Osler-Weber Syndrome
RQ: Relative Quantities
R-SMAD: Receptor-regulated Small Mother Against Decapentaplegic
SC35: serine and arginine rich splicing factor 2
SCAVMs: Spinal Cord Arteriovenous Malformations
SMAD: Small Mother Against Decapentaplegic
SMCs: Smooth Muscle Cells
Sp1: Specific Protein 1
TAK1: TGF-β-Activated Kinase 1
TGBR2: Transforming Growth factor beta Receptor 2

TGBRI/II: Transforming Growth factor beta Receptor type I/II
TGF-β: Transforming Growth Factor beta
TIA: Transient Ischemic Attack
TSR1: Ribosome Maturation Factor 1
TTCE: Transthoracic Contrast Echocardiography
US: Ultrasonography
UTR: Untranslated Region
VEGF: Vascular Endothelial Growth Factor
VUS: Variant of Uncertain Significance
WP361: dimethane sulfonate
WT: Wild Type
ZP: Zona Pellucida

Contents

1. Introduction

Hereditary Haemorrhagic Telangiectasia (HHT) is an autosomal dominant disease with a worldwide incidence of 1 in 5000 - 8000 individuals, even if variations can be observed in different regions (Shovlin 2010). Several authors reported the regional incidence of the disease underling, in some cases, a higher prevalence due to the presence of the founder effect. It was calculated that HHT incidence in both Danish and Japanese population is roughly comparable to the worldwide data; in fact, the disease affects around 1:6500 and 1:5000 – 1:8000 respectively (Dakeishi et al. 2002; Kjeldsen et al. 1999). The incidence is similar also in the French administrative area of Jura (1:5062), while it is higher in the areas of Ain (1:3345) and Deux-Sèvres (1:4287), and in the Afro-Caribbean population from the Leeward islands of Netherlands Antilles (1:1639) (Bideau et al. 1989; Gallione et al. 2000). Moreover, the founder effect was also described in a cohort of French and Italian patients, suggesting the diffusion of the disease among the two Countries (Lesca et al. 2008).
However, probably because of the diagnostic delay, these data underestimate the frequency of the disease (Pierucci et al. 2012; Shovlin 2010).

1.1 History and clinical features of the disease

HHT is also known as Rendu-Osler-Weber (ROW) syndrome, from the name of the scientists who initially described it. Rendu first described HHT in a 52 years old man with recurrent epistaxis and multiple haemangiomatous spots on face, especially on the lips, tongue and palate and hypothesised that similar lesions in the nose could account for the nosebleeds. Moreover, he reported a similar anamnesis for the mother of the patient (Rendu 1896). In 1901, Osler provided a complete description of the disease underling the hereditary nature, the spontaneous and recurrent epistaxis and the presence of numerous angiomas on the face and on the mucosa of nose, lips, cheeks and tongue (Osler 1901). Few years later, Weber increased the clinical description of HHT reporting other cases (Weber 1907). In a paper published in1909 about the characterization of the diseases, Hanes named the disorder “Hereditary Haemorrhagic Telangiectasia”, and provided several illustrations of the typical vascular lesions: the telangiectases (Hanes 1909).

Nowadays, HHT is described as a genetic disease characterised by severe and recurrent bleeding of nasal and gastrointestinal mucosae caused by the presence of abnormal vessels structures in which arteries are directly connected to veins, leading to the consequently loss of the normal capillary beds.
The smallest structures are called telangiectases and normally are observed on the skin of the face, lips, tongue, nasal and gastrointestinal mucosae and fingertips. The largest structures are called ArterioVenous Malformations (AVMs) and involve internal organs as lungs, liver and the central nervous system (Bayrak-Toydemir et al. 2004; Shovlin 2010). Both telangiectases and AVMs are characterized by thin walls of the vessels, that become prone to bleed when exposed to high perfusion pressures (Govani and Shovlin 2009). Indeed, chronic epistaxes are frequently observed in HHT patients and may cause the depletion of body's iron stores leading to anaemia and the requirement of transfusions in some cases (Bayrak-Toydemir et al. 2004). In the HHT spectrum are included other clinical features encompassing pulmonary hypertension due to hepatic AVMs (Brohée et al. 1984), Pulmonary Arterial Hypertension (PAH) (Trembath et al. 2001) and Juvenile Polyposis (JP) (Gallione et al. 2004).

1.1.1. Epistaxis

Recurrent and spontaneous epistaxis related to HHT is the consequence of the rupture of nasal telangiectases. Epistaxis is a major issues in up to 90% of patients, who often referred a reduction in their own quality of life (Faughnan et al. 2011; Hoag et al. 2010). Epistaxis appears before the age of 20 years in about 50% of patients, and nearly all patients present this symptom before the age of 40 years, even if there is high variability of the severity (Faughnan et al. 2011). In fact, the monthly occurrence, the duration and the intensity of epistaxis are quite mutable, varying from less than one bleeding per month to daily ones, from few seconds to several hours and from blood spots to gushes (Dupuis-Girod et al. 2010). In 2009, Pagella et al. established a grading score which aimed to classify the epistaxes severity, based on their frequency, intensity and duration. A score, ranging between 1 and 3, is calculated for each parameter and then added together to obtain the total score. Thus, total score of 7-9 defines “severe” epistaxis, between 4 and 6 it defines “moderate” epistaxis and the minimum score value, 3, defines “mild” epistaxis (Pagella et al. 2009). The year after, Hoag et al. established another score, called Epistaxis Severity Score (ESS), based on six predictor factors consisting of intensity, frequency, duration, need for medical attention, transfusion related to epistaxis and anaemia (Table 1). The ESS has a range of 0 to 10, organized in three different subgroups of severity: i) 1-4 mild; ii) 4-7 moderate and iii) 7-10 severe.

Data Sheet for the Calculation of the Epistaxis Severity Score for HHT.
INTENSITY How would you describe your typical nose bleeding intensity? (coefficient 0.25) 0 – Not typically gushing 1 – Typically gushing or pouring
FREQUENCY How often do you typically have nose bleeding? (coefficient 0.14) 0 – Less than monthly 3 – Several per week 1 – Once per month 4 – Once per day 2 – Once per week 5 – Several each day
DURATION How long do your typical nose bleeds last? (coefficient 0.25) 0 – <1 minute 3 – 16-30 minutes 1 – 1-5 minutes 4 – >30 minutes 2 – 6-15 minutes
NEED FOR MEDICAL ATTENTION Have you every sought medical attention for nose bleeding? (coefficient 0.30) 0 – No 1 – Yes
NEED FOR TRANSFUSION Have you ever received a red blood cell transfusion specifically because of nose bleeding? (coefficient 0.31) 0 – No 1 – Yes
ANAEMIA Are you anaemic (low blood count) currently? (coefficient 0.20) 0 – No 1 – Yes

Table 1. Data Sheet for the Calculation of the Epistaxis Severity Score for HHT. Patients answered to six questions and the number of the response is multiplied by the respective coefficient. The sum of these numbers gives the raw ESS (Hoag et al. 2010).

Moreover, patients reported that nosebleeds can get worse because of several foods, as red wine, spices, chocolate, coffee, berries, oily fish and other foods containing high level of salicylates or other anti-platelet agents (Silva et al. 2013). Also the replacement of the loss iron by appropriate integration may aggravate nosebleeds in about 1:20 people (Shovlin et al. 2016).

Up to date, there is no cure for epistaxes but only therapies that improve their management. Different therapeutic approaches have been proposed to destroy symptomatic telangiectases and reduce the bleeding. Surgical approaches include cauterization and coagulation systems, as Argon Plasma Coagulation (APC), CO laser, KTP laser and Nd:YAG laser (Pagella et al. 2013). More recently, it was proved the efficacy of thalidomide for the pharmacological treatment of severe epistaxes in HHT patients (Invernizzi et al. 2015).

1.1.2. Telangiectases

Telangiectases are small lesions of the vascular structures, in which there are direct arteriovenous connections and the loss of the capillary bed usually intervening between arteriole and venule (Shovlin 2010). These lesions arise because of the abnormal interaction between Endothelial Cells (ECs) and Smooth Muscle Cells (SMCs) of the vessels' walls (Pardali et al. 2010).
HHT related telangiectases are observed at typical sites, as oral and nasal mucosae, lips, tongue, skin of the face and of hands and fingertips (Figure 1). They are typically present from the age of 30 years and the number increases with age (Govani and Shovlin 2009). Moreover, telangiectases are conditioned by the environment as highlighted by the predominant involvement of the sun-exposed areas on the face (Dupuis-Girod et al. 2010).
About the 80% of HHT patients have gastrointestinal (GI) telangiectases; even if the whole gastrointestinal tract can be affected, they are most commonly found in the stomach and in the upper duodenum. However, the bleeding of these lesions is observed only in about 25-30% of patients over age 60 years, and can lead to melena and anaemia (Bayrak-Toydemir et al. 2004; Canzonieri et al. 2014). Esophago-Gastro-Duodenoscopy (EGD) is the standard test used to evaluate the presence of telangiectases in the GI tract, and can be complemented by small-bowel videocapsule endoscopy to explore the entire small bowel (Faughnan et al. 2011; Iddan and Swain 2004).

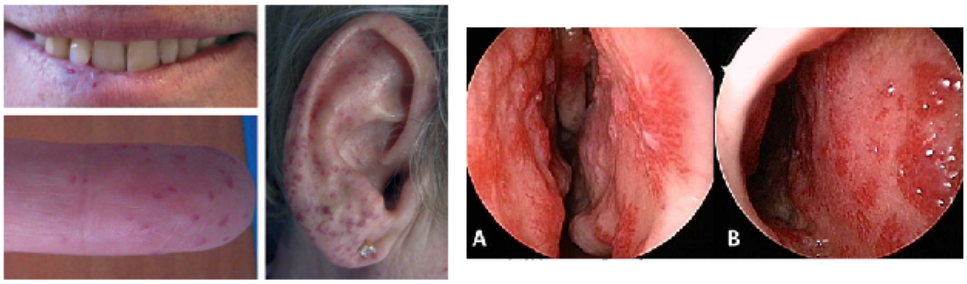


Figure 1. Typical HHT telangiectases. On the left, figures of mucocutaneous telangiectases (Dupuis-Girod et al. 2010). On the right, figures of nasal telangiectases (Pagella et al. 2013).

1.1.3. Arteriovenous malformations (AVMs)

It has been suggested that large AVMs arise from telangiectases by progressive vascular remodelling, and their symptoms are often not secondary to haemorrhage (Bayrak-Toydemir et al. 2004). As telangiectases, even HHT-related AVMs are observed at characteristic sites that include internal organs as lungs, liver, central

nervous system and GI tract (Dupuis-Girod et al. 2010). Rarely, AVMs have been described in other sites, including coronary arteries, vessels of eye, spleen, urinary tract and vagina (Brant et al. 1989; Hsi et al. 2003; Humphries et al. 1993; Lande et al. 1976; Ziani et al. 2000).

Pulmonary AVMs (PAVMs) provide direct communication between the pulmonary and the systemic circulations leading to a right to left shunt that impairs gas exchange and the filtration role of the pulmonary circulation (Gill et al. 2015; Shovlin 2014). About the 80-90% of patients with PAVMs are affected by HHT, while the others are sporadic cases. PAVMs are observed in 15-50% of HHT patients: in most cases PAVMs are multiple, bilateral and generally located at the bases of the lungs. PAVMs are probably congenital but may enlarge over time (Bayrak-Toydemir et al. 2004; Gossage and Kanj 1998).

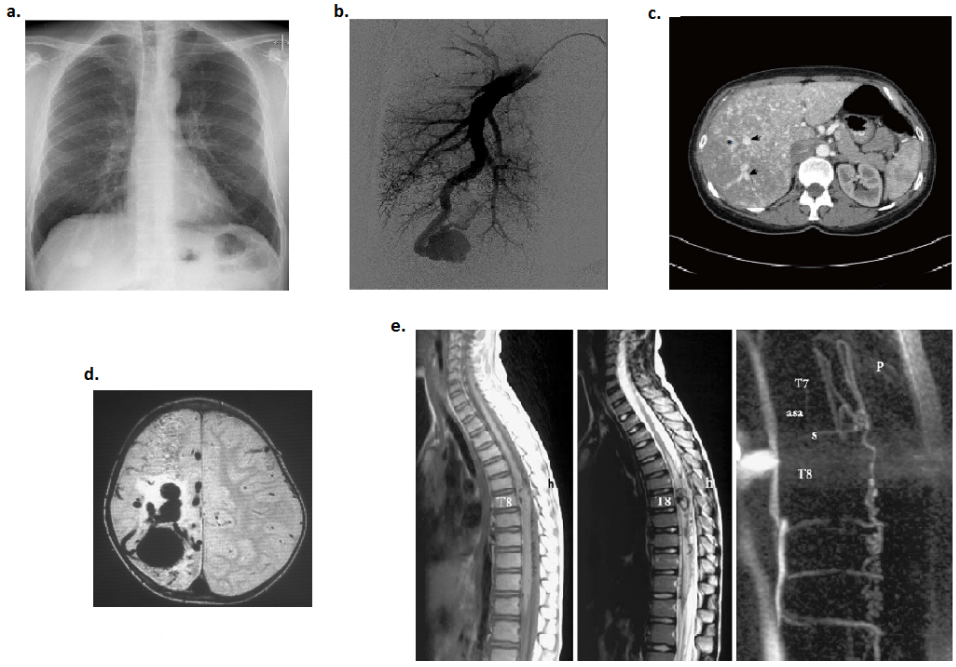


Figure 2. Arteriovenous malformations at characteristic sites. (a) Pulmonary AVM at the right lung base observed by frontal chest radiography. **(b)** Pulmonary arteriogram of the same patient shows the AVM structure consisting of the feeding artery, the aneurismal sac and the draining vein (Gill et al. 2015). **(c)** Liver CT scan showing dilated arteries (arrows) and a vascular malformation (star) (Vorselaars et al. 2015). **(d)** Brain magnetic resonance showing a cerebral AVM in a HHT patient (Dupuis-Girod et al. 2010). **(e)** Sagittal thoracic MRI showing a spinal AVM dorsal to the posterior surface of the T8 vertebrae (Poisson et al. 2009)

PAVMs consist of three anatomical components: i) feeding artery; ii) aneurismal sac and iii) draining vein (Figure 2a-b). There could be more than one feeding artery and draining vein, while the aneurismal sac could be substitute by a serpiginous network of vessels (Lacombe et al. 2013). The diagnosis of PAVMs is very important because of their association with life-threatening complications (Faughnan et al. 2011). Indeed, the right to left shunt can cause severe hypoxemia which is then compensated by polycythemia. Other complications are stroke, Transient Ischemic Attack (TIA) and cerebral abscess, present in about 10-19%, 6-37% and 5-19% of HHT patients with PAVMs, respectively, as a consequence of the paradoxical clot embolism due to the loss of the filtration role of the capillary bed (Bayrak-Toydemir et al. 2004; Parra et al. 2010). Moreover, PAVMs can haemorrhage subsequently to the rupture of the aneurismal sac, characterized by thin walls, and lead to haemoptysis and haemothorax, fatal in some cases (Lacombe et al. 2013; Shovlin 2014).
Generally, PAVMs are not symptomatic even prior to the development of complications, thus is important to follow up patients with screening test in order to supervise the lungs involvement in the progression of the disease (Faughnan et al. 2011). To date, PAVMs are diagnosed by both TransThoracic Contrast Echocardiography with agitate saline (TTCE) and Chest CT scanner examination, even if TTCE has higher sensitivity and lower risk than CT (Gazzaniga et al. 2009; Lacombe et al. 2013; Nanthakumar et al. 2001). In TTCE, during the echocardiography, the agitated saline solution is injected intravenous and micro-bubbles appear in the right heart. If there is the right to left shunt, after few heart beats, micro-bubbles emerge in the left cardiac cavities, making them opaque. Barzilai et al. proposed to use the opacity level to distinguish different grades of severity of the shunt:

1. grade 0 indicates the absence of micro-bubbles;
2. grade 1 indicates the minimal left ventricular opacity;
3. grade 2 indicates moderate opacity;
4. grade 3 indicates extensive opacity without outlining the endocardium;
5. grade 4 extensive opacity with endocardial definition.

TTCE is very sensitive for the diagnosis of PAVMs; in fact, up to 97% of PAVMs are identified with this technique (Lacombe et al. 2013).
The early diagnosis of PAVMs allows to treat them before the appearance of complications and, nowadays, the first-line treatment is the transcatheter embolization (Shovlin 2014). Embolization is evaluated successful if it helped to anatomically resolve the PAVMs, to reduce the right to left shunt and to improve the O_2 saturation. Long-term follow up is based on thorax CT, since around 80-90% of patients, successfully embolized, remain positive to TTCE after embolization (Lacombe et al. 2013). International guidelines for HHT management

recommend to treat PAVMs by embolization in adults and specially in women before pregnancy. During the pregnancy there is a 1% risk of maternal death, because of the haemorrhage of PAVMs (Shovlin et al. 2008; Wain et al. 2012).

Hepatic AVMs (HAVMs) are found in 41-74% of HHT patients and they can be divided in different groups according to the intrahepatic shunt. There are three shunts: i) from the hepatic artery to the portal vein; ii) from the hepatic artery to the hepatic vein and iii) from the portal vein to the hepatic vein (Buscarini et al. 2004, 2011). Only 8% of HHT patients show HAVMs symptoms, which include most commonly the High-Output Heart Failure (HOHF), the portal hypertension, the ischemic biliary disease and the encephalopathy (Buscarini et al. 2011; Nishioka et al. 2015). Moreover, liver vascular malformations in HHT have been related to a higher prevalence (100 fold increase than in the general population) of hepatocellular regeneration with the development of Nodular Regenerative Hyperplasia (NRH) or Focal Nodular Hyperplasia (FNH) (Buscarini et al. 2004; Gincul et al. 2008).
The diagnosis of HAVMs is based on several imaging techniques, such as Doppler Ultrasonography (US), Magnetic Resonance Imaging (MRI), triphasic spiral CT (Figure 2c) and mesenteric angiography. Doppler US is the least invasive test and it is not associated with procedural complications (Faughnan et al. 2011). The outputs of this diagnostic method can be used to classify the vascular malformations, eventually depicted, into different grades of severity that range from 0, denoting a minimal severity, to 4, that indicates the liver decompensation (Buscarini et al. 2011).
Treatment of HAVMs is decided according to the individual anamnesis. Generally, intense medical therapy, based on diuretics, beta-blockers, digoxin, Angiotensin Converting Enzyme (ACE) inhibitors and antiarrhythmic agents, can be given to patients with HOHF. Portal hypertension and encephalopathy are treated with antibiotics and, if necessary, with blood transfusions or iron administration in order to reduce anaemia and oppose to the bleeding if present (Buscarini et al. 2011; Geisthoff et al. 2015). Patients resistant to pharmacological therapies can be treated by hepatic artery embolization and, as last choice, liver transplantation. Embolization reduces the arteriovenous or arterioportal shunting, but whit a transient effect (Faughnan et al. 2011). Recently, Dupuis-Girod and collaborators demonstrated, in a preliminary study, that patients with severe HAVMs and HOHF treated with bevacizumab, a Vascular Endothelial Growth Factor (VEGF) inhibitor, reported a significant decrease in the cardiac output and in the duration and frequency of epistaxis (Dupuis-Girod et al. 2012). Bevacizumab was also successfully used to treat the bleeding of the GI tract (Kochanowski et al. 2015). An additional surgical approach has been proposed by Liu et al.: they performed

the double banding/ligation of hepatic arteries in a selected cohort of patients, who did not respond to other therapies, improving their quality of life(Liu et al. 2015).

The term Cerebral Vascular Malformations (CVMs) is referred to several vascular abnormalities, which differ for their morphology and that can be found in HHT patients. Among CVMs are included Cerebral AVMs (CAVMs) (Figure 2d), cavernous malformations, venous angiomas, capillary telangiectases, vein of Galen malformations, high flow pial fistulae and mixed malformations (Faughnan et al. 2011). CVMs have been described in about 1-23% of HHT patients, even if the rate of intracranial haemorrhage is 2-4% per year and is lower in HHT patients compared to sporadic cases of CVMs (Kim et al. 2015; Willemse et al. 2000). In fact, non-symptomatic CVMs are not treated because of the high risk of the intervention (Govani and Shovlin 2009). The International Guidelines suggest the catheter angiography as the best diagnostic modality, although there is a 0.5% risk of permanent stroke (Faughnan et al. 2011). Therapeutic approaches, as embolization, neurosurgical resection and stereotactic radiotherapy, are considered only if CVMs are symptomatic and managed on an individual basis (Faughnan et al. 2011; Geisthoff et al. 2015).

Only 3-4.5% of HHT patients develop Spinal Cord AVMs (SCAVMs-Figure 2e), and several cases are described in the paediatric age (Mahadevan et al. 2004; McDonald et al. 2000). SCAVMs clinical manifestations include headache, neurocognitive insufficiency, bladder insufficiency, sciatic pain, sphincter disturbance, back pain and in rare cases lead to acute, subacute or progressive paraplegia or tetraplegia due to subarachnoid haemorrhage (Krings et al. 2005; Poisson et al. 2009). SCAVMs can seriously impair the life-quality of patients, thus the early diagnosis is fundamental to prevent complications. Spinal angiography is the main technique used for the diagnosis and the morphologic characterization of spinal vascular lesions, and it is needed to decide the therapeutic strategy in both paediatric and adult populations (Flores et al. 2016; Kalani et al. 2011). There are no standardized approaches for the management of these vascular lesions and, equally to AVMs in other internal organs, there is a strictly correlation to the personal anamnesis of the patient. In any case, endovascular therapy is the first option if at least one of the feeding arteries is large enough to access to the shunt. In the event that embolization does not success, patient may undergo neurosurgery (Mont'Alverne et al. 2003; Rodesch and Lasjaunias 2003).

1.2. Clinical and molecular diagnosis

HHT exhibits incomplete penetrance until 40 years of age; this character accounts for physicians' difficulties in making the clinical diagnosis. Moreover, the average age of onset and the phenotypic manifestations of the disease vary among patients

leading to a wide heterogeneity. Interestingly, the phenotypic variability is observed not only between but also within families. For example, about 50% of patients develop epistaxes by the age of 10 years, almost all patients have recurrent nosebleeds, even if with different severity, and about 100% of patients have telangiectases of hands, lips, face and oral cavity after the third decade of life (McDonald et al. 2015).
In 2000, Shovlin and colleagues, on behalf of the Scientific Advisory Board of the HHT Foundation International Inc., presented the clinical diagnostic criteria, called "Curaçao criteria", that encompass:

1. spontaneous and recurrent epistaxis;
2. telangiectases at characteristic sites, as lips, oral cavity, nose, fingertips and gastrointestinal mucosa;
3. AVMs at characteristic sites, as lungs, liver and central nervous system;
4. family history, i.e. a first degree relative with HHT according to these criteria.

The diagnosis is "definite" if three criteria are present, "possible" or "suspected" if two criteria are present and "unlikely" if only one criterion is present (Shovlin et al. 2000).
However, it is necessary to make few observations about the "possible" or "suspected" diagnosis. If one out of the two criteria is the family history and the second criterion is the epistaxis, it is suggested to consider also other diagnoses since epistaxes are related to many different diseases, with high frequency in the general population. On the contrary, if the second criterion are AVMs in internal organs, which are very rarely described in sporadic cases, the diagnosis of HHT is essentially confirmed (Govani and Shovlin 2009).

In the last two decades several genes have been associated to HHT. Thus, mutation analyses may support the clinical diagnosis, helping in discussing about the risk assessment in HHT families by means of genetic counselling. Although HHT is clinical diagnosed, mutation analysis can be helpful in clarifying the phenotype of patients "possible" or "unlikely" affected. Furthermore, in patients with "definite" clinical HHT, molecular investigations are useful to early diagnose other family members, who do not meet the clinical criteria, in order to manage the disease in the best way (Faughnan et al. 2011; McDonald et al. 2015).

1.3. Genetics of HHT

Hereditary Haemorrhagic Telangiectasia is a genetically heterogeneous disease caused by mutations in some of the genes involved in the TGF-β/BMPs (Transforming Growth Factor-beta/Bone Morphogenetic Proteins) signalling

pathway (McDonald et al. 2015). Up to date, four genes have been correlated to HHT:

1. *ENG* (MIM*131195), coding for ENDOGLIN (ENG), associated to HHT1 (MIM#187300);
2. *ACVRL1,* coding for Activin A Receptor, Type II-Like Kinase 1 (ALK1),associated to HHT2 (MIM#600376);
3. *GDF2*, coding for Bone Morphogenetic Protein 9 (BMP9), associated to an HHT-like syndrome or HHT5 (MIM#615506);
4. *MADH4,* coding for Mothers Against Decapentaplegic Homolog 4 (SMAD4), associated to a syndrome called JPHT (MIM#175050) characterized by both HHT and Juvenile Polyposis symptoms.

Most of the patients (85%) carries mutation in either *ENG* or*ACVRL1*;2-3% of patients carry mutation in *MADH4* while mutations in *GDF2* were only described in few families (Gallione et al. 2010; Olivieri et al. 2007; Wooderchak-Donahue et al. 2013). Approximately 13% of individuals with HHT clinical diagnosis have no mutation in these genes; this result highlights the odds that other genes are involved in HHT pathogenesis (McDonald et al. 2015).
Other two loci, one on chromosome 5 and one on chromosome 7, have been related to HHT type 3 and type 4, respectively (Bayrak-Toydemir et al. 2006; Cole et al. 2005). However, until now no other disease-causing genes have been found in these loci.

1.3.1. The TGF-β superfamily

The TGF-β superfamily consists of a variety of more than 40 signalling cytokines including TGF-β 1, 2, and 3, BMPs and activins. Depending on the different cell lineages, all these proteins can be involved in the regulation of cell proliferation, differentiation, apoptosis and migration during both developmental processes and tissue homeostasis.
Cytokines of the TGF-β superfamily are generated as precursor dimer that, when activated by proteases, can bind to transmembrane receptors characterized by serine kinase activity. These receptors can be divided into three subgroups: the type I receptors (TGBRI); the type II receptors (TGBRII); and the type III or auxiliary receptors. In humans, researchers identified seven type I receptors, also named ALKs (ALK1, ALK2, ALK3, ALK4, ALK5, ALK6 and ALK7); five type II receptors (TGBR2, BMPR2, ACVR2A, ACVR2B and AMHR2) and two accessory receptors (Endoglin and Betaglycan) (Macias et al. 2015; Pardali et al. 2010). The functional receptor consists of the association of two TGBRI and two TGBRII, and can also include an auxiliary receptor that contributes to stabilize the ligand-receptor interaction.

The signal cascade starts when the ligand binds, with high affinity, the type II receptor dimer; this binding leads to a conformational change of type II receptor and to the recruitment of the type I receptor dimer. The ligand/TGBRII complex activates the type I receptor by phosphorylation at multiple serine and threonine residues located in the kinase domain. Once activated, the type I receptor propagates the signals through a cascade of intracellular effectors by either the SMAD-dependent or the SMAD-independent pathway (Bernabeu et al. 2007; Macias et al. 2015; Pardali et al. 2010). In ECs, Endoglin and ALK1 play an important role in transducing TGF-β and BMP signalling cascade; in particular BMP9, the physiological ligand of both receptors, is one of the players involved in Endoglin and ALK1-mediated angiogenesis (Figure 3) (Jonker 2014; Pardali et al. 2010).

SMAD proteins consist of two globular domains: a N-terminal domain called "Mad Homology 1" (MH1) and a C-terminal domain known as "Mad Homology 2" (MH2) that are connected by a linker region. The MH1 domain binds to the DNA, the MH2 domain mediates protein-protein interactions with many regulators and effectors, including the TGF-β ones, while the linker is a target for both positive and negative regulations (Macias et al. 2015). The SMAD family in humans includes eight members of intracellular mediators that are classified in three groups: i) receptor-regulated SMADs (R-SMAD-1, -2, -3, -5, and -8); ii) common-mediator SMAD (Co-SMAD-4) and iii) inhibitory SMADs (ISMAD-6 and -7).
RSMADs contain in their C-terminal tail the Ser-X-Ser motif that is phosphorylated by the TGBRI. The phosphorylation activates the RSMADs, allowing their interaction with the CoSMAD-4 and the generation of both homotrimers and heterotrimers. These trimers translocate into the nucleus where specific transcription factors are recruited in order to regulate the expression of target genes.
The ISMADs-6 and -7 interact with activated receptors and RSMADs with the aim of suppressing their activity, competing for the interaction with the TGBRI and recruiting specific ubiquitin ligases or phosphatases. Thus, the activated receptor complex is labelled for proteasomal degradation or dephosphorylation (Macias et al. 2015; Pardali et al. 2010).
The signalling specificity of the SMAD-dependent pathway is determined by the type 1 and the type 2 receptors of the TGF-β superfamily. In fact, ALK4, ALK5 and ALK7 mediate phosphorylation of RSMADs-2 and -3, whereas ALK1, ALK2, ALK3 and ALK6 mediate phosphorylation of RSMADs-1, -5 and -8.
The angiogenesis process is regulated by both ALK1 and ALK5 signalling pathways, by BMP9, and by the TGF-β1 different concentrations (Jonker 2014; Pardali and ten Dijke 2009). It has been demonstrated that low TGF-β1 concentrations promote endothelial cells proliferation and migration, whereas high

concentrations have the opposite effect (Pardali and ten Dijke 2009). Instead, the role of ALK1 and ALK5 has not yet completely clarified.
Several works show that ALK1 and ALK5 induce opposite endothelial cells responses both in terms of proliferation and migration, but conflicting results have been obtained by different research groups. Goumans et al. proved that ALK1 has a pro-angiogenic effect, promoting the proliferation and the migration of endothelial cells, while ALK5 inhibits these processes (Goumans et al. 2002). On the other hand, Larrivée and collaborators demonstrated that ALK1 activation has an anti-angiogenic effect through the inhibition of both the same pathways, confirming the results previously obtained by Lamouille et al. (Lamouille et al. 2002; Larrivée et al. 2012). In any case, it is supported the so called "balance model", which sustains that the balance between ALK1 and ALK5 signalling properly regulates the angiogenesis.

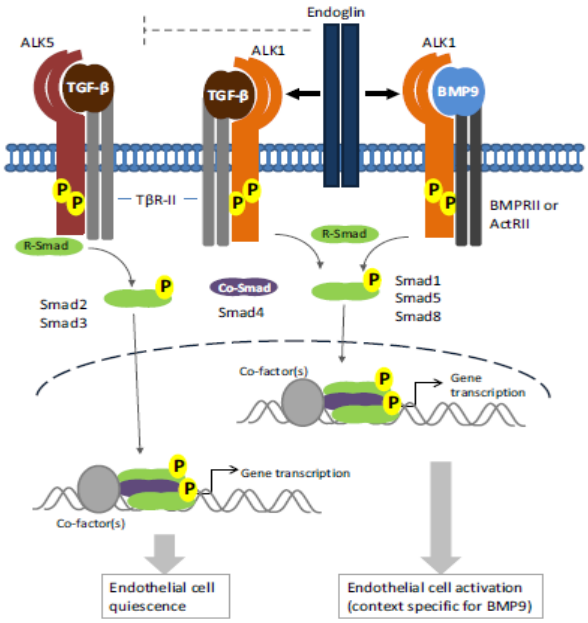


Figure 3. TGF-β/BMP signalling SMAD-dependent pathway. The figure shows the roleof Endoglin and ALK1 in promoting the angiogenesis process. On the contrary, ALK5 activity leads to the inhibition of the angiogenesis and to cells quiescence (Jonker 2014).

However, the "balance model" is called into questions by Park and collaborators who suggest that ALK5 is not required within ECs for the vessels formation. In fact, they demonstrated that, both in mice and zebrafish, Alk1 is the only TGF-β

type I receptor in ECs, while Alk5 is expressed only in vascular SMCs (Park et al. 2008; Seki et al. 2006).
The role of ENG is still not completely understood. It is known that this type III receptor interacts with ALK1, modulating its activity. Some authors proved that ENG leads to the ECs proliferation through the promotion of the TGF-β/ALK1 signalling pathways, while other researchers reported contrasting results that underline the increased activation of ALK1 signalling after the loss of ENG (Koleva et al. 2006). Furthermore, ENG plays a role in the regulation of the ALK5 signalling pathway: the binding to ALK5 inhibits the TGF-β/ALK5/SMAD3 pathway and enhances the TGF-β/ALK5/SMAD2 pathway (Bernabeu et al. 2009).
Also regarding the BMP9 role, conflicting results have been reported. Several studies show the pro-angiogenic effect of BMP9, driven by the induction of SMAD-1/-5/-8 phosphorylation that leads to the proliferation of ECs (Nolan-Stevaux et al. 2012; Suzuki et al. 2010). Differently, other studies highlight the anti-angiogenic effect of BMP9 (David et al. 2007; Scharpfenecker et al. 2007).

The SMAD-independent pathway is characterized by the activation of several other molecules, as TAK1, ERK, JNK, p38, Rho GTPase and PI3K/AKT, which crosstalk with the SMAD-dependent pathway (Pardali et al. 2010).

*1.3.2. ENG (MIM *131195)*

The 1994 was the year of the discovery of the first gene related to HHT. Initially, two different research groups simultaneously identified, by linkage analyses, the gene *locus* (*ORW1*) on the long arm of the chromosome 9, at the cytogenetic position 9q33-34 (McDonald et al. 1994; Shovlin et al. 1994). Subsequently, in December, McAllister and collaborators published on *Nature* a paper proving the existing relationship between HHT and the *ENG* gene that had been previously mapped to chromosome 9q34 (Fernandez-Ruiz et al. 1993; McAllister et al. 1994). Nowadays, it is known the exact localization of the gene on chromosome 9:127,815,017-127,854,636 (UCSC Genome Browser database – GRCh38/hg38). In the RefSeq database three different transcripts of the gene, coding for three isoforms of the protein are annotated. The first transcript (NM_001114753.2) encodes the longest isoform, called Long-Endoglin (L-ENG). The second transcript (NM_000118.3) has an additional segment in its 3'-end that includes an earlier stop codon compared to the first variant. This transcript encodes the shortest isoform of the protein, called Short-Endoglin (S-ENG). The third transcript (NM_001278138.1) is characterized by a distinct 5'UTR and by the loss of a portion of the 5' coding region which causes the use of a downstream start codon. Thus, the protein presents a shorter N-terminus tail compared to the other isoforms.

The gene and the protein

Endoglin is a TGF-β type III receptor and it is expressed predominantly in endothelial cells, activated monocytes, syncytiotrophoblasts and some stromal cells (Gougos et al. 1992; Lastres et al. 1992; St-Jacques et al. 1994). The activated form of Endoglin is a transmembrane homodimer in which the two monomers interact thanks to disulphide bonds in correspondence of Cys350 and Cys582 residues (Alt et al. 2012; Guerrero-Esteo et al. 2002). Endoglin is highly O-glycosylated (Asn88, Asn102, Asn121, Asn134 and Asn307) (Figure 4) and it is able to indirectly bind several ligands of the TGF-β superfamily through the TGF-β type I and II receptors (Barbara et al. 1999; Gougos and Letarte 1990). The homodimeric protein weighs 180kDa and each monomer contains three different domains, that are encoded by 15 exons:

1. an extracellular domain of 561 amino acids;
2. a hydrophobic transmembrane domain of 25 amino acids;
3. a cytoplasmic region of 47 amino acids rich in serine and threonine (in the long isoform) or14 amino acids (in the short isoform).

In the extracellular region there is a *zona pellucida* (ZP) domain, that consists of 260 amino acids (from Lys362 to Asp561) with eight conserved cysteine residues, localised close to the transmembrane region. The ZP domain is organized in two different subdomains, named ZP-N and ZP-C, that are probably involved in the protein oligomerization. Moreover, human Endoglin ZP domain contains also the RGD (Arg-Gly-Asp) tripeptide, that allows the interaction with other RGD-receptors or proteins (Gougos et al. 1992). The RGD tripeptide is a prototypic member of a family of motifs involved in the interaction between Integrins and the extracellular matrix or cell membrane surface proteins. In fact, Rossi et al. proved that Endoglin binds, with its RGD motif, to the integrin α5β1, expressed on leukocyte membrane. This interaction suggests that Endoglin plays a role in the transendothelial leukocyte infiltration (Rossi et al. 2013, 2016).
The N-terminus tail of the extracellular domain contains a fragment called "orphan" domain (OD), because it does not have any significant homology to other protein consensus domains and for many years its function was not known (Llorca et al. 2007). Subsequently, it has been demonstrated that the OD binds the ligand BMP9 (Figure 5) (Alt et al. 2012).

The cytosolic domain differs according to the isoform. The predominant isoform (L-ENG) is the longest one; it contains the 47-residues domain and is characterised by the higher conservation among mammalian species. The pre-mRNA coding for L-ENG may undergo alternative splicing, leading to the biosynthesis of the shorter isoform, S-ENG, with a different cytosolic tail of 14 amino acids (Bernabeu et al. 2007). Specifically, S-ENG is expressed when the 135bps intron between exons 14

and 15 is retained, introducing an earlier stop codon in the open reading frame (Bellón et al. 1993).
Endothelial cells predominantly express L-ENG isoform, while S-ENG isoform expression increases during cells senescence (Bellón et al. 1993). Because of the alternative splicing, only L-Endoglin contains the PDZ-binding motif (Ser-Ser-Met-Ala), which confers to the protein its adhesive properties (Bernabeu et al. 2007). Moreover, close to the PDZ motif there are several serine and threonine residues that can be phosphorylated. Koleva and collaborators showed that the serine residues 634 and 635 of L-ENG are constitutively phosphorylated by TGBR2. Ser634 phosphorylation is needed to allow the phosphorylation of threonine residues, preferentially Thr640, Thr647 and Thr654, by ALK1. Threonine phosphorylation by ALK1 is involved in modulating the ALK1-dependent adhesive and proliferative effects in endothelial cells, suggesting a starting point for the SMAD-independent signalling pathway (Koleva et al. 2006).
The difference in the cytosolic domain between S-ENG and L-ENG is related also to their capability of interact with ALK1 and ALK5. Indeed, Blanco et al. proved that the long isoform binds ALK1 with higher affinity compared to S-ENG and supports endothelial cells proliferation through the enhancing of TGF-β/ALK1 signalling pathway and the interference with the one regulated by TGF-β/ALK5. On the other hand, S-ENG strongly binds ALK5 and inhibits ECs proliferation. Thus, it has been proposed that L-ENG and S-ENG play a contrasting role in regulating the angiogenesis process. This hypothesis is supported by the high expression of S-ENG in senescent endothelial cells (Blanco et al. 2008).
Endoglin alternative splicing is regulated by the splicing factor ASF/SF2, which interacts with a *cis*-element centred in the IVS13-36G nucleotide, in order to regulate the intron retention mechanism. Intron retention takes place in the cytoplasm during the senescence, corroborating the connection of this process with both S-ENG and ASF/SF2 (Blanco and Bernabeu 2011). Indeed, it was yet reported the role of ASF/SF2 in senescence (Verduci et al. 2010).

Recently, a soluble form of Endoglin (sEndoglin) was detected in association with several diseases as preeclampsia and cancer (del Castillo et al. 2014; Hawinkels et al. 2010; Venkatesha et al. 2006). It has been suggested that the production of sEndoglin depends on the activity of the matrix metalloprotease 14 (MT1-MMP), that cleaves the transmembrane form of the protein at a putative site, localized in the extracellular region between the two ZP subdomains (Llorca et al. 2007). Other authors identified a different cleavage site close to the transmembrane region, at the position 586-587, corresponding to a GL (Gly-Leu) amino acidic sequence. A cut in this site generates a soluble protein containing the whole extracellular domain (Hawinkels et al. 2010). Several papers suggest that the sEndoglin has an anti-angiogenic role, in contrast with L-ENG (Castonguay et al. 2011; Hawinkels

et al. 2010; Venkatesha et al. 2006). The MT1-MMP function in promoting sEndoglin formation has been confirmed also in the vascular repair process after injury, when an over-expression of MT1-MMP and thus a higher release of sEndoglin are observed. The authors suppose that sEndoglin is necessary only in the early damage response to hinder the angiogenesis, while in the advanced response the transmembrane ENG is up-regulated, triggering the angiogenesis (Gallardo-Vara et al. 2016).

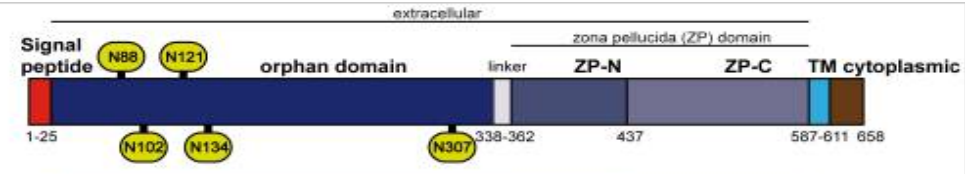


Figure 4. Schematic representation of human Endoglin. TM: transmembrane region. ZP: *zona pellucida* domain. Indicated residues are the putative sites for glycosylation (modified from Alt et al. 2012).

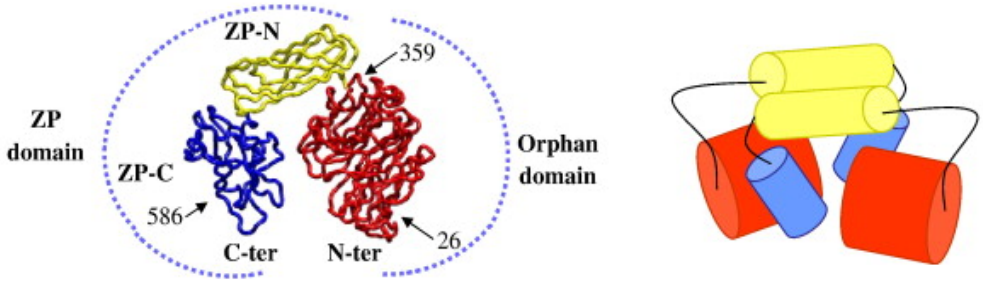


Figure 5. Endoglin. Prediction of Endoglin structure and model of the Endoglin dimer. Blue and yellow cylinders represent the ZP-C and the ZP-N domain, respectively, and the red cylinders represent the orphan domain of the protein (modified from Llorca et al. 2007).

ENG and HHT pathogenesis

Endoglin is a homodimeric transmembrane glycoprotein, mainly expressed on vessels' ECs, which operates as an auxiliary receptor of the TGF-β superfamily (Bernabeu et al. 2007). Many works highlighted the implication of ENG in the regulation of angiogenesis: it has been shown that a decrease in the expression level causes the formation of abnormal vascular structures (Arthur et al. 2000; Li et al. 1999). Indeed, Endoglin knock-out mice die at embryonic day 10.5-11.5 because of defects in the development of the cardiovascular system (Li et al. 1999). On the opposite, mice carrying Endoglin heterozygous mutation are viable and present an HHT-like phenotype (Mahmoud et al. 2010). However, the molecular and cellular Endoglin functions are not completely understood. To explain the

disease pathogenesis, a dominant-negative model was initially proposed. Thus, the mutated protein interferes with the wild type one, affecting the signalling pathway (McAllister et al. 1994). This model was supported by the high number of nonsense mutations resulting in premature stop codon, then in the synthesis of truncated proteins (Abdalla and Letarte 2006).
Nowadays, it is known that heterozygous *ENG* mutations are related to HHT because of the formation of unstable proteins which do not reach the cellular membrane (Bayrak-Toydemir et al. 2004; Castonguay et al. 2011; Fernandez-L et al. 2006). Studies on the expression level of mutated ENG on the cell surface, for both vascular ECs and monocytes, prove a 50% decrease compared to controls, suggesting the haploinsufficiency as the pathogenic model of the disease (Barbara et al. 1999; Pece et al. 1997).
In this scenario, the attention has been focused on the promoter of the gene. *ENG* promoter is characterised by the absence of the TATA and CAAT regions and by two different Sp1-binding site at the positions -37/-29 and -50/-24, suggesting that the transcription factor Sp1 (Specific Protein 1) plays a role in *ENG* expression. The -37/-29 site is necessary to the promoter function: in fact, mutations in this region and the activity of WP631, a Sp1 inhibitor, do not allow gene transcription. The second site, -50/-24, is important for the promoter responsiveness to the TGF-β/SMAD3 signalling. Both these findings sustain the important role of Sp1 in *ENG* transcription (Botella et al. 2001; Ríus et al. 1998).

Endoglin physiological ligands

ENG homology with Betaglycan, the other TGF-β auxiliary receptor, had an important role in the discovery of the ENG physiological ligands. Indeed, the transmembrane and the cytoplasmic domains of these proteins present 63% amino acid sequence identity (Cheifetz et al. 1992). Binding with high affinity all TGF-β isoforms and presenting them to their receptors, Betaglycan promotes TGF-β signalling pathway (López-Casillas et al. 1994). Betaglycan is expressed in almost all cell types, with few exceptions like endothelial, epithelial, and hematopoietic cells (Cheifetz and Massagué 1991). In ECs, Betaglycan function is replaced by Endoglin. In fact, it was demonstrated that ENG binds to TGF-β1 and TGF-β3 in HUVECs (Cheifetz et al. 1992). Furthermore, it was proved that Endoglin binds other ligands, as Activin A, BMP2 and BMP7, but only in the presence of their TGF-β receptors (Barbara et al. 1999). More recently, in 2007, it was discovered that ENG binds the ALK1 physiological ligands, BMP9 and BMP10, stressing the involvement of the two receptors in the same signalling pathway (David et al. 2007; Scharpfenecker et al. 2007).
Castonguay and collaborators observed the binding between ENG and either BMP9 or BMP10, even if in their experiments the extracellular domain of ENG does not

bind TFG-β1 and TGF-β3, neither in the presence nor absence of their specific receptors. They firstly map the binding site of BMP9 and BMP10 in the Endoglin OD amino acidic sequence through the construction of truncated forms of Endoglin, which differed because of their extracellular domain. The high affinity for the two ligands was preserved in all the truncated proteins in which the extracellular region containing only the OD was present. Moreover, they point out that Endoglin acts preventing BMP9 interaction with TGF-β type II receptors and blocking their signalling pathway. This happens because Endoglin and the TGF-β type II receptor bind to BMP9 at the same site (Castonguay et al. 2011). Few months later, Alt and colleagues confirmed that Endoglin binds to BMP9 through its orphan domain between amino acid 22 and 338 (Alt et al. 2012).

ENG mutations and polymorphisms

The International HHT Database (http://arup.utah.edu/database/hht/) has 525 variants related to *ENG* 67% of which are surely pathogenic. In particular, 111 deletions, 102 missense mutations, 66 splice defects, 58 insertions, 46 nonsense mutations, 39 intronic mutations, 22 large deletions, 15 silent mutations and 5 large duplications are listed. The mutations spread across the whole gene, except in exon 15, which presents only a benign variant; up to date, no mutations have been described in the cytosolic domain of the protein (Förg et al. 2014). According to Koleva and collaborators, it is probable that mutations in this ENG domain affect only the phosphorylation level of the protein, with any pathogenic effect (Koleva et al. 2006).
As mentioned before, several authors demonstrated that mutant forms of Endoglin do not reach the cytoplasmic membrane, suggesting that the underlying pathogenic mechanism for HHT type 1 is the haploinsufficiency (Bayrak-Toydemir et al. 2004; Castonguay et al. 2011; Fernandez-L et al. 2006). Recently, Forg and collaborators obtained partly opposite results by studying several ENG missense mutations. In agreement with the haploinsufficiency model, they observed that most of the mutated proteins are trapped within the rough Endoplasmic Reticulum (rER). The proteins with S480C or R571H mutations represent the exceptions: in fact they are found in the plasma membrane at high level, similarly to the wild type protein. All the mutant proteins analysed interact with the wild type protein, forming heterodimers in whom the mutant monomer exerts a dominant-negative effect on the wild type (Förg et al. 2014). Mallet et al. studied the pathogenic effect of ENG missense mutations, investigating their capability to affect the binding to BMP9. Sixteen out of 31 mutant proteins examined are able to reach the membrane surface and to normally interact with BMP9. Thus, even if these variants determine an amino acid change, they are benign. The remaining 15 mutants totally or partially impair the signalling pathway and are not expressed at the membrane

level; only S278P and F282V mutations are expressed, even if in these cases proteins are not able to interact with BMP9. According to the authors, the amino acids S278 and F282 likely localise in the ligand binding site, within the OD of the protein (Mallet et al. 2015). Both papers, by Förg and Mallet, pointed out the need to thoroughly study missense variants to distinguish polymorphisms from real mutations. Indeed, other authors have identified many polymorphisms, previously described as missense mutations because of the amino acid change (Abdalla and Letarte 2006; Harrison et al. 2003; Olivieri et al. 2007).
Many *ENG* mutations cause the formation of a premature stop codon, directly by nonsense mutation or indirectly by out of frame insertions and deletions. In both cases, mutations resulted in the biosynthesis of truncated protein, with loss of function (Abdalla and Letarte 2006).
A relative high amount of large deletions and duplications (about 5% of the total variants described) have been found in *ENG,* probably because of the presence of several repetitive elements, as the Alu sequences. In particular, 72% of the breakpoints localise in intron 8. Other Alu sequences, involved in large deletions and/or duplications, have been observed in introns 1, 2 and 3 (Lesca et al. 2006). Two additional Alu elements were identified 900bps and 9kbps upstream to the *ENG* start codon and were related to large deletions, including the promoter (Fontalba et al. 2013).
Since the 15% of HHT patients present no mutations in the exons of HHT-related genes, new efforts have been focused on the non coding regions, as introns, UTRs and regulatory elements. For example, Damjanovich et al. (2011) analysed the 5'UTR in a cohort of 154 patients with "definite" or "suspected" clinical diagnosis, and three different variants were found: c.-205A > C, c.-127C > T and c.-9G > A. The c.-205A>C variant does not affect the protein expression, thus it is considered as a benign variant. The c.-127C>T variant creates a new potential start codon, leading to the slippage of the translation initiation. This condition determines a high decrease of the ENG expression (74%) compared to the wild type protein. The c.-9G>A variant creates a new translation initiation sitewith the same reading frame of the wild type protein, but with three additional amino acids. The variant mildly reduces the expression of the protein (20% less than the wild type) and in one patient was observed in homozygosis. The authors hypothesised that the c.-9G>A variant generates an hypomorfic allele of *ENG* which might contribute to the severity of the disease, but only in presence of other genetic causes (Damjanovich et al. 2011).

Over the years, many polymorphisms have been identified in the *ENG* sequence and most of them localise in introns (Olivieri et al. 2007). Several papers report differences in allele frequencies comparing cohorts of HHT patients and controls (Lesca et al. 2004, 2008). Moreover, the polymorphisms can influence the

phenotype of the disease. For example, two independent research groups demonstrate the existing relationship between the *ENG* polymorphism rs1800956 (D366H) with the risk of sporadic Intracranial Aneurysms (IAs) (Joo et al. 2008; Lin et al. 2014).

*1.3.3. ACVRL1 (MIM *601284)*

The second locus related to HHT has been identified in the pericentromeric region of chromosome 12 and called *ORW2.* Within this region, *ACVRL1* (MIM *601284) has been identified as the second HHT-related gene. Indeed, three different mutations were found in three unrelated HHT patients (Johnson et al. 1996). Today the revised exact localization on chromosome 12 it is known (12:51,907,418-51,921,069 from UCSC Genome Browser database – GRCh38/hg38). In the RefSeq database are annotated two different variants of the transcript, both encoding for the same isoform of the protein. The first variant (NM_000020.2) is the longest one, while the second variant (NM_001077401.1) differs from the other in its 5'UTR because of the presence of a poorly conserved upstream start codon, that could lead to the synthesis of a 14 amino acids longer protein.

The gene and the protein

ALK1 is a TGF-β type I receptor, mainly expressed in vessels' endothelial cells like Endoglin. The gene is composed of 10 exons; exon one is not translated as the start codon lies in exon 2. Moreover, exon 2 contains the signal peptide needed to translocate the protein on the membrane surface (Berg et al. 1997).

The protein is a transmembrane receptor with serine-threonine kinase activity, weights 15kDa and consists of 503 amino acids organized in three different regions:

1. a short extracellular domain encoded by exons 3 and partly by exon 4;
2. a transmembrane domain encoded by the second part of exon 4 and partly by exon 5;
3. a long cytosolic domain encoded by the remaining exons, from 5 to 10.

ALK1 structure is similar to other TGF-β type I receptors, in fact, the members of the ALK family present high sequence identities (from 60 to 79%). The extracellular domain contains a potential N-linked glycosylation site and 10 conserved Cys residues (Cys34, Cys36, Cys41, Cys46, Cys51, Cys69, Cys77, Cys89, Cys90 and Cys95), essential to the formation of disulphide bonds (ten Dijke et al. 1993; Mahlawat et al. 2012). These bonds contribute to stabilize the β-strands structures, which fold in a particular shape called “three finger toxin fold”, from the name of the three-fold neurotoxins class, typical of both the TGF-β type I and

type II receptors. Recently, it was proposed a bioinformatic model of the extracellular domain of the protein. This model has a cupped left hand shape exhibiting the "three finger toxin fold", and contains only four out of the expected five disulphide bonds between the conserved Cys residues (Figure 6a) (Scotti et al. 2011).

The cytosolic domain presents a glycine and serine rich region (SGSGSGLP) called GS domain, characterized by the helix-loop-helix structure typical of the type I receptors; it is located in the juxtamembrane region of the protein, close to the enzymatic activity. The GS domain plays an important role in the protein function: mutant proteins without this domain do not take part to the phosphorylation process, and thus the signal is not propagated into the cell (Feng and Derynck 1997). Moreover, the change of only one amino acid in this region leads to either loss or gain of function (Saitoh et al. 1996; Wieser et al. 1995).

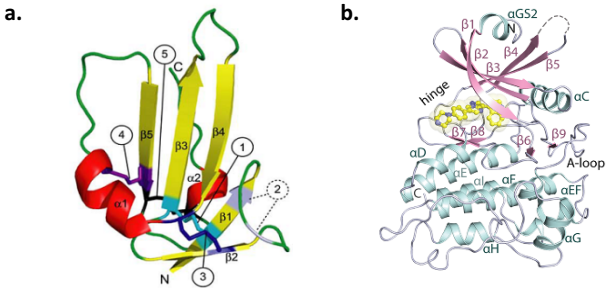


Figure 6. ALK1 structure. (a) Representation of the extracellular domain of the protein, characterized by the "three finger toxin fold". Numbers in circles indicate the disulphide bonds (Scotti et al. 2011). **(b)** The structure of the kinase domain of the protein (Kerr et al. 2015).

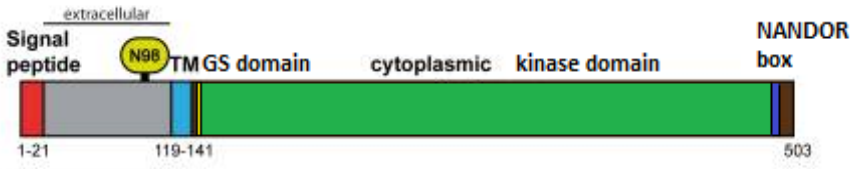


Figure 7. Schematic representation of ALK1 domain. TM: transmembrane region. The indicated amino acid, N98, is involved in the glycosylation of the protein (modified from Alt et al. 2012).

The cytosolic domain of ALK1 also owns the serine-threonine kinase activity, organized in 11 different subdomains which function depends on few highly conserved residues. For example, subdomain I presents the motif Gly-X-Gly-X-X-Gly, that is an ATP binding site; subdomain II has an invariant Lys involved in the

phosphorylation process; while subdomains VI to IX are the central core of the catalytic activity (Figure 6b). Noteworthy, regions with lower conserved amino acid sequences separate the subdomains from one another (Hanks and Quinn 1991).
Finally, a short C-terminal tail is the last part of the cytosolic domain of ALK1 and it is characterised by a highly conserved domain between residues 479 and 489, called NANDOR box (for "Non-Activating Non-Down-Regulating). This box is needed for type I receptor phosphorylation and therefore for the kinase activity (Figure 7) (Abdalla et al. 2003; Abdalla and Letarte 2006; Berg et al. 1997).

ACVRL1 and HHT pathogenesis

A large body of evidence supports the involvement of ALK1 in the regulation of the angiogenesis process through both the activation of the SMAD-dependent and the SMAD-independent signalling pathways. For example, knock out *Alk1* mice die at the embryonic day 11.5 because new vessels are not able to create a well organized structure including arteries, veins and capillary beds (Larrivée et al. 2012). Instead, when *Alk1* deletions are introduced in mice immediately after their birth, animals develop AVMs in the internal organs, severe haemorrhages and, in the worst cases, die (Park et al. 2008). Rochon and collaborators highlighted the ALK1 role in angiogenesis studying *alk1*-deficient zebrafish embryos, which develop CAVMs. The absence of *alk1* affects the endothelial cells migration and thus leads to a not balanced distribution of these cells and to the formation of the CAVMs (Rochon et al. 2015).
In 2010, Ricard and collaborators proved that haploinsufficiency is the underlying mechanism for HHT2. They functionally studied 19 different ALK1 mutations evaluating the capability of the mutant proteins to interact with BMP9 and to propagate its signal. They found that all the mutants localise in the membrane surface and interact with BMP9 but do not completely drive the signal. In fact, a 50% reduction in the BMP9 response mediated by ALK1 was observed in HHT2 patients (Ricard et al. 2010). Similar results were obtained more recently by another research group, enforcing that haploinsufficiency is the pathogenesis model for HHT2 (El Din et al. 2015). Both works enforce previously data about the reduced expression of ALK1 in HHT patients compared to controls (Abdalla et al. 2000; Abdalla and Letarte 2006).
However, at least for some mutations, a dominant-negative effect of the mutated allele was postulated, and a "second hit" model that leads to the complete ALK1 loss of function was hypothesized. According to this model, the inherited mutation predisposes the subject to the manifestation of the disease, which will appear only after the onset of a somatic mutation affecting the wild type allele (Berg et al. 1997; Lesca et al. 2006; Park et al. 2008). Since AVMs and telangiectases are

described only at characteristic sites, it is possible that their development depends on the "second hit" (Park et al. 2008).

ACVRL1 physiological ligands

ALK1 is predominantly expressed in endothelial cells, and for a long time it was considered as an orphan receptor because the ligands able to activate it were not identified. At a later time it was shown the binding between ALK1 with Activin and TGF-β, but only in the presence of the appropriate type II receptors (Attisano et al. 1993; Goumans et al. 2002). In 2007, it was shown that BMP9 binds ALK1 with higher affinity than the other ligands.
In ECs, ALK1 – BMP9 interaction activated the SMAD1/5/8 signalling, inducing their phosphorylation and the up-regulation of *ID1* (inhibitor of DNA binding 1), known as a target gene of the SMAD-dependent pathway (David et al. 2007; Korchynskyi and Ten Dijke 2002; Scharpfenecker et al. 2007). Moreover, it was demonstrated that the BMP9 response was strengthened by the over-expression of Endoglin, which bound BMP9 itself (Scharpfenecker et al. 2007).
However, the signal propagation needed a ternary complex consisting of the ligand and both type I and type II receptors. Thus, the next step forward was to identify the type II receptor that interacted with ALK1. David et al. demonstrated that both BMPRII and ACVR2A bound to ALK1 in order to form a functional signalling complex able to transmit the BMP9 response (David et al. 2007). On the other hand, Townson and collaborators sustained ACVR2B as the more selective type II receptor for ALK1, since they observed transient *in vitro* ALK1 binding to either BMPR2 or ACVR2A with high dissociation of the complex, even at physiological temperatures (Townson et al. 2012).
It has been demonstrated that also BMP10, which has the 65% amino acidic sequence identity with BMP9, binds to ALK1 with high affinity in endothelial cells, inhibiting both their growth and migration (David et al. 2007; Scharpfenecker et al. 2007).

ALK1 is involved in the regulation of both physiological and pathological angiogenesis, even if contrasting results have been obtained by several research groups about its capability to promote or inhibit this process (Goumans et al. 2002; Larrivée et al. 2012). The Notch pathway is another regulator of angiogenesis activated by VEGF. In 2012, Larrivèe and collaborators proved the existing synergy between ALK1 and Notch pathways in regulating VEGF-dependent angiogenesis.

ACVRL1 mutations and polymorphisms

The International HHT Database (http://arup.utah.edu/database/hht/) has 572 variants related to *ACVRL1* and 50% of them are surely pathogenic. In particular, 172 missense mutations, 63 deletions, 37 nonsense mutations, 29 intronic mutations, 28 insertions, 27 splice defect mutations, 7 large deletions and 6 silent variants are listed. The mutations spread across the whole gene, even if most of them localise in the kinase domain of the protein.
Compared to *ENG*, *ACVRL1* is characterised by a higher amount of missense mutations. Several authors demonstrated that missense mutations may damage the amino acidic residues shared by all the ALK family members. It was hypothesised that mutations in these sites affect the protein structure, making it not functional (Abdalla et al. 2003; Abdalla and Letarte 2006). Other mutations also impair the 10 conserved Cys residues in the ALK1 extracellular domain; these residues probably contribute to the interactions with other members of the TGF-β superfamily (Lesca et al. 2008; Scotti et al. 2011; Townson et al. 2012). Indeed, it was shown that some missense mutations modify the BMP9 binding site, affecting ALK1 response to its ligand (Ricard et al. 2010; Scotti et al. 2011).
The underlying pathogenic mechanism changes according to the protein domain affected by the mutation. In case of mutation affecting the extracellular domain, the protein does not reach the membrane surface and consequently does not bind BMP9. As reported above, when the mutation is in the kinase domain, the mutant protein localises at the membrane surface and binds to BMP9, but does not drive the signal (Ricard et al. 2010).
Moreover, it should not be forgotten the impact of both exonic and intronic mutations on the splicing process. In 2015, El Din et al. functionally studied 23 *ACVRL1* mutations spread across the whole gene sequence. They observed that a missense mutation in exon 6 (I245V), and a single nucleotide change in intron 7 (c.1048+5G>A), affect the correct splicing of the pre-mRNA (El Din et al. 2015).
HHT2 has been related also to the deletion of the whole *ACVRL1* gene (Shoukier et al. 2008).

Many polymorphisms have been identified in *ACVRL1* (Abdalla and Letarte 2006). Some of them determine an amino acid change without any pathogenic effect. For example, D179A, R386C, R454W and A482V are rare polymorphisms which have been initially described as missense mutations (Ricard et al. 2010).
Noteworthy the case of the common variant c.314-35A>G, in the third intron of the gene, that is significantly associated with PAVMs developed in HHT patients carrying *ENG* mutations, but not *ACVRL1* mutations. This result stresses the role of polymorphisms as genetic modifiers, which contribute to the clinical phenotype and to the severity of the disease (Pawlikowska et al. 2015).

1.3.4. GDF2 (MIM *605120)

The 2013 was another important year for HHT research, since it was discovered the relationship between mutations in *GDF2* and a HHT-like phenotype in three unrelated patients. The P85L and the R68L variants were identified as certainly pathogenic, while the third variant, R333Wwas defined as suspected pathogenic because it was already described in the general population, even if with a frequency of 0.004 (Wooderchak-Donahue et al. 2013). Later, other *GDF2* mutations were identified in HHT patients (Hernandez et al. 2015). Nowadays, in the OMIM database,*GDF2* is related to the clinical phenotype HHT type 5.
In 2000, the gene was mapped on chromosome 10, and the currently revised position is 10:47,322,490-47,326,270 (UCSC Genome Browser database – GRCh38/hg38). In the RefSeq database only one transcript of the gene is annotated (NM_016204.3).

GDF2 encodes for BMP9, belonging to the Bone Morphogenetic Protein of the TGF-β superfamily. All the TGF-β family members are secreted as large precursors, called procomplexes, consisting of two large prodomains linked by disulphide bonds to a smaller Growth Factor (GF) dimer. These domains are cleaved by Proprotein Convertases (PC), as Furin, at specific sites, but they remain linked thanks to non-covalent bindings (Mi et al. 2015; Tillet and Bailly 2014). The prodomains have a regulatory function; in fact, they contribute to maintain inactive the GF dimer in *in vivo* experiments (Adams et al. 2010). Pro-BMP9 has an open-armed conformation, characterised by a core containing two four-stranded β-sheets and the α4-helix, typical of the procomplexes of the TGF-β family members (Mi et al. 2015).
BMP9 is produced at higher level by hepatocytes and at lower level by lungs and brain (Bidart et al. 2012; Castonguay et al. 2011). BMP9 is required for the proper development and organization of blood and lymphatic vessels, but has also been described as a hepatogenic, osteogenic and chondrogenic factor. Moreover, BMP9 is important to regulate glucose metabolism and the expression of the hormone hepcidin, involved in the homeostasis of iron.
Within the HHT context, it is important to underline that BMP9 is the ALK1 physiological ligand. BMP9-ALK1 interaction leads to the activation of the SMAD1/5/8 pathway resulting in the regulation of the angiogenesis (David et al. 2008).

1.3.5. HHT and the Juvenile Polyposis

Juvenile Polyposis (JP) is an autosomal dominant syndrome that affects the gastrointestinal epithelium, leading to the formation of many polyps, specifically in the stomach, the small intestine, the colon, and the rectum (Larsen Haidle and

Howe 2015). The majority of polyps are benign, even if their presence is related to the risk of develop gastrointestinal cancer, with a higher incidence than the general population. In particular, JP patients develop colorectal cancer and the incidence approaches the 68% of patients by age 60 years (Brosens et al. 2007).
The term "juvenile" refers to the polyps' characteristics; they are hamartomas with smooth and unruffled appearance, and with distinct histology from that of adenomas. Usually, the first manifestation of the disease is the rectal bleeding, which often appears during the first decade of patients life, even if there is a huge variability in terms of age of onset and numbers of polyps (Gallione et al. 2006; Larsen Haidle and Howe 2015). JP is clinically diagnosed according to the following criteria:

- presence of more than 5 juvenile polyps in the colorectum;
- presence of multiple juvenile polyps throughout the GI tract;
- presence of juvenile polyps in subjects with a family history of JP.

The diagnosis is proved if the patient shows only one criterion (Gallione et al. 2004).

To date two genes have been associated to JP: *MADH4* (MIM *600993), coding for SMAD4, and *BMPR1A* (MIM *601299), coding for a TGF-β type I receptor. Mutations in these genes account for about the 50-60% of JP patients (Calva and Howe 2008; Zbuk and Eng 2007).

MADH4 is the joining link between JP and HHT, since mutations in this gene lead to the manifestation of a syndrome, called JPHT or JP-HHT. This syndrome brings together symptoms of both diseases and in particular the juvenile polyps from JP and the epistaxes and the AVMs from HHT (Gallione et al., 2006; Gallione et al., 2004). Before the identification of the JPHT syndrome, in the literature were described several cases of JP patients with HHT symptoms, mainly PAVMs, but the genetic cause was unknown (Conte et al. 1982; Cox et al. 1980; Desai et al. 1998; Radin 1994).
In JPHT, AVMs are predominantly found in young patients, while epistaxes and telangiectases are less severe than in HHT patients. Like HHT, JPHT is characterised by a high phenotypic heterogeneity among families and also among members of the same family. This situation suggests the presence of other genetic modifiers and/or environmental factors able to influence the disease phenotype (Gallione et al. 2010).

MADH4

MADH4 maps on 18q21.1 (Howe et al., 1998), and its exact genetic position is 18:51,029,614-51,085,045 (UCSC Genome Browser database – GRCh38/hg38). In

the RefSeq database only one transcript is annotated (NM_005359.5).The gene has 11 exons and codifies the Co-SMAD4, a 552 amino acids protein which consists of two highly conserved domains, called MH1 and MH2, and by a linker region between them. The MH1 domain is encoded by exons 1 and 2, the domain MH2 is encoded by exons 8, 9, 10 and 11, while the remaining exons encode the linker region (Gallione et al. 2010).

Up to date 88 different genetic variants, 97% certainly pathogenic, spanning the entire gene, are listed in the Arup database (http://www.arup.utah.edu/database/). In particular, there are 25 deletions, 24 missense mutations, 19 nonsense mutations, 12 silent variants, 9 insertions, 6 duplications and 4 splice site mutations. The database collects all the mutations involved in the pathogenesis of JP, JPHT or HHT.
At the beginning, *MADH4* mutations related to JPHT were observed in the MH2 domain of the protein, suggesting the presence of a mutational "hot spot" (Gallione et al. 2004, 2006). The identification of this "hot spot" was considered crucial for the correct diagnosis of the disease, especially to distinguish with any doubts JP and JPHT patients. Further investigations revealed that the majority of mutations lie in the MH2 domain and are related to the alteration of the coding reading frame, resulting in the formation of premature termination codon and in the protein loss of function. However, some mutations have been identified in the other exons, encoding for either the MH1 domain or the linker region of the protein.
There is no difference in the localization of JPHT-related and JP-related mutations alongside the gene; on the contrary, each mutation can cause both JP and JPHT. Therefore, any patient with *MADH4* mutation is at risk for the gastrointestinal cancer and for the HHT manifestations, and should be adequately monitored (Gallione et al. 2004, 2010).

1.4. miRNAs and HHT

microRNAs (miRNAs) are a family of short, single-stranded 21-22bps-long, non-coding RNAs, representing about 1% of all human genes and the most abundant class of small RNAs in animals. miRNAs modulate gene expression by translational repression or by mRNA cleavage in a sequence-specific manner (Bartel 2004). miRNAs localise within or between other genes, called intragenic and intergenic miRNAs, respectively, and are transcribed by the RNA Polymerase II as long primary transcripts. These are called pri-miRNAs and have a peculiar hairpin structure with three spiral turns flanked by single-stranded sequence. The pri-miRNAs undergo nuclear and cytoplasmic processing events in order to take on the mature form. Briefly, during the nuclear processing the single-stranded RNA is recognised by a complex consisting of the binding protein DGCR8 and the RNase III Drosha. The complex cleaves the pri-miRNAs to produce the 60bps-long pre-

miRNAs, with truncated hairpin shape. The pre-miRNAs interact with the protein Exportin5 and the GTPase Ran, assembling a complex that migrates into the cytoplasm, where is recognised and cleaved by the RNase III Dicer to generate the mature single-stranded miRNAs (Figure 8) (Finnegan and Pasquinelli 2013; Gulyaeva and Kushlinskiy 2016).
Over the years, it has been shown that miRNAs are also present in body fluids. These circulating miRNAs correlate to altered physiological conditions, representing new effective biomarkers (Gilad et al. 2008; Navickas et al. 2016; Del Vescovo et al. 2014). miRNAs are a promising class of disease biomarkers, which may potentially influence all the aspects of clinical care, from the early diagnosis of the disease to its prognosis (Landi et al. 2010).

Until now, the role of circulating miRNAs in HHT has been poorly investigated. Tabruyn et al. observed the down-regulation of miR-205 in plasma samples of both HHT1 and HHT2 patients. The authors demonstrated that miR-205 decreases the proliferation of HUVECs, affects the TGF-β signalling in endothelial cells, directly targets SMAD1 and SMAD4, and is modulated by TGF-β (Tabruyn et al. 2013). In the same year, the up-regulation of miR-210 in plasma samples of 15 HHT patients was demonstrated. In particular the misregulation of miR-210 has been related to the presence of PAVMs, observed in 8 patients (Zhang et al. 2013).
Taken together, these papers provide good reasons to further investigate the role of miRNAs in HHT pathogenesis, also because the miRNA "signature" in HHT remains to be identified.

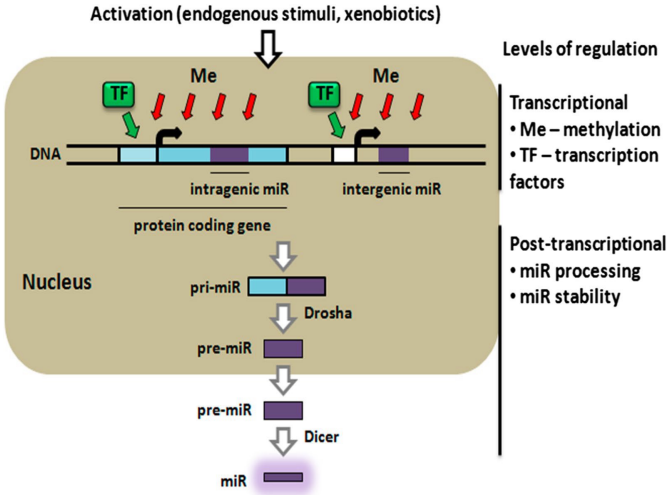


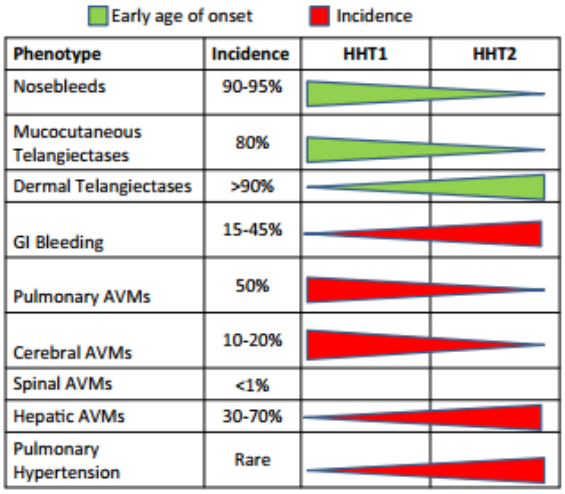
Figure 8. miRNAs maturation process (Gulyaeva and Kushlinskiy 2016).

1.5. Genotype – Phenotype correlations

First HHT studies were focused on the description of the disease phenotype without considering patients genotype. In 2003, Berg and collaborators published a questionnaire-based study which aimed to identify differences in the disease phenotype according to the mutated gene. The questionnaire was completed by 83 patients, 49 with *ENG* mutations and the remaining 34 with *ACVRL1* mutations, and both groups had similar percentages of males and females. The main result is that HHT1 patients present a worst phenotype than HHT2 patients. Indeed, the HHT1 group reported a significant earlier onset age of both epistaxes and telangiectases and a higher incidence of PAVMs than the HHT2 group (Berg et al. 2003). Since the publication of this work, several researchers focused their efforts on the study of genotype-phenotype correlations, in order to allow the clinical discrimination of HHT1 and HHT2.

Thus, results obtained by Berg et al. have been corroborated by other works, stressing the worst clinical condition of patients with *ENG* mutations compared to ones with *ACVRL1* mutations. For example, it is of interest that the epistaxes onset age is lower in HHT1 patients than HHT2 patients and that HHT1 patients normally develop a higher amount of telangiectases of the nasal mucosa (Bayrak-Toydemir et al. 2006; Lesca et al. 2007). However, Kjeldsen and collaborators did not observe significant difference in the severity of the epistaxes and their onset age between HHT1 and HHT2 patients (Kjeldsen et al. 2005). Other researches highlighted that the penetrance of the epistaxes is complete after the 4^{th} decade of life in both disease types and that there is no difference in the frequency of the bleeding (Lesca et al. 2007). It is also confirmed that HHT1 patients develop PAVMs and their complications more often than HHT2 patients (Sabbà et al. 2007). In particular, PAVMs have been related to patients carrying *ENG* mutations inducing the formation of premature stop codons and thus the synthesis of truncated proteins. It has been hypothesised that PAVMs development in HHT1 patients is due to the presence of another Endoglin function, which is not associated to the TGF-β signalling pathway (Lesca et al. 2007). Other differences between HHT1 and HHT2 patients are observed in the GI bleeding, more severe in HHT1 patients, in the CAVMs manifestation, at higher incidence in HHT1 patients, and in the liver involvement, more frequent in HHT2 patients (Figure 9) (Bayrak-Toydemir et al. 2006; Gincul et al. 2008; Kjeldsen et al. 2005; Lesca et al. 2007; Sabbà et al. 2007).

Probably, many genetic and/or environmental modifiers play a role in the clinical manifestation of the disease, as suggested by the huge phenotypic heterogeneity, between and within families, that characterised HHT (Lesca et al. 2007).



Phenotype	Incidence	HHT1	HHT2
Nosebleeds	90-95%		
Mucocutaneous Telangiectases	80%		
Dermal Telangiectases	>90%		
GI Bleeding	15-45%		
Pulmonary AVMs	50%		
Cerebral AVMs	10-20%		
Spinal AVMs	<1%		
Hepatic AVMs	30-70%		
Pulmonary Hypertension	Rare		

Figure 9. Genotype-phenotype correlations in HHT. The table reports the differences observed in HHT1 and HHT2 patients concerning the age of onset and the incidence of the symptoms. The base of triangles corresponds to either the earlier age of onset or the higher incidence of the symptoms (Arthur et al. 2015).

1.6. HHT and Pulmonary Arterial Hypertension

Pulmonary Hypertension (PH) is a hemodynamic state defined by mean Pulmonary Artery Pressure (PAPm) ≥25mmHg measured by right heart catheterization, and can be caused by different diseases (Galiè et al. 2016; Hoeper et al. 2013). According to the last update from the 5th World Symposium on Pulmonary Hypertension (Nice, 2013), the term PAH describes a PH subtype characterized by progressive increase of Pulmonary Vascular Resistance (PVR), due to mechanical obstruction of the pulmonary vascular bed in response to endothelial injury (Madonna et al. 2015). PAH is divided into subgroups that include: heritable (HPAH), idiopathic (IPAH), and PAH associated with other systemic diseases or drug/toxin exposures (Austin and Loyd 2013). Right heart catheterization remains essential for PH or PAH diagnosis (Hoeper et al. 2013).
During the past two decades important progress in understanding PAH genetics led to the discovery of the Bone Morphogenetic Protein Receptor Type 2 (*BMPR2* - MIM#178600) as the major heritable risk factor for development of PAH. In fact, mutations in *BMPR2* are responsible for 75% of HPAH patients and 15% of apparently IPAH patients (Austin and Loyd 2013; Deng et al. 2000). *BMPR2* encodes a member of the BMPs receptor family and its ligands are BMPs, members of the TGF-β superfamily (West et al. 2014).

BMPR2 implication on PAH pathogenesis highlights the relevance of the TGF-β superfamily. Indeed, some familial PAH cases are caused by mutations in other genes belonging to the TGF-β family receptors or signalling proteins, among which *ACVRL1* and *ENG*. Mutations in one of these genes lead to PAH in conjunction with HHT (Austin and Loyd 2013; Harrison et al. 2003).
Trembath and collaborators first described, in 2001, the association between PAH and HHT and nowadays several case reports have been published about patients with a diagnosis of both PAH and HHT (Miyake et al. 2016; Olivieri et al. 2006; Piao et al. 2016; Pousada et al. 2015). Moreover, in 2014 Ishiwata et al. described the case of a Japanese 17 years old girl who developed PAH as the first manifestation of HHT (Ishiwata et al. 2014). Considering the existing overlap between these two diseases, it is not surprising that PAH has been included in the possible clinical manifestations of HHT (Shovlin 2010).
Several research groups identified other PAH-related mutations in genes of the TGF-β superfamily, including *SMAD1*, *SMAD4* and *SMAD9* (Drake et al. 2011; Nasim et al. 2011; Shintani et al. 2009). Moreover, thanks to whole exome sequencing, other genes involved in HPAH were identified, i.e. *CAV1* (caveolin 1), *KCNK3* (potassium two pore domain channel subfamily K member 3) and *EIF2AK4* (eukaryotic translation initiation factor 2 alpha kinase 4) (Austin et al. 2012; Ma et al. 2013; Simonneau et al. 2013). Even if all these genes are related to heritable PAH, mutations in *BMPR2* are associated to a more severe phenotype (Girerd et al. 2015).
In 2014, Wang et al. reported the case of a 13 years old girl with two heterozygous mutations: one in *BMPR2* and one in *KCNA5* (potassium voltage-gated channel subfamily A member 5). The authors explained the presence of two mutations with the "two hit" model, suggesting that the second hit causes the early onset and the severity of the disease.
More recently, also *GDF2* has been related to PAH because an homozygous nonsense mutation was observed in a 5 years old boy with severe PAH and right heart failure, but neither in the boy nor in his family HHT clinical features were observed (Wang et al. 2016).

2. Aims of the research

Although the HHT diagnosis is based on "Curaçao" clinical criteria, mutation analysis of the disease-causing genes is important for several reasons. In fact, due to the genetic heterogeneity of the disease, one of the main goal of researchers and clinicians is the identification of genotype-phenotype correlations in order to clinically distinguish patients whit mutations in different genes and, in the future, to choose the appropriate management and follow up for each individual. Also, the identification of the disease-causing mutation in the proband allows researchers to make early diagnosis in other members of the same family. Moreover, mutation analyses provide information about all the variants localised in genes' sequences. Most of them are already known to be pathogenic, while the remaining could be new variants with a sure pathogenic effect, polymorphisms and Variants of Uncertain Significance (VUS), which need further investigation to understand if they are pathogenic.
In this context, my project aimed to:

1. Research mutations in either *ENG* or *ACVRL1* in patients with a "definite" HHT diagnosis;
2. Describe the HHT Italian population, focusing on the distribution of mutations in *ENG* and *ACVRL1* exons;
    i. Study an intronic VUS of *ENG* found in a patient with HHT and PAH to assess if it has a pathogenic role, leading to the manifestation of the disease.

Nowadays, it is well known the correlation between circulating miRNAs and several diseases. In fact, this type of small RNA is a promising class of disease biomarkers, which may be useful in the clinical care, from the diagnosis to the prognosis of the disease. Since the role of circulating miRNAs in HHT is not completely understood, I decided to dedicate part of my PhD project to the study of this topic, aiming to:

1. Identify miRNA profiling of HHT patients;
2. Evaluate the presence of difference in miRNA expression between HHT1 and HHT2 patients;

3. Evaluate the presence of miRNAs misregulated in patients having particular clinical symptoms, as PAVMs or HAVMs, in order to identify new potential biomarkers.

3. Materials and Methods

3.1. Patients

Patients' samples were collected from two Italian reference centres for HHT: the Otorhinolaryngology unit at IRCSS Fondazione Policlinico "San Matteo" in Pavia (executive director Prof. M. Benazzo) and the Gastroenterology unit at "Ospedale Maggiore" in Crema (executive director Prof. E. Buscarini). Peripheral blood samples were collected for each patient who had given informed consent to the analyses. The studies obtained the approval of the Ethical Committee of Fondazione IRCSS "Policlinico San Matteo".

3.2. DNA extraction

Genomic DNA was extracted from peripheral blood using the "GenElute™ Blood Genomic DNA Kit" by SIGMA, according to manufacturer's instructions. Once completed the protocol, DNA quality was evaluated by electrophoresis, using 1% agarose gel (running buffer: TBE1X – Tris-Boric acid-EDTA).

3.3. PCR (Polymerase Chain Reaction)

Primers used to amplify *ENG* and *ACVRL1* coding exons were drawn by our lab using the online software "Primer3" (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 2 and 3). The experiments reaction volume was 30µL and the reaction mix contained 3ng/µL of genomic DNA; 0.25pmol/µL of primers mix (forward + reverse); 200µM of dNTPs mix (dATPs + dCTPs + dGTPs + dTTPs); 1X enzyme reaction buffer; 1.5mM of $MgCl_2$; 1.5U PolyTaq polymerase (POLYMED) and sterile H_2O to volume.
The PCRs were performed with TRIO-Thermobloch™ (Biometra), PCR System 9700-GeneAmp® PE (Applied Biosystems) e C1000 Thermal Cycler (BIO-RAD Laboratories, USA), using the following amplification program:

1. 95°C → 5'
2. 95°C → 40"
3. 61°C to 67°C* → 40"
4. 72°C → 40"

} 35/40* times

5. 72°C → 5’
6. 4°C → pause

*different Tm and cycles number depend on the exon analysed

The size of PCR products was evaluated by electrophoresis, using 2% agarose gel (running buffer: TBE1X) and the Gene Ruler™ 100bps DNA Ladder (Fermentas Life Sciences).

Gene	*Exon*	*Primers*
ENG	1	F: 5’ – GCCCGTGGCACTTCCTCTAC – 3’ R: 5’ – AATACTTGGGGCCTGGTCCGTG – 3’
	2	F: 5’ – GTGACCTTGGGCAGGAGACG – 3’ R: 5’ – AGGGACAGCCACAAGGAAGG – 3’
	3	F: 5’ –GGCAGGGAGAGTGGAGTGGA – 3’ R: 5’ – ATTGGCTTCACCAGGCAGGA – 3’
	4	F: 5’ –GCAAGGGAGAAGGGGCTGAT – 3’ R: 5’ – CTCCTGCACTCTTGGTGCCC – 3’
	5	F: 5’ – TGAGGGAAGGGACTGAGGTG – 3’ R: 5’ – GTGGGGACTAGTGTCAGGGG – 3’
	6-7	F: 5’ – TCCCTATCCCATAAACCCACACCT – 3’ R: 5’ – GAGGCTGATGTACCTTGCCCA – 3’
	8	F: 5’– GGTGCGGGCACACAGTGAT– 3’ R : 5’ – TTACTGTGCCACGTGATTGGC – 3’
	9-10	F: 5’ – GACTTGGGACCCCTGGGTTGT – 3’ R : 5’ – CATGGCTTGCCAGGAGTTTCC – 3’
	11	F: 5’ – GTCAGGCAACTCCACAGGGC – 3’ R : 5’ – GGGAAGAAAGGCGGAGAGGA – 3’
	12	F: 5’ – GGGACACTGACAAGGATGTGGC – 3’ R : 5’ – TGATTAAGGCTCCGCCCCTC – 3’
	13	F: 5’ –GGTCACGGAGCCAGGGAG – 3’ R: 5’ – TCCCCCTTGCCATGTGCTAT – 3’
	14-15	F: 5’ – GCCTGGCTGTGATGAGCCC – 3’ R: 5’ – TAGGCGCGGAGAGCAGGC – 3’

Table 2. Primers used for the amplification of *ENG* exons. The F indicates the forward primer, while the R indicates the reverse one.

Gene	*Exon*	*Primers*
ACVRL1	2	F: 5' – GGAGTGCAGAGCTAGGGTTTCCC – 3' R: 5' – CCAGCTTCTCAAGTTCAGCCCCA – 3'
	3	F: 5' – GGGGTCAGACGAGAGGGACA – 3' R: 5'- GCCCCTTTATTGGCCAGAGC – 3'
	4	F: 5' – TGGGCAGGACTCTGGGATCT – 3' R: 5' – GCTCCCCACCGGCTCTAATC – 3'
	5	F: 5' – AGCTGGGCGAGTGAGGAGC – 3' R: 5' – CCCCAGCTGCCTCTCGTTCC – 3'
	6	F: 5'– AAACCTAAGGGTCTGGGGTTCTGT – 3' R: 5' – AGAGGTTGATGCTGCAGGTGGG – 3'
	7	F: 5'– CCCACCCCCAGACCTAGCTT – 3' R: 5' – GGGGTCTTGCGGAGGAGAA – 3'
	8	F: 5' – CCACTGCCTTCCAGCCCATC – 3' R: 5' – GGCTCCACAGGCTGATTCCCCTT – 3'
	9	F: 5' – ATTGCATTATACTGTCCCTCTCAGG – 3' R: 5' – GAGGTGCCCTAACCAGGACA – 3'
	10	F: 5' – CCCCATTACCGGCCATCCTCC – 3' R: 5' – TGCCCATCCCCAGCACACAC – 3'

Table 3. Primers used for the amplification of *ACVRL1* exons. The F indicates the forward primer, while the R indicates the reverse one.

3.4. Purification and Sequencing

The PCRs were purified using MultiScreen®$PCR_{\mu 96}$ Filter Plate (Merck Millipore) and prepared for the Sanger sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem™). The templates were loaded on the 3500 series Genetic Analyzer 8-Capillary (Applied Biosystem™) and sequences analysed by FynchTV (Geospiza™).

3.5. MLPA (Multiplex Ligation-dependent Probe Amplification)

MLPA was performed to indentify large deletions or duplications that cannot be detected by direct sequencing, using SALSA MLPA probemix P093-C2 HHT/PPH1 (MRC-Holland). The P093 probemix contains MLPA probes for each exons of *ENG*, *ACVRL1* and *BMPR2* genes and 8 reference probes with different autosomal locations. The experiment was performed using a total DNA quantity of 50ng, according to manufacturer's instructions. The thermocycler program for the MLPA reaction is shown below:

1. DNA denaturation
   1.1. 98°C → 5'
   1.2. 25°C → pause
2. Hybridization reaction
   2.1. 95°C → 1'
   2.2. 60°C → 18h

3. Ligation reaction
   3.1. 54°C → pause
   3.2. 54°C → 15'
   3.3. 98°C → 5'
   3.4. 20°C → pause
4. PCR reaction
   4.1. 95°C → 30" } 35 cycles
   4.2. 60°C → 30" }
   4.3. 72°C → 60" }
   4.4. 72°C → 20'
   4.5. 15°C → pause

The fragment separation was performed on the 3500 series Genetic Analyzer 8-Capillary (Applied Biosystem™), using GS500 size standard, and results were analysed using Coffalyser software (https://coffalyser.wordpress.com/).

3.6. Plasma preparation and RNA extraction

Plasma was obtained from peripheral blood samples by two centrifugations at 1500g per 10'. After the first centrifugation, plasma was collected and transferred to a clean 1.5mL tube and then it underwent the second centrifugation in order to avoid red blood cells contamination. Plasma samples were stored 24h in liquid nitrogen and then at -80°C until use.
Total RNA was extracted from plasma using miRCURY™ RNA Isolation Kit (EXIQON), according to manufacturer's instructions. The purified RNA samples were stored at -80°C.

3.7. miRNAs reverse transcription and quantification

miRNAs analyses were performed using miRCURY LNA™ Universal RT microRNA PCR (EXIQON) consisting of the Universal cDNA synthesis kit II and the ExiLENT SYBR® Green master mix kit with the human microRNA primer sets (PCR panels I+II ready to use). The reverse transcription was performed using Universal cDNA synthesis kit II. The reaction volume was 40µL and the reaction mix contained: 1X reaction buffer; 4µL of enzyme mix; 5ng/µL of template total RNA and nuclease free water. The reverse transcription program:

1. Incubation at 42°C → 60'
2. Heat-inactivation of the reverse transcriptase at 95°C → 5'
3. Cool at 4°C.

The samples were stored at -20°C until use.

The qPCR was carried out using the ExiLENT SYBR® Green master mix kit with serum/plasma focus microRNA PCR Panel (I+II), V4.M, according to manufacturer's instruction. Each 384-well PCR plates has LNA™ primer sets dried down (10µL reaction per well). Panel I contains 372LNA™ primer sets for the amplification of human microRNAs; 3 inter-plate calibrators; 3 primer sets for reference genes; 5 RNA Spike-in control primer sets and one blank well (H_2O). Panel II contains 380LNA™ primer sets for the amplification of human microRNAs; 3 inter-plate calibrators and one blank well (H_2O). Panels were analysed by CFX384 Touch™ Real-Time PCR Detection System (BIO-RAD Laboratories). The PCR amplification program is described below and was followed by melting curve analysis:

1. 95°C → 10'
2. 95°C → 10" } 40 cycles
3. 60°C → 1', ramp rate 1.6°C/s
4. Melting curve 65°C - 95°C → 5" (in 0.5°C increment + plate read)

Raw data were analysed using GenEx software by Multid analyses (http://genex.gene-quantification.info/). miRNAs presenting a *p-value* < 0.05 and a $\log_2$(fold-change) ≥+1 or ≤-1, at the same time, were considered as statistically significant and underwent gene/miRNA enrichment analysis with Cytoscape plugin "ClueGo" (v.2.2.5) and "CluePedia" (v.1.2.5) (Bindea et al. 2013). We analysed three miRNAs lists and everyone was enriched using the miRTaBase database (validated miRTaBase value = 0.6) in order to visualize the top 20 predicted target genes corresponding to each miRNA. Then, miRNAs and their predicted target genes were analysed to identify the affected pathway on the Reactome database (Fabregat et al. 2016).

3.8. Plasmids site-directed mutagenesis

Site-direct mutagenesis was performed on two constructs: a plasmid vector containing whole *ENG* coding sequence and intron 14, called pcEXV-ENG, and another one with *ENG* sequence from the beginning of exon 14 to the end of exon 15 including the intron between them, called pDisplay-MinigEND. The mutagenesis was carried out using primer specifically modified with the variant of interest (F: 5'– GGGCCCCT**T**CATCCACCC – 3' and R: 5'– GGGTGGATG**A**AG GGGCCC – 3'; in bold the nucleotide change).

3.9. Bacterial transformation and cell transfection

Mutated constructs were used to transform One Shot TOP10 Chemically Competent *E.coli.* Bacteria grew up in LB agar plate (100µg/mL ampicillin), then

single colonies were inoculated in LB medium (100µg/mL ampicillin) and incubated overnight at 37ºC.
DNA was extracted from bacteria using PureYield™ Plasmid Miniprep System (Promega) and the occurred mutagenesis was verified by Sanger sequencing. Mutated constructs were used to transfect cells. Human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco's Modified Eagle Medium (DMEM - Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 100U/mL penicillin and 100U/mL streptomycin (Gibco). Cell transfection was carried out using Lipofectamine® LTX Reagent (Invitrogen). In order to study variant effect on alternative splicing process, cells were also co-transfected with pDiplay-MinigEND mutated and vectors containing splicing factor SC35 or ASF/SF2.

3.10. Western Blot analyses

Forty-eight hours after transfection, cells were lysed at 4ºC for 10 minutes with a lysis solution containing Hepes 1M, NaCl 5M, EGTA 150mM, EDTA 500mM, Phosphatase Inhibitors 100X, Proteinase Inhibitors 25X, 10% TRITON 100X. Lysates were centrifuged at 13200g and 4ºC for 15 minutes then the supernatants were stored at -20°C. Protein amount was quantified by Bradford assay and 60µg of protein were separated by SDS-PAGE and electrotransferred to a PVDF membrane using iBlot Gel Transfer System (Invitrogen).
Immunodetection was performed with the following monoclonal primary antibodies:

- anti-CD105 1:1000 (ab169545; Abcam) for pcEXV-ENG;
- anti-HA 1:50 (12CA5; Roche) for pDisplay-minigEND;
- anti-V5 1:5000 (R960-25; Invitrogen) for splicing factors SC35 and ASF/SF2;
- anti-β-actin 1:4000 (AC-15; Sigma).

Overnight incubation at 4ºC with monoclonal antibodies was followed by 1h incubation with secondary polyclonal antibody, anti-mouse or anti-rabbit. Protein bands were revealed using SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific).

3.11. EMSA (Electrophoretic Mobility Shift Assay)

Nuclear Extracts (NE) were prepared from AG1522 fibroblasts by using Nuclear Extraction Kit (Cayman chemical) according to manufacturer's instruction. DNA probes were labelled at the 3'-end with biotin using Biotin 3'End DNA Labelling

Kit (Thermo Scientific) according to the manufacturer's instructions. The following three different double-stranded probes were used:

1. control (CTRL): 5' - GCAGGCGGCCTGGCCCAGCCCCTTCTC – 3' (Botella et al. 2001);
2. wild type (WT): 5' - GCATGCCGGGCCCCT**C**CATCCAAA – 3';
3. mutated (MUT): 5' - GCATGCCGGGCCCCT**T**CATCCAAA – 3'.

The difference between the WT and MUT probes is highlighted in bold. Each probe was labelled as single-stranded oligonucleotide and then annealed with the complementary probe with a thermocycler using the following program:

1. 95°C → 5'
2. 95°C – 1°C/cycle → 1'(28/36 cycles)*
3. 67/59°C* → 30'
4. 67/59°C* – 1°C/cycle → 1'(63/55 cycles)*
5. 4°C → pause

*changes depending on the probe: CTRL probe anneals at 67°C (step 2: 28 cycles and step 4: 63 cycles), while WT and MUT probes anneal at 59°C (step 2: 36 cycles and step 4: 55 cycles).

The DNA binding reaction was performed using the Lightshift Chemiluminescent EMSA Kit (Thermo Scientific). 30µg of NE were incubated at room temperature for 20 minutes with 20fmol of labelled probes and 200-fold molar excess of the unlabelled probe was added as competitor of the labelled probe. The samples were run on 6% non-denaturing polyacrilamide gel in TBE 0.5X (running buffer: pre-cooled TBE 0.5X). The electrophoretic transfer to nylon membrane was performed at 300mA for 90 minutes (transfer buffer: pre-cooled TBE 0.5X). Membrane was crosslinked on a transilluminator (312nm bulbs) for 15 minutes. Biotin-labelled DNA was detected using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific). Membranes were exposed in an equipped CCD camera for 45 minutes. Supershift experiments were performed incubating at room temperature for 20' 4µg of antibody anti-Sp1 (sc-59X – Santa Cruz) with the reaction mix before the addition of the labelled probe.

3.12. qRT-PCR (quantitative Real Time Polimerase Chain Reaction)

PBMCs (Peripheral Blood Mononuclear Cells) were isolated using Histopaque®-1077 (Sigma®). 2.5mL of peripheral blood was mixed with 2.5mL of RPMI 1640 medium (Gibco) and carefully layer onto 9mL of Histopaque®-1077. Then, the samples were centrifugated at 400g for 40' at room temperature. After centrifugation the opaque interface containing mononuclear cells was aspirated

with a Pasteur pipette, transferred into a clean tube and washed several times with RPMI 1640.
Total RNA was extracted from PBMCs using RNeasy® Mini Handbook (Qiagen) according to manufacturer's instructions. RNA samples were stored at -20°C until use. qPCR were performed using EXPRESS One-Step SYBR® GreenER™ with Premixed ROX (Invitrogen). The volume of the reaction was 20µL and the mix contained: 120ng of total RNA; 10µL of EXPRESS SYBR® GreenER™ qPCR SuperMix with Premixed ROX; 10pmol/µL of primers (forward and reverse); 0.5µL of EXPRESS SuperScript® Mix for One-Step SYBR® GreenER™ and DEPC-treated water. The thermalcycler program was:

1. cDNA synthesis
   1.1. 50°C → 5'
2. qPCR
   2.1. 95°C → 2'
   2.2. 95°C → 15" } 40 cycles
   2.3. 60°C → 1' }
3. Melting curve analysis
   3.1. 60°C–95°C

β-actin was used as reference gene. Primers for *ENG*, *ACVRL1* and *ACTB* were from Zucco et al.:

- *ENG*. F: 5' – AGCTGACTCTCCAGGCATCC – 3' and R: 5' – GCAGCT CTGTGGTGTTGACC – 3';
- *ACVRL1*. F: 5' – GTGAGAGTGTGGCCGTCAAG – 3' and R: 5' – CAT GTCTGAGGCGATGAAGC – 3';
- *ACTB*. F: 5' – AGCCTCGCCTTTGCCGA – 3' and R: 5' – CTGGTGCC TGGGGCG – 3'.

3.13. In silico analyses

In silico analyses were performed to predict pathogenic role of unknown variant and to identify putative binding site of transcription factors. The list of software used for this thesis:

- MutationTaster2;
- Polyphen-2;
- FATHMM/mklv2.3;
- Ensembl/VEP;
- TomTom4.11.2
- Patch1.0
- AliBaba2.1.

3.14.Statistics

Two-tails Student's t-test and one way ANOVA test were used for statistical computing (*p-value* ≤ 0.05 = *; *p-value* ≤ 0.005 = ** and *p-value* ≤ 0.0005 = ***).

4. Results

4.1. The HHT Italian population

In the three years of my PhD, DNA samples from several patients with a HHT clinical diagnosis were analysed in order to identify the disease-causing mutation in either *ACVRL1* or *ENG*. We first analysed 39 index cases with a "definite" diagnosis of HHT (Table 4) and, after identification of the mutation, their relatives.
We found the disease-causing mutation in 35 out of the 39 analysed patients (~90%). Twenty-three patients (~59%) carrying a mutation on *ACVRL1*; 12 patients (~31%) carrying a *ENG* mutation, three of them with a large deletion detected by MLPA and no mutation has been identified in the remaining 4 patients (~10%). In particular, we observed that most of the *ACVRL1* mutations localised in exons 3, 7 and 8 (14 mutations: 40% of total mutations) and that large deletions are more frequent in *ENG* (3 mutations: ~9% of total mutations).
Twenty-two mutations are listed in the HHT International database, while the other 13 are new mutations, highlighted in bold in Table 5. We demonstrated the pathogenic role of these unknown mutations using *in silico* prediction software, as MutationTaster and Polyphen, and proving the co-segregation of the mutation and the disease within the family. The only exception is the *ENG* mutation c.1852+42 C>T, carried by the patient P19, described in the next paragraph (*4.2. Case Report*).
Moreover, we observed that some mutations are shared by unrelated families; for example, *ACVRL1* mutations c.203dupG (p.G68fs) and c.1435C>T (p.R479X) were found in ALB, ON, DIN and in GIU, IGG, respectively, and *ENG* mutation c.360+1G>A (Splice Site) was found in LCV and NOA.

ID	*Family*	*Epi.*	*Tel.*	*PAVM*	*HAVM*	*Other*
AG	X	X	X	---	---	---
ALB	X	X	X	m.i.	m.i.	---
BcH	X	X	X	X	---	---
BLA	X	X	X	X	---	PH
CAZ	X	X	X	---	X	---
CLS	X	X	X	---	---	---
DDL	X	X	X	m.i.	m.i.	PH
DIN	X	X	X	m.i.	m.i.	---
ESP	X	X	X	---	X	---
FgLa	X	X	X	---	---	---
FV	X	X	X	m.i.	---	PH
GeA	X	X	X	---	---	---
GIU	X	X	X	m.i.	m.i.	PAH
HUN	X	X	X	X	X	---
IGG	X	X	X	---	---	---
LCV	X	X	X	X	---	---
LNL	X	X	X	---	---	---
MgP	X	X	X	X	---	---
NAT	X	X	X	m.i.	m.i.	---
NOA	X	X	X	m.i.	m.i.	---
ON	X	X	X	---	---	---
P19	X	X	X	---	---	PAH
PF	X	X	X	---	---	GI bleeding
PNK	X	X	X	m.i.	m.i.	---
PON	X	X	X	---	---	---
PRI	X	X	X	---	X	PAH
PSP	X	X	X	X	---	---
RAC	X	X	X	---	---	---
REL	X	X	X	---	X	PAH
RNL	X	X	X	---	---	---
SaD	X	X	X	---	---	---
SaGi	X	X	X	m.i.	m.i.	---
SIG	X	X	X	X	---	---
TcA	X	X	X	m.i.	m.i.	---
TOF	X	X	X	X	---	---
TUC	X	X	X	---	X	GI bleeding
ViS	X	X	X	X	---	---
VMG	X	X	X	m.i.	m.i.	---
ZAC	X	X	X	---	m.i.	---

Table 4. Description of clinical symptoms. All patients present at least three criteria for the HHT diagnosis. The term family refers to the presence of a first degree relative affected by HHT; Epi. refers to epistaxis; Tel. refers to telangiectases; m.i. refers to missing information.

ID	*Gene*	*Mutation*
ALB	A3	c.203dupG (p.G68fs)
CLS	A3	**c.107G>C (p.C36S)**
DDL	A3	c.150G>A (p.W50X)
DIN	A3	c.203dupG (p.G68fs)
ESP	A3	**c.68delC (p.P23fs)**
ON	A3	c.203dupG (p.G68fs)
FV	A4	c.430C>T (p.R114X)
PRI	A5	**c.595G>C (p.A199P)**
TcA	A5	c.567delG (p.G189fs)
TUC	A5	c.601C>T (p.Q201X)
BLA	A7	**c.1048G>T (p.G350C)**
HUN	A7	**c.1048+1G>T (Splice Site)**
PF	A7	**c.1042G>A (p.D348N)**
PNK	A7	**c.863T>C (p.F288S)**
PON	A7	c.1010T>C (p.L337P)
RNL	A7	c.1004A>G (p.N335S)
SaGi	A8	**c.1141_1180del39 (p.L381_K394del)**
ViS	A8	c.1135G>A (p.E379K)
REL	A9	**c.1377+4A>G (Splice Site)**
VMG	A9	c.1298C>G (p.P433R)
CAZ	A10	c.1451G>A (p.R484Q)
GIU	A10	c.1435C>T (p.R479X)
IGG	A10	c.1435C>T (p.R479X)
MgP	E1	c.1A>G (p.M1V)
SIG	E1	c.-127C>T
GeA	E3	c.277C>T (p.R93X)
LCV	E3	c.360+1G>A (Splice Site)
NOA	E3	c.360+1G>A (Splice Site)
TOF	E6	**c.816+5G>A (Splice Site)**
NAT	E8	**c.1134+1delG (Splice Site)**
ZAC	E8	**c.1014delC (p.P338PfsX21)**
P19	E14	**c.1852+42 C>T**
AG	ENG	del E1
FgLa	ENG	del E9_E14
LNL	ENG	del E9_E14
BcH	NF	---
PSP	NF	---
RAC	NF	---
SaD	NF	---

Table 5. Mutations identified in HHT patients. Mutations written in bold are not listed in the HHT International database. The letters A and E refer to *ACVRL1* and *ENG* genes, respectively, while the number indicates the exon in which the mutation localized. NF means Not Found.

The results of the screening have been added to the data previously obtained by the Medical Genetics Laboratory, headed by Prof. C. Danesino. Until October 2016, 1884 samples were collected and 1270 of them were analysed, leading to the identification of the disease-causing mutation in 859 samples from 340 unrelated families. In collaboration with Prof. C. Sabbà (Centro di Assistenza e Ricerca Sovraziendale per le Malattie Rare, University of Bari) we organized data about Italian patients in order to describe the genetics of the HHT Italian population.
The disease-causing mutation was identified in 383 HHT families and about 68% (259 families) of them have HHT type 2 versus 32% of families (124) having HHT type 1. By direct sequencing, 235 different mutations were identified and they spread among the whole coding sequence and flanking introns of both genes (141 mutation on *ACVRL1* and 94 mutations on *ENG* – Table 6 and 7), with the exceptions of *ACVRL1* exon 2 and *ENG* exons 11 and 15, where no mutations were detected. Noteworthy, about 47% of patients have a mutation in either exons 3, 7 or 8 of *ACVRL1*. The comparison between the number of families and the number of mutations highlighted that several mutations are shared by different families, especially in exons 3, 4, 8 and 10 of *ACVRL1* and in exon 3 and 6 of *ENG* (Figure 10).

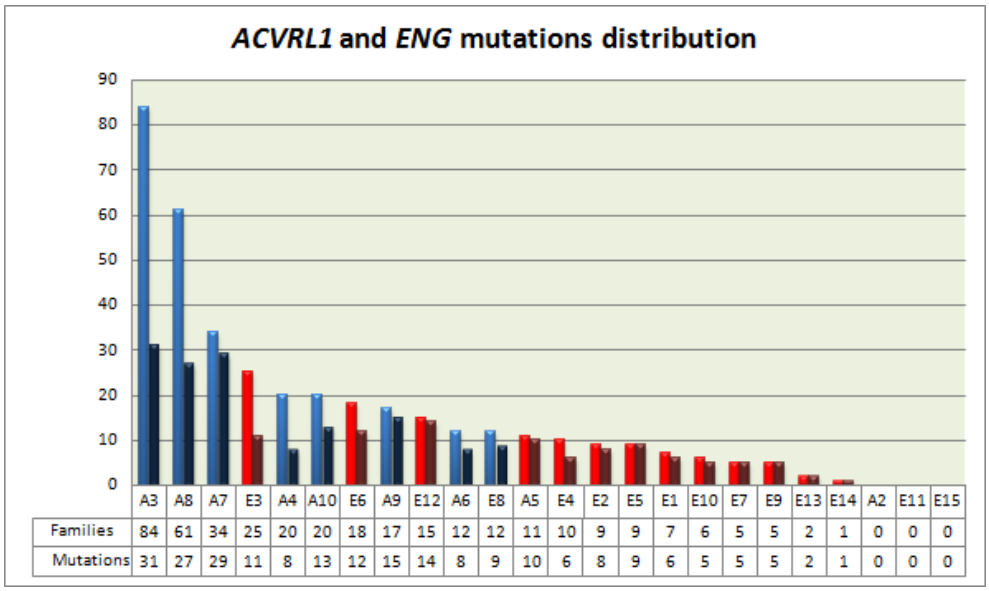


Figure 10. Mutations distribution in different *ACVRL1* and *ENG* exons. Color blue has been used to indicate *ACVRL1* while color red indicates *ENG*. Light bars refer to the number of families and dark bars refer to the number of different mutations.

In many cases, *ACVRL1* frequent mutations were found in families with the same geographical origin. In fact we observed:

- c.152 G>A (p.C51Y) in 4 families from Sicily;
- c.164_169 delTGGTGC (p.L55_V56del) in 4 families from Emilia Romagna;
- c.200 G>A (p. R67Q) in 4 families from Sardinia;
- c.203dupG (p.G68GfsX101) in 4 families from Veneto;
- c.205_209dupTGCGG (p.G70GfsX54) in other 4 Sardinian families;
- c.1199 C>A (p.A400D) in 4 families from Lombardy.

In particular, we found the mutation c.289_294delCACAAC (p.H97_N98del), never observed in other parts of the world, in 16 apparently unrelated families from the Bergamo district (Lombardy).
On the contrary, mutations as c.430 C>T (p. R144X); c.1120 C>T (p. R374W); c.1121 G>A (p. R374Q) and c.1231 C>T (p.R411W) were found in families with different geographical origin (Figure 11). Similar results were obtained also for *ENG* frequent mutations, as:

- c.210delG (p.E70DfsX10) described in 2 families from Veneto;
- c.277 C>T (p.R93X) described in 3 families from Campania and 2 from Lombardy;
- c.1134 G>A (p. A378A) described in 2 families from Lombardy.

ENG mutations c.360+1 G>A (Splice Site); c.771dupC (p.Y258LfsX76) and c.1465 C>T (p.Q489X) were found in families from different Italian Regions (Figure 12).

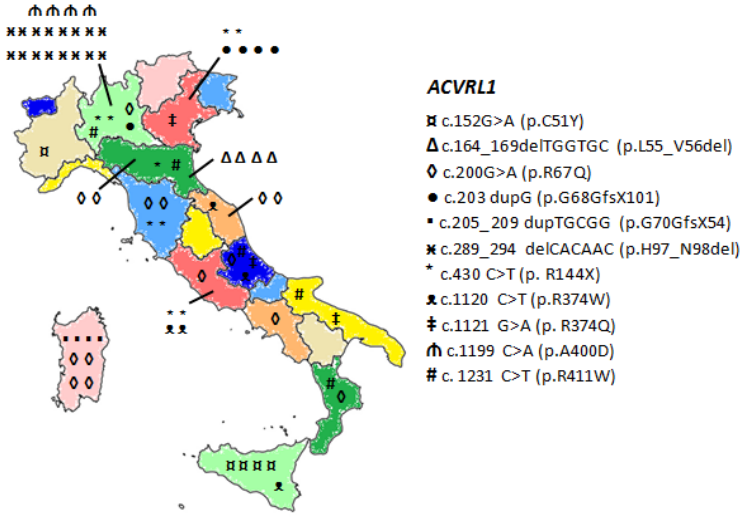


Figure11. Geographic distribution of *ACVRL1* frequent mutations.

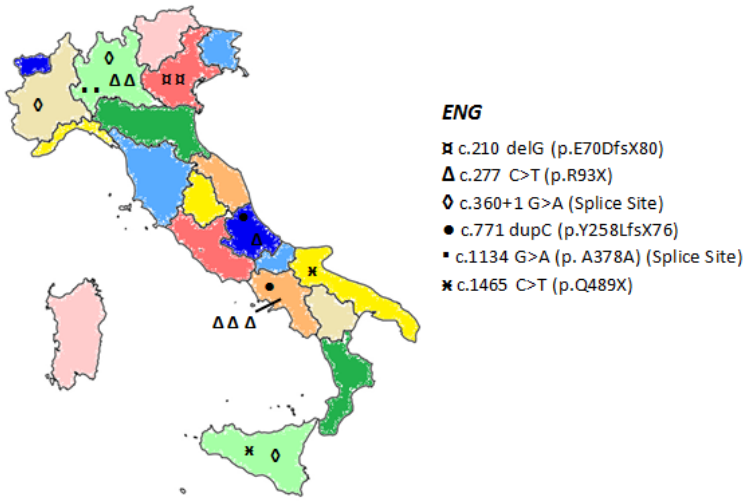


Figure 12. Geographic distribution of *ENG* frequent mutations.

Moreover, we observed that several mutations of both genes affect the same nucleotide or amino acid. For example, *ENG* mutations c.360+1 G>A (Splice Site) and c.360+1 G>C (Splice Site); c.771dupC (p.Y258LfsX76), c.771delC (p.Y258fsX101) and c.772delT (p.Y258fsX101) and *ACVRL1* mutations c.145delG (p.A49PfsX5) and c.145dupG (p.A49PfsX120); c.152 G>A (p.C51Y) and c.152 G>T (p.C51F); c.625 G>C (p.G209R) and c.625 G>A (p.G209X); c.858 C>A (p.Y286X) and c.858 C>G (p.Y286X); c.988 G>A (p.D330N) and c.988 G>T (p.D330Y); c.1031 G>A (p.C344Y) and c.1031 G>T (p.C344F); c.1048 G>C (p. G350R), c.1048 G>A (p.G350S) and c.1048 G>T (p.G350C); c.1218 G>A (p.W406X) and c.1218 G>T (p.W406C); c.1298 C>T (p. P433L) and c.1298 C>G (p.P433R).

Comparing the mutations found by our group with the mutations listed in the international database, we observed that 36 mutations on *ENG* and 45 on *ACVRL1* are new ones. *In silico* analyses and variants co-segregation with the disease proved their pathogenic role. In addition, 10 out of the 36 *ENG* mutations and 19 out of the 45 *ACVRL1* mutations affect a specific nucleotide and/or amino acid, already associated to a different mutation in the international database (Table 6 and 7).

ACVRL1 mutations		
c.68del (p.P23fs)	c.625+5 G>A (Spl)	c.1123 T>G (p.Y375D)
c.89del (p.P30RfsX3)	c.526-6 C>G (Spl)	c.1127 T>A (p.M376K)
c.107 G>C (p.C36S)	c.626-3 C>G (Spl)	c.1129 G>A (p. A377T)
c.113 G>T (p.S38I)	c.650 G>A (p.W217X)	c.1132 C>T (p.P378S)
c.115_118dup (p.H40PfsX130)	c.656 G>A (p.G219D)	c.1133 C>T (p.P378L)
c.121 T>C (p.C41R)	c.673_674del (p.S225X)	c.1135 G>A (p.E379K)
c.139dup (p.R47PfsX122)	c.686 A>T (p.L229M)	c.1139 T>G (p.V380G)
c.143 G>A (p.G48E)	c.743_744del (p.T248X)	c.1141_1180del (p.L381_Y393del)
c.145del (p.A49PfsX5)	c.760del (p.Asp254ThrFsX4)	c.1184 G>A (p.W395X)
c.145dup (p.A49PfsX120)	c.676 G>A (p.V226M)	c.1190 A>G (p.D397G)
c.150 G>A (p.W50X)	c.773-1 G>T (Spl)	c.1199 C>A (p.A400D)
c.152 G>A (p.C51Y)	c.811_823del (p.T271SfsX26)	c.1208 T>C (p.L403P)
c.152 G>T (p.C51F)	c.824_827dup (p.L275LfsX118)	c.1218 G>A (p.W406X)
c.164_169 del (p.L55_V56del)	c.837 C>G (p.Y279X)	c.1218 G>T (p.W406C)
c.172 G>T (p.E58X)	c.847_853del7insTT (p. G283Ffs)	c.1221 G>T (p. E407D)
c.197 A>C (p.H66P)	c.853dup (p.L285PfsX107)	c.1231 C>T (p.R411W)
c.199 C>T (p.R67W)	c.858 C>A (p.Y286X)	c.1232 G>A (p. R411Q)
c.200 G>A (p. R67Q)	c.858 C>G (p.Y286X)	c.1246+2T>G (Spl)
c.203dup (p.G68GfsX101)	c.863T>C (p.F288S)	c.1070A>C (p.Q357P)
c.205_209dup (p.G70GfsX54)	c.878del (p.T293SfsX)	c.1261 T>A (p.Y421N)
c.207C>A (p.C69X)	c.918_920dup (p.A307dup)	c.1270 C>T (p.P424S)
c.218_219insAA (p.H73QfsX50)	c.924 C>A (p.C308X)	c.1271 C>A (p.P424H)
c.230 G>A (p.C77Y)	c.938 T>C (p.L313P)	c.1280 A>T (p.D427V)
c.235 G>A (p.G79R)	c.946_948 del (p. E316del)	c.1297 C>A (p. P433T)
c.247 G>T (p.E83X)	c.961 C>T (p.Q321X)	c.1298 C>T (p. P433L)
c.266 G>A (p.C89Y)	c.982 C>T (p.H328Y)	c.1298C>G (p.P433R)
c.283 T>C (p.C95R)	c.988 G>A (p.D330N)	c.1321 G>A (p.V441M)
c.284G>A (p.C95Y)	c.988 G>T (p.D330Y)	c.1324_1332del (p.V442_V444del)
c.289_294del (p.H97_N98del)	c.1000del (p. R334fsX20)	c.1331_1332del (p.V444X)
c.313+1 G>A (Spl)	c.1004 A>G (p.N335S)	c.1339 C>T (p.Q447X)
c.313+2 T>A (Spl)	c.1010 T>C (p.L337P)	c.1347del (p.Thr450ProFsX15)
c.314-3 C>G (Spl)	c.1031 G>A (p.C344Y)	c.1348 A>G (p.T450A)
c.321delA(p.Q107X)	c.1031 G>T (p.C344F)	c.1377+4 A>G (Spl)
c.400_409del (p.A134X)	c.1042G>A (p.D348N)	c.1297 C>T (p. P433S)
c.430 C>T (p. R144X)	c.1048 G>C (p. G350R)	c.1378-2 A>G (Spl)
c.435del (p.R145RfsX20)	c.1048 G>A (p.G350S)	c.1385 C>G (p.S462X)
c.448 C>T (p.Q150X)	c.1048 G>T (p.G350C)	c.1427 C>T (p.P476L)
c.505 C>T (p.Q169X)	c.1048+1 G>T (Spl)	c.1435 C>T (p.R479X)
c.525+1 G>A (Spl)	c.1048+5 G>T (Spl)	c.1436 G>A (p.R479Q)
c.526-1 G>T (Spl)	c.1049-4_1049-2del3insCC (Spl)	c.1438 C>T (p.L480F)
c.590 C>T (p.T197I)	c.1054 G>C (p.A352P)	c.1445 C>A (p.A482E)
c.595 G>C (p.A199P)	c.1062_1080 dup (p. Y361AfsX37)	c.1450 C>T (p.R484W)
c.601 C>T (p.Q201X)	c.1086_1089dup (p.I364GfsX51)	c.1451 G>A (p.R484Q)
c.617 A>G (p.E206G)	c.1112 G>A (p.G371D)	c.1468 C>T (Q490X)
c.617_625del (p.E206_V208del)	c.1115 C>T (p.T372I)	c.1411 T>C (p.C471R)
c.625 G>C (p.G209R)	c.1120 C>T (p. R374W)	c.1426 C>A (p.P476T)
c.625 G>A (p.G209X)	c.1121 G>A (p. R374Q)	c.1432 G>C (p.A478P)

ENG mutations		
c.-127 C>T	c.524-4_524-1del insAAA (Spl)	c.1098_1099insT (p.A367C)
c.1 A>G (p.M1V)	c.526 C>T (p.Q176X)	c.1134 G>A (p. A378A)
c.3 G>A (p.M1I)	c.572_646dup (p.H215QfsX31)	c.1134+1del (Spl)
c.63delC (p.T22QfsX31)	c.574del (p.191 fs:X221)	c.1134+1 G>A (Spl)
c.67+1T>C (Spl)	c.581_582delinsATG (p.L194HfsX140)	c.1088 G>C (p.C363S)
c.27_44del (p.A9_A15del)	c.582_600del insTGGTCCGCAAG (p.L194fs)	c.1135del (p.H379IfsX380)
c.68-1 G>C (Spl)	c.591_619del (p.R197fs)	c.1144 T>G (p.C382G)
c.97 C>T (p.Q33X)	c.604dup (p.A202fs)	c.1190dup (p.D398RfsX24)
c.119del (p.G40X)	c.674del (p.Pro225ArgFsX9)	c.1195del (p.R339fs)
c.125_129del (p. V42VfsX4)	c.736del (p.D246X)	c.1208 T>C (p.F403S)
c.145 G>T (p.V49F)	c.737del (p.D246VfsX113)	c.1292 C>A (p.S431X)
c.155 G>T (p.G52V)	c.771dup (p.Y258LfsX76)	c.1306 C>T (p.Q436X)
c.166C>T (p.Q56X)	c.771del (p.Y258fs)	c.1309 C>T (p.R437W)
c.210del (p.E70DfsX10)	c.772del (p.Y258 fsX101)	c.1311+2 T>A (Spl)
c.220-6_227del (p.G74TfsX71)	c.774 C>A (p. Y258X)	c.1311+5 G>A (Spl)
c.220-1 G>C (Spl)	c.781 insCCCCTACG (p.S260_W261insPLS)	c.1429 -18_-1del (Spl)
c.229 C>T (p.Q77X)	c.808 C>T (p.Q270X)	c.1465 C>T (p.Q489X)
c.250del (p.A84HfsX18)	c.816+2 T>A (Spl)	c.1468_1471del (p.L490TfsX26)
c.277 C>T (p.R93X)	c.816+5 G>A (Spl)	c.1470_1471insA (p.D491fsX)
c.287 T>C (p.L96P)	c.816+5 G>C (Spl)	c.1478del (p. C493SfsX25)
c.298_299del (p.S100CfsX48)	c.790 G>A (p.D264N)	c.1513 G>T (p.E505X)
c.309_311del (p. S104del)	c.817-2 A>T (Spl)	c.1513del (p.E505NfsX12)
c.360 C>A (p.Tyr210X)	c.832_834del (p.S278del)	c.1550_1551del (p.V517EfsX10)
c.360+1 G>A (Spl)	c.889 C>T (p.Q297X)	c.1586 G>C (p.R529P)
c.360+1 G>C (Spl)	c.909_929del (p.R304_I310del)	c.1586_1587dup (p.R529AfsX)
c.380delT (p.F127X)	c.937 T>C (p.S313P)	c.1672_1684dup (Q562RfsX9)
c.392delC (p. P131fs)	c.1014del (p.P338PfsX21)	c.1686+2 T>C (Spl)
c.432_441del (p.T144fsX159)	c.1080_1083del (p.T361SfsX7)	c.1585C>T (p.R529C)
c.511 C>T (p.R171X)	c.1085_1086insA (p.T361fsX395)	c.1477T>C (p.C493R)
c.523 G>A (p.A175E)	c.1088 G>A (p.C363Y)	c.1687-1 G>C (Spl)
c.502 A>T (p.I168F)	c.1097_1119del (p.D365EfsX22)	c.1687del (p.E563KfsX9)
		c.1852+42 C>T

Table 6 (previous page) and Table 7. *ENG* and *ACVRL1* mutations found in Italian patients. Black has been used for mutations listed in the HHT International database; green describes new mutations; orange refers to those new mutations affecting a nucleotide or amino acid in which other mutation has already been described and red indicates mutations with a pendant classification.

Then, mutations have been grouped according to their effect at the protein level; we observed that missense mutations are the most common type in ALK1 (52%) (Figure 13.a), while deletion are predominant in Endoglin (32%) (Figure 13.b). On the contrary, deletion/insertion, silent and intronic mutations are the less represented in both proteins. These results agree with data from the HHT International database (http://arup.utah.edu/database/hht/) (Figure 13.c-d).

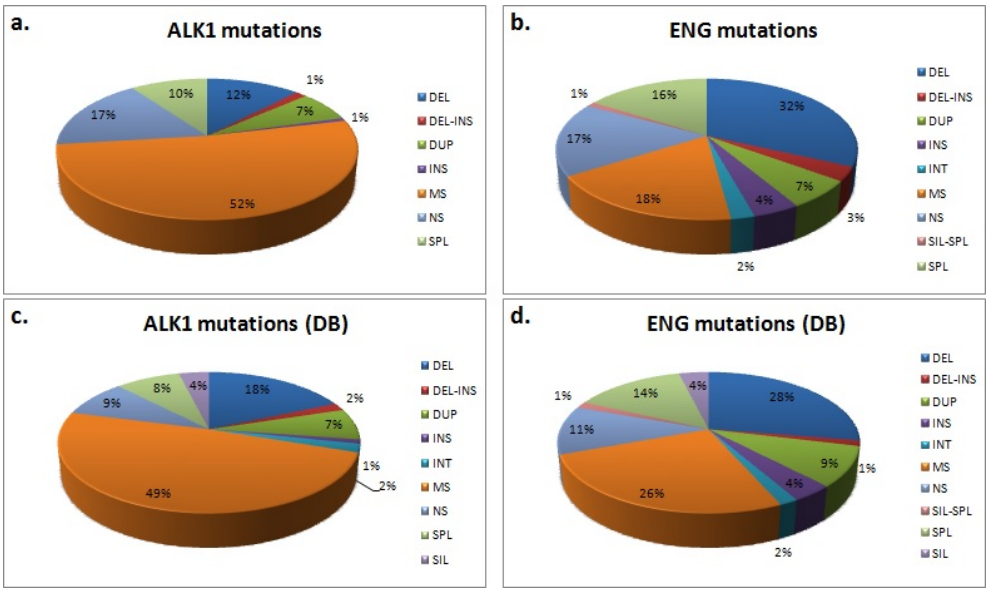


Figure 13. Classification of mutations according to their effect at the protein level. (a-b) Data concerning the HHT Italian population. **(c-d)** Data obtained from the HHT International database. DEL: Deletion; DEL-INS: Deletion/Insertion; DUP: Duplication; INS: Insertion; INT: Intronic; MS: Missense; NS: Nonsense; SIL-SPL: Silent effect on Splicing; SPL: Splicing affected; SIL: Silent.

The HHT Italian population consists of other 5 families carrying large deletions or duplications in *ACVRL1* or *ENG*, listed in Table 8.

	ACVRL1	***ENG***
Deletions	Exon3_8 del (1 family)	Exon1 del (1 family) Exon9_14 del (2 families)
Duplications		Exon5_7 dup (1 family)

Table 8. List of *ACVRL1* and *ENG* large deletions and duplications detected by MLPA.

To complete the description of the HHT Italian population, we list in Table 9 the polymorphisms observed in patients and in bold we highlight those not present in the International database. We observed that all the *ACVRL1* polymorphisms localised in introns, while *ENG* polymorphisms localised also in exons, leading sometimes to an amino acid change, as c.14 C>T (p.T5H) and c.322 C>T

(p.H108L). The non-pathogenic role of these variants was proved by the fact that the patient carries a mutation with a known disease-causing effect.

ACVRL1	***ENG***
Exon 3. c. 313+11 C>T **Exon 4.** c. 314-35 A>G **Exon 5.** c. 526-22 A>G c. 625+110_625+130del c. 625+164 T>C **Exon 9**. c. 1377+45 T>C c. 1377+65 A>G	**Exon 1.** c. 14 C>T (p.T5H) **Exon 2.** c. 207 G>A (p.L69L) c. 219+25 G>T **Exon 3. c. 321 G>T (p.L107L)** **c. 322 C>T (p.H108Y)** **Exon 8.** c. 1029 C>T (p.T343T) **Exon 14.** c. 1742-72 T>C **Exon 15. c. 1937+424 G>A**

Table 9. List of polymorphisms found in HHT patients.

4.2. Case Report

A 63 years old Italian man was hospitalized in 1996 for the evaluation of an atrial flutter. Right heart catheterization revealed severe precapillary PH, with a mean pulmonary artery pressure of 58mmHg (83/33mmHg). No right to left shunt was identified. The first diagnosis was of PAH and mutation analyses of *BMPR2* did not identify mutations.
In 2014, the patient was hospitalized in the otorhinolaryngology unit for the presence of epistaxes; telangiectases of nasal mucosa, lips and fingertips were also observed. These symptoms led to the diagnosis of "suspected" HHT, and a genetic counselling was offered to the patient. In reconstructing the genealogical tree, it was discovered the presence of nosebleeds in other family members.
ENG and *ACVRL1* coding regions were analysed by direct sequencing and MLPA (Figure 14) but no known disease-causing mutation was found. We only identified the variant c.1852+42 C>T in the intron 14 of *ENG,* 16bps after the STOP codon (TGA) of the short isoform of the protein (Figure 15).

4.2.1. In silico analyses of the variant and MLPA

The variant is not listed in the HHT International database, but is present in dbSNP (rs 779974705) and ExAC, that reports a very low frequency (0.0001331), since it was described only in 4 out of the 30058 genomes taking into account by this database and only as heterozygous allele. We analysed the variant *in silico* by using several software, as MutationTaster, FATHMM/mkl and Ensembl/VEP, in order to evaluate the hypothetical pathogenic role of this base change. MutationTaster classified the variant as a polymorphism able to change a splice site. According to FATHMM/mkl, the variant had a damaging effect. Ensembl/VEP made the deepest analysis. It differentiated between the two mature transcripts derived by the alternative splicing showing that the variant is in 3'UTR of the longest mRNA (encoding S-ENG) while it is in the intron of the shortest mRNA (encoding L-ENG). In both cases it described the impact of the variant as modifier, but underlined that prediction were complicated by the localization of the VUS within a non-coding region. We asked to other family members to approve the analysis of their DNA in order to verify the co-segregation of the variant and the disease. At the moment, we are still collecting these samples but, until now, all the analysed patients with clinical symptoms carry the variant (Figure 16).

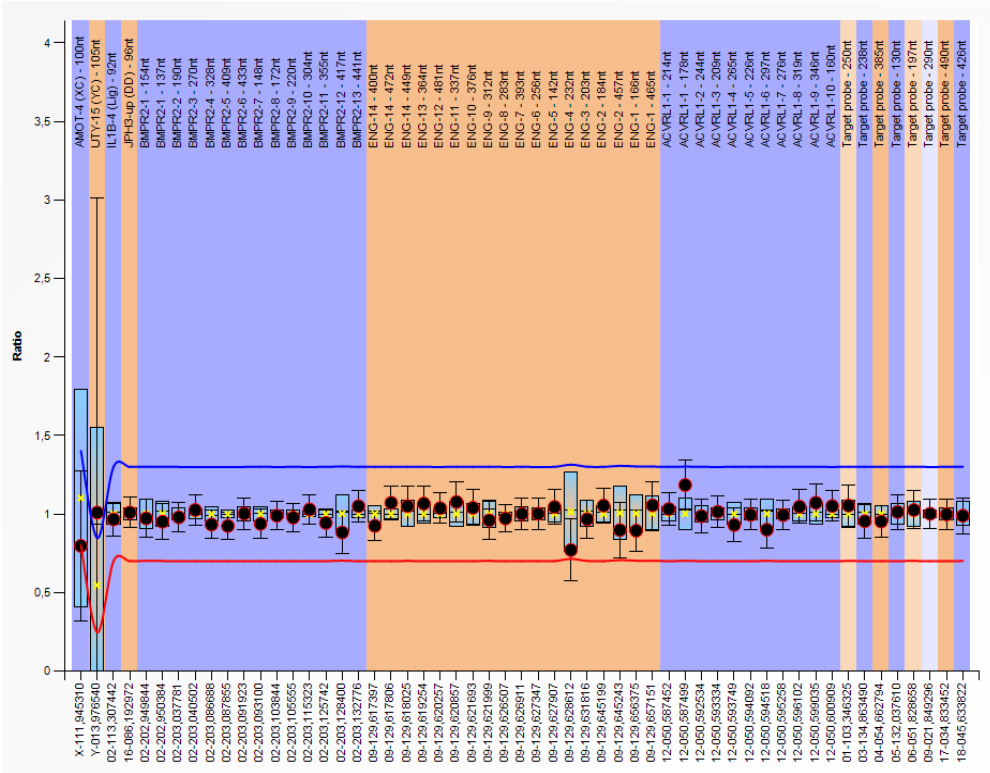


Figure 14. MLPA results: calculated probe ratios. The blue and the red lines define the upper and the lower significant limit, respectively. Probes that exceed these limits are duplicated (above the blue line) or deleted (under the red line). The name of the probes are indicated in the upper part of the graph and their corresponding chromosomal location is in the bottom part.

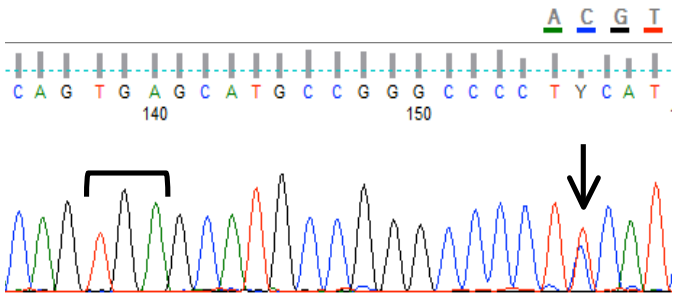


Figure 15. A portion of the electropherogram of the exon 14 of *ENG*. The bracket indicates the stop codon of the short isoform, while the arrow indicates the nucleotide C>T change.

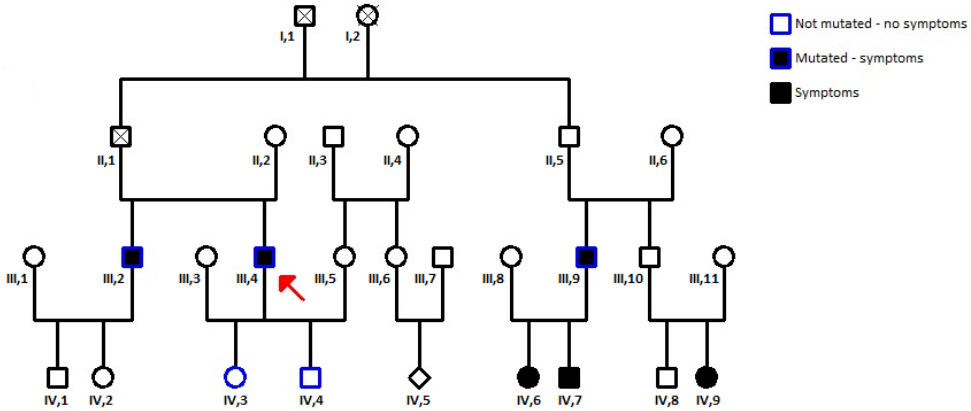


Figure 16. Genealogical tree of the proband (red arrow) family.

Taking these data into account, we considered the variant as a VUS, and we studied it in collaboration with Prof. C. Bernabeu (Centro de Investigaciones Biologicas, CSIC, Madrid).

4.2.2. Protein expression

Protein expression experiments were performed using the plasmid pcEXV-ENG (Figure 17.a). Mutated and wild type constructs were transfected in HEK293T cells and western blotting was used to evaluate ENG expression. We observed that ENG was less expressed by the mutated samples, with a mean reduction of more than 40% when compared to the control (*p-value* = 0.0001; SD = ± 0.035) (Figure 17.b-c). The experiments were repeated with the plasmid pDisplay-MinigEND (Figure 17.d). Results obtained were in agreement with the previous ones. In fact, the construct carrying the variant was expressed less than the wild type: we observed a mean difference of more than 25% among the two types of sample (*p-value* = 0.02; SD = ±0.07) (Figure 17.e-f).

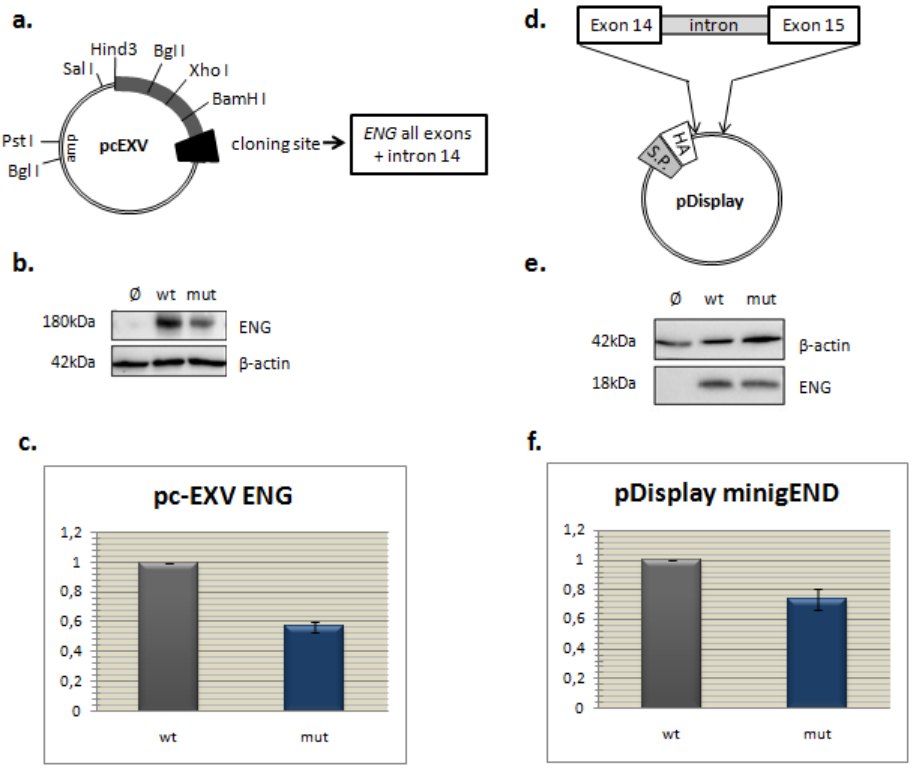


Figure 17. VUS affected *ENG* expression in vitro. (a) The pcEXV construct, inside whom all *ENG* exons and the intron 14 were cloned. **(b-c)** The construct was site-directed mutated, to introduce the C>T change to study, and then transfected in HEK293T cells. The empty vector (Ø) was used as control of the transfection procedure. The comparison between the wt and mut Endoglin showed a reduction of about 40% in the expression of the protein carrying the mutation (*p value* = 0.0001). **(d)** The pDisplay minigEND construct was used to repeat the experiment and to confirmed data obtained with the other construct. This construct contains only a region of *ENG*, from exon 14 to exon 15. **(e-f)** Results of the transfection experiment, showing that mut Endoglin is less expressed (about 25%) than the wt one (*p value* = 0.02). Also in this case, the empty vector was used as control.

Since the VUS lies in the intron that could be retained (leading to the biosynthesis of S-ENG) we evaluated if the alternative splicing process was affected, causing the misregulation of the protein isoforms. To test our hypothesis, HEK293T cells were co-transfected with pDisplay construct, wild type or mutated, and with either ASF/SF2 or SC35 expression vector. SC35 was used as a control, because it is a splicing factor which does not contribute to the alternative splicing of Endoglin.

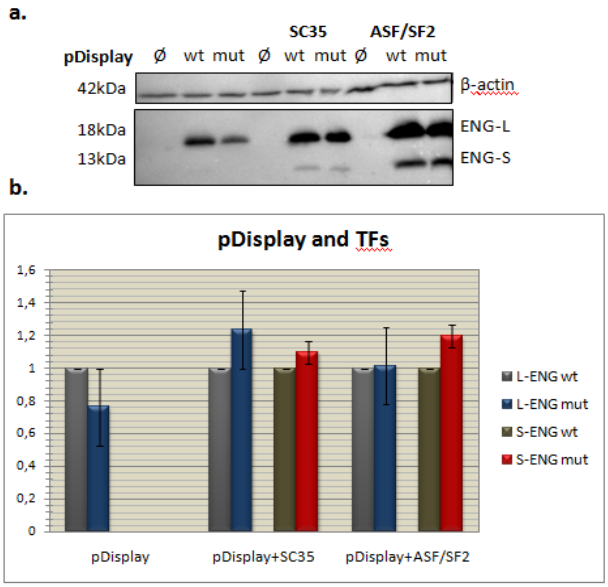


Figure 18. The VUS and the alternative-splicing of Endoglin.(a-b) HEK293T cells were co-transfected with pDisplay and either SC35 or ASF/SF2 expression vector. The co-transfection with ASF/SF2 led to the expression of both L-ENG and S-ENG, but there was any difference comparing the wt and the mut proteins.

Obtained results showed a significant increase of S-ENG when ASF/SF2 was over-expressed, in contrast with the null effect of SC35 over-expression, according with literature data (Blanco and Bernabeu 2011). However, there were no differences between the expression of the wild type and the mutated isoforms of the protein (L-ENG *p-value* = 0.96 and SD = ±0.24; S-ENG *p-value* = 0.20 and SD = ±0.07) (Figure 18.a-b).

4.2.3. Sp1 and ENG expression

To confirm data obtained by western blotting, *ENG* expression was evaluated by qRT-PCR as summarized in Figure 19.a. We observed that *ENG* was transcribed

65% less in the patient compared to the control, with a SD of ±0.20. Even for *ACVRL1*we observed a 25% decrease in the mRNA transcription of the patient compared to the control, with a SD of ±0.47.

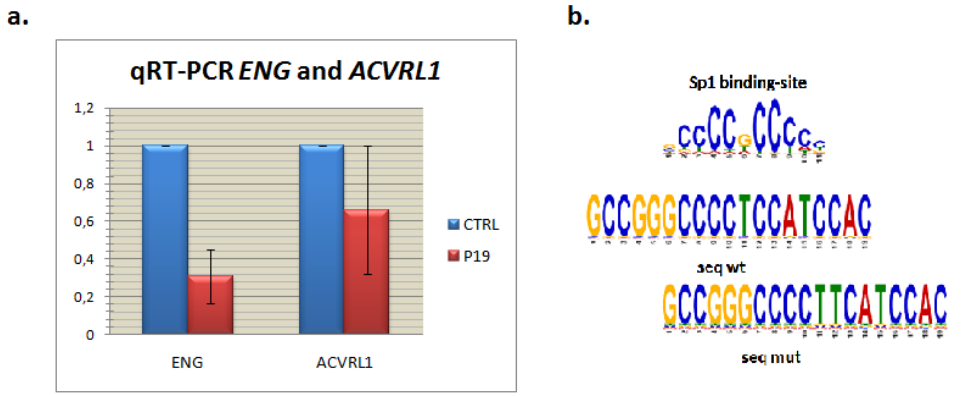


Figure 19. (a) qRT-PCR results. The qRT-PCR was made on the total RNA extracted from a peripheral blood sample of the patient and of a healthy control. Both *ENG* and *ACVRL1* are expressed less in the patient, with a higher reduction concerning *ENG* (about 65%). **(b) The output of the Tomtom Motif Comparison Tool.** The first line is the Sp1 binding-site, and the second and the third lines are respectively the wild type and the mutated *ENG* region. The wt sequence fits well with the binding-site; while the VUS is not included within the Sp1 predicted binding-site, because the nucleotide change causes the slippage behind of the binding-site.

To verify this hypothesis we compared wild type and mutated sequences with *in silico* prediction tools (TomTom4.11.2; Patch1.0 and AliBaba2.1) which aim to identify putative transcription factor binding-site. Each prediction tool identified several differences between the two sequences and, noteworthy, all the software predicted that the C>T change leads to the alteration or to the completely loss of a putative Sp1 binding-site (Figure 19.b). Even if binding-sites for many other transcription factors were predicted, we decided to focus on Sp1 because its involvement in the regulation of *ENG* expression has been already demonstrated (Botella et al. 2001).

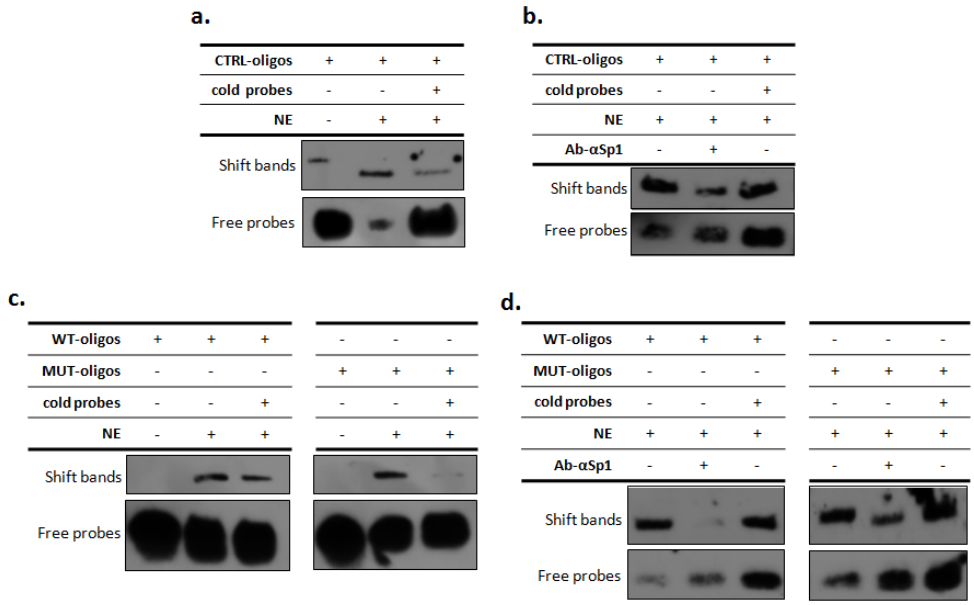


Figure 20. EMSA. (a) Nuclear extract derived from AG1522 cells was incubated with the biotin-labelled CTRL probes. Sample without the NE was used as control for contaminations. The shifted band revealed that the oligonucleotides bind to a protein within the NE. The binding is inhibited by the 200X cold probes. **(b)** Supershift was performed adding to the reaction mix 4µg of Ab-αSp1. It was observed a reduction in the intensity of the shift band, proving that the shift is due to the Sp1-binding. **(c)** The experiment was repeated with the WT and MUT probes. Both oligonucleotides bound to Sp1. **(d)** The supershift showed that the WT probes bound to Sp1 with higher affinity.

We tested the wild-type and the mutated sequences for their ability to bind Sp1 by using the EMSA. We first used the oligonucleotide 5' – GCAGGCGGCCTGGCC CAGCCCCTTCTC – 3' (CTRL), as internal control. This sequence is present in the *ENG* promoter and contains a known Sp1 binding-site (Botella et al., 2001). A shift band appeared when the CTRL probes were incubated with the Nuclear Extract of AG1522 cells (Figure 20.a). To demonstrate that the oligonucleotides shifted due to the binding to Sp1, it was performed a supershift, by adding in the reaction mix the antibody anti-Sp1. We observed a reduction in the intensity of the shift band, confirming that Sp1 bound to the CTRL oligonucleotide (Figure 20.b).
The same experiments were repeated with the wild type (WT) and mutated (MUT) oligonucleotides, 5' – GCATGCCGGGCCCCT**C**CATCCAAA – 3' and 5' – GCA TGCCGGGCCCCT**T**CATCCAAA – 3', respectively (in red the nucleotide changed). Both oligonucleotides shifted, revealing that they bound to Sp1 but with different affinity, as shown by the supershift. In the WT sample the shifted band almost disappeared in the presence of the Ab-αSp1, while the supershift of the

MUT probes was smaller. In fact, we observed a little reduction in the intensity of the shifted band of the sample incubated with the antibody (Figure 20.c-d).

4.3. miRNAs profiling in HHT patients

miRNAs profiling was performed in collaboration with Prof. M. Denti (RNA Biology and Biotechnology Laboratory at CIBIO, University of Trento). We included in the experiment 15 samples, consisting of 5 HHT1 patients, 5 HHT2 patients and 5 controls.
The genetic characteristics, age and sex of patients included in miRNAs analyses are summarized in Table 10.

Group	*ID*	*Age(yrs)*	*Sex*	*Gene*	*Mutation*
HHT1	DAU422	46	F	*ENG*	c.277 C>T (p.R93X)
	FA	65	M	*ENG*	c.392 delC (p.P131RfsX32)
	RR	43	F	*ENG*	c.816+5 G>C (Splice Site)
	ZV	25	F	*ENG*	c.1429-18_1429-1 del18bps (Splice Site)
	RES5	55	M	*ENG*	c.1135 delC (p.H379IfsX1)
HHT2	IS	55	M	*ACVRL1*	c.1135 G>A (p.E379K)
	GzLu	59	F	*ACVRL1*	c.235 G>A (p.G79R)
	ZA	53	F	*ACVRL1*	c.924 C>A (p.C308X)
	BuF1	53	F	*ACVRL1*	c.1121 G>A (p.R374Q)
	PRI1	61	F	*ACVRL1*	c.595 G>C (p.A199P)
CTRL	CTRL1	68	M	---	---
	CTRL2	56	F	---	---
	CTRL3	56	F	---	---
	CTRL4	45	F	---	---
	CTRL5	53	M	---	---

Table 10. Genetics data of samples included in the study of miRNAs profiling.

Samples were sex and age matched with controls. The HHT1 group included three females and two males, with mean age of 46.8 ±14.9 years; the HHT2 group consisted of four females and one male, with mean age of 56.2 ±3.6 years and the control group included three females and two males, with mean age of 55.6 ±8.3 years.
Three out of the ten mutations are not listed in the HHT International database (*ENG* c.1429-18_1429-1 del18bps (Splice Site); *ENG* c.1135 delC (p.H379IfsX1) and *ACVRL1* c.595 G>C (p.A199P)). For these last mutations, pathogenicity was demonstrated using MutationTaster, Polyphen and co-segregation inside the family.

Human miRNome panels from EXIQON include 752 miRNAs, among which there are specific miRNAs useful to evaluate haemolysis of samples. Figure 21 shows absence of an irregular red blood cells contamination in the analysed samples.

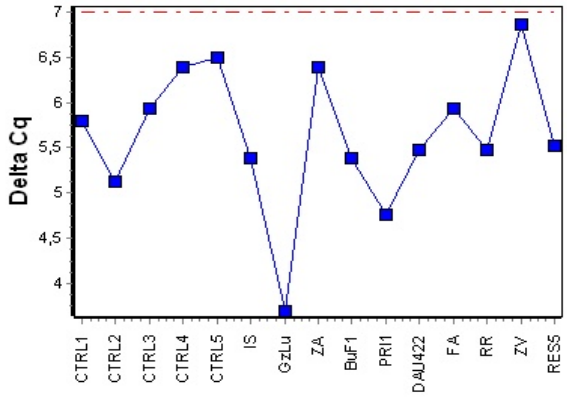
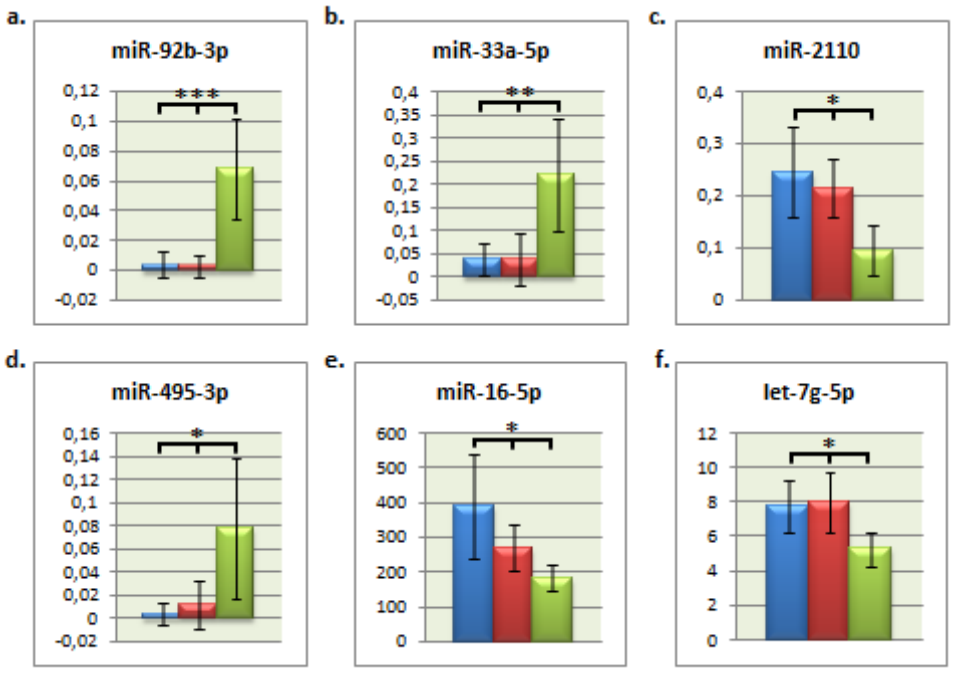


Figure 21. Haemolysis plot. Red line in the upper part of the graph indicates the threshold limit value. Larger values are associated to red blood cells contamination. In the y-axis, delta Cq values, calculated comparing the level of miR-451a, highly expressed in red blood cells, with miR-23a-3p, which is unaffected by the haemolysis (ΔCq = Cq miR-23a-3p – Cq miR-451a) (Blondal et al. 2013).

Raw data obtained by qRT-PCR revealed that 502 miRNAs were detected in at least one sample and that 111 miRNAs were expressed in all samples. We cleaned raw data excluding 252 miRNAs expressed in less than four samples or at very low concentration. The remaining 250 were analysed in order to identify the miRNAs differentially expressed in the three groups.
Statistical analyses were initially performed using one way ANOVA test in order to compare the three groups concurrently. The ANOVA found 15 miRNAs with significant *p-value*, which are listed in Table 11. Considering mRQ (mean Relative Quantities) values of the three groups it was possible to identify miRNAs equally misregulated in both groups of patients: miR-2110; miR-16-5p; miR-19b-3p; miR-20a-5p. On the contrary, the remaining miRNAs were differently expressed by HHT1 and HHT2 patients, as showed by the histograms (Figure 22).

	CTRL		HHT1		HHT2		
miRNA	***mRQ***	***dev.st***	***mRQ***	***dev.st***	***mRQ***	***dev.st.***	***p-value***
miR-92b-3p	0.003889	0.008	0.003140	0.007	0.068695	0.033	0.000307
miR-33a-5p	0.040068	0.035	0.040590	0.056	0.220168	0.121	0.005308
miR-2110	0.246431	0.085	0.215250	0.056	0.096265	0.048	0.008327
miR-495-3p	0.004523	0.010	0.012699	0.021	0.077763	0.060	0.017672
miR-16-5p	391.354168	149.169	272.572312	66.758	184.920602	38.860	0.018132
let-7g-5p	7.802560	1.506	8.013154	1.783	5.303657	0.975	0.022133
let-7i-5p	3.338723	1.392	3.881737	1.048	1.908586	0.275	0.026278
miR-223-3p	47.266019	12.796	32.834831	8.277	53.330037	10.461	0.028348
miR-26a-5p	0.618238	0.183	0.629693	0.207	1.181972	0.050	0.030368
miR-20a-5p	18.027262	2.528	15.167952	2.374	12.706041	3.297	0.032069
miR-98-5p	0.125167	0.109	0.223767	0.083	0.077937	0.021	0.039937
miR-491-5p	0.015301	0.015	0.125375	0.128	0.004553	0.007	0.046123
miR-502-5p	0.030093	0.017	0.252130	0.236	0.048237	0.037	0.047696
miR-214-3p	0.021463	0.032	0.029921	0.029	0.090779	0.059	0.048191
miR-19b-3p	29.266463	9.639	24.290276	2.512	17.828864	5.265	0.049976

Table 11. ANOVA results. mRQ indicates the mean relative quantities of each samples' groups.



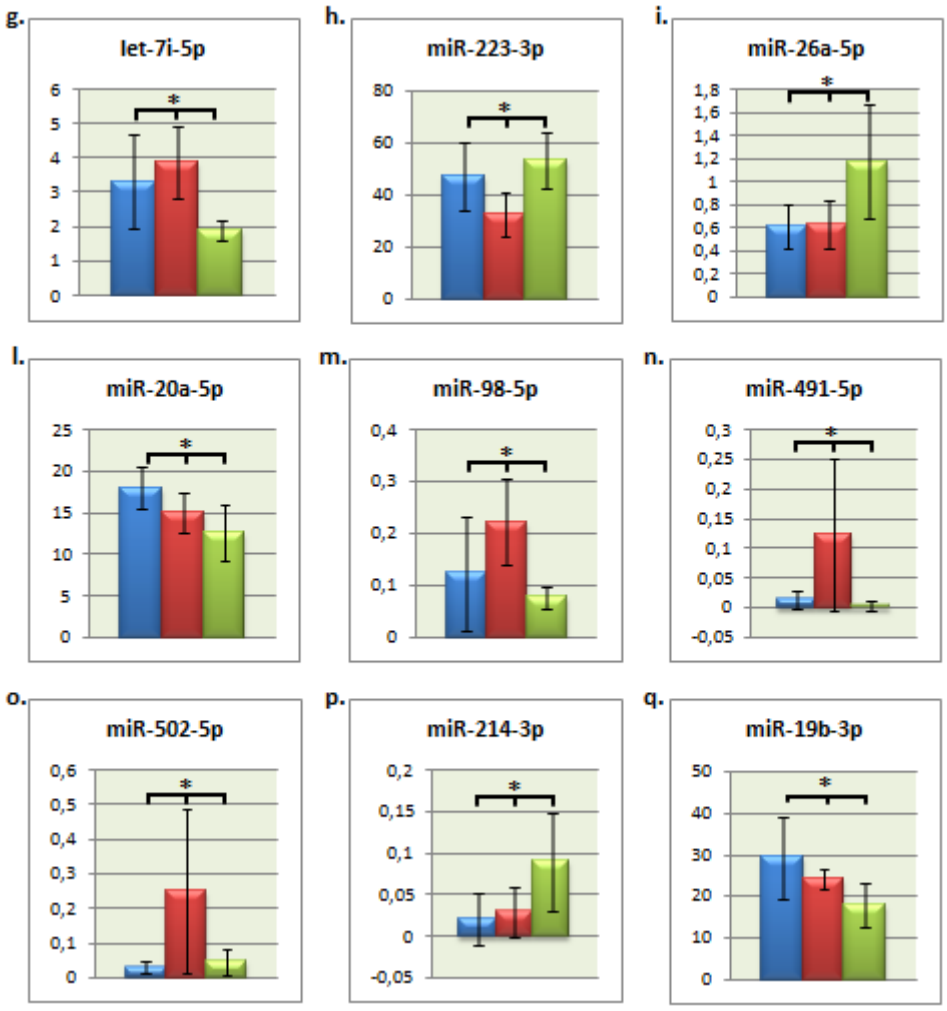


Figure 22. Histograms showing misregulated miRNAs identified by ANOVA. Blue bars indicate the CTRL group; red bars indicate the HHT1 group and the green bar indicate the HHT2 group. The star (*) indicates *p-value* ≤ 0.05; the double star (**) indicates *p-value* ≤ 0.005 and the triple star (***) indicates *p-value* ≤ 0.0005.

By Student's t-test, the samples' groups were compared two by two. The analysis performed combines the *p-value* with the value of the $\log_2$(fold-change), arbitrary considered as significant for values ≥+1 or ≤-1, which identify those miRNAs expressed at least twice or half, respectively, in patients when compared to controls.

Firstly, we compared the control group with the patients group, including both HHT1 and HHT2 samples. We observed 62 misregulated miRNAs: 48 were up-regulated while 14 were down-regulated in patients. The t-test identified 12 miRNAs with a *p-value* ≤ 0.05, even if only 8 of them had a significant $\log_2$(fold-change), as shown in the volcano plot (Figure 23). These miRNAs are listed in Table 12 and all of them were up-regulated in the patients. Noteworthy, some miRNAs were almost exclusively observed in the HHT group, as miR-1537-3p, miR-181b-5p, miR-92b-3p and miR495-3p, which were found only in one out of five controls (Figure 24). The 4 miRNAs that did not exceed the fold-change threshold were miR-20a-5p (*p-value*: 0.020); miR-29c-3p (*p-value*: 0.035); miR-93-5p (*p-value*: 0.040) and miR-148b-3p (*p-value*: 0.044).

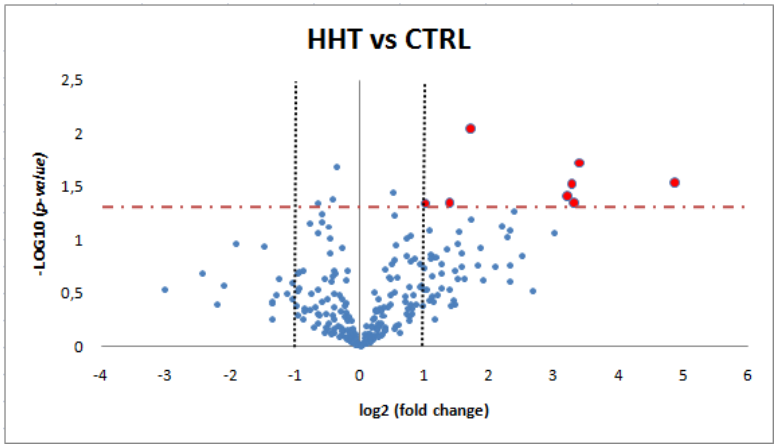


Figure 23. Volcano plot. This graph shows the similarities and dissimilarities among HHT and CTRL groups. The horizontal red line indicates the threshold for the *p-value*, while the vertical black lines indicate the threshold for the fold change. miRNAs with significant *p-value* and fold change are red and localize on the upper right panel. miRNAs among the two black lines and above the red line have a significant *p-value* but their expression level is similar in patients and controls. miRNAs on the lower right and left panels are, at least, twice or half expressed in patients compared to controls but without statistical significance.

HHT vs CTRL						
miRNA	***mRQ CTRL***	***St. dev***	***mRQ HHT***	***St. dev***	***log_2(fold change)***	***p-value***
miR-1537-3p	0.001709	0.003	0.050108	0.059	4.873452	0.029357
let-7g-3p	0.005261	0.007	0.055313	0.055	3.394217	0.018703
miR-495-3p	0.004523	0.010	0.045231	0.054	3.322086	0.045735
miR-181b-5p	0.009956	0.022	0.095657	0.103	3.264300	0.030298
miR-92b-3p	0.003889	0.008	0.035917	0.041	3.207269	0.039825
miR-144-5p	0.073326	0.047	0.241011	0.156	1.716710	0.009139
miR-100-5p	0.067461	0.006	0.175172	0.119	1.376657	0.045787
miR-30c-5p	0.410452	0.245	0.823114	0.474	1.003877	0.045115

Table 12. Misregulated miRNAs in HHT patients. mRQ CTRL and mRQ HHT indicate the mean relative quantities of miRNAs of controls and patients, respectively. To calculate the log_2(fold change) the ratio mRQ CTRL/mRQ HHT has been used. *p-value* was calculated by Student's t-test.

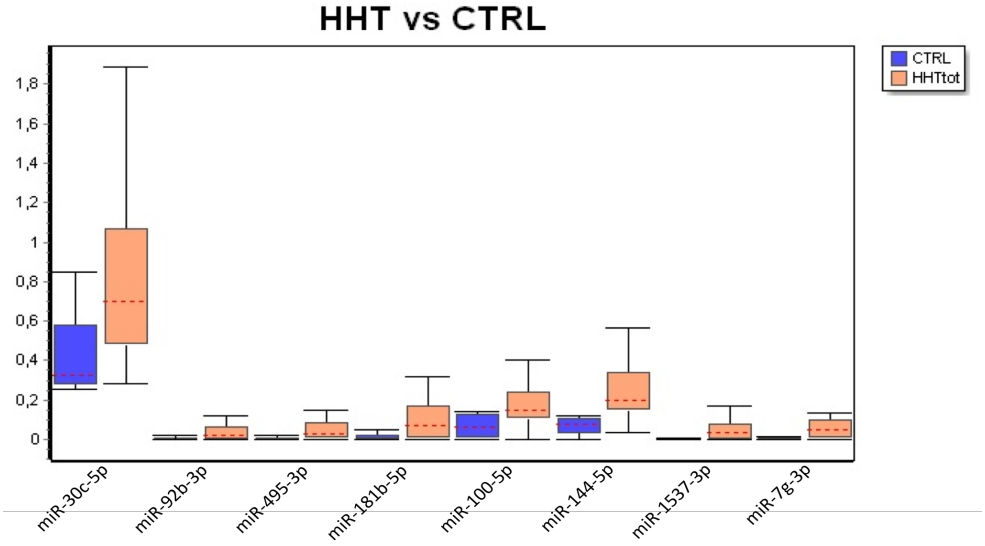


Figure 24. Bar plot. miRNAs relative quantities are indicated in the y-axis. The red dashed lines indicate the median.

Then, we compared the control group with either HHT1 or HHT2 group to evaluate the presence of miRNAs/genotype correlations (Table 13).
The comparison between HHT1 patients and controls revealed the presence of 71 misregulated miRNAs, among which 30 were down-regulated in patients, while the remaining 41 were up-regulated. However, only 3 out of the 71 identified miRNAs had a significant *p-value* and only one of them, miR-100-5p, exceeded the fold-change threshold; in fact, it was detected as significantly up-regulated also in the

previous analysis, including all HHT patients (Figures 25 and 27). The other two miRNAs under the fold-change threshold were miR-23a-3p and miR-24-3p (*p-value*: 0.034 and 0.004, respectively).

The HHT2 group differentially expressed 71 miRNAs; 23 of them were down-regulated and 48 of them were up-regulated in patients. Student's t-test analysis identified 14 miRNAs with *p-value* $\leq$ 0.05 and 7 had a significant $\log_2$(fold-change) value. In particular, miR-92b-3p, miR-33a-5p, miR-186-5p and miR-144-5p were up-regulated and miR-2110, miR-16-5p and miR-1224-5p were down-regulated in patients compared to controls (Figures 26 and 28). The other 7 significant miRNAs were: miR-20a-5p (*p-value*: 0.006); miR-25-3p (*p-value*: 0.017); miR-148a-3p (*p-value*: 0.022); miR-363-3p (*p-value*: 0.025); miR-93-5p (*p-value*: 0.027); let-7b-5p (*p-value*: 0.046) and let-7g-5p (*p-value*: 0.049).
Noteworthy, we observed that miR-92b-3p was expressed only by HHT2 patients; in fact, it was detected in all HHT2 patients, while only one out of the five controls and HHT1 samples expressed this miRNA. Moreover, miR-92b-3p and miR-144-5p were already described as up-regulated in the group including all the HHT patients.

HHT1 vs CTRL						
miRNA	***mRQ CTRL***	***St. dev***	***mRQ HHT1***	***St. dev***	***log_2(fold change)***	***p-value***
miR-100-5p	0.067461	0.006	0.145293	0.080	1.603869	0.019793

HHT2 vs CTRL						
miRNA	***mRQ CTRL***	***St. dev***	***mRQ HHT2***	***St. dev***	***log_2(fold change)***	***p-value***
miR-92b-3p	0.003889	0.008	0.068695	0.033	4.142793	0.010513
miR-2110	0.246431	0.085	0.096265	0.048	-1.356096	0.013186
miR-33a-5p	0.040068	0.035	0.220168	0.121	2.458088	0.027029
miR-16-5p	391.3542	149.169	184.9206	38.860	-1.081568	0.034194
miR-186-5p	0.073326	0.030	0.130789	0.062	1.494618	0.036727
miR-1224-3p	0.046414	0.052	0.020181	0.015	-2.126645	0.041306
miR-144-5p	0.088129	0.047	0.231371	0.129	1.657819	0.049767

Table 13. Misregulated miRNAs in HHT1 and HHT2 patients.

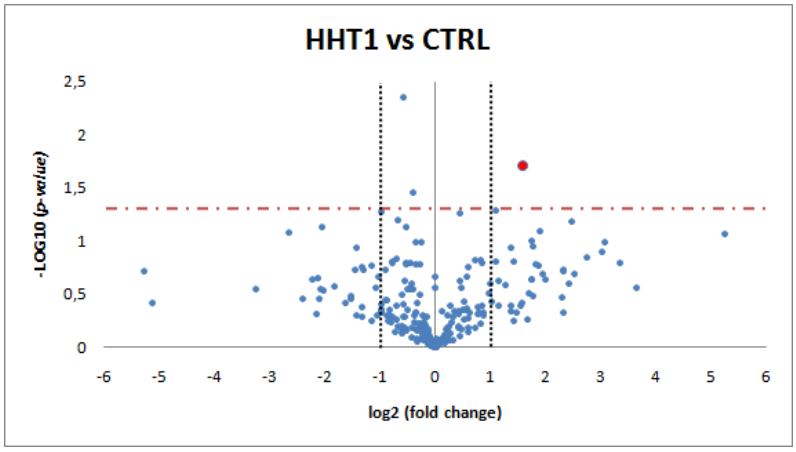


Figure 25. Volcano plot. HHT1 patients were very similar to the control group. Only the miRNA miR-100-5p, red spot, has a significant *p-value* and exceeds the $\log_2$(fold change) threshold. The other two miRNAs with *p-value* ≤ 0.05 were miR-23a-3p and miR-24-3p, but they localized in the middle panel of the graph because of the fold change.

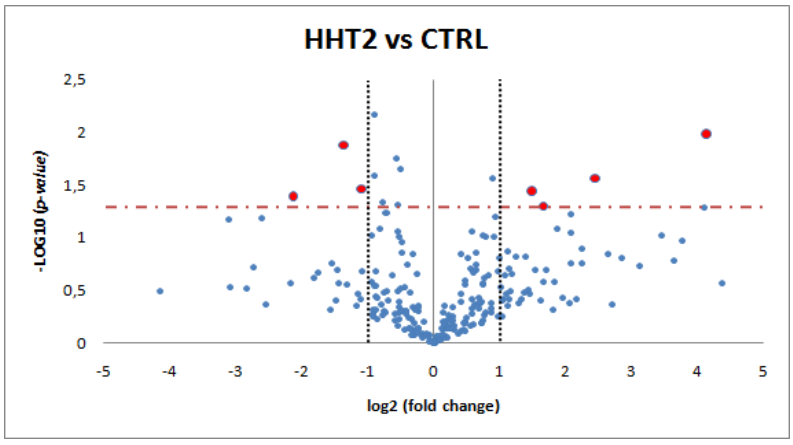


Figure 26. Volcano plot. The comparison between HHT2 and CTRL groups highlighted the misregulation of several miRNAs. In particular we observed in the upper right panel the over-expressed miRNAs (miR-92b-3p; miR-33a-5p; miR-186-5p and miR-144-5p), while in the upper left panel the down-regulated miRNAs (miR-2110; miR-16-5p and miR-1224-3p). The middle upper panel includes those miRNAs with significant *p-value* and under the threshold of the fold change (miR-20a-5p; miR-25-3p; miR-148a-3p; miR-363-3p; miR-93-5p; let-7b-5p and let-7g-5p).

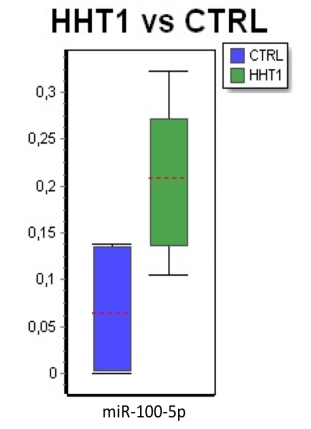


Figure 27. Bar plot. miR-100-5p relative quantities are indicated in the y-axis.. The red dashed lines indicate the median.

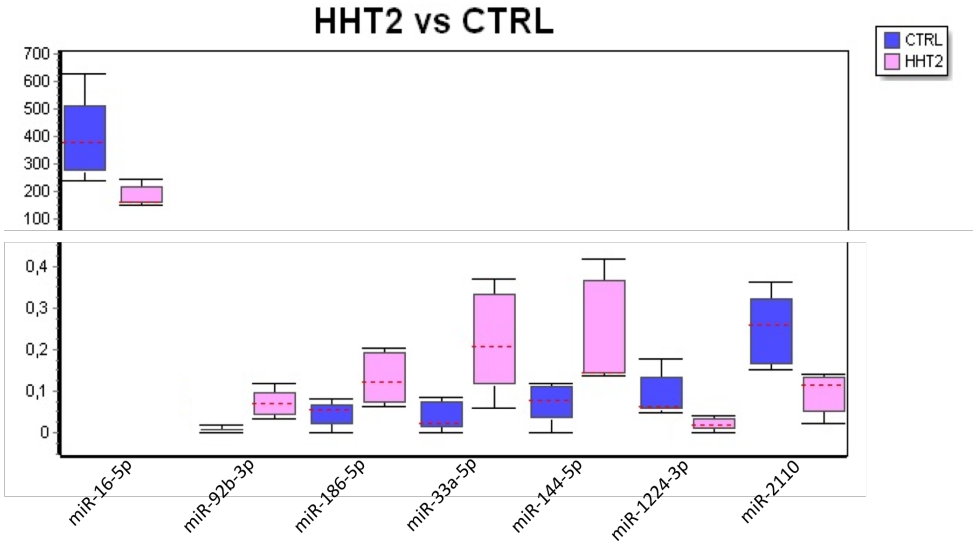


Figure 28. Bar plot. miRNAs relative quantities are indicated in the y-axis. The red dashed lines indicate the median. We observed 4 up-regulated miRNAs and 3 down-regulated miRNAs. In particular we detected a over-expression of miR-92b-3p which was not expressed at all in the control samples.

To evaluate the presence of miRNAs differentially expressed according to patients genotype, HHT1 and HHT2 groups were directly compared. We identified 78 miRNAs: 29 were down-regulated in HHT2 patients, while the other 49 were down-regulated in HHT1 patients. The statistical analysis recognized 13 miRNAs with a *p-value* ≤ 0.05 and Table 14 contains the 7 ones which fitted for the fold-change exclusion criterion. In particular, miR-2110 and let-7i-5p were down-regulated in HHT2 patients, miR-92b-3p, miR-33a-5p, miR-200c-3p and miR-133b were up-regulated in HHT2 patients and miR-98-5p was up-regulated in HHT1 patients. We observed that miR-92b-3p and miR-200c-3p were detected only in the HHT2 group. The other 6 miRNAs showing only a significant *p-value* were: miR-233-3p (*p-value*: 0.009); let-7g-5p (*p-value*: 0.023); miR-33b-5p (*p-value*: 0.033); miR-28-3p (*p-value*: 0.038); miR-16-5p (*p-value*: 0.041) and miR-19b-3p (*p-value*: 0.049) (Figures 29 and 30).

HHT2 vs HHT1						
miRNA	***mRQ HHT2***	***St. dev***	***mRQ HHT1***	***St. dev***	***log_2(fold change)***	***p-value***
miR-2110	0.096265	0.048	0.215250	0.056	-1.160927	0.007407
miR-92b-3p	0.068695	0.033	0.003139	0.007	4.451509	0.010593
let-7i-5p	1.908586	0.275	3.881737	1.048	-1.024197	0.011730
miR-98-5p	0.077937	0.021	0.223767	0.083	-1.521620	0.015814
miR-33a-5p	0.220168	0.121	0.040589	0.056	2.439428	0.026115
miR-200c-3p	0.066882	0.045	0.001392	0.003	5.586390	0.033214
miR-133b	0.185002	0.108	0.042760	0.058	2.113187	0.040927

Table 14. Comparison between HHT1 and HHT2 patients. The two groups differ in the expression of seven miRNAs. miRNAs with negative log_2(fold change) value are over-expressed in HHT1 patients, while miRNAs with positive values characterize the HHT2 group.

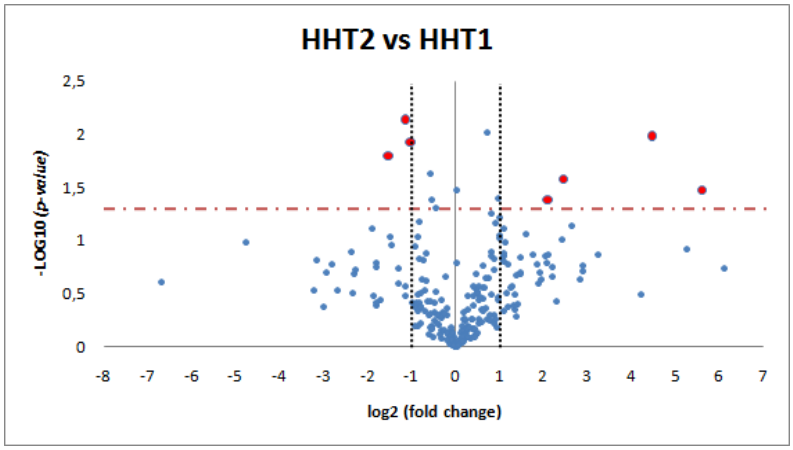


Figure 29. Volcano plot. Red dots indicate miRNAs differentially expressed in the groups analysed. In particular, on the upper left panel there are miRNAs over-expressed in HHT1 patients, while in the upper right panel there are the ones over-expressed by HHT2 patients.

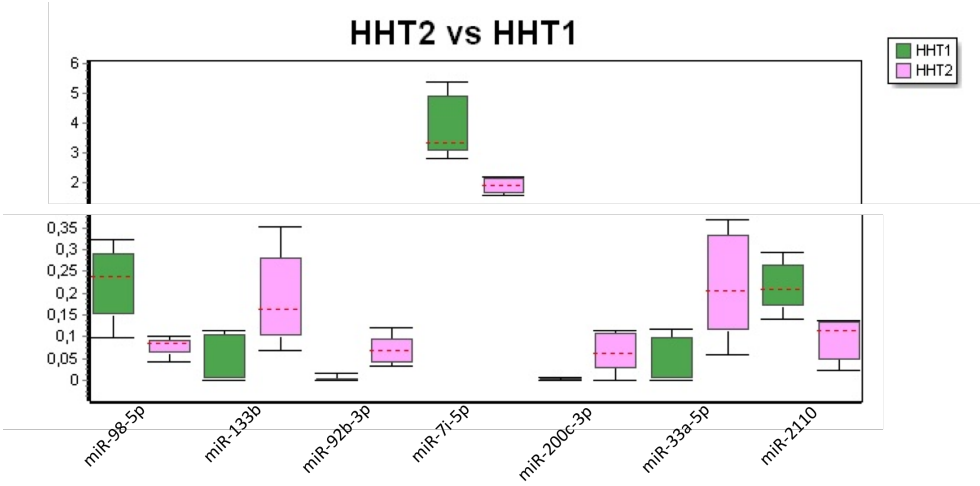


Figure 30. Bar plot. The y-axis indicates values of miRNAs relative quantities and red dashed lines indicate the median. miR-2110, let-7i-5p and miR-98-5p better characterize the HHT1 group, while the HHT2 group over-expressed miR-92b-3p, miR-33a-5p, miR-200c-3p and miR-133b. The most significant difference concerns miR-92b-3p and miR-200c-3p, which were not expressed in HHT1 group.

Each patient included in this study has at least three "Curaçao criteria", i.e. positive family history, epistaxes and telangiectases. Moreover, seven patients have lesions in internal organs, in particular 5 individuals have a PAVM, one has a HAVM, one has GI telangiectases and one has PAH (Table 15). Patients were divided into two groups depending on the presence or the absence of PAVMs, called PAVM and NO-P, respectively. Thus, we evaluated correlations among the deregulated miRNAs and this clinical symptom.

Genotype	*ID*	*Family*	*Epi.*	*Tel.*	*PAVM*	*HAVM*	*Other*
HHT1	DAU422	X	X	X	---	---	---
	FA	X	X	X	X	---	---
	RR	X	X	X	X	---	---
	ZV	X	X	X	X	---	---
	RES5	X	X	X	X	---	---
HHT2	IS	X	X	X	---	---	---
	GzLu	X	X	X	---	---	---
	ZA	X	X	X	---	---	---
	BuF1	X	X	X	X	---	GI bleeding
	PRI1	X	X	X	---	X	PAH

Table 15. Patients' clinical symptoms. Family refers to the presence of the disease in other members of the family; Epi. Refers to epistaxes; Tel. Refers to telangiectases.

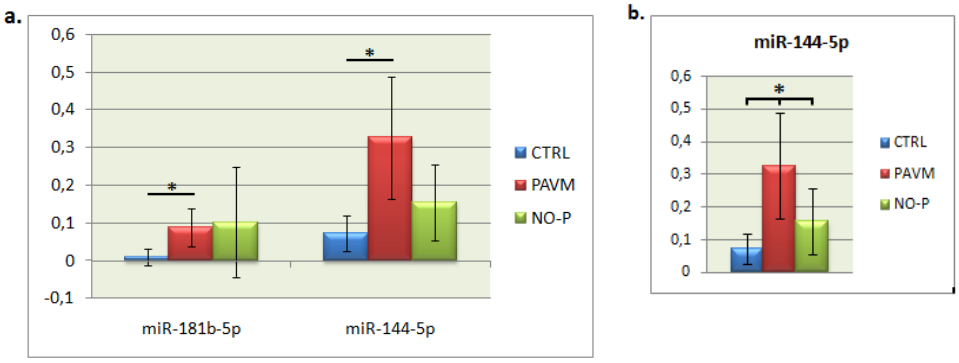


Figure 31. (a) Student's t-test results. miR-181b-5p and miR-144-5p were up-regulated only in PAVM group compared to CTRL group (*p-values*: 0.02). **(b) One way ANOVA results.** miR-144-5p is up-regulated in PAVM group compared to CTRL and NO-P groups concurrently (*p-value*: 0.01).

The comparison between PAVM and CTRL groups showed that miR-181b-5p and miR-144-5p were significantly up-regulated in patients with lung involvement (*p-values*: 0.02). No significant differences were found between PAVM and NO-P groups (miR-181b-5p *p-value*: 0.9; miR-144-5p *p-value*: 0.09). However, miR-144-5p up-regulation was observed comparing PAVM group with CTRL+NO-P group (*p-value*: 0.04) and comparing CTRL, PAVM and NO-P groups concurrently by ANOVA (*p-value*: 0.01) (Figure 31).

Then, we decided to compare our results to literature. We evaluated if the miRNAs already published as misregulated in HHT (miR-210-3p, up-regulated in HHT patients with PAVM; miR-205-5p, down-regulated in patients versus controls) were differently expressed in our samples. The expression of miR-210-3p and miR-205-5p was analysed in samples groups obtained according to either clinical or genetic data, respectively. We observed that miR-210-3p was down-regulated in the PAVM group, while both CTRL, NO-P and CTRL+NO-P groups were very similar (Figure 32.a). Data concerning the miR-205-5p were uniform, as both HHT1 and HHT2 patients had lower miRNA concentrations compared to controls (Figure 32.b). However in our study neither of the two miRNAs reached statistical significance (Table 16 and Figure 33).

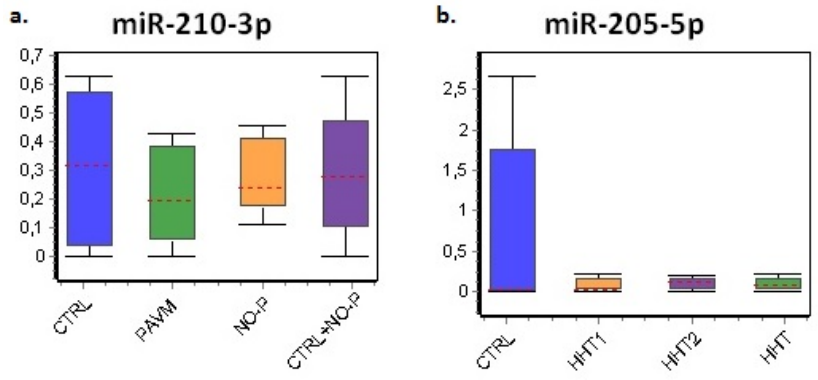


Figure 32. Bar plot of miR-210-3p and miR-205-5p expression. The y-axis indicates miRNAs relative quantities and red dashed lines indicate the median. miR-210-3p was down-regulated in PAVM patients and miR-205-5p was down-regulated in HHT patients, both HHT1 and HHT2, compared to controls.

miRNA	*mRQ CTRL±SD*	*mRQ PAVM±SD*	*mRQ NO-P±SD*	*mRQ CTRL+NO-P ±SD*
miR-210-3p	0.307972±0.052	0.213736±0.175	0.282059±0.135	0.295015±0.206

miRNA	*mRQ CTRL±SD*	*mRQ HHT±SD*	*mRQ HHT1±SD*	*mRQ HHT2±SD*
miR-205-5p	0.715558±1.152	0.088231±0.089	0.075557±0.102	0.100906±0.084

Table 16. Mean relative quantities of miR-210-3p and miR-205-5p in CTRL and patients' groups.

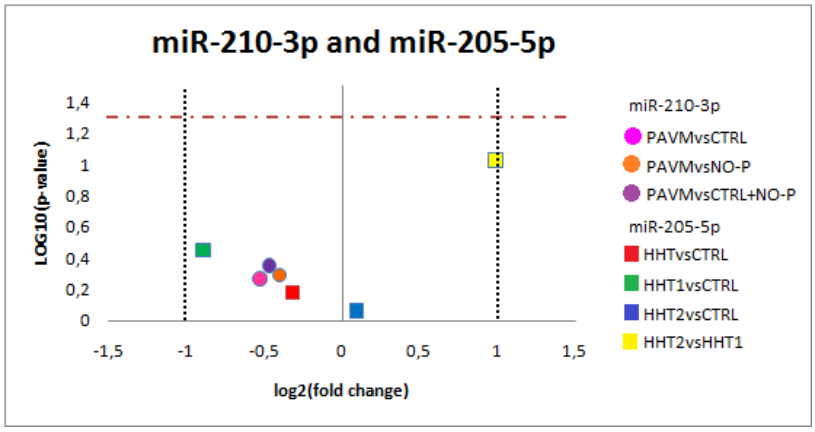


Figure 33. Scatter plot of miR-210-3p and miR-205-5p distribution. Rounded coordinates indicate miR-210-3p and squared ones indicate miR-205-5p. Different colors are used to indicate the $\log_2$(fold change) value obtained comparing two by two samples' groups. Results show that miR-210-3p is lightly down-regulated in PAVM group. miR-205-5p is down-regulated in both HHT1 and HHT2 groups, in fact the $\log_2$(HHT2/HHT1) is almost equal to zero (blue squared coordinate). However, no coordinates exceed neither the *p-value* nor the fold change threshold.

4.3.1. miRNAs enrichment analysis

The enrichment analysis was performed to identify predicted target genes of statistically significant miRNAs, which were grouped in three different lists, as described in Table 12 (HHT versus CTRL), Table 13 (HHT2 versus CTRL) and Table 14 (HHT2 versus HHT1). The enrichment analysis identified the top 20 target genes (Figure 34) for each miRNA, despite miR-1537, which was not related to any putative target. Then, the enriched gene/miRNA data were used to identify the affected pathways, using Reactome database, and results are shown in Figures 35 and 36.

Pathway analysis revealed that deregulated miRNAs, of both HHT versus CTRL and HHT2 versus CTRL lists, affected a limited number of pathways and the most

significant ones are the SMAD2/SMAD3:SMAD4 transcriptional activity and the signalling by NOTCH1 (Figures 35.a-b, 36.a-b). In particular, miRNAs involved in the regulation of these pathways are:

- miR-16-5p which regulates *SMAD3*, *SP1*and *KOR*;
- miR-186-5p which regulates *SP1*, *MYC* and *NCSTN*;
- miR-92b-3p which regulates *SMAD3*, *MYC*, *PTEN* and *ADAM10*;
- miR-33a-5p which regulates *SRC*;
- miR-30c-5p which regulates *TGIF2*, *NCOR2*, *DLL4* and *NOTCH1*;
- miR-181-5p which regulates *CREB1*, *SIRT1* and *PTEN*;
- miR-100-5p which regulates *NCOR2*.

The analysis of HHT2 versus HHT1 miRNAs list put in evidence the involvement of the PI3K/AKT activation pathway (Figures 35c, 36c). Specially, miRNAs involved in this pathway are:

- miR-92b-3p which regulates *PSME3* and *PTEN*;
- let-7i-5p which regulates *AGO1* and *CDKN1A*;
- miR-133b which regulates *AKT1*, *EGFR* and *FGFR1*;
- miR-98-5p which regulates *IRS2* and *CBL*;
- miR-33a-5p which regulates *IRS2*.

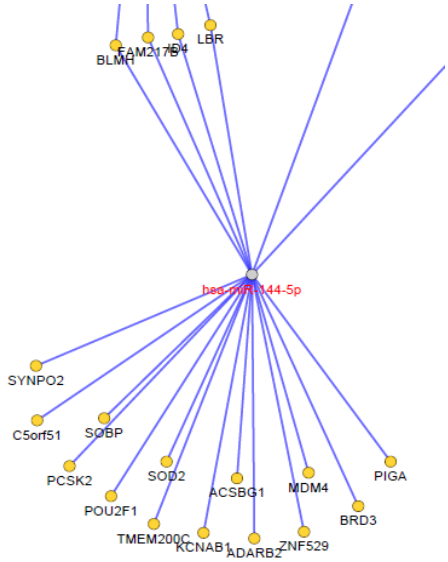
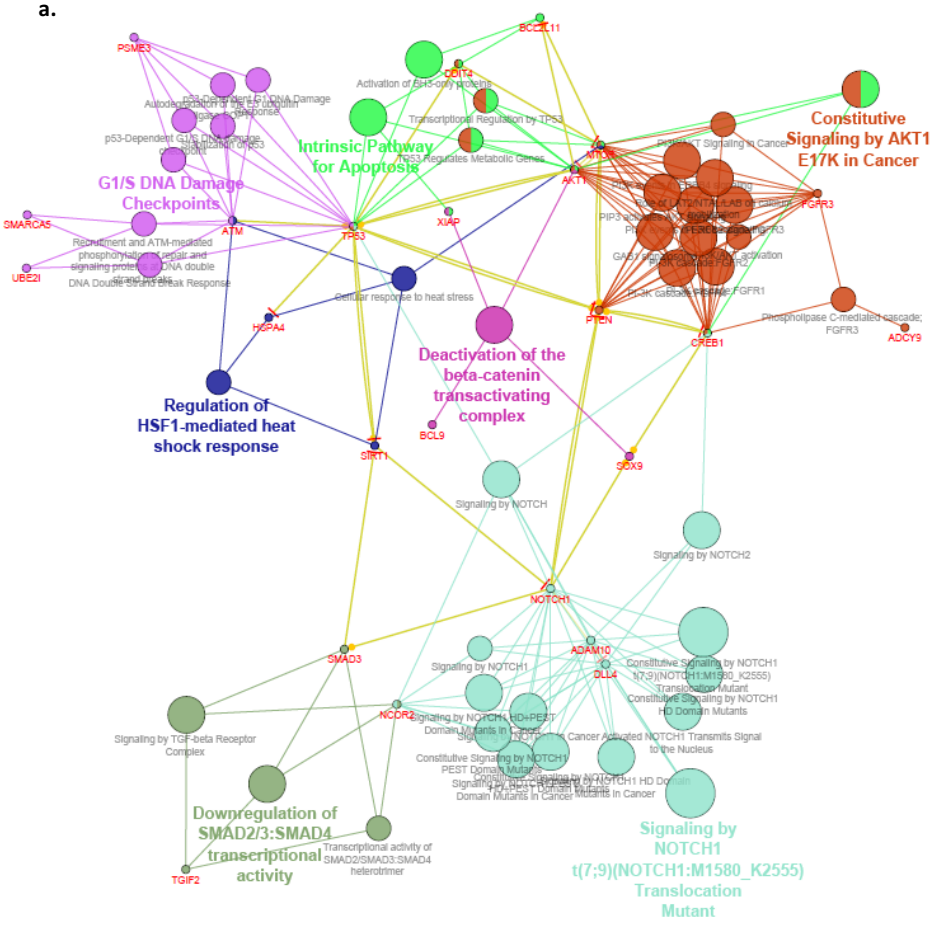
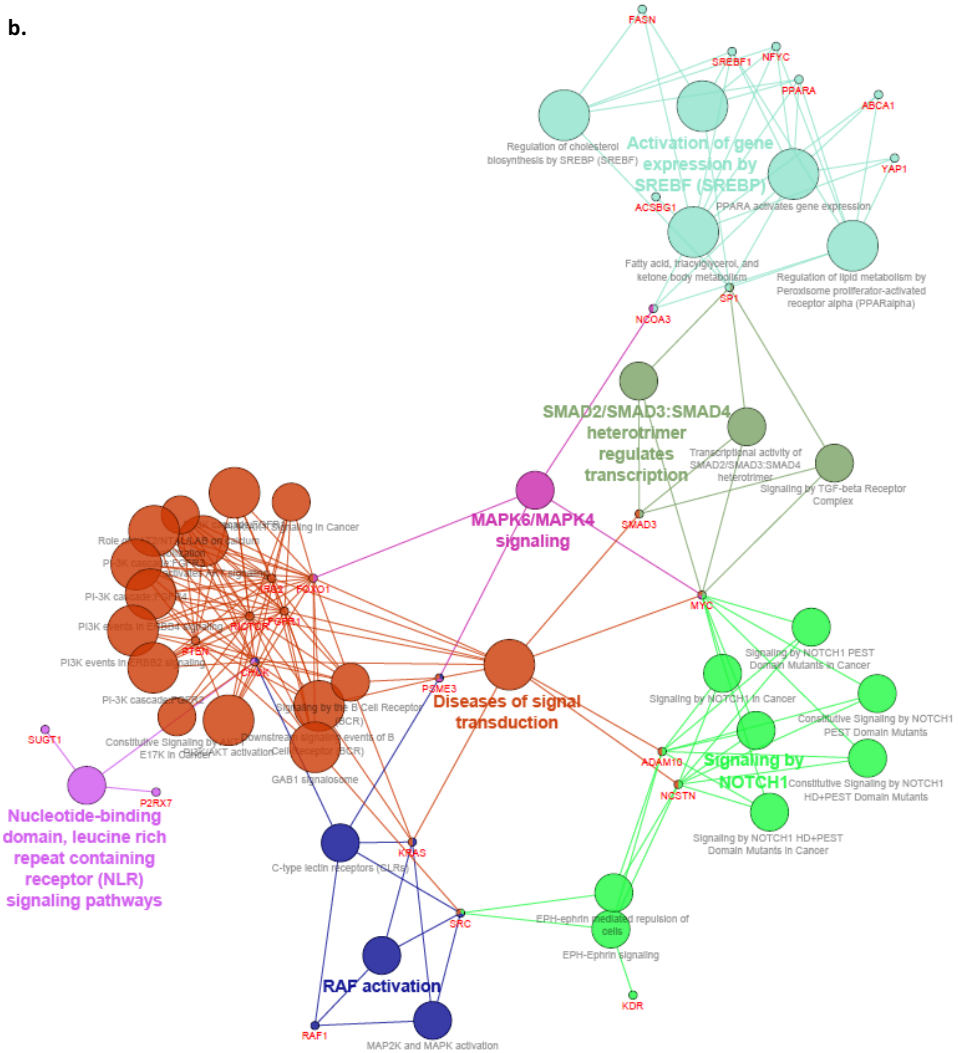


Figure 34. Example of the gene/miRNA enrichment analysis. The figure shows a portion of the gene/miRNA enrichment analysis, focused on 18 out of 20 top targets of miR-144-5p belonging to the HHT2vsCTRL miRNAs list.

a.


Intrinsic Pathway for Apoptosis
Constitutive Signaling by AKT1 E17K in Cancer
G1/S DNA Damage Checkpoints
Deactivation of the beta-catenin transactivating complex
Regulation of HSF1-mediated heat shock response
Downregulation of SMAD2/3:SMAD4 transcriptional activity
Signaling by NOTCH1 t(7;9)(NOTCH1:M1580_K2555) Translocation Mutant
Signaling by NOTCH
Signaling by NOTCH2
Signaling by NOTCH1
Signaling by TGF-beta Receptor Complex
Cellular response to heat stress
Phospholipase C-mediated cascade; FGFR3
PSME3
BCL2L11
CDK2
SMARCA5
ATM
TP53
XIAP
AKT1
FGFR3
UBE2I
HSPA4
PTEN
CREB1
ADCY9
BCL9
SIRT1
SOX9
NOTCH1
SMAD3
ADAM10
DLL4
NCOR2
TGIF2

b.


FASN
SREBF1
NFYC
PPARA
ABCA1
Regulation of cholesterol biosynthesis by SREBP (SREBF)
Activation of gene expression by SREBF (SREBP)
YAP1
ACSBG1
PPARA activates gene expression
Fatty acid, triacylglycerol, and ketone body metabolism
Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)
SP1
NCOA3
SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription
Transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer
Signaling by TGF-beta Receptor Complex
MAPK6/MAPK4 signaling
SMAD3
MYC
Signaling by NOTCH1 PEST Domain Mutants in Cancer
Signaling by NOTCH1 in Cancer
Constitutive Signaling by NOTCH1 PEST Domain Mutants
ADAM10
Signaling by NOTCH1
Constitutive Signaling by NOTCH1 HD+PEST Domain Mutants
NCSTN
Signaling by NOTCH1 HD+PEST Domain Mutants in Cancer
PI3K events in ERBB2 signaling
PTEN
CHUK
PSME3
Diseases of signal transduction
Signaling by the B Cell Receptor (BCR)
Downstream signaling events of B Cell Receptor (BCR)
GAB1 signalosome
SUGT1
P2RX7
Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways
KRAS
C-type lectin receptors (CLRs)
SRC
EPH-ephrin mediated repulsion of cells
EPH-Ephrin signaling
KDR
RAF activation
RAF1
MAP2K and MAPK activation

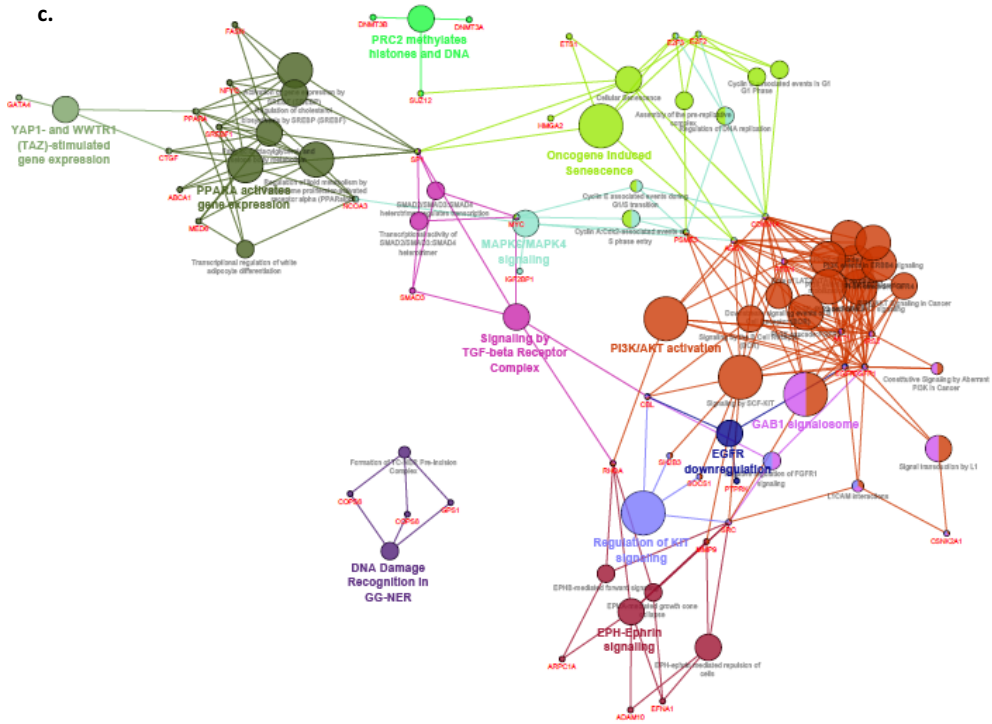
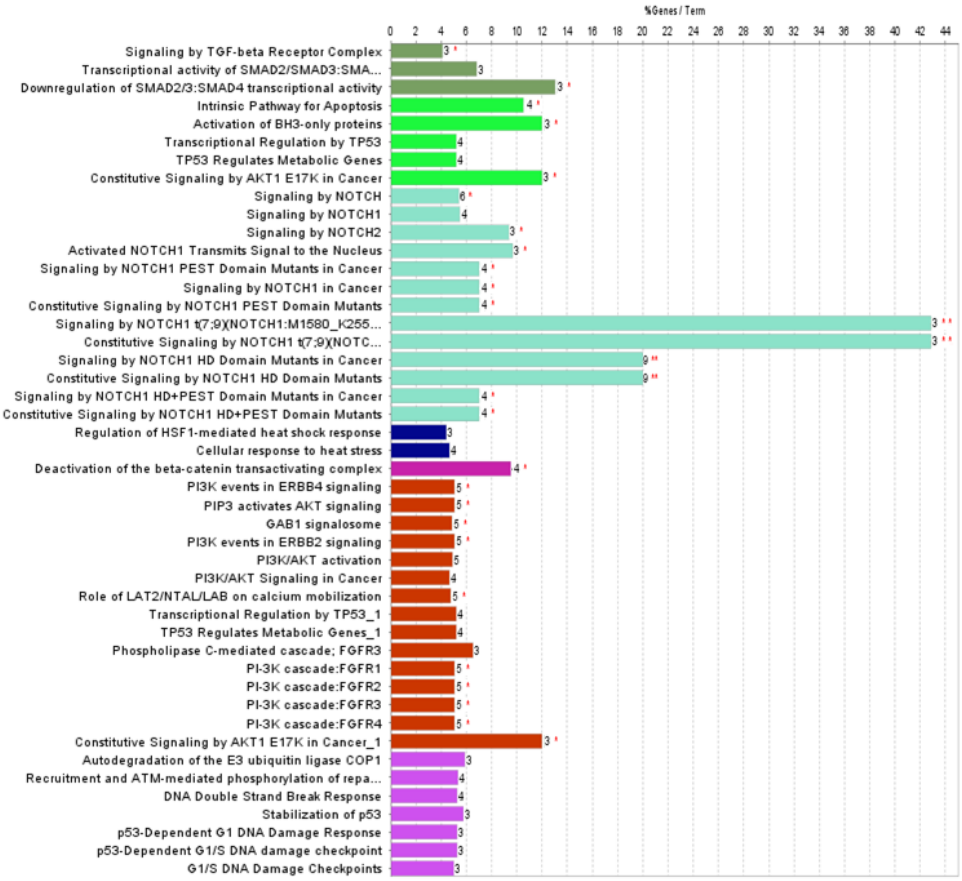
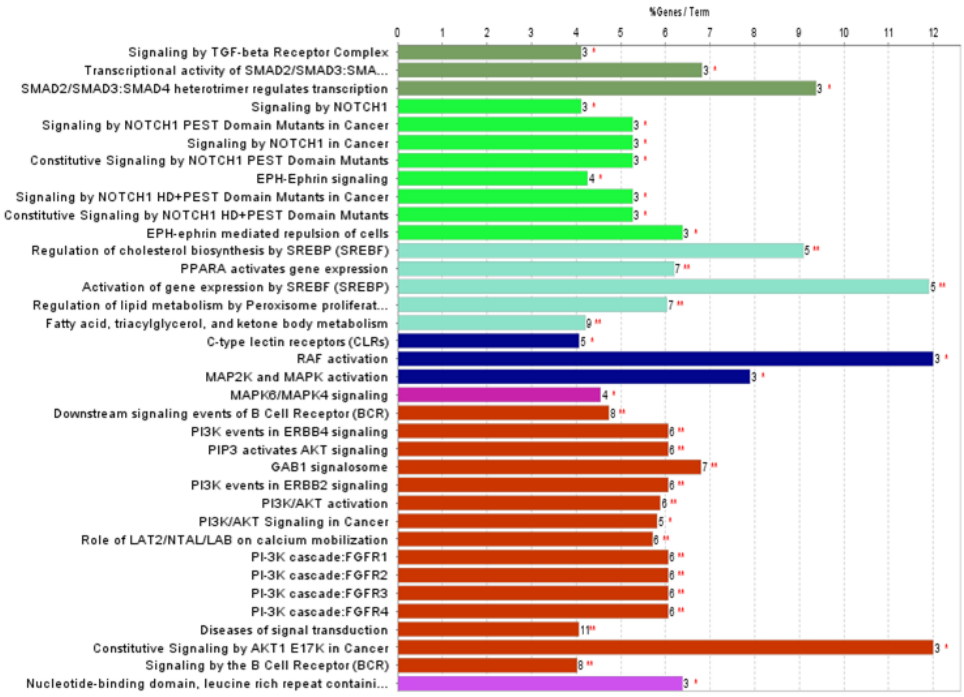


Figure 35. Pathway enrichment analysis of deregulated miRNAs. Panels show the results obtained from the analysis of miRNAs deregulated in **(a)** HHTvsCTRL, **(b)** HHT2vsCTRL and **(c)** HHT2vsHHT1. The target genes of deregulated miRNAs are indicated in red and linked to their corresponding pathways identified by Reactome database and indicated as circles. Circles dimensions reproduce the number of genes within the pathway that were identified as miRNAs target. Distances between circles indicate the correlation level of different pathways.

a.


%Genes / Term
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Signaling by TGF-beta Receptor Complex
Transcriptional activity of SMAD2/SMAD3:SMA...
Downregulation of SMAD2/3:SMAD4 transcriptional activity
Intrinsic Pathway for Apoptosis
Activation of BH3-only proteins
Transcriptional Regulation by TP53
TP53 Regulates Metabolic Genes
Constitutive Signaling by AKT1 E17K in Cancer
Signaling by NOTCH
Signaling by NOTCH1
Signaling by NOTCH2
Activated NOTCH1 Transmits Signal to the Nucleus
Signaling by NOTCH1 PEST Domain Mutants in Cancer
Signaling by NOTCH1 in Cancer
Constitutive Signaling by NOTCH1 PEST Domain Mutants
Signaling by NOTCH1 t(7;9)(NOTCH1:M1580_K255...
Constitutive Signaling by NOTCH1 t(7;9)(NOTC...
Signaling by NOTCH1 HD Domain Mutants in Cancer
Constitutive Signaling by NOTCH1 HD Domain Mutants
Signaling by NOTCH1 HD+PEST Domain Mutants in Cancer
Constitutive Signaling by NOTCH1 HD+PEST Domain Mutants
Regulation of HSF1-mediated heat shock response
Cellular response to heat stress
Deactivation of the beta-catenin transactivating complex
PI3K events in ERBB4 signaling
PIP3 activates AKT signaling
GAB1 signalosome
PI3K events in ERBB2 signaling
PI3K/AKT activation
PI3K/AKT Signaling in Cancer
Role of LAT2/NTAL/LAB on calcium mobilization
Transcriptional Regulation by TP53_1
TP53 Regulates Metabolic Genes_1
Phospholipase C-mediated cascade; FGFR3
PI-3K cascade:FGFR1
PI-3K cascade:FGFR2
PI-3K cascade:FGFR3
PI-3K cascade:FGFR4
Constitutive Signaling by AKT1 E17K in Cancer_1
Autodegradation of the E3 ubiquitin ligase COP1
Recruitment and ATM-mediated phosphorylation of repa...
DNA Double Strand Break Response
Stabilization of p53
p53-Dependent G1 DNA Damage Response
p53-Dependent G1/S DNA damage checkpoint
G1/S DNA Damage Checkpoints

b.


%Genes / Term
0 1 2 3 4 5 6 7 8 9 10 11 12
Signaling by TGF-beta Receptor Complex
Transcriptional activity of SMAD2/SMAD3:SMA...
SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription
Signaling by NOTCH1
Signaling by NOTCH1 PEST Domain Mutants in Cancer
Signaling by NOTCH1 in Cancer
Constitutive Signaling by NOTCH1 PEST Domain Mutants
EPH-Ephrin signaling
Signaling by NOTCH1 HD+PEST Domain Mutants in Cancer
Constitutive Signaling by NOTCH1 HD+PEST Domain Mutants
EPH-ephrin mediated repulsion of cells
Regulation of cholesterol biosynthesis by SREBP (SREBF)
PPARA activates gene expression
Activation of gene expression by SREBF (SREBP)
Regulation of lipid metabolism by Peroxisome proliferat...
Fatty acid, triacylglycerol, and ketone body metabolism
C-type lectin receptors (CLRs)
RAF activation
MAP2K and MAPK activation
MAPK6/MAPK4 signaling
Downstream signaling events of B Cell Receptor (BCR)
PI3K events in ERBB4 signaling
PIP3 activates AKT signaling
GAB1 signalosome
PI3K events in ERBB2 signaling
PI3K/AKT activation
PI3K/AKT Signaling in Cancer
Role of LAT2/NTAL/LAB on calcium mobilization
PI-3K cascade:FGFR1
PI-3K cascade:FGFR2
PI-3K cascade:FGFR3
PI-3K cascade:FGFR4
Diseases of signal transduction
Constitutive Signaling by AKT1 E17K in Cancer
Signaling by the B Cell Receptor (BCR)
Nucleotide-binding domain, leucine rich repeat containi...

c.

Figure 36. List of pathways affected by deregulated miRNAs. Panels show the results obtained from the pathways analysis of miRNAs deregulated in **(a)** HHTvsCTRL, **(b)** HHT2vsCTRL and **(c)** HHT2vsHHT1. All the pathways are listed in the y-axis. The bars indicate the percentage value calculated as the number of genes identified as miRNAs target compared to the total number of genes involved in that pathway. The numbers next to the bars indicate the number of target genes and the red stars indicate the statistical significance (* = *p-value* < 0.05; ** = *p-value*< 0.005). Same colours were used for bars and corresponding circles on figure 35.

5. Discussion

Hereditary Haemorrhagic Telangiectasia (HHT) is a rare genetic syndrome, inherited as an autosomal dominant trait, characterised by abnormal vascular structures, called telangiectases or arteriovenous malformations (AVMs), in which arteries are directly connected to veins leading to the loss of the capillary bed. Concerning telangiectases, HHT patients usually develop these vascular dysplasia at typical sites, as lips, oral cavity, nose, fingertips and gastrointestinal mucosa: on the other hand, liver, lungs and central nervous system are characterized by the presence of AVMs. In fact, their presence is a condition for the clinical diagnosis of the disease, based on the "Curaçao criteria" (Shovlin 2010).

Up to date mutations in four genes have been related to HHT. About the 85% of patients carry a mutation in either *ENG* or *ACVRL1* which cause two different disease subtypes called HHT1 and HHT2, respectively. Since Berg and collaborators described for the first time the HHT genotype-phenotype correlations, researchers and clinicians have focused their efforts on this topic, acquiring a large amount of knowledge. It is known that HHT1 patients show a worse clinical picture compared to HHT2 patients, presenting a higher incidence of PAVMs and CAVMs, while HAVMs and gastrointestinal involvement are observed more frequently in HHT2 phenotype (Berg et al. 2003; McDonald et al. 2015).

HHT has a worldwide incidence of around 1:5-8000, even if several authors reported a higher prevalence of the disease in different regions as a consequence of the presence of regional founder effect. For example, in the French areas of Ain and Deux-Sèvres, the incidence is 1:3345 and 1:4287 respectively, while the Afro-Caribbean population from the Leeward islands of Netherlands Antilles present an incidence of 1:1639 (Bideau et al. 1989; Gallione et al. 2000). However, these data probably underestimate the frequency of the disease, since there is a diagnostic delay partly due to the incomplete penetrance of the symptoms until the 4th decade of patients' life (Pierucci et al. 2012; Shovlin 2010).

Although HHT is clinically diagnosed, the molecular diagnosis plays an important role, specially because of the genotype-phenotype correlations. The early diagnosis of the disease may help in choosing the appropriate follow up for each patient and therefore in preventing complications.

5.1. Genetic features and common mutations in HHT Italian patients

The first part of my PhD project aimed to describe the state of the art of HHT genetics in Italian patients. At the beginning, I contributed to increase the knowledge regarding data already collected over the years by the Medical Genetics laboratory. I performed a molecular screening of *ACVRL1* and *ENG* coding regions in a group of 39 patients, who were clinically assessed as certainly affected by HHT. In fact, as shown in Table 4, all of them manifest at least three out of the four "Curaçao" criteria. By Sanger sequencing and MLPA techniques, the disease-causing mutation was identified in 35 patients (almost 90%), proving the efficiency of the "Curaçao" criteria.

Up to date, 235 different HHT-causing mutations have been identified in 388 families from all Italian regions, with a higher mutability of *ACVRL1*; these data are in agreement with the literature data, published by our group, about the preponderance of the HHT type 2 in the Mediterranean populations (Olivieri et al. 2007). Among these families, five carry a large deletion or duplication, as listed in Table 8. Even if the number of families is small, a higher involvement of *ENG* (4 families versus only one family with an *ACVRL1* mutation) was observed, in agreement with literature data; in fact, the International database lists 8 large deletions on *ACVRL1* compared to 22 large deletions and 5 large duplication on *ENG*. The similarity between the Italian population and the data reported in the International database also involves the distribution of the different types of mutations. In fact, as shown in Figure 13, if we classify mutations according to the effect at the protein level, we observe analogous frequency in Italian patients compared to International data.

We found that mutations are spread on the whole coding sequences of both *ACVRL1* and *ENG*; however, not all *ACVRL1* exons are equally involved in the pathogenesis of the disease: almost half of the families, in fact, carries a mutation in either exons 3, 7 or 8. To investigate the meaning of this uneven distribution, the number of mutations per exon was compared with the number of families and it emerged the presence of recurrent mutations in exons 3, 4 and 8 for *ACVRL1* while recurrent *ENG* mutations were observed in exons 3 and 6. In *ENG* this situation is less pronounced, probably because of the higher percentage of HHT2 patients.

The most likely explanation is that families having the same disease-causing mutation are actually related. To test this hypothesis, we analysed the genealogical tree of these families in order to find a connection branch. We looked for common names but we did not obtain any results, thus, we decided to evaluate the geographical origin, as reported in Figure 11 and Figure 12. Results confirmed our hypothesis only for some mutations, including *ACVRL1* c.152G>A (p.C51Y); c.164_169delTGGTGC (p.L55_V56del); c.200G>A (p.R67Q); c.203dupG (p.G68GfsX101); c.205_209dupTGCGG (p.G70CfsX54) and c.1199C>A

(p.A400D) and *ENG* c.210delG (p.E70DfxX10); c.277C>T (p.R93X) and c.1134 G>A (p.A378A). Very particular is the case of the mutation observed in the Bergamo district: in this case, 16 apparently unrelated families carries the mutation c.289_294 delCACAAC (p.H97_N98del), that has never been described in other part of the world. The regional founder effect associated to this mutation has been described by our laboratory in the past (Olivieri et al. 2007).
On the other hand, our hypothesis has been rejected for other analysed mutations, including *ACVRL1* c.430 C>T (p.R144X); c.1120 C>T (p.R374W); c.1121 G>A (p.R374Q) and c.1231 C>T (p.R411W) and *ENG* c.360+1 G>A (Splice Site); c.771 dupC (p.Y258LfsX76) and c.1465 C>T (p.Q489X), which were found in families hailing from different geographic areas, far from one another.
Existing relations among families partly explains the high number of recurrent mutations observed in few exons. Another possible explanation may concern the role of the different protein domains encoded by these exons. For example, *ACVRL1* exon 3 encodes for the extracellular domain (implicated in interaction with BMP9), exon 4 encodes for part of the extracellular and transmembrane domains and exon 8 codes for the kinase domain localised in the cytosolic tail. Thus, it is possible that recurrent mutations affect residues having an important role in the interaction with BMP9 or other signalling proteins, in the kinase activity, and in the signal transduction process. Indeed, the comparison of the amino acidic sequences of ALK1 and all the other TGF-β type I receptors (ALK2-7) revealed that many of the mentioned mutations affect conserved residues, as C51, G68, R374, A400 and R411 (see Appendix). Moreover, we observed in our population the presence of at least two different mutations affecting some of these amino acids, such as C51Y and C51F, R374W and R374Q, R411W and R411Q. For this reason, we may consider these residues as “hot spots”: this is in agreement with the knowledge about other residues which have already been described as mutational “hot spots” (Abdalla and Letarte 2006). We did not observe the same comparing Endoglin and Betaglycan sequences (see Appendix).
Taking together, all these findings underline that conserved residues, involved in the protein function, often present a high propensity to mutate. In particular, the fact that C51, R374 and R411 are recurrently mutated in unrelated families and may be affected by several mutations proves that they are essential for the correct protein function. Our group previously demonstrates that C51 takes part to the formation of the disulphide bonds that stabilize the structure of the extracellular domain of ALK1. In the same paper it was demonstrated that missense mutations, affecting the *ACVRL1* exon 3, may change the correct molecular conformation of the extracellular domain and/or the protein interactions at the ligand-receptor interface (Scotti et al. 2011).
Nucleotides involved in the disease pathogenesis can also localise in the regulatory domains of the genes. For example, the *ENG* mutation c.-127 C>T, which lies in

the 5'UTR of the gene and affects the initiation of the translation was observed (Damjanovich et al. 2011). Other observed mutations lie in introns, very close to introns' boundaries, and exert their pathogenic role affecting the splicing of the protein.
Mutation analysis is also useful to discover new variants, which can be classified as mutations with a sure pathogenic effect, or polymorphisms and variants of uncertain significance which need further investigation. We encountered all these three different situations analysing Italian patients; as mentioned in the Results chapter we observed 45 and 39 new variants of *ACVRL1* and *ENG*, respectively. Some of them are surely mutations, as *ACVRL1* c.247 G>T (p.E83X) and c.1339 C>T (p.Q477X), and *ENG* c.380 delT (p.F127X), c.808 C>T (p.Q270X) and c.1465 C>T (p.Q489X), which directly or indirectly induce the formation of a premature stop codon. On the contrary, other new variants have been classified as polymorphisms, including *ENG* c.321 G>T (p.L107L), c.322 C>T (p.H108Y) and c.1937+424 G>A. We had no doubt in making this classification for several reasons. In fact, we identified a different disease-causing mutation in patients carrying these variants and, moreover, *in silico* analyses suggested their role as polymorphisms. Finally, we also found the Variants of Uncertain Significance (VUS) c.1852+42 C>T in a patient who does not carry any other mutations, as demonstrated both by Sanger sequencing and MLPA.
The study of VUS is very important because it adds new information about the role of specific nucleotide and/or amino acid residue in the protein function and about the involvement of this variant in the pathogenesis of the disease. We could learn new information about regulation of protein expression, alternative splicing, interactions with other proteins and, in general, about the pathogenic mechanism from a molecular point of view. Thus, we decided to functionally study the variant c.1852+42 C>T and obtained results are discussed in the next paragraph.

5.2. A novel intronic mutation affecting ENG transcription

The patient P19 was diagnosed as affected by HHT in 2014 at the age of 61 years old, after an initial diagnosis of PAH made in 1996 at the age of 43 years old. The diagnostic delay was partly due to the late onset of the typical HHT symptoms and partly to the fact that the overlap between PAH and HHT was unknown at the age of the first diagnosis. This is the reason why the patient underwent a first mutation analysis focused on the *BMPR2* gene and only subsequently *ACVRL1* and *ENG* were analysed. The analyses, both by direct sequencing and MLPA, revealed the absence of known disease-causing mutations and identified only the variant c.1852+42 C>T localised in intron 14 of *ENG*. This variant is not listed in the International database, and results from *in silico* analyses were contrasting. The databases dbSNP and ExAC describe this nucleotide change, reporting a very low

frequency in the population (0.0001331) for the T allele. This result could be the first evidence of the putative pathogenic effect of the variant, considering the threshold of 1% that has been established to discern mutations (frequency <1%) from polymorphisms (frequency >1%) (Brookes 1999). More recently, Karki and collaborators highlight that it is not correct to classify a variant according to its frequency in the general population. In fact, in the era of the next generation sequencing the 1% threshold becomes inappropriate, since it has been described the presence of mutations with higher frequency, rare variants which do not correlate with human diseases and variants with different effect in separated populations (Auer et al. 2012; Karki et al. 2015; Myles et al. 2008).

Then, to evaluate the co-segregation with the disease, we analysed samples from other relatives, both with and without symptoms. The variant was found only in members with clinical symptoms. Unfortunately, data about co-segregation are still incomplete, since we could not collect samples from the cousin (Figure 16, III,10) and from nephews and nieces (Figure 16, IV,1; IV,2; IV,6; IV,7; IV,8 and IV,9). Taking together, these data suggest that the variant may have a pathogenic effect and, thus, it could be the cause of PAH and other HHT symptoms in the patient. The results, obtained by western blotting, sustain this hypothesis; in fact, the site-directed mutagenesis of the two plasmids pcEXV and pDisplay migEND, used in the experiments, led to a statistically significant decrease in the expression level of the protein when compared to the wild-type. The down-regulation of *ENG* was confirmed by qPCR. In this last experiment we also analysed *ACVRL1* expression, because it has been recently demonstrated that both *ACVRL1* and *ENG* are down-regulated in HHT patients irrespective of the gene affected by the disease-causing mutation (Zucco et al. 2014). Obtained results agreed with literature data: as expected *ACVRL1* is down-regulated, although less than *ENG*.

Further investigations were needed to understand the underling mechanism involved in the reduction of the protein expression.

As stated in the Introduction, Endoglin is an auxiliary receptor of the TGF-β/BMPs signalling pathway involved in the regulation of the angiogenesis. It has been proved the presence of two isoforms, called short- and long-Endoglin. ECs predominantly expressed L-ENG and the over-expression of S-ENG induces cell senescence via TGF-β/ALK5 signalling (Blanco et al. 2008). The expression of S-ENG depends on the activity of the splicing factor ASF/SF2, which promotes the alternative splicing of the premature mRNA inducing the retention of intron 14 and thus the early termination of the protein biosynthesis (Blanco and Bernabeu 2011). Since the VUS lies in the intron that could be retained, we hypothesised that it could affect the alternative splicing of the protein, leading to the concurrently expression of a higher quantity of S-ENG and a lower quantity of L-ENG. The high level of S-ENG could consequently lead to the inhibition of endothelial cells proliferation and then could simulate the haploinsufficiency model of HHT

pathogenesis, which has been suggested by several authors (Abdalla and Letarte 2006; Fernandez-L et al. 2006). Results showed that S-ENG expression increases in presence of the splicing factor ASF/SF2 (Figure 18), in agreement with literature data (Blanco and Bernabeu 2011). However, we did not observed a higher expression of the short isoform of the mutated protein compared to the wild type. Obtained results prove that the variant affects the expression of the protein but it does not impair the alternative splicing.

Another possible explanation is that the variant impairs an earlier stage of the gene expression process, as the mRNA transcription. For this purpose, we identified several transcription factors able to bind both the wild type and the mutated sequences through an *in silico* analyses. In some cases, the putative binding sites changed according to the presence of the variant. In particular, all the tools identified a Sp1-binding site affected by the presence of the variant, that we investigated using the EMSA. We decided to further investigate Sp1 because it is known its involvement in the regulation of *ENG* transcription (Botella et al. 2001). The experiment evaluates the shift of the 3'-biotin-labelled double-stranded oligonucleotides, which is due to their binding to a protein from the cellular nuclear extracts. The wild-type and the mutated probes were concurrently tested and we observed that both of them bound to a protein. Then, by the supershift experiments it was demonstrated that Sp1 was the protein involved in the binding, even if with different affinity for the two sequences. In fact, Sp1 bound with higher affinity the wild type sequence, while the mutation reduced the interaction with *ENG* sequence because of the change in Sp1 binding site.

These findings validate our hypothesis about the pathogenic role of the variant c.1852+42 C>T and suggest that the transcription of the gene is impaired. Moreover, the results prove the existence of a Sp1-binding site (5' – GGAGGGGC – 3'), localised in the intron 14 of *ENG*. Probably, this binding-site lies within a regulatory element which plays a role in the transcription of the gene, as an enhancer.

Enhancers are an important class of regulatory element which can promote the expression of target genes thanks to their ability to communicate with other regulatory elements across long distances, up to hundreds of kilobases, as the promoters (Engel et al. 2016). A model proposed to explain the enhancer activity is the "DNA looping" that hypothesised the presence of a molecular bridge consisting of macromolecular complexes that join the enhancer to the gene promoter. Several authors have been validated this model visualizing the DNA loop by scanning transmission electron microscopy, even concerning the activity of Sp1 (Mastrangelo et al. 1991; Su et al. 1991).

The transcription factor Sp1 was generally described as a promoter-specific binding protein, involved in the regulation of many biological processes, as cell growth, differentiation, carcinogenesis and apoptosis; however, it can also bind to

enhancers, distal from the promoter. Sp1 recognises and binds GC-rich sequences (consensus sequence: 5' – GGGCGG(G/A)(G/A)(C/T) – 3') through three zinc fingers located at its C-terminal tail (Beishline and Azizkhan-Clifford 2015; Vizcaíno et al. 2015). Thanks to the multidimerization domain, localised in the C-terminal tail, Sp1 molecules can self-associate forming tetramers, which in turn interact leading to the formation of large multimers. These multimers are the molecular bridge which connects enhancer to promoter binding-site forming a DNA loop (Mastrangelo et al. 1991; Su et al. 1991).
We could speculate that a similar mechanism likely concerns the regulation of *ENG* transcription; therefore, Sp1 polymerises and produces the molecular bridge which joins the known promoter site and the putative distal enhancer, in order to promote the maximal transcription of the gene. The presence of the variant c.1852+42 C>T reduces the Sp1 binding-affinity to the enhancer leading to a down-regulation of the gene and thus to a reduced biosynthesis of protein. The wild-type allele ensures the biosynthesis of functional protein, but not enough to compensate the deficiency caused by the mutated allele. Thus, the haploinsufficiency occurs, leading to the onset of the disease. This hypothesis is supported by literature, as a similar Sp1-dependent mechanism has been demonstrated for the maximal transcription level of the human topoisomerase-IIα (Williams et al. 2007).

5.3. Misregulation of circulating miRNAs identifies new mechanisms involved in HHT-dependent impairment of angiogenesis

Circulating miRNAs were described for the first time in 2008 in a group of patients affected by large B-cell lymphoma (Lawrie et al. 2008) and, since their discovery, they have been largely investigated. However, their role is still not completely understood and researchers sustain two main school of thoughts. Many authors have demonstrated that circulating miRNAs are specifically released by cells as signalling molecules having functional effect on acceptor cells (Kosaka et al. 2010; Mittelbrunn et al. 2011; Skog et al. 2008). On the contrary, another research group proved that miRNAs are released from cells during apoptosis, necrosis or basal secretion activity without any particular function (Turchinovich et al. 2011; Turchinovich and Burwinkel 2012). The fact remains that in both theories circulating miRNAs, at least some of them, may have a role in cell-cell communication both at short- and long-range (Turchinovich et al. 2016).
To the best of our knowledge, until now only two papers have been published about circulating miRNAs in HHT. Thus, we decided to contribute to the study of this topic, analysing the miRNAs signature in a cohort of 15 samples (5 HHT1 patients, 5 HHT2 patients and 5 controls). In particular, the choice of the samples number was made taking into account the known information about miRNAs in

HHT. The aim was to analyse the minimum number of samples needed to obtain data with a higher statistically significance if compared with literature.
The data were first analysed by one way ANOVA, in order to compare the three groups simultaneously, and then by Student t-test to better appreciate differences between patients and controls, HHT1 and HHT2 groups, and patients showing particular symptoms and the other individuals. The overall result is that 23 miRNAs were misregulated in HHT patients compared to controls, in particular 15 of them were up-regulated and 8 of them were down-regulated.
Thanks to the first analysis with ANOVA, we identified 15 deregulated miRNAs with a *p-value* < 0.05 and observed a different trend for HHT1 and HHT2 patients, as only four out of 15 miRNA shad a similar misregulation in both groups. On the other hand, the remaining 11 miRNAs were differentially expressed either in HHT1 or in HHT2 group. This situation was further stressed thanks to t-test analyses. In fact, comparing in different ways the patients and the controls, we identified for the first time several miRNAs misregulated only in one out of two patients groups. In particular, we observed a strong association between miR-92b-3p and miR-33a-5p with the HHT2 group, since these two miRNAs were detected only in samples from patients carrying an *ACVRL1* mutation, and between miR-144-5p and the PAVM group. The first conclusion is that HHT2 patients show a more peculiar miRNAs profile. We hypothesise that this condition might be related to the fact that ALK1 function is highly restricted to the angiogenesis process, while ENG activity has been described in other molecular mechanisms, such as macrophage aging (Aristorena et al. 2014), diabetes (Bilir et al. 2016), and osteogenic differentiation (Ishibashi and Inui 2014).
Then, thanks to the enrichment and pathways analyses, we identified the top 20 target genes of those miRNAs misregulated in patients compared to controls (HHTvsCTRL and HHT2vsCTRL miRNAs lists) and in the two different groups of patients (HHT2vsHHT1 miRNAs list). We identified a limited number of affected pathways, related to the regulation of transcription via SMAD2/SMAD3:SMAD4, the NOTCH signalling and the activation of the PI3K/AKT signalling. All these pathways have already been described in association with HHT.
In particular, the SMAD proteins are the intracellular effectors involved in the regulation of the angiogenesis through the BMP/ALK1 signalling cascade (Pardali et al. 2010) and, furthermore, SMAD4 mutations are related to JPHT, the syndrome characterised by the overlap of HHT with Juvenile Polyposis (Gallione et al. 2004). This is congruent with the identification of up-regulated miRNAs, as miR-92b-3p, miR-30c-5p, miR-100-5p and miR-186-5p, which may reduce the biosynthesis of the proteins involved in the SMAD pathway. The main predicted target belonging to this pathway is SMAD3. This protein, together with SMAD2, transmits the ALK5 signalling, promoting the regulation of angiogenesis with an opposite effect when compared to ALK1 (Goumans et al. 2002). miR-16-5p is the only down-

regulated miRNA identified by the enrichment analysis as involved in the regulation of SMADs signalling pathway. This miRNA plays also a role in the regulation of VEGF expression. It was proved that its *in vitro* down-regulation, under hypoxia, leads to the over-expression of VEGF and consequently to the activation of angiogenesis (Hua et al. 2006). Thus, it can be speculated that the up-regulated miRNAs may be involved in the down-regulation of the ALK5/SMAD3 dependent pathway. On the other hand, miR-16-5p down-regulation may be involved in the increased VEGF plasma concentrations that have been observed in HHT patients (Sadick et al. 2005). Both these conditions could lead to a greater impairment of angiogenesis caused by *ACVRL1* or *ENG* mutations.

The second affected pathway is the NOTCH signalling, which is involved in the regulation of several cellular processes, including embryonic and post-natal development, stem cell maintenance, cell differentiation and death (Borggrefe et al. 2016). Its role in the regulation of angiogenesis was demonstrated. Briefly, the angiogenesis starts when tissues release specific growth factor, as the VEGF, leading to a series of molecular processes that involved different types of endothelial cells, including tip and stalk cells. VEGF induces the ECs switch to tip cells that, in turn, switch to stalk cells able to proliferate and generate new vascular tubes. The NOTCH pathway is a regulator of the tip/stalk cells switch, in particular, because of the activity of the ligand DLL4 localised in the membrane of the tip cells. Recently, it has been demonstrated that BMP9/ALK1 and NOTCH signalling pathways cooperate during this switch in order to promote the development of normal vessels. It is possible to speculate about the role of up-regulated miRNAs in the inhibition of the NOTCH signalling members, which may lead to a greater impairment of angiogenesis. Moreover, our hypothesis further underlines the known synergism between ALK1 and NOTCH signalling pathways. In fact, it has been shown that the canonical NOTCH targets *HES1*, *HEY1* and *HEY2* and the ligand JAG1 are over-expressed at the end of the ALK1 signalling cascade (Larrivée et al. 2012). On the other side, NOTCH pathway regulates SMAD1/2/3 expression levels in endothelial cells (Fu et al. 2009).

The enrichment analysis also identified the activation of the PI3K/AKT signalling pathway. This pathway is involved in the regulation of several processes as cell survival, cell cycle progression and cellular growth. Both PI3K and AKT present a kinase activity and their function can be inhibited by the phosphatase protein PTEN, that may prevent cell growth and promote apoptosis exerting a tumour suppressor effect. Studies on PTEN activity correlate the protein loss of function with the constitutively PI3K/AKT signalling activation (Fresno Vara et al. 2004). The PI3K/AKT pathway cooperates with TGF-β/BMP superfamily in the SMAD-independent signalling cascade (Pardali et al. 2010). In particular, the PI3K/AKT signalling may be activated by Endoglin in order to stabilise endothelial capillaries (Lee et al. 2012). In this scenario, we observed the up-regulation of miR-92b-3p

and miR-133b which may target *PTEN* and *AKT1*, respectively. AKT1 is one out of 3 different protein isoforms that ECs predominantly express(Yu et al. 2015).
Thus, the down-regulation of *PTEN* might lead to the permanent activity of PI3K/AKT, even if this result is in contrast with the concurrently down-regulation of *AKT1*. Indeed, Lee and colleagues demonstrated that the suppression of AKT signalling promotes the apoptosis and the capillary regression. A possible explanation might be that *PTEN* down-regulation leads to a worsening of angiogenesis regulation. Cells try to compensate this condition by the down-regulation of *AKT1* and, thus, inducing the regression of capillary structures. Obviously, we aware that PI3K/AKT pathway has been revealed by the enrichment analysis of HHT2vsHHT1 miRNAs list (Figures 35c and 36c). To understand if miRNAs belonging to this list are really misregulated, the comparison with their expression in the CTRL group is needed. For example, miR-133b was described as up-regulated in the HHT2 group (Table 14). However comparing its expression in HHT2 and CTRL samples we did not observe any significant difference, thus we cannot prove the misregulation of miR-133b.
For this reason, we may hypothesise that only *PTEN* is down-regulated, leading to the permanent activation of the PI3K/AKT signalling and thus to an inappropriate growth of capillaries.
We decided not to enrich miR-100-5p and miR-144-5p because they were detected as individual misregulated miRNA in HHT1vsCTRL and PAVMvsCTRL comparisons, respectively. Moreover, these miRNAs were described as significant in other lists; in fact, we have speculated about the miR-100-5p putative role in the inhibition of the SMADs pathway.
miR-114-5p was detected up-regulated in both HHT2 and PAVM groups. This result seems in contrast with the fact that 4 out of 5 PAVM patients belong to the HHT1 group. The analysis of the miRNA relative quantities detected in each patient revealed that only the HHT1 patient without PAVM presents a low miR-144-5p expression similar to controls. Moreover, in all HHT2 patients we detected elevated miR-144-5p relative quantities and the highest value was observed in the single patient with PAVM. The gene/miRNA enrichment analysis identified miR-144-5p top 20 target genes (Figure 34). Among them, only *ACSBG1* was described in correlation to a specific pathway (fatty acid, triacylglycerol and keton metabolism). However, this pathway has not evident implication in HHT pathogenesis. Looking for the function of the 20 targets in GeneCards database (www.genecards.org), the involvement of *ID4* in the TGF-β signalling pathway emerged. Inhibitors of DNA-binding (ID) proteins are helix-loop-helix transcription factors. Recently, it has been proposed the mediator function of ID4 in the BMP-2-induced Endoglin-dependent osteogenic differentiation of periodontal ligament cells (Ishibashi and Inui 2014). These results suggest to

further investigate *ID4* functions in order to evaluate the involvement of this transcription factor in the pathogenesis of HHT.
The final step was the comparison of our results with literature (Table 16 and Figure 33). We did not observe the up-regulation of miR-210-3p in PAVM patients reported by Zhang and colleagues. In fact, miRNA relatives quantities were similar in all groups. On the other side, we observed the down-regulation of miR-205-5p in both HHT1 and HHT2 groups in agreement with data by Tabruyn and colleagues, even if our results did not present any statistical significance. It is probable that these results are due to the differences between our cohort of patients and the ones described in literature.

These preliminary data about circulating miRNAs profile in HHT provide new insights about the molecular mechanisms underlying this disease. Certainly, it is needed to validate obtained results in a larger cohort of patients and to functionally study misregulated miRNAs to confirm or reject our hypothesis.

6. Conclusions and perspectives

The results of my PhD thesis on Hereditary Haemorrhagic Telangiectasia give new insights about two molecular features of this syndrome.
First of all, the importance of mutation analysis is stressed; it remains a fundamental support for a better care of patients and, furthermore, a powerful method to investigate pathogenic mechanisms of the disease. Concerning this last aspect, it was demonstrated that a new intronic variant can be associated with a molecular mechanism, able to cause haploinsufficiency, that has never been described for HHT.
The second feature concerns the role of circulating miRNAs in HHT.I presented the first results of this project, which contributes to increase the knowledge since the analysed cohort of patients is the largest one studied until now. Obtained results suggest new molecular mechanisms underpinning the disease, and new genotype-phenotype correlations. Obviously, the validation of these results is mandatory; with this aim, the Ethical Committee of Fondazione IRCCS, Policlinico "San Matteo", has already approved the second phase of the project on 25 HHT1 and 25 HHT2 patients. Moreover, functional studies will be performed to validate the hypothesis about the involvement of these miRNAs in cellular processes that can be related to HHT.
This study establishes the basis for the identification and use of miRNAs as new potential biomarkers. In particular, the PAVM/HAVM-related miRNAs will help the early diagnosis of the symptoms, reducing their medical complications and thus improving the patients' quality of life.

Appendix

ALKs alignment

```
ALK1 ----------------mtlgs--prkgl-lm--------llmalvtqgdpvkpsrgplvt 33
ALK2 ----------------mvdgv--milpvlim--------ialpspsmedekpkvnpklym
ALK3 mpqlyiyirllgaylfiisrvqgqnldsmlhgtgmksdsdqkksengvtlapedtlpflk
ALK4 ----------------maes--aga-ssffp--------lvvlllagsggsgprgvqall
ALK5 ----------------meaavaapr-prlll--------lvlaaaaaaaaallpgatalq
ALK6 --------------------------mllrsagkl--nvgtkkedgestaptprpkvlr
ALK7 ------------------------------------------------------------

ALK1 ctcesphc--kg-ptc-rgawctvvlvreegrhpqehrgcgnl-------hrelcrgrpt 82
ALK2 cvceglsc--gnedhc-egqqcfsslsindgfhvy-qkgcfqv----yeqgkmtcktpps
ALK3 cycsghcpddainntcitnghcfaiieeddqgettlasgcmk-----yegsdfqckdspk
ALK4 cactscl---qanytcetdgacmvsifnldgmehhv-rtcipkvelvpagkpfyclssed
ALK5 cfchlct---kdnftcvtdglcfvsvtettdkvihn-smciaeidliprdrpfvcapssk
ALK6 ckchhhcpedsvnnicstdgycftmieeddsglpvvtsgclg-----legsdfqcrdtpi
ALK7 --------------------------mltngkeqvi-kscvslpe---lnaqvfchssn-

ALK1 e--fvnhyccd-shlcnhnvslvleatqp-pseqpgtdgq---lal-ilgpvlallalva 134
ALK2 p--gqaveccq-gdwcnrnitaqlptkgk---sfpgtqnfhlevgliilsvvfavcllac
ALK3 aqlrrtieccr-tnlcnqy--lqpt--lppvvigpffdgsirwlvllismavciiamiif
ALK4 ---lrnthccy-tdycnrid-lrvpsghlkepehpsmwgpve-lvgiiagpvfllfliii
ALK5 tgsvtttyccn-qdhcnkie-l-----------------------------------
ALK6 phqrrsieccternecnkd--lhpt--lpplknrdfvdgpihhrallisvtvcslllvl-
ALK7 --nvtkteccf-tdfcnnit-lhlptaspnap----klgpme-laiiitvpvcllsiaam

ALK1 lgvlglwhv-rrrqekqrglhselgesslilkaseqgdsmlgdlldsdcttgsgsglpfl 193
ALK2 llgvalrkfkrrnqerlnprdveygtie-glittnvgdstladlldhsctsgsgsglpfl
ALK3 sscf-cykhycksissrrrynr-dleqdeaf---ipvgeslkdlidqsqssgsgsglpll
ALK4 ivflvinyhqrv-yhnrqrldmedpscemc----lskdktlqdlvydlstsgsgsglplf
ALK5 ------------------------------------------------pttglpll
ALK6 iilf-cyfrykr-qetrprysi-gleqdety---ippgeslrdlieqsqssgsgsglpll
ALK7 ltvwacqgrqcs-yrkkkrpnveeplsecnl---vnagktlkdliydvtasgsgsglpll

ALK1 vqrtvarqvalvecvgkgrygevwrglwhgesvavkifssrdeqswfreteiyntvllrh 253
ALK2 vqrtvarqitllecvgkgrygevwrgswqgenvavkifssrdekswfretelyntvmlrh
ALK3 vqrtiakqiqmvrqvgkgrygevwmgkwrgekvavkvfftteeaswfreteiyqtvlmrh
ALK4 vqrtvartivlqeiigkgrfgevwrgrwrggdvavkifssreerswfreaeiyqtvmlrh
ALK5 vqrtiartivlqesigkgrfgevwrgkwrgeevavkifssreerswfreaeiyqtvmlrh
ALK6 vqrtiakqiqmvkqigkgrygevwmgkwrgekvavkvfftteeaswfreteiyqtvlmrh
ALK7 vqrtiartivlqeivgkgrfgevwhgrwcgedvavkifssrderswfreaeiyqtvmlrh
```

```
ALK1 dnilgfiasdmtsrnsstqlwlithyhehgslydflqrqtlephlalrlavsaacglahl 313
ALK2 enilgfiasdmtsrhsstqlwlithyhemgslydylqlttldtvsclrivlsiasglahl
ALK3 enilgfiaadikgtgswtqlylitdyhengslydflkcatldtrallklaysaacglchl
ALK4 enilgfiaadnkdngtwtqlwlvsdyhehgslfdylnrytvtiegmiklalsaasglahl
ALK5 enilgfiaadnkdngtwtqlwlvsdyhehgslfdylnrytvtvegmiklalstasglahl
ALK6 enilgfiaadikgtgswtqlylitdyhengslydylksttldaksmlklayssvsglchl
ALK7 enilgfiaadnkdngtwtqlwlvseyheqgslydylnrnivtvagmiklalsiasglahl

ALK1 hveifgtqgkpaiahrdfksrnvlvksnlqcciadlglavmhsqgsdyldignnprvgtk 373
ALK2 hieifgtqgkpaiahrdlksknilvkkngqcciadlglavmhsqstnqldvgnnprvgtk
ALK3 hteiygtqgkpaiahrdlksknilikkngscciadlglavkfnsdtnevdvplntrvgtk
ALK4 hmeivgtqgkpgiahrdlksknilvkkngmcaiadlglavrhdavtdtidiapnqrvgtk
ALK5 hmeivgtqgkpaiahrdlksknilvkkngtcciadlglavrhdsatdtidiapnhrvgtk
ALK6 hteifstqgkpaiahrdlksknilvkkngtcciadlglavkfisdtnevdippntrvgtk
ALK7 hmeivgtqgkpaiahrdiksknilvkkcetcaiadlglavkhdsilntidipqnpkvgtk

ALK1 rymapevldeqirtdcfesykwtdiwafglvlweiarrtivngivedyrppfydvvpndp 433
ALK2 rymapevldetiqvdcfdsykrvdiwafglvlwevarrmvsngivedykppfydvvpndp
ALK3 rymapevldeslnknhfqpyimadiysfgliiwemarrcitggiveeyqlpyynmvpsdp
ALK4 rymapevldetinmkhfdsfkcadiyalglvyweiarrcnsggvheeyqlpyydlvpsdp
ALK5 rymapevlddsinmkhfesfkradiyamglvfweiarrcsiggihedyqlpyydlvpsdp
ALK6 rymppevldeslnrnhfqsyimadmysfglilwevarrcvsggiveeyqlpyhdlvpsdp
ALK7 rymapemlddtmnvnifesfkradiysvglvyweiarrcsvggiveeyqlpyydmvpsdp

ALK1 sfedmkkvvcvdqqtptipnrlaadpvlsglaqmmrecwypnpsarltalrikktlqkis 493
ALK2 sfedmrkvvcvdqqrpnipnrwfsdptltslaklmkecwyqnpsarltalrikktltkid
ALK3 syedmrevvcvkrlrpivsnrwnsdeclravlklmsecwahnpasrltalrikktlakmv
ALK4 sieemrkvvcdqklrpnipnwwqsyealrvmgkmmrecwyangaarltalrikktlsqls
ALK5 sveemrkvvceqklrpnipnrwqscealrvmakimrecwyangaarltalrikktlsqls
ALK6 syedmreivcikklrpsfpnrwssdeclrqmgklmtecwahnpasrltalrvkktlakms
ALK7 sieemrkvvcdqkfrpsipnqwqscealrvmgrimrecwyangaarltalrikktisqlc

ALK1 nspekpkviq 503
ALK2 nsldklktdc
ALK3 esqdvki---
ALK4 vqedvki---
ALK5 qqegikm---
ALK6 esqdikl---
ALK7 vkedcka---
```

ENG-BGL alignment

```
ENG mdrgtlplavalllascslsptslaetvhcdlqpvgperg-evtyttsqvskgcvaq-ap 58
BGL mtshyviaif-almsscl-atagpepgalcelspvsashpvqalmesftvlsgcasrgtt

ENG nailevhvlflef----ptgpsq-leltlqaskqngtwprevllvlsvnssvflhlqalg 113
BGL glpqevhvlnlrtagqgpgqlqrevtlhlnpissvhihhksvvfllnsphplvwhlkter

ENG iplhlaynssl-------vtfqeppgvnttelpsfpkt-q-ilewaaer-gpitsaaeln 163
BGL latgvsrlflvsegsvvqfssanfsltaeteernfphgnehllnwarkeygavtsftelk

ENG dpqsillrlgqaqgslsfcmleasqdmgrtlewrprtpalvrgchlegvaghkeahilrv 223
BGL iarniyikvgedqvfppkcnigknflslnyl-aeylqpkaaegcvmssqpqneevhiiel
```

```
ENG lpghsa--gprtvtvkvelscapgdld----avlilqgppyvswlidanh---nmqiwtt 274
BGL itpnsnpysafqvditidirpsqedlevvknlililkckksvnwviksfdvkgslkiiap

ENG geysfkifpeknirg--fklpdtp---qgllgearmlnasivasfvelplasivslhass 329
BGL nsigfgkesersmtmtksirddipstqgnlvkwaldngyspitsytmapvanrfhlrlen

ENG --c-----------------------------------------------------ggr 333
BGL naeemgdeevhtippelrilldpgalpalqnppirggegqngglpfpfpdisrrvwneeg

ENG lqtspapiqtt------------ppkdtcspellmsliqtkcaddamtlvlkkelva-hl 380
BGL edglprpkdpvipsiqlfpglrepeevqgsvdialsv---kcdnekmivavekdsfqa-s

ENG kctitgltfwdpsceaedrgdkfvlrsaysscgmqv----sasmi--sneavvnilsss- 433
BGL gysgmdvtlldptckakmngthfvlesplngcgtrprwsaldgvvyy-nsiviqvpalgd

ENG ----------------------------spqrkk---vhcl-------------nmdsls 449
BGL ssgwpdgyedlesgdngfpgdmdegdaslftrpeivvfncslqqvrnpssfqeqphgnit

ENG fqlglylsphf---lqasntiepgqqsfvqvrvspsvsefllqldschldlgpegg---t 503
BGL fnmelyntdlflvpsqgvfsvpenghvyvevsvtkaeqelgfaiqtcfispysnpdrmsh

ENG veliqgraakgncvslls------pspegdp---rfsfllhfytvp-ipktgtlsctval 553
BGL ytiienicpkdesvkfyspkrvhfpipqadmdkkrfsfvfkpvfnts-ll--flqceltl

ENG rpktg--sqdqe----------------------vhrtvfmrlniis------------- 576
BGL ctkmekhpqklpkcvppdeactsldasiiwammqnkktftkplavihheaeskekgpsmk

ENG -pdls--gctskglvlpavlgitfgafligalltaalwyiysh-trsp-skrepvvavaa 631
BGL epnpisppif-hgldtltvmgiafaafvigalltgalwyiysht-ge-tagrqqv--pts

ENG passessstnhsigstqstpcstssma 658
BGL ppasenssaahsigstqstpcsssta
```

References

Abdalla SA, Cymerman U, Johnson RM, Deber CM, Letarte M. Disease-associated mutations in conserved residues of ALK-1 kinase domain. Eur. J. Hum. Genet. 2003;11:279–287.

Abdalla SA, Letarte M. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. J. Med. Genet. 2006;43:97–110.

Abdalla SA, Pece-Barbara N, Vera S, Tapia E, Paez E, Bernabeu C, Letarte M. Analysis of ALK-1 and endoglin in newborns from families with hereditary hemorrhagic telangiectasia type 2. Hum. Mol. Genet. 2000;9:1227–37.

Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC et al. A comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. Sect. D Biol. Crystallogr. 2010;66:213–221.

Alt A, Miguel-Romero L, Donderis J, Aristorena M, Blanco FJ, Round A, Rubio V, Bernabeu C, Marina A. Structural and functional insights into endoglin ligand recognition and binding. PLoS One. 2012;7:1-12.

Aristorena M, Blanco FJ, de Las Casas-Engel M, Ojeda-Fernandez L, Gallardo-Vara E, Corbi A, Botella LM, Bernabeu C. Expression of endoglin isoforms in the myeloid lineage and their role during aging and macrophage polarization. Journal of cell science. 2014;127:2723-2735.

Arthur H, Geisthoff U, Gossage JR, Hughes CCW, Lacombe P, Meek ME, Oh P, Roman BL, Trerotola SO, Velthuis S, Wooderchak-Donahue W. Executive summary of the 11th HHT international scientific conference. Angiogenesis. 2015;18:511–524.

Arthur HM, Ure J, Smith J, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk D, Burn J, Diamond G. Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. Dev. Biol. 2000;217:42–53.

Attisano L, Carcamo J, Ventura F, Weis FMB, Massague J, Wrana JL. Identification of human activin and TGF-beta type I receptors that form heteromeric kinase complexes with type II receptors. Cell. 1993;75:671–680.

Auer PL, Johnsen JM, Johnson AD, Logsdon BA, Lange LA, Nalls MA, Zhang G, Franceschini N, Fox K, Lange EM, Rich SS, O'Donnell CJ, Jackson RD, Wallace RB, Chen Z et al. Imputation of exome sequence variants into population-based samples and blood-cell-trait-associated loci in african americans: NHLBI GO exome sequencing project. Am. J. Hum. Genet. 2012;91:794–808.

Austin E, Loyd J. Heritable forms of pulmonary arterial hypertension. Semin. Respir. Crit. Care Med. 2013;34:568–580.

Austin ED, Ma L, LeDuc C, Rosenzweig EB, Borczuk A, Phillips JA, Palomero T, Sumazin P, Kim HR, Talati MH, West J, Loyd JE, Chung WK. Whole exome sequencing to identify a novel gene (Caveolin-1) associated with human pulmonary arterial hypertension. Circ. Cardiovasc. Genet. 2012;5:336–343.

Barbara NP, Wrana JL, Letarte M, Barbara NP, Wrana JL, Letarte M. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor- β. J. Biol. Chem. 1999;274:584–594.

Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. Cell. 2004;116:281–297.

Bayrak-Toydemir P, Mao R, Lewin S, McDonald J. Hereditary hemorrhagic telangiectasia: an overview of diagnosis and management in the molecular era for clinicians. Genet. Med. 2004;6:175–191.

Bayrak-Toydemir P, McDonald J, Akarsu N, Toydemir RM, Calderon F, Tuncali T, Tang W, Miller F, Mao R. A fourth locus for hereditary hemorrhagic telangiectasia maps to chromosome 7. Am. J. Med. Genet. 2006;140:2155–2162.

Beishline K, Azizkhan-Clifford J. Sp1 and the "hallmarks of cancer." FEBS J. 2015;282:224–258.

Bellón T, Corbí A, Lastres P, Calés C, Cebrián M, Vera S, Cheifetz S, Massague J, Letarte M, Bernabéu C. Identification and expression of two forms of the human transforming growth factor-beta-binding protein endoglin with distinct cytoplasmic regions. Eur. J. Immunol. 1993;23:2340–2345.

Berg J, Porteous M, Reinhardt D, Gallione C, Holloway S, Umasunthar T, Lux A, McKinnon W, Marchuk D, Guttmacher A. Hereditary haemorrhagic telangiectasia: a questionnaire based study to delineate the different phenotypes caused by endoglin and ALK1 mutations. J. Med. Genet. 2003;40:585–90.

Berg JN, Gallione CJ, Stenzel TT, Johnson DW, Allen WP, Schwartz CE, Jackson CE, Porteous ME, Marchuk DA. The activin receptor-like kinase 1 gene: genomic structure and mutations in hereditary hemorrhagic telangiectasia type 2. Am. J. Hum. Genet. 1997;61:60–7.

Bernabeu C, Conley BA, Vary CPH. Novel biochemical pathways of endoglin in vascular cell physiology. J. Cell. Biochem. 2007;102:1375–1388.

Bernabeu C, Lopez-Novoa JM, Quintanilla M. The emerging role of TGF-β superfamily coreceptors in cancer. Biochim. Biophys. Acta - Mol. Basis Dis. 2009;1792:954–973.

Bidart M, Ricard N, Levet S, Samson M, Mallet C, David L, Subileau M, Tillet E, Feige JJ, Bailly S. BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain. Cell. Mol. Life Sci. 2012;69:313–324.

Bideau A, Plauchu H, Brunet G, Robert J. Epidemiological investigation of Rendu-Osler disease in France: its geographical distribution and prevalence. Popul. 1989;44:3–22.

Bilir B, Ekiz Bilir B, Yilmaz I, Soysal Atile N, Yildirim T, Kara SP, Gumustas SA, Orhan AE, Aydin M. Association of apelin, endoglin and endocan with diabetic peripheral neuropathy in type 2 diabetic patients. Eur. Rev. Med. Pharmacol. Sci. 2016;20:892–8.

Bindea G, Galon J, Mlecnik B. CluePedia Cytoscape plugin: Pathway insights using integrated experimental and in silico data. Bioinformatics. 2013;29(5):661–663.

Blanco FJ, Bernabeu C. Alternative splicing factor or splicing factor-2 plays a key role in intron retention of the endoglin gene during endothelial senescence. Aging Cell. 2011;10:896–907.

Blanco FJ, Grande MT, Langa C, Oujo B, Velasco S, Rodriguez-Barbero A, Perez-Gomez E, Quintanilla M, López-Novoa JM, Bernabeu C. S-endoglin expression is induced in senescent endothelial cells and contributes to vascular pathology. Circ. Res. 2008;103:1383–1392.

Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, Dahlsveen IK. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods. 2013;59:164–169.

Borggrefe T, Lauth M, Zwijsen A, Huylebroeck D, Oswald F, Giaimo BD. The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGFβ/BMP and hypoxia pathways. Biochim. Biophys. Acta - Mol. Cell Res. 2016;1863:303–313.

Botella LM, Sánchez-Elsner T, Rius C, Corbí A, Bernabéu C. Identification of a critical Sp1 site within the Endoglin promoter and its involvement in the transforming growth factor-β Stimulation. J. Biol. Chem. 2001;276:34486–34494.

Brant A, Schachat A, White R. Ocular manifestations in hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber disease). Am. J. Ophthalmol. 1989;107:642–646.

Brohée D, Franken P, Fievez M, Baudoux M, Hénuzet C, Brasseur P, Struyven J. High-output right ventricular failure secondary to hepatic arteriovenous microfistulae. Selective arterial embolization treatment. Arch. Intern. Med. 1984;144:1282–4.

Brookes AJ. The essence of SNPs. Gene. 1999;234:177–186.

Brosens LAA, van Hattem A, Hylind LM, Iacobuzio-Donahue C, Romans KE, Axilbund J, Cruz-Correa M, Tersmette AC, Offerhaus GJA, Giardiello FM. Risk of colorectal cancer in juvenile polyposis. Gut. 2007;56:965–7.

Buscarini E, Danesino C, Plauchu H, de Fazio C, Olivieri C, Brambilla G, Menozzi F, Reduzzi L, Blotta P, Gazzaniga P, Pagella F, Grosso M, Pongiglione G, Cappiello J, Zambelli A. High prevalence of hepatic focal nodular hyperplasia in subjects with hereditary hemorrhagic telangiectasia. Ultrasound Med Biol. 2004;30:1089–1097.

Buscarini E, Leandro G, Conte D, Danesino C, Daina E, Manfredi G, Lupinacci G, Brambilla G, Menozzi F, De Grazia F, Gazzaniga P, Inama G, Bonardi R, Blotta P, Forner P et al. Natural history and outcome of hepatic vascular malformations in a large cohort of patients with hereditary hemorrhagic teleangiectasia. Dig. Dis. Sci. 2011;56:2166–2178.

Calva D, Howe J. Hamartomatous polyposis syndromes. Surg Clin North Am. 2008;88:779–817.

Canzonieri C, Centenara L, Ornati F, Pagella F, Matti E, Alvisi C, Danesino C, Perego M, Olivieri C. Endoscopic evaluation of gastrointestinal tract in patients with hereditary hemorrhagic telangiectasia and correlation with their genotypes . Genet. Med. 2014;16:3–10.

del Castillo G, Sánchez-Blanco E, Martín-Villar E, Valbuena-Diez AC, Langa C, Pérez-Gómez E, Renart J, Bernabeu C, Quintanilla M. Soluble endoglin antagonizes Met signaling in spindle carcinoma cells. Carcinogenesis. 2014;36:212–222.

Castonguay R, Werner ED, Matthews RG, Presman E, Mulivor AW, Solban N, Sako D, Pearsall RS, Underwood KW, Seehra J, Kumar R, Grinberg AV. Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. J. Biol. Chem. 2011;286:30034–30046.

Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J. Biol. Chem. 1992;267:19027–19030.

Cheifetz S, Massagué J. Isoform-specific transforming growth factor-beta binding proteins with membrane attachments sensitive to phosphatidylinositol-specific phospholipase C. J. Biol. Chem. 1991;266:20767–72.

Cole SG, Begbie ME, Wallace GMF, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J. Med. Genet. 2005;42:577–82.

Conte W, Rotter J, Schwartz A, Congleton J. Hereditary generalized juvenile polyposis, arteriovenous malformations and colonic carcinoma. Clin. Res. 1982;30:93A.

Cox KL, C. Jr FR, Wong A, Gandhi G. Hereditary generalized juvenile polyposis associated with pulmonary arteriovenous malformation. Gastroenterology. 1980;78:1566–1570.

Dakeishi M, Shioya T, Wada Y, Shindo T, Otaka K, Manabe M, Nozaki JI, Inoue S, Koizumi A. Genetic epidemiology of hereditary hemorrhagic telangiectasia in a local community in the northern part of Japan. Hum. Mutat. 2002;19:140–148.

Damjanovich K, Langa C, Blanco FJ, McDonald J, Botella LM, Bernabeu C, Wooderchak-Donahue W, Stevenson DA, Bayrak-Toydemir P. 5'UTR mutations of ENG cause Hereditary Hemorrhagic Telangiectasia. Orphanet J. Rare Dis. 2011;6:85.

David L, Mallet C, Keramidas M, Lamandé N, Gasc JM, Dupuis-Girod S, Plauchu H, Feige JJ, Bailly S. Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circ. Res. 2008;102:914–922.

David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood. 2007;109:1953–1961.

Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA. Familial primary pulmonary hypertension (gene *PPH1*) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am. J. Hum. Genet. 2000;67:737–44.

Desai DC, Murday V, Phillips RK, Neale KF, Milla P, Hodgson SV. A survey of phenotypic features in juvenile polyposis. J Med Genet. 1998;35:476–481.

ten Dijke P, Ichijo H, Franzén P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K. Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene. 1993;8:2879–2887.

El Din FA, Patri S, Thoreau V, Rodriguez-Ballesteros M, Hamade E, Bailly S, Gilbert-Dussardier B, Abou Merhi R, Kitzis A. Functional and splicing defect analysis of 23 *ACVRL1* mutations in a cohort of patients affected by hereditary hemorrhagic telangiectasia. PLoS One. 2015;10:1–18.

Drake KM, Zygmunt D, Mavrakis L, Harbor P, Wang L, Comhair SA, Erzurum SC, Aldred MA. Altered MicroRNA processing in heritable pulmonary arterial hypertension: An important role for Smad-8. Am. J. Respir. Crit. Care Med. 2011;184:1400–1408.

Dupuis-Girod S, Bailly S, Plauchu H. Hereditary hemorrhagic telangiectasia (HHT): from molecular biology to patient care. J. Thromb. Haemost. 2010;8:1447-1456.

D**upuis-Girod S, Ginon I, Saurin JC, Marion D, Guillot E, Decullier E, Roux A, Carette MF, Gilbert-Dussardier B, Hatron PY, Lacombe P, Lorcerie B, Riviere S, Corre R, Giraud S, Bailly S et al.** Bevacizumab in patients with hereditary hemorrhagic telangiectasia and severe hepatic vascular malformations and high

cardiac output. Jama. 2012;307:948–955.

Engel KL, Mackiewicz M, Hardigan AA, Myers RM, Savic D. Decoding transcriptional enhancers: Evolving from annotation to functional interpretation. Semin. Cell Dev.Biol. 2016;57:40–50.

Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, Jassal B, Jupe S, Korninger F, McKay S, Matthews L, May B, Milacic M, Rothfels K, Shamovsky V et al. The reactome pathway knowledgebase. Nucleic Acids Res. 2016;44:481–487.

Faughnan ME, Palda V, Garcia-Tsao G, Geisthoff UW, McDonald J, Proctor DD, Spears J, Brown DH, Buscarini E, Chesnutt MS, Cottin V, GangulyA, Gossage JR, Guttmacher E, Hyland RH, Kennedy SJ et al. International guidelines for the diagnosis and management of hereditary haemorrhagic telangiectasia. J. Med. Genet. 2011;48:73–87.

Feng XH, Derynck R. A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity. EMBO J. 1997;16:3912–23.

Fernandez-L A, Sanz-Rodriguez F, Zarrabeitia R, Perez-Molino A, Morales C, Restrepo CM, Ramirez JR, Coto E, Lenato GM, Bernabeu C, Botella LM. Mutation study of spanish patients with hereditary hemorrhagic telangiectasia and expression analysis of endoglin and ALK1. Hum. Mutat. 2006;27:1–10.

Fernandez-Ruiz E, St-Jacques S, Bellon T, Letarte M, Bernabeu C. Assignment of the human endoglin gene (END) to 9q34-->qter. Cytogenet Cell Genet. 1993;64:204–207.

Finnegan EF, Pasquinelli AE. MicroRNA biogenesis: regulating the regulators. Crit. Rev. Biochem. Mol. Biol. 2013;48:51–68.

Flores BC, Klinger DR, White JA, Batjer HH. Spinal vascular malformations: treatment strategies and outcome. Neurosurg. Rev. 2016:1–14.

Fontalba A, Fernández-Luna JL, Zarrabeitia R, Recio-Poveda L, Albiñana V, Ojeda-Fernández ML, Bernabéu C, Alcaraz LA, Botella LM. Copy number variations in endoglin locus: mapping of large deletions in Spanish families with hereditary hemorrhagic telangiectasia type 1. BMC Med. Genet. 2013;14:121.

Förg T, Hafner M, Lux A. Investigation of endoglin wild-type and missense mutant protein heterodimerisation using fluorescence microscopy based IF, BiFC and FRET analyses. PLoS One. 2014;9:1–22.

Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev. 2004;30:193–204.

Fu Y, Chang A, Chang L, Niessen K, Eapen S, Setiadi A, Karsan A. Differential regulation of transforming growth factor beta signaling pathways by Notch in human endothelial cells. J. Biol. Chem. 2009;284:19452–19462.

Galiè N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M, Ghofrani A, Gomez Sanchez MA, Hansmann G, Klepetko W, Lancellotti P et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension. Eur. Heart J. 2016;37:67–119.

Gallardo-Vara E, Blanco FJ, Roqué M, Friedman SL, Suzuki T, Botella LM, Bernabeu C. Transcription factor KLF6 upregulates expression of metalloprotease MMP14 and subsequent release of soluble endoglin during vascular injury. Angiogenesis. 2016;19:155–171.

Gallione C, Aylsworth AS, Beis J, Berk T, Bernhardt B, Clark RD, Clericuzio C, Danesino C, Drautz J, Fahl J, Fan Z, Faughnan ME, Ganguly A, Garvie J, Henderson K et al. Overlapping spectra of SMAD4 mutations in juvenile polyposis (JP) and JP-HHT syndrome. Am. J. Med. Genet. 2010;152:333–339.

Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin É, Westermann CJJ, Marchuk DA. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in *MADH4* (SMAD4). Lancet. 2004;363:852–859.

Gallione CJ, Richards JA, Letteboer TGW, Rushlow D, Prigoda NL, Leedom TP, Ganguly A, Castells A, Ploos van Amstel JK, Westermann CJJ, Pyeritz RE, Marchuk DA. SMAD4 mutations found in unselected HHT patients. J. Med. Genet. 2006;43:793–7.

Gallione CJ, Scheessele EA, Reinhardt D, Duits AJ, Berg JN, Westermann CJJ, Marchuk DA. Two common endoglin mutations in families with hereditary hemorrhagic telangiectasia in the Netherlands Antilles: evidence for a founder effect. Hum. Genet. 2000;107:40–44.

Gazzaniga P, Buscarini E, Leandro G, Reduzzi L, Grosso M, Pongiglione G, Pedrinazzi C, Lanzarini L, Portugalli V, Blotta P, Forner P, Boccardi E, Pagella F, Manfredi G, Olivieri C et al. Contrast echocardiography for pulmonary arteriovenous malformations screening: does any bubble matter? Eur. J. Echocardiogr. 2009;10:513–518.

Geisthoff UW, Nguyen HL, Röth A, Seyfert U. How to manage patients with hereditary haemorrhagic telangiectasia. Br. J. Haematol. 2015;171:443–452.

Ghai V, Wang K. Recent progress toward the use of circulating microRNAs as clinical biomarkers. Arch. Toxicol. 2016:1–20.

Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A. Serum microRNAs are promising novel biomarkers. PLoS One. 2008;3:1–7.

Gill SS, Roddie ME, Shovlin CL, Jackson JE. Pulmonary arteriovenous malformations and their mimics. Clin. Radiol. 2015;70:96–110.

Gincul R, Lesca G, Gelas-Dore B, Rollin N, Barthelet M, Dupuis-Girod S, Pilleul F, Giraud S, Plauchu H, Saurin JC. Evaluation of previously nonscreened hereditary hemorrhagic telangiectasia patients shows frequent liver involvement and early cardiac consequences. Hepatology. 2008;48:1570–1576.

Girerd B, Coulet F, Jais X, Eyries M, Van Der Bruggen C, De Man F, Houweling A, Dorfmuller P, Savale L, Sitbon O, Vonk-Noordegraaf A, Soubrier F, Simonneau G, Humbert M, Montani D. Characteristics of pulmonary arterial hypertension in affected carriers of a mutation located in the cytoplasmic tail of bone morphogenetic protein receptor type 2. Chest. 2015;147:1385–1394.

Gossage J, Kanj G. Pulmonary arteriovenous malformations. A state of the art review. Am. J. Respir. Crit. Care Med. 1998;158:643–661.

Gougosa, Letarte M. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J. Biol. Chem. 1990;265:8361–4.

Gougos A, St Jacques S, Greaves A, O'Connell PJ, d'Apice AJ, Buhring HJ, Bernabeu C, van Mourik JA, Letarte M. Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukemic cells, and syncytiotrophoblasts. Int Immunol. 1992;4:83–92.

Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J. 2002;21:1743–53.

Govani FS, Shovlin CL. Hereditary haemorrhagic telangiectasia: a clinical and scientific review. Eur. J. Hum. Genet. 2009;17:860–71.

Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C. Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. J. Biol. Chem. 2002;277:29197–29209.

Gulyaeva LF, Kushlinskiy NE. Regulatory mechanisms of microRNA expression. *J.* Transl. Med. 2016;14:143.

Gusev A, Lee SH, Trynka G, Finucane H, Vilhjálmsson BJ, Xu H, Zang C, Ripke S, Bulik-Sullivan B, Stahl E, Kähler AK, Hultman CM, Purcell SM, McCarroll SA, Daly M et al. Partitioning heritability of regulatory and cell-type-specific variants across 11 common diseases. Am. J. Hum. Genet. 2014;95:535–552.

Hanes F. Multiple hereditary telangiectases causes hemorrhage (hereditary hemorrhagic telangiectasia). Bull. Johns Hopkins Hosp. 1909;20:63–73.

Hanks S, Quinn A. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol. 1991.

Harrison RE, Flanagan J, Sankelo M, Abdalla S, Rowell J, Machado RD, Elliott CG, Robbins IM, Olschewski H, McLaughlin V, Gruenig E, Kermeen F, Halme M, Räisänen-SokolowskiA, Laitinen T, Morrell NW, Trembath RC. Molecular and

functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. J. Med. Genet. 2003;40:865–871.

Hawinkels LJAC, Kuiper P, Wiercinska E, Verspaget HW, Liu Z, Pardali E, Sier CFM, Ten Dijke P. Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. Cancer Res. 2010;70:4141–4150.

Hernandez F, Huether R, Carter L, Johnston T, Thompson J, Gossage JR, Chao E, Elliott AM. Mutations in *RASA1* and *GDF2* identified in patients with clinical features of hereditary hemorrhagic telangiectasia. Hum. Genome Var. 2015;2:1–6.

Hoag JB, Terry P, Mitchell S, Reh D, Merlo C. An epistaxis severity score for hereditary hemorrhagic telangiectasia. Laryngoscope. 2010;120:838–843.

Hoeper MM, Bogaard HJ, Condliffe R, Frantz R, Khanna D, Kurzyna M, Langleben D, Manes A, Satoh T, Torres F, Wilkins MR, Badesch DB. Definitions and diagnosis of pulmonary hypertension. Journal of the American College of Cardiology. 2013;62:42-50.

Hsi DH, Ryan GF, Hellems SO, Cheeran DC, Sheils L. Large aneurysms of the ascending aorta and major coronary arteries in a patient with hereditary hemorrhagic telangiectasia. Mayo Clin. Proc. 2003;78:774–6.

Hua Z, Lv Q, Ye W, Wong CKA, Cai G, Gu D, Ji Y, Zhao C, Wang J, Yang BB, Zhang Y. MiRNA-directed regulation of VEGF and other angiogenic under hypoxia. PLoS One. 2006;1.

Humphries J, Jr FH, Underwood PJ. Vaginal telangiectasias: unusual presentation of the Osler-Weber-Rendu syndrome. Obs. Gynecol. 1993;81:865–866.

I**ddan GJ, Swain CP.** History and development of capsule endoscopy. Gastrointest. Endosc. Clin. N. Am. 2004;14:1–9.

Invernizzi R, Quaglia F, Klersy C, Pagella F, Ornati F, Chu F, Matti E, Spinozzi G, Plumitallo S, Grignani P, Olivieri C, Bastia R, Bellistri F, Danesino C, Benazzo M, Balduini CL. Efficacy and safety of thalidomide for the treatment of severe recurrent epistaxis in hereditary haemorrhagic telangiectasia: Results of a non-randomised, single-centre, phase 2 study. Lancet Haematol. 2015;2:465–473.

Ishibashi O, Inui T. Dataset of microarray analysis to identify endoglin-dependent bone morphogenetic protein-2-responsive genes in the murine periodontal ligament cell line PDL-L2. Genomics Data. 2014;2:24–26.

Ishiwata T, Terada J, Tanabe N, Abe M, Sugiura T, Tsushima K, Tada Y, Sakao S, Kasahara Y, Nakanishi N, Morisaki H, Tatsumi K. Pulmonary Arterial Hypertension as the First Manifestation in a Patient with Hereditary Hemorrhagic Telangiectasia. Intern. Med. 2014;53:2359–2363.

Johnson DW, Berg JN, Baldwin M, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance M, Diamond A, Guttmacher E, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk D. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat. Genet. 1996;13:189–195.

Jonker L. TGF-β & BMP receptors endoglin and ALK1: Overview of their functional role and status as antiangiogenic targets. Microcirculation. 2014;21:93–103.

Joo SP, Lee JK, Kim TS, Kim MK, Lee IK, Seo BR, Kim JH, Kim SH, Oh CW. A polymorphic variant of the endoglin gene is associated with increased risk for intracranial aneurysms in a Korean population. Surg. Neurol. 2008;70:39–44.

Kalani MYS, Ahmed AS, Martirosyan NL, Cronk K, Moon K, Albuquerque FC, McDougall CG, Spetzler RF, Bristol RE. Surgical and endovascular treatment of pediatric spinal arteriovenous malformations. World Neurosurg. 2011;78:348–54.

Karki R, Pandya D, Elston RC, Ferlini C. Defining "mutation" and "polymorphism" in the era of personal genomics. 2015;8:1–7.

Kerr G, Sheldon H, Chaikuad A, Alfano I, von Delft F, Bullock AN, Harris AL. A small molecule targeting ALK1 prevents Notch cooperativity and inhibits functional angiogenesis. Angiogenesis. 2015;18:209–217.

Kim H, Nelson J, Krings T, Karel G, Mcculloch CE, Lawton MT, Young WL, Faughnan ME. Hemorrhage rates from brain arteriovenous malformation in patients with hereditary hemorrhagic telangiectasia. Stroke. 2015;46:1362–1364.

Kjeldsen AD, Møller TR, Brusgaard K, Vase P, Andersen PE. Clinical symptoms according to genotype amongst patients with hereditary haemorrhagic telangiectasia. J. Intern. Med. 2005;258:349–355.

Kjeldsen AD, Vase P, Green A. Hereditary haemorrhagic telangiectasia: A population-based study of prevalence and mortality in Danish patients. J. Intern. Med. 1999;245:31–39.

Kochanowski J, Sobieszczańska M, Tubek S, Żurek M, Pawelczak J. Successful therapy with bevacizumab in a case of hereditary hemorrhagic telangiectasia. Hum. Vaccines Immunother. 2015;11:680–681.

Koleva RI, Conley BA, Romero D, Riley KS, Marto JA, Lux A, Vary CPH. Endoglin structure and function: Determinants of endoglin phosphorylation by transforming growth factor-β receptors. J. Biol. Chem. 2006;281:25110–25123.

Korchynskyi O, Ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the *Id1* promoter. J. Biol. Chem. 2002;277:4883–4891.

Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J. Biol. Chem. 2010;285:17442–17452.

Krings T, Ozanne A, Chng SM, Alvarez H, Rodesch G, Lasjaunias PL. Neurovascular phenotypes in hereditary haemorrhagic telangiectasia patients according to age. Review of 50 consecutive patients aged 1 day-60 years. Neuroradiology. 2005;47:711–720.

Lacombe P, Lacout A, Marcy PY, Binsse S, Sellier J, Bensalah M, Chinet T, Bourgault-Villada I, Blivet S, Roume J, Lesur G, Blondel JH, Fagnou C, Ozanne A, Chagnon S, El Hajjam M. Diagnosis and treatment of pulmonary arteriovenous malformations in hereditary hemorrhagic telangiectasia: An overview. Diagn. Interv. Imaging. 2013;94:835–848.

Lamouille S, Mallet C, Felge JJ, Bailly S. Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. Blood. 2002;100:4495–4501.

Lande A, Bedford A, Schechter L. The spectrum of arteriographic findings in Osler-Weber-Rendu disease. Angiology. 1976;27:223–240.

Landi MT, Zhao Y, Rotunno M, Koshiol J, Liu H, Bergen AW, Rubagotti M, Goldstein AM, Linnoila I, Marincola FM, Tucker MA, Bertazzi PA, Pesatori AC, Caporaso NE, McShane LM, Wang E. MicroRNA expression differentiates histology and predicts survival of lung cancer. Clin. Cancer Res. 2010;16:430–441.

Larrivée B, Prahst C, Gordon E, del Toro R, Mathivet T, Duarte A, Simons M, Eichmann A. ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. Dev. Cell. 2012;22:489–500.

Larsen Haidle J, Howe J. Juvenile Polyposis Syndrome. Gene Reviews. 2015:1–17.

Lastres P, Bellon T, Cabañas C, Sanchez-Madrid F, AcevedoA, GougosA, Letarte M, Bernabeu C. Regulated expression on human macrophages of endoglin, an Arg-Gly-Asp-containing surface antigen. Eur. J. Immunol. 1992;22:393–397.

L**awrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultwood J, Wainscoat JS, Hatton CSR, Harris AL.** Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br. J. Haematol. 2008;141:672–675.

Lee NY, Golzio C, Gatza CE, Sharma A, Katsanis N, Blobe GC. Endoglin regulates PI3-kinase/Akt trafficking and signaling to alter endothelial capillary stability during angiogenesis. Mol. Biol. Cell. 2012;23:2412–23.

Lesca G, Burnichon N, Raux G, Tosi M, Pinson S, Marion MJ, Babin E, Gilbert-Dussardier B, Rivière S, Goizet C, Faivre L, Plauchu H, Frébourg T, Calender A, Giraud S. Distribution of *ENG* and *ACVRL1* (ALK1) mutations in French HHT patients. Hum. Mutat. 2006;27.

Lesca G, Genin E, Blachier C, Olivieri C, Coulet F, Brunet G, Dupuis-Girod S, Buscarini E, Soubrier F, Calender A, Danesino C, Giraud S, Plauchu H. Hereditary hemorrhagic telangiectasia: evidence for regional founder effects of *ACVRL1* mutations in French and Italian patients. Eur. J. Hum. Genet. 2008;16:742–

749.

Lesca G, Olivieri C, Burnichon N, Pagella F, Carette MF, Gilbert-Dussardier B, Goizet C, Roume J, Rabilloud M, Saurin J-C, Cottin V, Honnorat J, Coulet F, Giraud S, Calender A, Danesino C, Buscarini E, Plauchu H. Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. Genet. Med. 2007;9:14–22.

Lesca G, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Rivière S, Leheup B, Goizet C, Carette MF, Cordier JF, Pinson S, Soubrier F, Calender A, Giraud S. Molecular screening of ALK1/*ACVRL1* and *ENG* genes in hereditary hemorrhagic telangiectasia in France. Hum. Mutat. 2004;23:289–299.

Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP. Defective angiogenesis in mice lacking endoglin. Science. 1999;284:1534–1537.

Lin Y, Yu H, Song W, Zhang Y, Zhang C, Zhu Y, Pang Q, Hui R, Chen J. A variant in the endoglin gene is associated with the development of sporadic intracranial aneurysms. Curr Neurovasc Res. 2014;11:294–301.

Liu ZC, Lu XF, Yang H, Liu HD, Song X, Ning SL, Xu YF, Chen YX. Clinical outcomes of patients with severe hepatic hereditary hemorrhagic telangiectasia after banding of the hepatic artery and banding/ligation of branches of the hepatic artery. Eur. J. Vasc. Endovasc. Surg. 2015:594–601.

Llorca O, Trujillo A, Blanco FJ, Bernabeu C. Structural Model of Human Endoglin, a Transmembrane Receptor Responsible for Hereditary Hemorrhagic Telangiectasia. J. Mol. Biol. 2007;365:694–705.

López-Casillas F, Payne HM, Andres JL, Massagué J. Betaglycan can act as a dual modulator of TGF-β access to signaling receptors: Mapping of ligand binding and GAG attachment sites. J. Cell Biol. 1994;124:557–568.

Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson KS, Soubrier F, Germain M, Trégouët DA, Borczuk A, Rosenzweig EB, Girerd B, Montani D, Humbert M, Loyd JE, Kass RS, Chung WK. A Novel Channelopathy in Pulmonary Arterial Hypertension. N. Engl. J. Med. 2013;369:351–361.

Macias MJ, Martin-Malpartida P, Massague J. Structural determinants of Smad function in TGF-beta signaling. Trends Biochem. Sci. 2015;40:296–308.

Madonna R, Cocco N, De Caterina R. Pathways and drugs in pulmonary arterial hypertension - focus on the role of endothelin receptor antagonists. Cardiovasc. Drugs Ther. 2015;29:469–479.

Mahadevan J, Ozanne A, Yoshida Y, Weon YC, Alvarez H, Rodesch G, Lasjaunias P. Hereditary haemorrhagic telangiectasia cerebrospinal localization in adults and children. Review of 39 cases. Interv. Neuroradiol. 2004;10:27–35.

Mahlawat P, Ilangovan U, Biswas T, Sun LZ, Hinck AP. Structure of the Alk1 extracellular domain and characterization of its bone morphogenetic protein (BMP)

binding properties. Biochemistry. 2012;51:6328–6341.

Mahmoud M, Allinson KR, Zhai Z, Oakenfull R, Ghandi P, Adams RH, Fruttiger M, Arthur HM. Pathogenesis of arteriovenous malformations in the absence of endoglin. Circ. Res. 2010;106:1425–1433.

Mallet C, Lamribet K, Giraud S, Dupuis-Girod S, Feige JJ, Bailly S, Tillet E. Functional analysis of endoglin mutations from hereditary hemorrhagic telangiectasia type 1 patients reveals different mechanisms for endoglin loss of function. Hum. Mol. Genet. 2015;24:1142–1154.

Mastrangelo IA, Coureyt AJ, Wall JS, Jackson SP, Hough PVC. DNA looping and SpI multimer links: A mechanism for transcriptional synergism and enhancement. Biochemistry. 1991;88:5670–5674.

McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat. Genet. 1994;8:345–51.

McDonald J, Wooderchak-Donahue W, VanSant Webb C, Whitehead K, Stevenson DA, Bayrak-Toydemir P. Hereditary hemorrhagic telangiectasia: Genetics and molecular diagnostics in a new era. Front. Genet. 2015;5:1–8.

McDonald JE, Miller FJ, Hallam SE, Nelson L, Marchuk DA, Ward KJ. Clinical manifestations in a large hereditary hemorrhagic telangiectasia (HHT) type 2 kindred. Am. J. Med. Genet. 2000;93:320–327.

McDonald MT, Papenberg KA, Ghosh S, Glatfelter AA, Biesecker BB, Helmbold EA, Markel DS, Zolotor A, McKinnon WC, Vanderstoep JL,Jackson CE, Iannuzzi M, Collins FS, BoehnkeM, PorteousME et al. A disease locus for hereditary haemorrhagic telangiectasia maps to chromosome 9q33-34. Nat Genet. 1994;6:197–204.

Mi L, Brown CT, Gao Y, Tian Y, Le VQ, Walz T, Springer TA. Structure of bone morphogenetic protein 9 procomplex. Proc. Natl. Acad. Sci. 2015;112:3710–3715.

Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, González MÁ, Bernad A, Sánchez-Madrid F. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat. Commun. 2011;2:282.

Miyake R, Fujino T, Abe K, Hosokawa K, Ohtani K, Morisaki H, Yamada O, Higo T, Ide T. Pulmonary arterial hypertension associated with hereditary hemorrhagic telangiectasia successfully treated with sildenafil. Int. J. Cardiol. 2016;214:275–276.

Mont'Alverne F, Musacchio M, Tolentino V, Belzile F, Riquelme C, Tournade A. Giant spinal perimedullary fistula in hereditary haemorrhagic telangiectasia: Diagnosis, endovascular treatment and review of the literature. Neuroradiology. 2003;45:830–836.

Myles S, Davison D, Barrett J, Stoneking M, Timpson N. Worldwide population differentiation at disease-associated SNPs. BMC Med. Genomics. 2008;1:22.

Nanthakumar K, Graham AT, Robinson TI, Grande P, Pugash RA, Clarke JA, Hutchison SJ, Mandzia JL, Hyland RH, Faughnan ME. Contrast echocardiography for detection of pulmonary arteriovenous malformations. Am. Heart J. 2001;141:243–246.

Nasim MT, Ogo T, Ahmed M, Randall R, Chowdhury HM, Snape KM, Bradshaw TY, Southgate L, Lee GJ, Jackson I, Lord GM, Gibbs JSR, Wilkins MR, Ohta-Ogo K, Nakamura K et al. Molecular genetic characterization of SMAD signaling molecules in pulmonary arterial hypertension. Hum. Mutat. 2011;32:1385–1389.

Navickas R, Gal D, Laucevičius A, Taparauskaitė A, Zdanytė M, Holvoet P. Identifying Circulating MicroRNAs as Biomarkers of Cardiovascular Disease: A Systematic Review. Cardiovasc. Res. 2016:322–337.

Nishioka Y, Akamatsu N, Sugawara Y, Kaneko J, Arita J, Sakamoto Y, Hasegawa K, Kokudo N. Hereditary Hemorrhagic Telangiectasia with Hepatic Vascular Malformations. Case Rep. Med. 2015;2015:917818.

Nolan-Stevaux O, Zhong W, Culp S, Shaffer K, Hoover J, Wickramasinghe D, Ruefli-Brasse A. Endoglin Requirement for BMP9 Signaling in Endothelial Cells Reveals New Mechanism of Action for Selective Anti-Endoglin Antibodies. PLoS One. 2012;7.

Olivieri C, Lanzarini L, Pagella F, Semino L, Corno S, Valacca C, Plauchu H, Lesca G, Barthelet M, Buscarini E, Danesino C. Echocardiographic screening discloses increased values of pulmonary artery systolic pressure in 9 of 68 unselected patients affected with hereditary hemorrhagic telangiectasia. Genet. Med. 2006;8:183–90.

Olivieri C, Pagella F, Semino L, Lanzarini L, Valacca C, Pilotto A, Corno S, Scappaticci S, Manfredi G, Buscarini E, Danesino C. Analysis of ENG and ACVRL1 genes in 137 HHT Italian families identifies 76 different mutations (24 novel). Comparison with other European studies. J. Hum. Genet. 2007;52:820–829.

Osler W. On a family form of recurring epistaxis, associated with multiple telangiectasis of the skin and mucous membranes. Bull Johns Hopkins Hosp. 1901;28:401–403.

Pagella F, Colombo A, Matti E, Giourgos G, Tinelli C, Olivieri C, Danesino C. Correlation of severity of epistaxis with nasal telangiectasias in hereditary hemorrhagic telangiectasia (HHT) patients. Am. J. Rhinol. Allergy. 2009;23:52–58.

Pagella F, Matti E, Chu F, Pusateri A, Tinelli C, Olivieri C, Canzonieri C, Boeri L, Ornati F, Danesino C. Argon plasma coagulation is an effective treatment for hereditary hemorrhagic telangiectasia patients with severe nosebleeds. Acta Otolaryngol. 2013;133:174–180.

Pardali E, ten Dijke P. Transforming growth factor-beta signaling and tumor angiogenesis. Front. Biosci. 2009;14:4848-4861.

Pardali E, Goumans MJ, ten Dijke P. Signaling by members of the TGF-β family in vascular morphogenesis and disease. Trends Cell Biol. 2010;20:556–567.

Park SO, Young JL, Seki T, Hong KH, Fliess N, Jiang Z, Park A, Wu X, Kaartinen V, Roman BL, Oh SP. ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2. Blood. 2008;111:633–642.

Parra JA, Bueno J, Zarauza J, Fariñas-Alvarez C, Cuesta JM, Ortiz P, Zarrabeitia R, Pérez del Molino A, Bustamante M, Botella LM, Delgado MT. Graded contrast echocardiography in pulmonary arteriovenous malformations. Eur. Respir. J. 2010;35:1279–85.

Pawlikowska L, Nelson J, Guo D, McCulloch C, Lawton M, Kim H, Faughnan M. The *ACVRL1* c.314-35A>G polymorphism is associated with organ vascular malformations in hereditary hemorrhagic telangiectasia patients with *ENG* mutations, but not in patients with *ACVRL1* mutations. Am. J. Med. Genet. 2015;167:1262–1267.

Pece N, Vera S, Cymerman U, White RI, Wrana JL, Letarte M. Mutant endoglin in hereditary hemorrhagic telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. J. Clin. Invest. 1997;100:2568–2579.

Piao C, Zhu Y, Zhang C, Xi X, Liu X, Zheng S, Li X, Guo J, Jia L, Nakanishi T, Cai T, Gu H, Du J. Identification of multiple *ACVRL1* mutations in patients with pulmonary arterial hypertension by targeted exome capture. Clin. Sci. 2016;130:1559–1569.

Pierucci P, Lenato GM, Suppressa P, Lastella P, Triggiani V, Valerio R, Comelli M, Salvante D, Stella A, Resta N, Logroscino G, Resta F, Sabbà C. A long diagnostic delay in patients with Hereditary Haemorrhagic Telangiectasia: a questionnaire-based retrospective study. Orphanet J. Rare Dis. 2012;7:33.

Poisson A, Vasdev A, Brunelle F, Plauchu H, Dupuis-Girod S. Acute paraplegia due to spinal arteriovenous fistula in two patients with hereditary hemorrhagic telangiectasia. Eur. J. Pediatr. 2009;168:135–139.

Pousada G, Baloira A, Valverde D. Pulmonary arterial hypertension and portal hypertension in a patient with hereditary hemorrhagic telangiectasia. Med. Clin. 2015;144:261–4.

Radin DR. Hereditary generalized juvenile polyposis: association with arteriovenous malformations and risk of malignancy. Abdom Imaging. 1994;19:140–142.

Rendu H. Épistaxis répétées chez un sujet porteur de petits angiomes cutanés et muqueux. Bull Soc méd Hosp. 1896;13:731–733.

Ricard N, Bidart M, Mallet C, Lesca G, Giraud S, Prudent R, Feige JJ, Bailly S. Functional analysis of the BMP9 response of ALK1 mutants from HHT2 patients: A diagnostic tool for novel *ACVRL1* mutations. Blood. 2010;116:1604–1612.

Ríus C, Smith JD, Almendro N, Langa C, Botella LM, Marchuk DA, Vary CP, Bernabéu C. Cloning of the promoter region of human endoglin, the target gene for hereditary hemorrhagic telangiectasia type 1. Blood. 1998;92:4677–4690.

Rochon ER, Wright DS, Schubert MM, Roman BL. Context-specific interactions between Notch and ALK1 cannot explain ALK1-associated arteriovenous malformations. Cardiovasc. Res. 2015;107:143–52.

Rodesch G, Lasjaunias P. Spinal cord arteriovenous shunts: From imaging to management. Eur. J. Radiol. 2003;46:221–232.

Rossi E, Sanz-Rodriguez F, Eleno N, Düwell A, Blanco FJ, Langa C, Botella LM, Cabañas C, Lopez-Novoa JM, Bernabeu C. Endothelial endoglin is involved in inflammation: Role in leukocyte adhesion and transmigration. Blood. 2013;121:403–415.

Rossi E, Smadja DM, Boscolo E, Langa C, Arevalo MA, Pericacho M, Gamella-Pozuelo L, Kauskot A, Botella LM, Gaussem P, Bischoff J, Lopez-Novoa JM, Bernabeu C. Endoglin regulates mural cell adhesion in the circulatory system. Cell. Mol. Life Sci. 2016;73:1715–1739.

Sabbà C, Pasculli G, Lenato GM, Suppressa P, Lastella P, Memeo M, Dicuonzo F, Guanti G. Hereditary hemorrhagic telangiectasia: Clinical features in ENG and ALK1 mutation carriers. J. Thromb. Haemost. 2007;5:1149–1157.

Sadick H, Riedel F, Naim R, Goessler U, Hörmann K, Hafner M, Lux A, Hormann K, Hafner M, Lux A. Patients with hereditary hemorrhagic telangiectasia have increased plasma levels of vascular endothelial growth factor and transforming growth factor-beta1 as well as high ALK1 tissue expression. Haematologica. 2005;90:818–828.

Saitoh M, Nishitoh H, Amagasa T, Miyazono K, Takagi M, Ichijo H. Identification of important regions in the cytoplasmic juxtamembrane domain of type I receptor that separate signaling pathways of transforming growth factor-β J. Biol. Chem. 1996;271:2769–2775.

Sakabe N, Savic D, Nobrega MA. Transcriptional enhancers in development and disease. Genome Biol. 2012;13:238.

Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L, Löwik CWGM, ten Dijke P. BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J. Cell Sci. 2007;120:964–72.

Scotti C, Olivieri C, Boeri L, Canzonieri C, Ornati F, Buscarini E, Pagella F, Danesino C. Bioinformatic analysis of pathogenic missense mutations of activin receptor like kinase 1 ectodomain. PLoS One. 2011;6.

Seki T, Hong KH, Oh SP. Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. Lab. Invest. 2006;86:116–29.

Shintani M, Yagi H, Nakayama T, Saji T, Matsuoka R. A new nonsense mutation of SMAD8 associated with pulmonary arterial hypertension. J. Med. Genet. 2009;46:331–7.

Shoukier M, Teske U, Weise B, Engel W, Argyriou L. Characterization of five novel large deletions causing hereditary haemorrhagic telangiectasia. Clin. Genet. 2008;73:320–330.

Shovlin CL. Hereditary haemorrhagic telangiectasia: pathophysiology, diagnosis and treatment. Blood Rev. 2010;24:203–219.

Shovlin CL. Pulmonary arteriovenous malformations. Am. J. Respir. Crit. Care Med. 2014;190:1217–1228.

Shovlin CL, Gilson C, Busbridge M, Patel D, Shi C, Dina R, Abdulla FN, Awan I. Can iron treatments aggravate epistaxis in some patients with hereditary hemorrhagic telangiectasia? Laryngoscope. 2016;0:1–7.

Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H. Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). Am. J. Med. Genet. 2000;91:66–67.

Shovlin CL, Hughes JMB, Tuddenham EGD, Temperley I, Perembelon YFN, Scott J, Seidman CE, Seidman JG. A gene for hereditary haemorrhagic telangiectasia maps to chromosome 9q3. Nat. Genet. 1994;6:205–209.

Shovlin CL, Sodhi V, McCarthy A, Lasjaunias P, Jackson JE, Sheppard MN. Estimates of maternal risks of pregnancy for women with hereditary haemorrhagic telangiectasia (Osler-Weber-Rendu syndrome): Suggested approach for obstetric services. BJOG An Int. J. Obstet. Gynaecol. 2008;115:1108–1115.

Silva BM, Hosman AE, Devlin HL, Shovlin CL. Lifestyle and dietary influences on nosebleed severity in hereditary hemorrhagic telangiectasia. Laryngoscope. 2013;123:1092–1099.

Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, Gomez Sanchez MA, Krishna Kumar R, Landzberg M, Machado RF, Olschewski H, Robbins IM, Souza R. Updated clinical classification of pulmonary hypertension. Jour. Am. Col. Card. 2013;62:34-41.

Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat. Cell Biol. 2008;10:1470–6.

St-Jacques S, Forte M, Lye SJ, Letarte M. Localization of endoglin, a transforming growth factor-beta binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. Biol. Reprod. 1994;51:405–413.

Su W, Jackson S, Tijan R, Echols H. DNA looping between sites for transcriptional activation - self-association of DNA bound SP1. Genes Dev. 1991;5:820–826.

Suzuki Y, Ohga N, Morishita Y, Hida K, Miyazono K, Watabe T. BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. J. Cell Sci. 2010;123:1684–1692.

Tabruyn SP, Hansen S, Ojeda-Fernández ML, Bovy N, Zarrabeitia R, Recio-Poveda L, Bernabéu C, Martial JA, Botella LM, Struman I. MiR-205 is downregulated in hereditary hemorrhagic telangiectasia and impairs TGF-beta signaling pathways in endothelial cells. Angiogenesis. 2013;16:877–887.

Tillet E, Bailly S. Emerging roles of BMP9 and BMP10 in hereditary hemorrhagic telangiectasia. Front. Genet. 2014;5:1–7.

Townson SA, Martinez-Hackert E, Greppi C, Lowden P, Sako D, Liu J, Ucran JA, Liharska K, Underwood KW, Seehra J, Kumar R, Grinberg AV. Specificity and structure of a high affinity activin receptor-like kinase 1 (ALK1) signaling complex. J. Biol. Chem. 2012;287:27313–27325.

Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Morrell NW, Berg J, Manes A, McGaughran J et al. Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N. Engl. J. Med. 2001;345:325–334.

Turchinovich A, Burwinkel B. Distinct AGO1 and AGO2 associated miRNA profiles in human cells and blood plasma. RNA Biol. 2012;9:1066–75.

Turchinovich A, Tonevitsky AG, Burwinkel B. Extracellular miRNA: A Collision of Two Paradigms. Trends Biochem. Sci. 2016;41:883–892.

Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res. 2011;39:7223–7233.

Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat. Med. 2006;12:642–9.

Verduci L, Simili M, Rizzo M, Mercatanti A, Evangelista M, Mariani L, Rainaldi G, Pitto L. MiRNA mediated interaction between leukaemia/lymphoma-related factor (LRF) and alternative splicing factor/splicing factor2 (ASF/SF2) affects MEF senescence and apoptosis. J. Biol. Chem. 2010;285:39551–39563.

Del Vescovo V, Grasso M, Barbareschi M, Denti MA. MicroRNAs as lung cancer biomarkers. World J. Clin. Oncol. 2014;5:604–620.

Vizcaíno C, Mansilla S, Portugal J. Sp1 transcription factor: A long-standing target in cancer chemotherapy. Pharmacol. Ther. 2015;152:111–124.

Vorselaars VM, Velthuis S, Snijder RJ, Vos JA, Mager JJ, Post MC. Pulmonary hypertension in hereditary haemorrhagic telangiectasia. World J. Cardiol. 2015;7:230–237.

Wain K, Swanson K, Watson W, Jeavons E, Weaver A, Lindor N. Hereditary hemorrhagic telangiectasia and risks for adverse pregnancy outcomes. Am. J. Med. Genet. 2012;158:2009–2014.

Wang G, Fan R, Ji R, Zou W, Penny DJ, Varghese NP, Fan Y. Novel homozygous BMP9 nonsense mutation causes pulmonary arterial hypertension: a case report. BMCPulm. Med. 2016;16:17.

Weber F. Multiple hereditary developmental angiomata (telangiectases) of the skin and mucous membranes associated with recurring haemorrhages. Lancet. 1907;2:160–162.

West J, Austin E, Fessel JP, Loyd J, Hamid R. Rescuing the BMPR2 signaling axis in pulmonary arterial hypertension. Drug Discov. Today. 2014;19:1241–1245.

Wieser R, Wrana JL, Massague J. GS domain mutations that constitutively activate T-beta-R-I, the downstream signalling component in the TGF-beta receptor complex. Embo J. 1995;14:2199–2208.

Willemse RB, Mager JJ, Westermann CJ, Overtoom TT, Mauser H, Wolbers JG. Bleeding risk of cerebrovascular malformations in hereditary hemorrhagic telangiectasia. J. Neurosurg. 2000;92:779–784.

Williams AO, Isaacs RJ, Stowell KM. Down-regulation of human topoisomerase IIalpha expression correlates with relative amounts of specificity factors Sp1 and Sp3 bound at proximal and distal promoter regions. BMC Mol. Biol. 2007;8:36.

Wooderchak-Donahue WL, McDonald J, O'Fallon B, Upton PD, Li W, Roman BL, Young S, Plant P, Fülöp GT, Langa C, Morrell NW, Botella LM, Bernabeu C, Stevenson DA, Runo JR, Bayrak-Toydemir P. BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia. Am. J. Hum. Genet. 2013;93:530–537.

Yu H, Littlewood T, Bennett M. Akt isoforms in vascular disease. Vascul. Pharmacol. 2015;71:57–64.

Zbuk K, Eng C. Hamartomatous polyposis syndromes. Nat clin Pr. gastroenterol Hepatol. 2007;4:492–502.

Zhang Q, Kandic I, Faughnan ME, Kutryk MJ. Elevated circulating microRNA-210 levels in patients with hereditary hemorrhagic telangiectasia and pulmonary arteriovenous malformations: a potential new biomarker. Biomarkers. 2013;18:23–9.

Ziani M, Valignat C, Lopez J, Ruffion A, Plauchu H, Perrin P. Renal arteriovenous malformation requiring surgery in Rendu-Osler-Weber disease (hereditary hemorrhagic telangiectasia). J Urol. 2000;164:1292–1293.

Zucco L, Zhang Q, Kuliszewski MA, Kandic I, Faughnan ME, Stewart DJ, Kutryk MJ. Circulating angiogenic cell dysfunction in patients with hereditary hemorrhagic telangiectasia. PLoS One. 2014;9:1–8.

List of original manuscripts

Articles

Efficacy and safety of thalidomide for the treatment of severe recurrent epistaxis in hereditary haemorrhagic telangiectasia: results of a non-randomised, single-centre, phase 2 study

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Summary

Background Hereditary haemorrhagic telangiectasia is a genetic disease that leads to multiregional angiodysplasia. Severe recurrent epistaxis is the most common presentation, frequently leading to severe anaemia. Several therapeutic approaches have been investigated, but they are mostly palliative and have had variable results. We aimed to assess the efficacy of thalidomide for the reduction of epistaxis in patients with hereditary haemorrhagic telangiectasia that is refractory to standard therapy.

Methods We recruited patients aged 17 years or older with hereditary haemorrhagic telangiectasia who had severe recurrent epistaxis refractory to minimally invasive surgical procedures into an open-label, phase 2, non-randomised, single-centre study at IRCCS Policlinico San Matteo Foundation (Pavia, Italy). We gave patients thalidomide at a starting dose of 50 mg/day orally. If they had no response, we increased the thalidomide dose by 50 mg/day increments every 4 weeks, until a response was seen, up to a maximum dose of 200 mg/day. After patients had achieved a response, they continued treatment for 8–16 additional weeks. The primary endpoint was the efficacy of thalidomide measured as the percentage of patients who had reductions of at least one grade in the frequency, intensity, or duration of epistaxis. We followed up patients each month to assess epistaxis severity score and transfusion need, and any adverse events were reported. We included all patients who received any study drug and who participated in at least one post-baseline assessment in the primary efficacy population. The safety population consisted of all patients who received any dose of study treatment. This trial is registered with ClinicalTrials.gov, number NCT01485224.

Findings Between Dec 1, 2011, and May 12, 2014, we enrolled 31 patients. Median follow-up was 15·9 months (IQR 10·1–22·3). Three (10%, 95% CI 2–26) patients had a complete response, with bleeding stopped, 28 (90%, 95% CI 74–98) patients had partial responses. Overall, all 31 (100%, 89–100) patients responded to therapy with a significant decrease in all epistaxis parameters ($p<0·0001$ for frequency, intensity, and duration). A response was achieved by 25 (81%) patients at 50 mg/day of thalidomide, five (16%) patients at 100 mg/day, and one (3%) patient at 150 mg/day. Patients had only non-serious, grade 1 adverse effects, the most common of which were constipation (21 patients), drowsiness (six patients), and peripheral oedema (eight patients). One patient died a month after the end of treatment, but this was not deemed to be related to treatment.

Interpretation Low-dose thalidomide seems to be safe and effective for the reduction of epistaxis in patients with hereditary haemorrhagic telangiectasia. Our findings should be validated by further studies with larger patient populations, longer follow-up, and that also assess the benefit for quality of life.

Funding Telethon Foundation.

Lancet Haematol 2015; 2: e465–73

Published Online October 27, 2015 http://dx.doi.org/10.1016/S2352-3026(15)00195-7

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Introduction

Hereditary haemorrhagic telangiectasia (also known as Rendu-Osler-Weber syndrome) is an autosomal-dominantly inherited vascular malformation syndrome that is characterised by telangiectasias and large arteriovenous malformations. With a prevalence of one case in every 5000–8000 people depending on the population,[1] hereditary haemorrhagic telangiectasia is one of the most common inherited disorders.

The two major genes implicated in hereditary haemorrhagic telangiectasia are *ENG*, which codes for endoglin, a type 3 transforming growth factor-beta (TGFβ) receptor, and *ACVRL1*, which codes for activin A receptor type II-like kinase 1 (ACVRL1), a type 1 TGFβ and bone morphogenetic protein (BMP) receptor. Mutations in *ENG* lead to type 1 hereditary haemorrhagic telangiectasia (OMIM 187300), and mutations in *ACVRL1* lead to type 2 disease (OMIM 600376). About 85% of patients with hereditary haemorrhagic telangiectasia have a disease-causing mutation in either of these genes.[2] Mutations in other genes, such as *MADH4* (also known as *SMAD4*) and *BMP9* (also known as *GDF2*) are present in 2% or less of cases.[3,4] Although the pathogenesis of hereditary haemorrhagic telangiectasia is poorly

Articles

Research in context

Evidence before this study

From Dec 1, 2010, to March 31, 2015, we searched PubMed with the search terms "hereditary hemorrhagic telangiectasia", "HHT", "epistaxis", "thalidomide", and "clinical trial", without data or language restrictions. The studies that we identified showed that all approaches used for the management of epistaxis in hereditary haemorrhagic telangiectasia, including surgical options, were largely palliative, with variable results and side-effects. There have been no well designed studies to support the effectiveness of medical treatments and most studies had been limited by the absence of a validated sensitive outcome measure. Because angiogenesis had been implicated in the pathogenesis of hereditary haemorrhagic telangiectasia, suggestions were made that anti-angiogenic substances could be effective in the treatment of vascular malformations in this disease. Bleeding inhibition had been reported in patients with hereditary haemorrhagic telangiectasia who received thalidomide as an anti-angiogenic cancer therapy. Moreover, thalidomide treatment induced vessel maturation in an experimental model of hereditary haemorrhagic telangiectasia and reduced severe nosebleeds in small case series and in isolated case reports. Overall, we identified 20 cases from five studies of the use of thalidomide for epistaxis in hereditary haemorrhagic telangiectasia refractory to standard medical and local surgical treatments. On the basis of this evidence, we designed this study to investigate the effectiveness of thalidomide in the reduction of epistaxis and to identify the lowest effective dose in patients with hereditary haemorrhagic telangiectasia.

Added value of this study

The results of our study support the efficacy and safety of low-dose thalidomide for patients with hereditary haemorrhagic telangiectasia. Treatment provided a rapid, often sustained, clinical improvement.

Implications of all the available evidence

Our findings could greatly improve patients' quality of life and promote the search for additional drugs with even better safety and efficacy profiles.

understood, suggestions have been made[5] that mutations in *ENG* or *ACVRL1*, which are mostly expressed on endothelial cells, lead to haploinsufficiency, causing an imbalance in the TGFβ and BMP signalling pathways. This imbalance leads to decreased TGFβ activation and increased VEGF production,[5] which in turn leads to excessive proliferation and migration of endothelial cells, resulting in reduced vessel maturation and thinning of the vascular wall.

The clinical manifestations of hereditary haemorrhagic telangiectasia derive from the vascular malformations, which range from small telangiectasias in the nasal, oral, and gastrointestinal mucosa, to large arteriovenous malformations in the lung, brain, and liver.[1] Recurrent and severe epistaxis caused by the rupture of nasal telangiectasias is the most common presentation of hereditary haemorrhagic telangiectasia. Less often, rupture of telangiectasias in the digestive tract causes gastrointestinal bleeding. Haemorrhages usually worsen with age and lead to severe anaemia requiring intravenous iron and blood transfusions. Furthermore, large arteriovenous malformations can cause morbidity because of rupture or arteriovenous blood shunting.

Although transcatheter embolotherapy with occluder devices is a well established and effective treatment for large arteriovenous malformations in the lung and brain, local treatment is difficult for largely disseminated telangiectasias, and no medical therapy to prevent their rupture has been approved.[6] Because angiogenesis has been implicated in the pathogenesis of hereditary haemorrhagic telangiectasia,[5] anti-angiogenic drugs have been suggested to be effective for the treatment of vascular malformations. Inhibition of bleeding has been reported in patients with hereditary haemorrhagic telangiectasia who received thalidomide as an anti-angiogenic drug for cancer therapy.[7,8] In another study, thalidomide treatment was reported to induce vessel maturation by enhancing pericyte and vascular smooth muscle cell coverage in an animal model of hereditary haemorrhagic telangiectasia, and severe nosebleeds were reduced in length, intensity, and frequency in six of seven patients included in the study.[9]

We aimed to investigate the effectiveness of thalidomide in the prevention of epistaxis and to identify the lowest effective dose for patients with severe forms of hereditary haemorrhagic telangiectasia that were refractory to local therapy.

Methods

Study design and participants

This study was an open-label, phase 2, dose-finding, single-group, non-randomised, single-centre study done at the IRCCS Policlinico San Matteo Foundation (Pavia, Italy). The study protocol was approved by the Ethical Committee of IRCCS Policlinico San Matteo Foundation (Pavia, Italy) and all patients provided signed informed consent before treatment.

We included patients who had a diagnosis of hereditary haemorrhagic telangiectasia according to the Curaçao criteria[10] and severe recurrent epistaxis (grade 2–3 of any epistaxis parameter[11] during the past month) that was refractory to minimally invasive surgical procedures, and who were older than 17 years and able to provide written informed consent. Participants were recruited from all patients who presented to the study centre and met the inclusion criteria. Women who were able to have children

had to agree to follow acceptable birth control methods to avoid conception throughout the study and for 4 weeks after the date of the last dose of thalidomide, to have a negative serum pregnancy test obtained within 48 h before the first dose of thalidomide, and to declare their intention to undergo pregnancy tests periodically while on the study medication. Men with a female partner able to have children had to agree to use an effective method of contraception throughout the study and for 1 week after the date of the final dose of thalidomide. The estimated life expectancy of each patient had to be longer than 10 months.

Women who were pregnant or lactating were excluded. Neurological diseases, psychiatric illness, active cardiovascular disease, and being at high risk for thromboembolic events (diabetes or uncontrolled infections, malignancy, immobility, prior history of thromboembolic events, use of erythropoietic drugs or other drugs such as hormone replacement therapy, central venous catheter, anti-cardiolipin, or anti-beta 2 glycoprotein antibodies) were also exclusion criteria. Patients with hereditary galactose intolerance, the Lapp lactase deficiency, or glucose-galactose malabsorption were also excluded because thalidomide capsules contain lactose.

Procedures

Eligible patients received thalidomide capsules (50 mg per capsule) at a starting dose of 50 mg/day orally before going to sleep, for 4 weeks. If patients had no response, we increased thalidomide dosage by 50 mg/day increments every 4 weeks, until the patient achieved a complete or partial response, up to a maximum dose of 200 mg/day (ie, this phase was potentially up to 16 weeks if a response was only achieved at 200 mg/day or if no response was achieved). Treatment was then continued for 8 additional weeks after a complete response was achieved, 16 additional weeks after a partial response was achieved, and 24 weeks if no response was achieved. The maximum dose was 100 mg/day if patients were older than 74 years. Treatment discontinuation and dose reduction could occur in the case of thromboembolic events, haematological side-effects, peripheral neuropathy, bradycardia, or skin reactions. The appendix (p 1) contains full details of the dose reductions and interruptions. Concomitant use of recombinant human erythropoietin or combined hormonal contraceptive were not allowed during the study. Drugs that cause drowsiness or those that are potentially associated with peripheral neuropathy were used with caution and monitored during the study. After the end of treatment, we followed up patients for at least 52 weeks.

We did clinical assessments and laboratory tests at baseline, every 4 weeks during treatment, and every 4 weeks for at least 52 weeks after the end of treatment. We monitored epistaxis severity, blood counts, number of blood transfusions, endoscopy of nasal mucosa (size and number of telangiectasias), symptoms, complaints, and adverse events. Before treatment, we directly interviewed patients to obtain data for their grades of epistaxis frequency, intensity, and duration during the previous 4 weeks, and afterwards we interviewed them once per month in person or by telephone.[11]

We assessed toxic effects with medical interviews, physical examination, and laboratory tests, including tests of thyroid-stimulating hormone concentrations. Adverse events or adverse drug reactions were recorded in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. We assessed treatment compliance with medical interviews and as the remaining pill count.

DNA extraction was done with the GenElute Blood Genomic DNA Kit (Sigma-Aldrich, St Louis, MO, USA). We did mutational analysis of *ENG* and *ACVRL1* by direct sequence analysis (primers are listed in the appendix p 2). Because cytochrome P450 (CYP)2C subfamily polymorphisms have been reported to partly explain the interpatient variability of in-vivo thalidomide concentrations by altering its metabolism,[12] we also searched for associations between *CYP2C19* and *CYP2C9* polymorphisms and both efficacy and possible side-effects of the drug. We examined three *CYP450* variant sequences via the PCR-RFLP method:[13,14] *CYP2C19*2*, a transition (+681G→A) in exon 5 that produces an aberrant splice site; *CYP2C9*2*, a transition (+430C→T) in exon 3 that produces an amino acid change (Arg to Cys), and *CYP2C9*3*, a transition (+1075A→C) in exon 7 that produces an amino acid change (Ile to Leu).

Outcomes

The primary endpoint was to assess the efficacy of thalidomide, measured as the proportion of patients showing a reduction of at least one grade in the frequency, intensity, or duration of epistaxis. For each epistaxis parameter, there were three levels of severity for each 4-week period (table 1).[11] Secondary endpoints were patients' need for blood transfusions, minimum dose of thalidomide needed to reduce bleeding, time to response, time to relapse after end of treatment, and the safety and tolerability of the drug. We also searched for associations between the mutations responsible for hereditary haemorrhagic telangiectasia and response to treatment. We planned to evaluate size and number of telangiectasias by endoscopy of the nasal mucosa (recording images of the size and localisation of telangiectasias) at baseline and every 8 weeks.

See Online for appendix

	Frequency	Intensity	Duration
Grade 1	Less than one episode per week	Slight stains on the handkerchief	<10 min
Grade 2	At least one episode per week	Soaked handkerchief	10–30 min
Grade 3	More than one episode per day	Bowl or similar utensil necessary	>30 min

If patients reported no epistaxis during the past 4 weeks, zero points were awarded for each parameter.

Table 1: Epistaxis parameter grades[11]

Articles

Statistical analysis

We did the sample size calculation using Fleming's single-stage procedure.[15] We assumed that treatment would yield responses in about 75% of patients on the basis of the results reported in small case series and that, in view of a possible high frequency of side-effects, a proportion of responses less than 50% would be of no interest. Recruitment of 31 patients would give the trial a power of 90% to detect an effects size of 25% or more, with a one-sided α of 5%.

	Patients (n=31)
Age (years)	62·6 (11·1)
Sex	
Male	20 (65%)
Female	11 (35%)
Epistaxis severity grade	
2	5 (16%)
3	26 (84%)
RBC transfusion dependence	23 (74%)
Previous treatments	
Argon plasma coagulation	17 (55%)
Electrocauterisation	15 (48%)
Embolisation	6 (19%)
Laser coagulation	2 (6%)
Septodermoplasty	3 (10%)
Arterial ligation	1 (3%)
Organ involvement	
Telangiectasias	
Nose*	31 (100%)
Skin	29 (94%)
Gastrointestinal tract*	9 (29%)
Large arteriovenous malformations	
Lung†	9 (29%)
Liver‡	13 (42%)
CNS†	1 (3%)

Data are mean (SD) or n (%). RBC=red blood cell. *Identified by endoscopy. †Identified by computed tomography. ‡Identified by ultrasound.

Table 2: **Presenting characteristics**

The full analysis population consisted of all patients who met the inclusion and exclusion criteria, received any study drug, and participated in at least one post-baseline assessment. We used this population as the primary efficacy population. The primary safety population consisted of all patients who received any dose of study treatment, excluding those who dropped out before receiving any treatment.

We calculated the primary endpoint as the percentage of patients showing a reduction of at least one grade in one of the epistaxis parameters. We calculated the difference between baseline scores and frequency, intensity, and duration scores at each timepoint during treatment. If any of these differences was more than zero, the patient was deemed a responder. A complete responder was defined as a patient with all three epistaxis scores equal to zero. Reductions in the severity of any bleeding parameter to anything other than zero represented a partial response. Both complete and partial response had to be maintained for at least 4 weeks. Failure to achieve at least a partial response was defined as no response, whereas relapse after complete or partial response was defined as the regression from complete response to any other degree of response or return from partial response to pretreatment severity of bleeding parameters.

We summarised continuous variables using n (non-missing sample size), mean, SD, median, and IQR. The frequency and percentages (based on the non-missing sample size) of reported outcome levels were reported for all categorical measures.

We calculated the overall numbers and percentage of responders to treatment with binomial 95% CIs. Cumulative relapse-free survival after the end of treatment was plotted on a Kaplan-Meier curve. We calculated the mean number of blood transfusions during the study period, together with a Poisson 95% CI, and we analysed the change in the number of transfused units per month using a repeated measures negative binomial regression model. We assessed the difference between baseline and end-of-treatment haemoglobin concentrations with the

	Frequency				Intensity				Duration			
	0	1	2	3	0	1	2	3	0	1	2	3
Week 0	0/31	2/31 (6%)	10/31 (32%)	19/31(61%)	0/31	1/31 (3%)	4/31 (13%)	26/31 (84%)	0/31	5/31 (16%)	15/31 (48%)	11/31 (35%)
Week 4	1/31 (3%)	6/31 (19%)	20/31 (65%)	4/31 (13%)	1/31 (3%)	10/31 (32%)	12/31 (39%)	8/31 (26%)	1/31 (3%)	15/31 (48%)	10/31 (32%)	5/31 (16%)
Week 8	0/31	10/31 (32%)	17/31 (55%)	4/31 (13%)	0/31	16/31 (52%)	11/31 (35%)	4/31 (13%)	0/31	25/31 (81%)	6/31 (19%)	0/31
Week 12	1/31 (3%)	11/31 (36%)	18/31 (58%)	1/31 (3%)	1/31 (3%)	17/31 (55%)	8/31 (26%)	5/31 (16%)	1/31 (3%)	24/31 (77%)	5/31 (16%)	1/31 (3%)
Week 16	2/30 (7%)	11/30 (37%)	16/30 (53%)	1/30 (3%)	2/30 (7%)	18/30 (60%)	6/30 (20%)	4/30 (13%)	2/30 (7%)	22/30 (73%)	5/30 (17%)	1/30 (3%)
Week 20	2/28 (7%)	14/28 (50%)	11/28 (39%)	1/28 (4%)	2/28 (7%)	17/28 (61%)	6/28 (21%)	3/28 (11%)	2/28 (7%)	21/28 (75%)	5/28 (18%)	0/28
Week 24	0/7	1/7 (14%)	5/7 (71%)	1/7 (14%)	0/7	4/7 (57%)	3/7 (43%)	0/7	0/7	6/7 (86%)	1/7 (14%)	0/7
Week 28	0/1	1/1 (100%)	0/1	0/1	0/1	1/1 (100%)	0/1	0/1	0/1	1/1 (100%)	0/1	0/1

Data are n/N (%).

Table 3: **Response to therapy on the basis of epistaxis parameters**

paired Student's *t* test. We fitted a linear regression model for repeated measures to describe changes with time.

We assessed the associations of mutations and polymorphisms of cytochromes *CYP2C19* and *CYP2C9* with response by use of Fisher's exact test. We assessed the association with relapse with the log-rank test.

Patients were deemed to represent a protocol deviation if they dropped out or had their study drug treatment interrupted for reasons other than those outlined by protocol (eg, unacceptable toxicity). These patients could not be assessed for a response.

We used Stata 13.1 for all analyses. The study was monitored by the Clinical Trial Quality Team of the IRCCS Policlinico San Matteo Foundation (Pavia, Italy).

This trial is registered with ClinicalTrials.gov, number NCT01485224, and EudraCT, number 2011-004096-36.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Dec 1, 2011, and May 12, 2014, we enrolled 31 patients, including 20 men and 11 women, with median age 64 years (range 44–84; table 2). We detected various types of mutations in *ACVRL1* (25 [81%] patients) or *ENG* (four [13%] patients). Median follow-up was 15·9 months (IQR 10·1–22·3). 14 (45%) patients were receiving iron therapy. During the study period, no other drugs for epistaxis were used by patients.

We assessed all patients for response. Treatment caused bleeding to stop for three (10%, 95% CI 2–26) patients and 28 (90%, 95% CI 74–98) patients had a significant

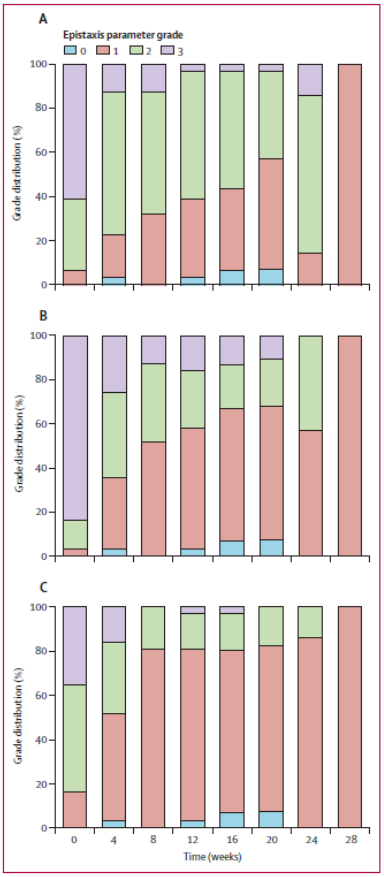

Figure 1: **Change in patient epistaxis parameters with time**
Epistaxis parameters are frequency (A), intensity (B), and duration (C).

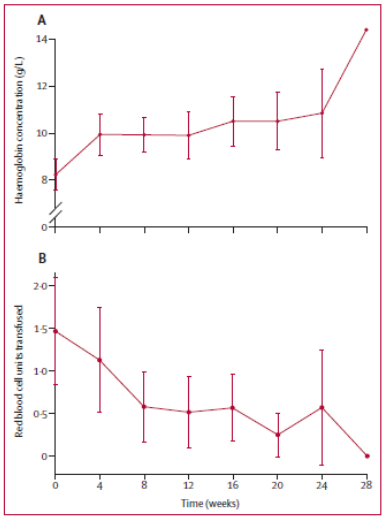

Figure 2: **Haemoglobin concentrations (A) and transfusion need (B) with time**
Values are means of all patients at each timepoint (95% CI).

decrease in all epistaxis parameters with 31 (100%, 95% CI 89–100) achieving a response (table 3, figure 1). All patients had a significant decrease in all epistaxis parameters ($p<0·0001$). We detected significant differences in the distribution of the scores for epistaxis frequency ($p=0·00031$), intensity ($p<0·0001$), and duration ($p=0·019$) within 4 weeks after patients started thalidomide. Complete or partial responses were achieved by 25 (81%) patients at 50 mg/day of thalidomide, five (16%) patients achieved partial responses at 100 mg/day, and one (3%) patient achieved a partial response at 150 mg/day. 25 (81%) patients achieved a response after 4 weeks, five (16%) following 8 weeks, and one (3%) after 12 weeks of treatment. Time to response was associated with dose of thalidomide.

Treatment significantly increased patients' haemoglobin concentrations ($p=0·00011$), with a maximum increase of 22·7 g/L (95% CI 11·3–34·2), and removed or greatly reduced the need for transfusion ($p<0·0001$), with a maximum decrease of 1·77 (95% CI 0·70–2·84) transfused red blood cell units per month (figure 2). 20 (87%, 95% CI 66–97) of the 23 patients who were dependent on transfusions became transfusion independent. During thalidomide treatment, patients' doses of iron therapy were not changed, except for three patients for whom iron therapy was discontinued because of the rapid improvement of their haemoglobin concentrations.

Assessment of the recordings obtained during rhinoscopy did not allow us to show notable differences in the morphology and number of telangiectasias of the nasal mucosa before and after treatment (data not shown). However, at enrolment, the examination was limited by the presence of crusting that prevented us from obtaining optimum images to be compared with those after treatment. We tried to examine 15 patients, but it was not possible to compare the results before treatment with those after treatment for any patients because of the poor image quality.

Patients had only non-serious, grade 1, adverse effects during treatment, the most common of which were constipation and drowsiness (table 4). Thalidomide did not need to be discontinued for any patients. Three patients had their thalidomide dosages temporarily reduced because of paraesthesia (two patients) or marked asthenia and dizziness (one patient). These patients could be assessed for a response and they were not excluded because the thalidomide dose was reduced only for a short time. Slight increases in thyroid-stimulating hormone (TSH) concentrations were detected in five patients, but without any clinical symptoms. No patients had thromboembolic events. Good treatment compliance was recorded for all patients.

30 patients completed treatment. One patient, who achieved a partial response after 8 weeks of treatment at a dose of 100 mg/day thalidomide, noticed progressive worsening of epistaxis in the following weeks and underwent surgical treatment. An 80-year-old male patient suddenly died a month after the end of treatment for unknown reasons. We cannot completely exclude an association with therapy, although no side-effects had been recorded for this patient during treatment. At a median follow-up after the end of therapy of 14·1 months

	Cumulative number of patients (%)						
	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks	28 weeks
Constitutional symptoms							
Asthenia	0/31	2/31 (6%)	2/31 (6%)	3/30 (10%)	3/28 (11%)	3/7 (43%)	0/1
Peripheral oedema	2/31 (6%)	5/31 (16%)	7/31 (23%)	8/30 (27%)	7/28 (25%)	0/7	0/1
Gastrointestinal symptoms							
Constipation	9/31 (29%)	15/31 (48%)	19/31 (61%)	21/30 (70%)	20/28 (71%)	4/7 (57%)	1/1 (100%)
Vomiting	0/31	1/31 (3%)	0/31	0/30	0/28	0/7	0/1
Neurological symptoms							
Dizziness	3/31 (10%)	4/31 (13%)	5/31 (16%)	5/30 (17%)	5/28 (18%)	1/7 (14%)	0/1
Drowsiness	2/31 (6%)	5/31 (16%)	6/31 (19%)	6/30 (20%)	6/28 (21%)	1/7 (14%)	0/1
Peripheral neuropathy	0/31	0/31	1/31 (3%)	2/30 (7%)	3/28 (10·7%)	1/7 (14%)	0/1
Psychiatric symptoms							
Depression	0/31	0/31	1/31 (3%)	1/30 (3%)	1/28 (4%)	0/7	0/1
Cardiovascular symptoms							
Bradycardia	0/31	1/31 (3%)	1/31 (3%)	1/30 (3%)	1/28 (4%)	1/7 (14%)	0/1
Haematological symptoms							
Leucopenia	1/31 (3%)	1/31 (3%)	1/31 (3%)	2/30 (7%)	2/28 (7%)	1/7 (14%)	0/1
Increase in thyroid-stimulating hormone	2/31 (6%)	3/31 (10%)	4/31 (13%)	4/30 (13%)	5/28 (18%)	1/7 (14%)	0/1

Data are n/N (%). All adverse events were grade 1.

Table 4: **Safety endpoints by each timepoint**

(IQR 10·9–21·9), eight (27%) patients maintained a response, whereas 21 (70%) patients relapsed, with a median relapse-free survival of 7·0 months (95% CI 6·1–9·6; figure 3). We detected no associations between clinical features, such as age, sex, epistaxis severity, and time to response, response duration, or toxicity (data not shown). Neither causative gene mutations nor *CYP2C19*/*CYP2C9* polymorphisms were significantly associated with response to therapy or toxic effects (appendix pp 3, 4).

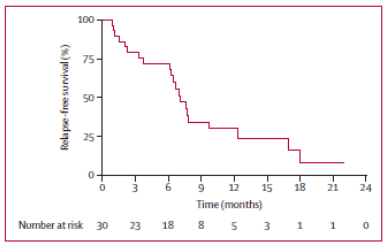

Figure 3: **Relapse-free survival after end of treatment**
One patient was censored from the analysis, because they relapsed before the end of treatment.

Discussion

To our knowledge, this is the first prospective study to assess the efficacy of thalidomide in a consistent group of patients with hereditary haemorrhagic telangiectasia. Our results suggest that low-dose thalidomide is safe and effective in the prevention of severe epistaxis in patients who have not benefited from traditional treatment approaches, providing a rapid and often sustained clinical improvement.

Despite advances in the understanding of the pathogenesis of hereditary haemorrhagic telangiectasia, treatments for affected individuals have not substantially improved. Control of epistaxis still represents a major problem; as many as 90% of affected individuals have recurrent nosebleeds by age 30 years.[16] Moreover, the nosebleeds worsen with age, causing repeated hospital admissions, chronic anaemia with severe iron deficiency, and transfusion dependency. Various treatment options allow otorhinolaryngologists to control nosebleeds, but these interventions often lose efficacy over time and patients eventually need risky or debilitating treatments, such as embolisation of the nasal vasculature or nose closure.[6]

Thus, a need exists for medical treatments that prevent epistaxis. Systemic administration of antifibrinolytic drugs has been used empirically, but two prospective clinical trials[17,18] have concluded that the efficacy of these drugs was low and they did not lead to increases in haemoglobin concentrations. Conflicting results have been reported for hormone therapy: case reports and small case series[19] have suggested that systemic oestrogens, oestrogens plus progesterone, and progesterone alone might be effective to prevent epistaxis. However, a randomised clinical trial[20] reported no difference in bleeding between patients who received oestrogen and those who received placebo. Moreover, severe side-effects from hormone therapy have been reported, especially in men and postmenopausal women, reducing the appeal of such an approach. Finally, in a double-blind, placebo-controlled clinical trial,[21] nine of ten patients receiving the anti-oestrogenic drug tamoxifen subjectively judged that both the frequency and intensity of their nosebleeds had been reduced during treatment, while only three of 11 patients who received a placebo reported favourable responses. However, haemoglobin concentrations and need for blood transfusions were not different between the two groups of patients, which raises doubts about the real effectiveness of this treatment.[21]

Advances in knowledge about the pathophysiology of vascular malformations in hereditary haemorrhagic telangiectasia have offered the potential for intervention in the regulation of the mechanisms of angiogenesis to not only change already formed abnormalities, but also to prevent their formation. Because VEGF concentrations are substantially increased in hereditary haemorrhagic telangiectasia and are implicated in its pathogenesis, bevacizumab, a humanised recombinant monoclonal antibody against VEGF, has been investigated for both systemic and topical use. Systemic bevacizumab given to 25 patients for 2·5 months reduced the duration of epistaxes but did not significantly change their frequency.[22] Moreover, results from two controlled trials did not show any significant benefit for submucosal injection or nasal spray administration of bevacizumab,[23,24] and therefore contradicted previous uncontrolled studies that reported the efficacy of local administration of this drug.[25–28]

Thalidomide is another molecule of potential interest for hereditary haemorrhagic telangiectasia treatment. Thalidomide was introduced in the late 1950s to prevent nausea during pregnancy, but was withdrawn from the market because of teratogenicity.[29] However, subsequent discoveries that thalidomide has immunomodulatory, anti-inflammatory, and anti-angiogenic activity stimulated clinical trials of thalidomide for immune and inflammatory disorders, and in various malignancies. On the basis of the results of these studies,[30] thalidomide was approved by the US Food and Drug Administration (FDA) for erythema nodosum leprosum and multiple myeloma. The observation that thalidomide inhibited bleeding in a few individuals with hereditary haemorrhagic telangiectasia who received the drug as an anti-angiogenic cancer therapy[7,8] encouraged researchers to try giving it to other patients with hereditary haemorrhagic telangiectasia. Five studies reported favourable effects for the treatment of epistaxis in 17 of 20 patients receiving doses of thalidomide ranging from 50 to 200 mg/day.[9,31]

Importantly, treatment was well tolerated and the only notable side-effect was deep vein thrombosis in one patient who received 100 mg of thalidomide per day.[32]

The results of our study support the efficacy of thalidomide for hereditary haemorrhagic telangiectasia because nosebleeds were reduced for all 31 treated patients. More importantly, patients' average haemoglobin concentrations were increased and their need for transfusions was reduced. We detected no differences in response between patients with mutations in *ENG* and *ACVRL1* or in patients with different *CYP450* polymorphisms. However, the study was not powered to detect such associations. Another finding was that 25 of 31 patients achieved remission with 50 mg/day thalidomide, a dose that is much lower than that used in all other disorders for which the drug has been approved or used experimentally. This low dosage is probably the reason why thalidomide was well tolerated in our case series. In particular, no patients had deep vein thromboses, the side-effect of most concern for patients receiving thalidomide for haematological malignancies. Furthermore, peripheral neuropathy, another common side-effect of thalidomide, occurred in only two patients. Neuropathy was mild and disappeared after dosage reduction. Despite these reassuring data, we cannot conclude that patients with hereditary haemorrhagic telangiectasia who are receiving low-dose thalidomide are not at risk of thromboembolic events and severe neuropathy, because patients with high thrombotic risk and pre-existing neurological disorders were excluded from our study.

Understanding the mechanism of action of thalidomide in hereditary haemorrhagic telangiectasia would provide opportunities for the development of more effective therapies, but the exact mechanism remains a matter of debate. Decades of investigation have identified several biological effects of thalidomide. The drug suppresses TNFα and affects the generation of pro-inflammatory cytokines. Moreover, thalidomide inhibits production of VEGF and basic fibroblast growth factor, thus antagonising angiogenesis and modifying the bone marrow micoenvironment.[33] Although thalidomide has previously been hypothesised to benefit patients with hereditary haemorrhagic telangiectasia by directly inhibiting endothelial cell proliferation and migration, more recent data have supported the notion that it modulates the activation of mural cells, enhancing both their proliferation and ability to envelop blood vessels[9]—ie, thalidomide makes blood vessels of people with hereditary haemorrhagic telangiectasia firmer and less prone to breaking. We have no data that directly supports this theory, but our finding that the median time to relapse after the end of treatment was more than 6 months fits with this notion. If thalidomide promotes the formation of firmer vessel walls, we would expect the reduction in bleeding to last even after the drug is discontinued.

Our finding that a course of low-dose thalidomide seems to be effective for transient reduction of epistaxis in hereditary haemorrhagic telangiectasia suggests the possibility of identifying treatment schedules that permanently reduce epistaxis. In this respect, continuous treatment with further reduced dosages might be effective and without significant side-effects. Alternatively, the same result might be achieved with treatments given in cycles rather than continuously. Furthermore, another important question is whether thalidomide is also effective for the prevention of gastrointestinal bleeding in hereditary haemorrhagic telangiectasia and whether it has any effect on arteriovenous malformations and cardiac output or pulmonary hypertension.

The most important weakness of our study is that it was not randomised; the rarity of hereditary haemorrhagic telangiectasia and the absence of a drug known to be effective to compare with thalidomide discouraged us from doing a controlled study. Although hereditary haemorrhagic telangiectasia is one of the most common forms of inherited disorder, it is still rare; moreover, patients with phenotypically severe disease, refractory to previous standard treatments and who do not have contraindications to the use of thalidomide represent only a small subset. In this setting, we designed a single-arm study with a 90% power. The second weakness is that we were unable to assess the effect of thalidomide on telangiectasias of the nasal mucosa. Finally, we did not formally investigate the effect of thalidomide on patients' quality of life. However, reduction of epistaxis was reported by the patients themselves, and this is very likely to be associated with their perceived quality of life.

Our findings should be validated by further studies with larger patient populations, longer follow-up, and that also assess the benefit for quality of life. We expect our finding that thalidomide might be effective for the reduction of epistaxis in hereditary haemorrhagic telangiectasia to promote the search for additional drugs with even better safety and efficacy profiles.

Contributors

RI contributed to the conception and design of the study, patient enrolment and treatment, analysis and interpretation of the data, and the writing of the manuscript. FQ collected clinical data and participated in the data analysis and interpretation. CK did the statistical analysis and contributed to the design of the study, the writing of the protocol, and the data interpretation. FP, FC, EM, and GS contributed to patient screening and recruitment, data collection and interpretation, and did the endoscopy of nasal mucosa. FO, SP, PG, and CO did the molecular biology studies, collected data, and participated in the data analysis and interpretation. FB and RB collected data and contributed to the data analysis and interpretation. MB contributed to study design, analysis, and interpretation of the data. CD contributed to the study design, coordination of molecular biology studies, analysis and interpretation of data, and reviewing of the manuscript. CLB contributed to the study conception and design, clinical trial coordination, data analysis and interpretation, writing and reviewing the manuscript.

Declaration of interests

We declare no competing interests.

Acknowledgments

The sponsor of the study was the IRCCS Policlinico San Matteo Foundation (Pavia, Italy). The study was an academic study sponsored by a non-profit scientific foundation. There was no commercial support for

the trial. The costs of the study were covered with research funds from the Telethon Foundation, an Italian charity organisation that invests in the research of genetic diseases (Telethon Grant number GGP13036). We thank all patients, their families and physicians for participation in the study, and the Associazione Fondazione HHT "Onilde Carini" and HHT Onlus.

References

1 Shovlin CL. Hereditary hemorrhagic telangiectasia: pathophysiology, diagnosis and treatment. *Blood Rev* 2010; **24:** 203–19.

2 McDonald J, Wooderchak-Donahue W, VanSant Webb C, Whitehead K, Stevenson DA, Bayrak-Toydemir P. Hereditary hemorrhagic telangiectasia: genetics and molecular diagnostics in a new era. *Front Genet* 2015; **6:** 1.

3 Gallione CJ, Repetto GM, Legius E, et al. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). *Lancet* 2004; **363:** 852–59.

4 Wooderchak-Donahue WL, McDonald J, O'Fallon B, et al. BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia. *Am J Hum Genet* 2013; **93:** 530–37.

5 Sadick H, Hage J, Goessler U, et al. Does the genotype of HHT patients with mutations of the ENG and ACVRL1 gene correlate to different expression levels of the angiogenic factor VEGF? *Int J Mol Med* 2008; **22:** 575–80.

6 Faughnan ME, Palda VA, Garcia-Tsao G, et al. International guidelines for the diagnosis and management of hereditary hemorrhagic telangiectasia. *J Med Genet* 2011; **48:** 73–87.

7 Kurstin R. Using thalidomide in a patient with epithelioid leiomyosarcoma and Osler-Weber-Rendu disease. *Oncology (Williston Park)* 2002; **16:** 21–24.

8 Pérez-Encinas M, Rabuñal Martínez MJ, Bello López JL. Is thalidomide effective for the treatment of gastrointestinal bleeding in hereditary hemorrhagic telangiectasia? *Haematologica* 2002; **87:** ELT34.

9 Lebrin F, Srun S, Raymond K, et al. Thalidomide stimulates vessel maturation and reduces epistaxis in individuals with hereditary hemorrhagic telangiectasia. *Nat Med* 2010; **16:** 420–28.

10 Shovlin CL, Guttmacher AE, Buscarini E, et al. Diagnostic criteria for hereditary hemorrhagic telangiecta (Rendu-Osler-Weber syndrome). *Am J Med Genet* 2000; **91:** 66–67.

11 Pagella F, Colombo A, Matti E, et al. Correlation of severity of epistaxis with nasal telangiectasias in hereditary hemorrhagic telangiectasia (HHT) patients. *Am J Rhinol Allergy* 2009; **23:** 52–58.

12 Ando Y, Fuse E, Figg WD. Thalidomide metabolism by the CYP2C subfamily. *Clin Cancer Res* 2002; **8:** 1964–73.

13 Ando Y, Price DK, Dahut WL, Cox MC, Reed E, Figg WD. Pharmacogenetic associations of CYP2C19 genotype with in vivo metabolisms and pharmacological effects of thalidomide. *Cancer Biol Ther* 2002; **1:** 669–73.

14 Goldstein JA. CYP2C9 polymorphisms and CYP2C9*2 genotyping primers. *Br J Clin Pharmacol* 2002; **53:** 409–10.

15 Fleming TR. One-sample multiple testing procedure for phase II clinical trials. *Biometrics* 1982; **38:** 143–51.

16 Lesca G, Olivieri C, Burnichon N, et al. Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. *Genet Med* 2007; **9:** 14–22.

17 Gaillard S, Dupuis-Girod S, Boutitie F, et al. Tranexamic acid for epistaxis in hereditary hemorrhagic telangiectasia patients: a European cross-over controlled trial in a rare disease. *J Thromb Haemost* 2014; **12:** 1494–502.

18 Geisthoff UW, Seyfert UT, Kübler M, Bieg B, Plinkert PK, König J. Treatment of epistaxis in hereditary hemorrhagic telangiectasia with tranexamic acid—a double-blind placebo-controlled cross-over phase IIIB study. *Thromb Res* 2014; **134:** 565–71.

19 Jameson JJ, Cave DR. Hormonal and antihormonal therapy for epistaxis in hereditary hemorrhagic telangiectasia. *Laryngoscope* 2004; **114:** 705–09.

20 Vase P. Estrogen treatment of hereditary hemorrhagic telangiectasia. A double-blind controlled clinical trial. *Acta Med Scand* 1981; **209:** 393–96.

21 Yaniv E, Preis M, Hadar T, Shvero J, Haddad M. Antiestrogen therapy for hereditary hemorrhagic telangiectasia: a double-blind placebo-controlled clinical trial. *Laryngoscope* 2009; **119:** 284–88.

22 Dupuis-Girod S, Ginon I, Saurin JC, et al. Bevacizumab in patients with hereditary hemorrhagic telangiectasia and severe hepatic vascular malformations and high cardiac output. *JAMA* 2012; **307:** 948–55.

23 Dupuis-Girod S, Ambrun A, Decullier E, et al. ELLIPSE Study: a phase I study evaluating the tolerance of bevacizumab nasal spray in the treatment of epistaxis in hereditary hemorrhagic telangiectasia. *MAbs* 2014; **6:** 794–99.

24 Riss D, Burian M, Wolf A, Kranebitter V, Kaider A, Arnoldner C. Intranasal submucosal bevacizumab for epistaxis in hereditary hemorrhagic telangiectasia: a double-blind, randomized, placebo-controlled trial. *Head Neck* 2015; **37:** 783–87.

25 Simonds J, Miller F, Mandel J, Davidson TM. The effect of bevacizumab (Avastin) treatment on epistaxis in hereditary hemorrhagic telangiectasia. *Laryngoscope* 2009; **119:** 988–92.

26 Guldmann R, Dupret A, Nivoix Y, Schultz P, Debry C. Bevacizumab nasal spray: noninvasive treatment of epistaxis in patients with Rendu Osler disease. *Laryngoscope* 2012; **122:** 953–55.

27 Karnezis TT, Davidson TM. Treatment of hereditary hemorrhagic telangiectasia with submucosal and topical bevacizumab therapy. *Laryngoscope* 2012; **122:** 495–97.

28 Rohrmeier C, Sachs HG, Kuehnel TS. A retrospective analysis of low dose, intranasal injected bevacizumab (Avastin) in hereditary hemorrhagic telangiectasia. *Eur Arch Otorhinolaryngol* 2012; **269:** 531–36.

29 Speirs AL. Thalidomide and congenital abnormalities. *Lancet* 1962; **1:** 303–05.

30 Melchert M, List A. The thalidomide saga. *Int J Biochem Cell Biol* 2007; **39:** 1489–99.

31 Franchini M, Frattini F, Crestani S, Bonfanti C. Novel treatments for epistaxis in hereditary hemorrhagic telangectasia: a systematic review of the clinical experience with thalidomide. *J Thromb Thrombolysis* 2013; **36:** 355–57.

32 Penaloza A, Vekemans MC, Lambert C, Hermans C. Deep vein thrombosis induced by thalidomide to control epistaxis secondary to hereditary haemorrhagic telangiectasia. *Blood Coagul Fibrinolysis* 2011; **22:** 616–18.

33 Thalgott J, Dos-Santos-Luis D, Lebrin F. Pericytes as targets in hereditary hemorrhagic telangiectasia. *Front Genet* 2015; **6:** 37.

Atlas of Genetics and Cytogenetics
in Oncology and Haematology

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Gene Section
Review

ACVRL1 (activin A receptor type II-like 1)

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Published in Atlas Database: March 2014

Online updated version : http://AtlasGeneticsOncology.org/Genes/ACVRL1ID569ch12q13.html
DOI: 10.4267/2042/54160

Abstract

Activin A receptor, type II-like kinase 1 (ALK1 is a serine-threonine kinase) predominantly expressed on endothelial cells surface. Mutations in its ACVRL1 encoding gene (12q11-14) cause type 2 Hereditary Haemorrhagic Telangiectasia (HHT2), an autosomal dominant multisystem vascular dysplasia. Its involvement in cancer neoangiogenesis has lead to the recent development of novel anti-cancer drugs, which are now in clinical trials.

Identity

Other names: ACVRLK1, ALK-1, ALK1, HHT, HHT2, ORW2, SKR3, TSR-I

HGNC (Hugo): ACVRL1

Location: 12q13.13

DNA/RNA

Note

Starts at 52300692 and ends at 52307134 bp from pter (according to hg19- Feb_2009).

Description

ACVRL1 is a protein coding gene and in human it is constituted by 10 exons. All exons but the first are coding exons. ACVRL1 transcript variants mRNA3 and mRNA4 include 11 exons, through the presence of a cryptic non-translated exon upstream of the canonical exon 1 (Garrido-Martin et al., 2010).

Transcription

Gene database underlines the presence of two different ACVRL1 transcripts, which both translate into the same protein isoform. The second transcript variant is the shortest one and differs from the first one in the 5'UTR region, due to the presence of an upstream in-frame start codon, poorly conserved in the population. Nevertheless, in 2010 two new transcripts were discovered in HUVEC cells. These new variants, called mRNA3 and mRNA4, begin the transcription +1 nucleotide upstream , respectively at -510 and -470 positions, adding a cryptic non translated exon, that doesn't affect the protein ORF (Garrido-Martin et al., 2010).

The promoter region of ACVRL1 (5' proximal region: -1035/+210) was characterized by Garrido-Martin et al., 2010. This region lacks TATA/CAAT boxes but contains a high number of GC-rich Sp1 consensus sites. It also shares different putative regulatory elements with other endothelial-specific genes. These motifs includes: Ets (E26-Transformation-Specific), KLF (Krüppel-Like Factor), NFkB (Nuclear Factor kappa-light-chain-enhancer of activated B cells), E2F (Elongation Factor 2), one Smad binding element (SBE), RXR (Retinoid X Receptor) and HIF (Hypoxia Inducible Factor). Moreover, the authors demonstrated that methylation status of CpG islands modulates Sp1 transcription of ACVRL1 in endothelial cells.

In 2013, it has been demonstrated that ubiquitin E3 ligase, EDD, can down-regulate ACVRL1 expression in HeLa and HUVEC cells (Chien et al., 2013).

Protein

Description

Activin A receptor, type II-like kinase 1 (also called ALK1, Uniprot entry P37023, protein family (pfam) 01064 of Activin types I and II receptor domains), is a serine-threonine kinase and it acts as a type I receptor for the Transforming Growth Factor-β / Bone Morphogenetic Protein (TGF-β/BMP) superfamily of ligands. It includes 503 amino acids, with residues 1-21 forming a leader sequence which targets the protein to the membrane. The extracellular domain includes amino acids 22-118 and it is followed by a 23 amino acid long transmembrane domain (residues 119-141). The intracellular domain comprises residues 142-503, with a GS domain (residues 172-201) and the protein kinase domain (residues 202-492).

The crystal structure of ALK1 ectodomain (Figure 1a) and of the intracellular kinase domain (Figure 1b) have been recently determined (PDB ID: 4FAO and 3MY0, respectively) (Townson et al., 2012).

Like all type I and type II receptors, ALK1 shows a general fold resembling a class of neurotoxins known as three-finger toxins and hence called "three-finger toxin fold". This fold is comprised from β-strands stabilised by disulphide bonds formed by conserved Cys residues. Three pairs of anti-parallel β-strands are curved to generate a concave surface. Despite the common architecture and the cluster of conserved Cys residues, very little sequence identity and no functional overlap exist between the two types of receptors.

BMPs consist of a Cys knot characterised by three pairs of highly conserved disulphide bonds in which one traverses through a ring formed by the other 2. This fold can be described as a hand with a concave palm side and two parallel β-sheet forming 4 fingers, with each β-strand being likened to a finger. Finger 2 leads to a helix "wrist" region. In the dimeric ligand the 4 fingers extend from the Cys core of the protein like butterfly wings. Binding of type I receptors occurs near the α-helix on the concave side at the junction between the two subunits (Kirsch et al., 2000), whereas binding to type II receptors happens on the convex side of the hand near the "fingertips" (Greenwald et al., 2003; Thompson et al., 2003).

Expression

ALK1 is predominantly expressed on the endothelial cells surface of arteries. According to EBI gene expression database, ALK1 levels are reduced in non-small cell lung cancer tissue, and increased in monocytes exposed to infections by Francisella tularensis novocida and by Porphyromonas gengivalis.

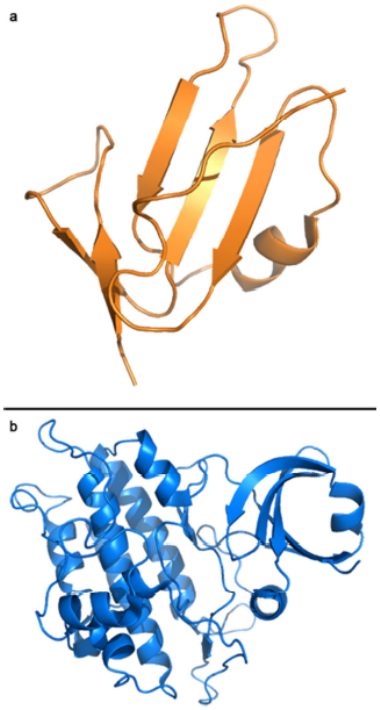

Figure 1: Structures of ALK1 ectodomain (a) and of the kinase domain (b).

Function

ALK1 activation, triggered by its physiological ligand BMP-9, can be pro-angiogenic or anti-angiogenic, depending on the experimental system considered. Thus, inhibition of primary cells (HMVEC-D, HUVEC and endothelial cells) proliferation was observed upon activation of the receptor, suggesting that this signaling pathway is involved in the resolution phase of angiogenesis, during which endothelial cell proliferation and migration stop. Disruption of the pathway would therefore lead to persistent proliferation of endothelial cells with the lack of a correct morphogenesis.

On the other hand, MESEC (mouse embryonic-stem-cell-derived endothelial cells) and MEEC (mouse embryonic endothelial cells) cells are stimulated to proliferate by ALK1 activation and BMP9 stimulates angiogenesis in a matrigel plug assay and in a tumour model in vivo. Also, cancer cells produced tumours whose size and

vascularization were reduced by 50% in ALK1+/- heterozygous mice compared with tumours implanted in wild-type littermates.
In addition, a soluble ALK1-Fc fusion protein known as Dalantercept (ACE-041) showed an anti-angiogenic effect by reducing vascular density and perfusion of the tumour burden in model mice of endocrine pancreatic tumorigenesis and mice bearing 786-0 and A498 human renal cell carcinoma (Wang et al., 2012).
This contradictory findings may be explained by the site- and context-dependent balance of the synergic proangiogenic effects of BMP-9 and the lower affinity ALK1 ligand TGF-β, but the assumption has to be confirmed.
Recent studies also report a role for ALK1 in cancer independent from its effects on angiogenesis, enhancing the cell migration and invasion potential in cancers like squamous cell carcinomas of the head and the neck or haepatocarcinomas (Hu-Lowe et al., 2011; Chien et al., 2013; Sun et al., 2013).
ALK1 signalling through SMAD 1/SMAD 5/SMAD 8 seems to induce chondrocytes hypertrophy in cartilages by an effect mediated by the interaction with the canonical Wnt signaling (van den Bosch et al., 2014).
Again, in other kind of cancers ALK1 activation seems to be protective, as assessed for instance in in vitro models of pancreatic cancers (Ungefroren et al., 2007).

Homology

ALK1 shares with other type I receptors a high degree of similarity in the GS domain, in the following serine-threonine kinase subdomains and in the short C-terminal tail (ten Dijke et al., 1994), but the extracellular domain shows a peculiar amino acidic sequence.

Mutations

Germinal

Mutations in the ACVRL1 gene result in Hereditary Hemorrhagic Telangiectasia Type 2 (HHT2). A germinal mosaic with two mutant alleles in hereditary hemorrhagic telangiectasia associated with pulmonary arterial hypertension was described (Eyries et al., 2011).
A germline heterozygous ACVRL1 polymorphisms (p. A482V) has been reported in a patient with a gonadotroph pituitary tumour by D'Abronzo et al., 1999.

Somatic

No somatic mutations of ACVRL1 have been found in human cancers.

Implicated in

Solid tumours

Note

As a receptor mainly expressed on the surface of endothelial cells, ALK1 overexpression and unbalances in its signalling are implicated in many solid tumours, despite the origin and specific features of the latter. Thus, they will be discussed in a single paragraph.
In a study (Hu-Lowe et al., 2011) performed on 3000 human tumour specimens representing more than 100 tumour types, ALK1 resulted particularly expressed in the vasculature of prostate cancers, malignant melanomas of the skin, follicular cancers of the tyroid, renal clear cell cancers and endometrioid ovarian cancers.
A reduced expression of ACVRL1 by qRT-PCR and immunohistochemistry was demonstrated in nasopharingeal carcinomas by Zhang et al., 2012.
An increased ALK1 expression in papillary thyroid carcinomas with bone formation was increased if compared to that in normal thyroid tissue and tumors without bone formation, as assessed using immunohistochemistry and quantitative real-time polymerase chain reaction (Na et al., 2013).
In Head and Neck Squamous Cell Carcinomas (HNSCC), using immunohistochemistry and qRT-PCR, Chien et al. found a correlation between a high ACVRL1 expression and an advanced T classification, a positive N classification, an advanced TNM stage, the presence of lymphovascular invasion, an extracapsular spread of lymph node metastasis and a poorer prognosis (Chien et al., 2013).
As a therapeutic target, anti-ALK1 drugs (both in the form of an Fc-fusion protein acting as a soluble receptor for BMP9 and of an anti-ALK1 monoclonal antibody) are under investigation in phase I and phase II clinical trials in a wide range of solid tumours (Vecchia et al., 2013). Phase II studies clinical trials encompass particularly squamous cell carcinoma of the head and neck, endometrial cancer, epithelial ovarian cancer, fallopian tube cancer and primary peritoneal carcinoma for ACE-041 (also known as Dalantercept, the Fc-receptor fusion protein). Dalantercept displayed promising antitumour activity particularly in patients with advanced refractory cancer (Bendell et al., 2014). PF-03446962 (the anti-ALK1 monoclonal antibody), is up to now studied in phase II clinical trials particularly in malignant mesoteliomas of the pleura and transitional cell carcinomas of the bladder.

A recent study showed that PF-03446962 has no activity as a single drug in refractory urothelial cancer as is thus suggested, for this kind of cancer, only as a combination therapy with other agents against the VEGF receptor axis (Necchi et al., 2014). Both Dalantercept and PF-03446962 are currently under investigation in phase II trials particularly in advanced and refractory hepatocarcinomas.

As assessed by Hosman et al., 2013, mutations in ACVRL1 gene, as the ones observed in HHT2 patients, seem to reduce the prevalence of some types of solid tumours and account for the unexpected good life expectancy of HHT patients older than 60 years of age.

Although it is important to take with care the results of the study due to the methodology used for the assessment (for the statistical and logistic difficulties to perform a longitudinal study in a rare disease, the authors used a questionnaire, inevitably biased), HHT patients older than 60 presented an apparent reduction in lung, liver and colorectal cancer compared to controls.

This could potentially be related to the ALK1 haploinsuffiency present in ALK1 HHT mutations, opposite to the overexpression usually showed in cancers.

On the other hand, colorectal cancer was instead more frequent in younger HHT patients, particularly in the subgroup with SMAD4 mutations and juvenile polyposis.

Hereditary hemorrhagic telangiectasia type 2 (HHT2)

Note

Hereditary Hemorrhagic Telangiectasia (HHT), or Rendu-Osler-Weber disease, is a vascular dysplasia inherited as an autosomal dominant trait (Shovlin, 2010; McDonald et al., 2011). It affects approximately 1 in 5-8000 individuals (Faughnan et al., 2011) with regional differences due to founder effects (Westermann et al., 2003; Lesca et al., 2008). The clinical diagnosis of HHT is based on the presence of at least three of the following "Curaçao criteria" (Shovlin et al., 2000): (1) spontaneous, recurrent epistaxis; (2) mucocutaneous telangiectases at characteristic sites as nose, lips, oral cavity, finger tips and gastrointestinal (GI) mucosa; (3) visceral arteriovenous malformations (AVMs) in lungs, liver, GI, brain and spinal cord; (4) family history of first-degree relative in whom HHT has been diagnosed using these criteria. Significant clinical variability was observed in HHT (Lesca et al., 2007; Govani and Shovlin, 2009), with both intra- and interfamilial variations in age-of-onset, localization of lesions, and severity of complications, whereas it usually shows a high penetrance. HHT is usually not apparent at birth, but evolves with age into a recognizable phenotypic pattern. Spontaneous recurrent nosebleeds are the most common and usually earliest clinical manifestation. HHT telangiectases develop and get worse with age. Complete penetrance was found to be by 40 years of age (Porteous et al., 1992). HHT patients show approximately 15-50% of pulmonary AVMs (PAVMs), 32-78% of liver AVMs (HAVMS) and approximately 23% will harbor AVMs in the brain (CAVMs). Although 80% of patients with HHT have gastric or small intestinal telangiectases, only 25-30% of patients will develop symptomatic GI bleeding which usually does not present until the fifth or sixth decades of life (Faughnan et al., 2011).

HHT arises from heterozygous mutations in ENG (HHT1, OMIM #187300) coding for ENDOGLIN (ENG) (McAllister et al., 1994) and ACVRL1 (HHT2, OMIM #600376) coding for ALK1 (Johnson et al., 1996), Type III and Type I TGF-β receptors, respectively. Certain HHT2 patients develop a Pulmonary Artery Hypertension (PAH)-like syndrome, suggesting that ACVRL1 mutations are also likely to be involved in PAH (Trembath et al., 2001; Olivieri et al., 2006). A subset of patients with juvenile polyposis, carrying mutations in SMAD4/MADH4 (JPHT, OMIM #175050), can also develop HHT (Gallione et al., 2004). Recently, mutations in BMP9 were reported in three unrelated families affected by a vascular-anomaly syndrome presenting with phenotypic overlap with HHT (Wooderchak-Donahue et al., 2013). Additional as-yet-unknown HHT genes have been suggested by linkage analysis in two affected kindred on chromosome 5 and on chromosome 7 (Cole et al., 2005; Bayrak-Toydemir et al., 2006). Molecular genetic testing of the three known genes detects mutations in approximately 85% of patients. As reported above, the mutated genes encode proteins that mediate signaling by TGF-β family.

More than 375 ACVRL1 variants are present in the international HHT mutation database and more than 185 are demonstrated to be pathogenic for HHT.

TGF-β ligands regulate angiogenesis through their actions either on endothelial cells (EC) and/or mural cell, demonstrating that they play important roles in both activation (via ALK1) and resolution (via ALK5) phases of angiogenesis. It has been reported that BMP9, rather than BMP10, might be the specific ALK1 ligand and activator of the Smad1/5/8 signaling pathway in endothelial cells and that they are potent inhibitors of EC migration and growth (David et al., 2007). Previous studies have suggested the synergy between Notch and TGF-β, and that Notch signaling modulates the balance between TGF-β/ALK1 and TGF-β/ALK5 signaling pathways (Fu et al., 2009).

ALK1-1 and pulmonary arterial hypertension

Note

Pulmonary arterial hypertension (PAH) is a severe and rare disease affecting small pulmonary arteries, with progressive remodeling leading to elevated pulmonary vascular resistance and right ventricular failure, and is a major cause of progressive right-sided heart failure and premature death (Trembath et al., 2001). PAH is defined as the sustained elevation of mean pulmonary artery pressure (PA) above 25 mmHg at rest or 30 mmHg during exercise (Rabinovitch, 2012). The histopathology is marked by vascular proliferation/fibrosis, remodeling, and vessel obstruction (Chan and Loscalzo, 2008).

In the second World Symposium held in Evian, France, in 1998, was proposed a clinical classification for pulmonary hypertension. The first category was defined PAH and includes two subgroups, the first incorporates both the idiopathic form (IPAH) that the inherited (HPAH) of the disease. The second subgroup includes a number of conditions associated with various diseases (APAH), including connective tissue diseases, human immunodeficiency virus infection, congenital heart disease, and portal hypertension (Simonneau et al., 2004; Machado et al., 2009).

Heterozygous mutations in the transforming growth factor-β receptor (TGF-β receptor) super family have been genetically linked to PAH and likely play a causative role in the development of disease. Particularly, mutations in the bone morphogenetic factor receptor type 2 (BMPR2) gene account for approximately 70% of all familial pedigrees of PAH (HPAH) and 10-30% of idiopathic PAH cases (IPAH) (Chan and Loscalzo, 2008; Machado et al., 2009).

Much less commonly (5%) two other members of the TGF-β superfamily are also recognized as uncommon causes of PAH: activine A receptor type II-like kinase 1 (ALK1) and, at significant lower frequency, endoglin (ENG) (Harrison et al., 2003). Heterozygous mutations of these genes cause the autosomal dominant vascular disorder hereditary haemorrhagic teleangiectasia (HHT) (Shovlin, 2010). In fact, in a small proportion of HHT patients, was observed a form of pulmonary arterial hypertension that is associated with a model of precapillary pulmonary hypertension that is histopathologically indistinguishable from idiopathic form of PAH. Since the publication by Trembath et al. in 2001 (Trembath et al., 2001), who first reported patients with a mutation in the gene ACVRL1 with clinical features of both PAH and HHT, subsequently, have been recognized several other mutations in the ALK1 gene that seem to predispose patients with HHT development of PAH. This observation was further confirmed by other studies (Olivieri et al., 2006) and extensively discussed by Machado et al. (Machado et al., 2009).

The exact prevalence of PAH in the HHT population has not been systematically evaluated, but most authors agree that it is a rare complication found in less than 1% of HHT patients (Cottin et al., 2007). In rare cases, ACVRL1 mutations have been reported to cause IPAH or HPAH without HHT (Harrison et al., 2003).

Both ALK1 and BMPR2 belong to the family of TGF-β receptors, they have different specific ligands but share a common intracellular pathway based on the activation of the SMAD proteins 1/5/8 (Faughnan et al., 2009).

The formation of an heteromeric complex with BMPR2 and ALK1 could at least in part explain why any dysregulation of this pathway may promote pulmonary endothelial and/or smooth muscle cell dysfunction and proliferative characteristic of PAH, in subjects carrying mutation either in BMPR2 or in ACVRL1 gene.

Mutations identified in several studies on ALK1 associated with PAH are all likely to disrupt activation of this intracellular pathway and the majority of these comprise missense mutations. Particularly, mutations in exon 10 of ACVRL1 are relevant because they occur in functional domains of the receptor within a conserved carboxyl-terminal region of ALK1 (the non-activating non-down regulating box) NANDOR BOX (Faughnan et al., 2009; Machado et al., 2009).

Of note, the NANDOR BOX, located from codon 479 to 489, is necessary for regulation of TGF-β signaling, accordingly any alteration may have effects on TGF-β-induced receptor signaling (Girerd et al., 2010).

Moreover, recent studies in animal models have shown that Alk1 heterozygous mice spontaneously develop signs of pulmonary hypertension in the early months of life, and with increasing age show more occluded vessels and pulmonary vascular remodeling, indicating a progression of the disease. These mice had also higher ROS levels in adult lungs contributing to PAH development compared to control mice.

Whereas Bmpr2 heterozygous mouse model requires additional factors, such as hypoxia and serotonin or inflammation, to elicit a pulmonary hypertensive phenotype (Jerkic et al., 2011).

Finally, Girerd B. et al. hypothesized that mutated ACVRL1 status might be associated with distinct PAH phenotypes, as compared with patients PAH without ALK1 mutations.

The authors analyzed clinical, functional characteristic, hemodynamic features and outcomes for patients with PAH carrying ACVRL1 mutation. Of notice, these patients were significantly younger at diagnosis (P

Pulmonary arterial hypertension is therefore a complex disease that involves the interaction between genetic predisposition and environmental risk factors. The identification of human mutations in components of the TGF-β receptor different from each other but somehow bound by common intracellular signaling pathways, which may lead to the development of pulmonary vascular disease, has provided important targets for further investigation.

Hematological malignancies

Note

Roughly 80% of non-Hodgkin's lymphomas and 60% of Hodgin lymphomas express ALK1 in their vasculature (Hu-Lowe et al., 2011). The expression of ALK1 in haematological cancers was further confirmed in an exploratory study on patients affected with Acute Myeloid leukemia (AML) (Otten et al., 2011).

Using qRT-PCR, ALK1 was demonstrated to be expressed by 82% of patients' samples (pretherapeutic bone marrow or peripheral blood from 93 patients with newly diagnosed AML). Furthermore, formalin-fixed, paraffin-embedded trephine bone marrow specimens from two arbitrarily selected patients with AML and from two patients with non-leukemic reactive changes were analyzed for ALK-1 expressions by immunohistochemistry. Endothelial cells from two AML patients and those with reactive disorders were strongly positive and a fraction of AML blasts stained positively for ALK-1 in AML bone marrows, whereas normal hematopoietic cells were negative.

Anyway, ALK1 alterations, opposite to those in ALK5, seemed not to have a significant impact on survival. Furthermore, the prevalence of haematological cancers in HHT patients as assessed by Hosman et al., 2013 showed no difference compared to controls.

References

Porteous ME, Burn J, Proctor SJ. Hereditary haemorrhagic telangiectasia: a clinical analysis. J Med Genet. 1992 Aug;29(8):527-30

McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet. 1994 Dec;8(4):345-51

ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K. Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem. 1994 Jun 24;269(25):16985-8

Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat Genet. 1996 Jun;13(2):189-95

D'Abronzo FH, Swearingen B, Klibanski A, Alexander JM. Mutational analysis of activin/transforming growth factor-beta type I and type II receptor kinases in human pituitary tumors. J Clin Endocrinol Metab. 1999 May;84(5):1716-21

Kirsch T, Sebald W, Dreyer MK. Crystal structure of the BMP-2-BRIA ectodomain complex. Nat Struct Biol. 2000 Jun;7(6):492-6

Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H. Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). Am J Med Genet. 2000 Mar 6;91(1):66-7

Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Morrell NW, Berg J, Manes A, McGaughran J, Pauciulo M, Wheeler L. Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N Engl J Med. 2001 Aug 2;345(5):325-34

Greenwald J, Groppe J, Gray P, Wiater E, Kwiatkowski W, Vale W, Choe S. The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. Mol Cell. 2003 Mar;11(3):605-17

Harrison RE, Flanagan JA, Sankelo M, Abdalla SA, Rowell J, Machado RD, Elliott CG, Robbins IM, Olschewski H, McLaughlin V, Gruenig E, Kermeen F, Halme M, Räisänen-Sokolowski A, Laitinen T, Morrell NW, Trembath RC. Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. J Med Genet. 2003 Dec;40(12):865-71

Thompson TB, Woodruff TK, Jardetzky TS. Structures of an ActRIIB:activin A complex reveal a novel binding mode for TGF-beta ligand:receptor interactions. EMBO J. 2003 Apr 1;22(7):1555-66

Westermann CJ, Rosina AF, De Vries V, de Coteau PA. The prevalence and manifestations of hereditary hemorrhagic telangiectasia in the Afro-Caribbean population of the Netherlands Antilles: a family screening. Am J Med Genet A. 2003 Feb 1;116A(4):324-8

Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). Lancet. 2004 Mar 13;363(9412):852-9

Simonneau G, Galiè N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, Gibbs S, Lebrec D, Speich R, Beghetti M, Rich S, Fishman A. Clinical classification of pulmonary hypertension. J Am Coll Cardiol. 2004 Jun 16;43(12 Suppl S):5S-12S

Cole SG, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet. 2005 Jul;42(7):577-82

Bayrak-Toydemir P, McDonald J, Akarsu N, Toydemir RM, Calderon F, Tuncali T, Tang W, Miller F, Mao R. A fourth locus for hereditary hemorrhagic telangiectasia maps to chromosome 7. Am J Med Genet A. 2006 Oct 15;140(20):2155-62

Olivieri C, Lanzarini L, Pagella F, Semino L, Corno S, Valacca C, Plauchu H, Lesca G, Barthelet M, Buscarini E,

Danesino C. Echocardiographic screening discloses increased values of pulmonary artery systolic pressure in 9 of 68 unselected patients affected with hereditary hemorrhagic telangiectasia. Genet Med. 2006 Mar;8(3):183-90

Cottin V, Dupuis-Girod S, Lesca G, Cordier JF. Pulmonary vascular manifestations of hereditary hemorrhagic telangiectasia (rendu-osler disease). Respiration. 2007;74(4):361-78

David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood. 2007 Mar 1;109(5):1953-61

Lesca G, Olivieri C, Burnichon N, Pagella F, Carette MF, Gilbert-Dussardier B, Goizet C, Roume J, Rabilloud M, Saurin JC, Cottin V, Honnorat J, Coulet F, Giraud S, Calender A, Danesino C, Buscarini E, Plauchu H. Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. Genet Med. 2007 Jan;9(1):14-22

Ungefroren H, Schniewind B, Groth S, Chen WB, Müerköster SS, Kalthoff H, Fändrich F. Antitumor activity of ALK1 in pancreatic carcinoma cells. Int J Cancer. 2007 Apr 15;120(8):1641-51

Chan SY, Loscalzo J. Pathogenic mechanisms of pulmonary arterial hypertension. J Mol Cell Cardiol. 2008 Jan;44(1):14-30

Lesca G, Genin E, Blachier C, Olivieri C, Coulet F, Brunet G, Dupuis-Girod S, Buscarini E, Soubrier F, Calender A, Danesino C, Giraud S, Plauchu H. Hereditary hemorrhagic telangiectasia: evidence for regional founder effects of ACVRL1 mutations in French and Italian patients. Eur J Hum Genet. 2008 Jun;16(6):742-9

Faughnan ME, Granton JT, Young LH. The pulmonary vascular complications of hereditary haemorrhagic telangiectasia. Eur Respir J. 2009 May;33(5):1186-94

Fu Y, Chang A, Chang L, Niessen K, Eapen S, Setiadi A, Karsan A. Differential regulation of transforming growth factor beta signaling pathways by Notch in human endothelial cells. J Biol Chem. 2009 Jul 17;284(29):19452-62

Govani FS, Shovlin CL. Hereditary haemorrhagic telangiectasia: a clinical and scientific review. Eur J Hum Genet. 2009 Jul;17(7):860-71

Machado RD, Eickelberg O, Elliott CG, Geraci MW, Hanaoka M, Loyd JE, Newman JH, Phillips JA 3rd, Soubrier F, Trembath RC, Chung WK. Genetics and genomics of pulmonary arterial hypertension. J Am Coll Cardiol. 2009 Jun 30;54(1 Suppl):S32-42

Garrido-Martin EM, Blanco FJ, Fernandez-L A, Langa C, Vary CP, Lee UE, Friedman SL, Botella LM, Bernabeu C. Characterization of the human Activin-A receptor type II-like kinase 1 (ACVRL1) promoter and its regulation by Sp1. BMC Mol Biol. 2010 Jun 29;11:51

Girerd B, Montani D, Coulet F, Sztrymf B, Yaici A, Jaïs X, Tregouet D, Reis A, Drouin-Garraud V, Fraisse A, Sitbon O, O'Callaghan DS, Simonneau G, Soubrier F, Humbert M. Clinical outcomes of pulmonary arterial hypertension in patients carrying an ACVRL1 (ALK1) mutation. Am J Respir Crit Care Med. 2010 Apr 15;181(8):851-61

Shovlin CL. Hereditary haemorrhagic telangiectasia: pathophysiology, diagnosis and treatment. Blood Rev. 2010 Nov;24(6):203-19

Faughnan ME, Palda VA, Garcia-Tsao G, Geisthoff UW, McDonald J, Proctor DD, Spears J, Brown DH, Buscarini E, Chesnutt MS, Cottin V, Ganguly A, Gossage JR, Guttmacher AE, Hyland RH, Kennedy SJ, Korzenik J, Mager JJ, Ozanne AP, Piccirillo JF, Picus D, Plauchu H, Porteous ME, Pyeritz RE, Ross DA, Sabba C, Swanson K, Terry P, Wallace MC, Westermann CJ, White RI, Young LH, Zarrabeitia R. International guidelines for the diagnosis and management of hereditary haemorrhagic telangiectasia. J Med Genet. 2011 Feb;48(2):73-87

Hu-Lowe DD, Chen E, Zhang L, Watson KD, Mancuso P, Lappin P, Wickman G, Chen JH, Wang J, Jiang X, Amundson K, Simon R, Erbersdobler A, Bergqvist S, Feng Z, Swanson TA, Simmons BH, Lippincott J, Casperson GF, Levin WJ, Stampino CG, Shalinsky DR, Ferrara KW, Fiedler W, Bertolini F. Targeting activin receptor-like kinase 1 inhibits angiogenesis and tumorigenesis through a mechanism of action complementary to anti-VEGF therapies. Cancer Res. 2011 Feb 15;71(4):1362-73

Jerkic M, Kabir MG, Davies A, Yu LX, McIntyre BA, Husain NW, Enomoto M, Sotov V, Husain M, Henkelman M, Belik J, Letarte M. Pulmonary hypertension in adult Alk1 heterozygous mice due to oxidative stress. Cardiovasc Res. 2011 Dec 1;92(3):375-84

McDonald J, Bayrak-Toydemir P, Pyeritz RE. Hereditary hemorrhagic telangiectasia: an overview of diagnosis, management, and pathogenesis. Genet Med. 2011 Jul;13(7):607-16

Otten J, Schmitz L, Vettorazzi E, Schultze A, Marx AH, Simon R, Krauter J, Loges S, Sauter G, Bokemeyer C, Fiedler W. Expression of TGF-β receptor ALK-5 has a negative impact on outcome of patients with acute myeloid leukemia. Leukemia. 2011 Feb;25(2):375-9

Eyries M, Coulet F, Girerd B, Montani D, Humbert M, Lacombe P, Chinet T, Gouya L, Roume J, Axford MM, Pearson CE, Soubrier F. ACVRL1 germinal mosaic with two mutant alleles in hereditary hemorrhagic telangiectasia associated with pulmonary arterial hypertension. Clin Genet. 2012 Aug;82(2):173-9

Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. J Clin Invest. 2012 Dec 3;122(12):4306-13

Townson SA, Martinez-Hackert E, Greppi C, Lowden P, Sako D, Liu J, Ucran JA, Liharska K, Underwood KW, Seehra J, Kumar R, Grinberg AV. Specificity and structure of a high affinity activin receptor-like kinase 1 (ALK1) signaling complex. J Biol Chem. 2012 Aug 10;287(33):27313-25

Zhang W, Zeng Z, Fan S, Wang J, Yang J, Zhou Y, Li X, Huang D, Liang F, Wu M, Tang K, Cao L, Li X, Xiong W, Li G. Evaluation of the prognostic value of TGF-β superfamily type I receptor and TGF-β type II receptor expression in nasopharyngeal carcinoma using high-throughput tissue microarrays. J Mol Histol. 2012 Jun;43(3):297-306

Chien CY, Chuang HC, Chen CH, Fang FM, Chen WC, Huang CC, Huang HY. The expression of activin receptor-like kinase 1 among patients with head and neck cancer. Otolaryngol Head Neck Surg. 2013 Jun;148(6):965-73

Hosman AE, Devlin HL, Silva BM, Shovlin CL. Specific cancer rates may differ in patients with hereditary haemorrhagic telangiectasia compared to controls. Orphanet J Rare Dis. 2013 Dec 20;8:195

Na KY, Kim HS, Lee SK, Jung WW, Sung JY, Kim YW, Park YK. Papillary thyroid carcinoma with bone formation. Pathol Res Pract. 2013 Jan 15;209(1):14-8

Sun C, Sun L, Jiang K, Gao DM, Kang XN, Wang C, Zhang S, Huang S, Qin X, Li Y, Liu YK. NANOG promotes liver cancer cell invasion by inducing epithelial-mesenchymal transition through NODAL/SMAD3 signaling pathway. Int J Biochem Cell Biol. 2013 Jun;45(6):1099-108

Vecchia L, Olivieri C, Scotti C. Activin Receptor-like kinase 1: a novel anti-angiogenesis target from TGF-β family. Mini Rev Med Chem. 2013 Aug;13(10):1398-406

Wooderchak-Donahue WL, McDonald J, O'Fallon B, Upton PD, Li W, Roman BL, Young S, Plant P, Fülöp GT, Langa C, Morrell NW, Botella LM, Bernabeu C, Stevenson DA, Runo JR, Bayrak-Toydemir P. BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia. Am J Hum Genet. 2013 Sep 5;93(3):530-7

Bendell JC, Gordon MS, Hurwitz HI, Jones SF, Mendelson DS, Blobe GC, Agarwal N, Condon CH, Wilson D, Pearsall AE, Yang Y, McClure T, Attie KM, Sherman ML, Sharma S. Safety, pharmacokinetics, pharmacodynamics, and antitumor activity of dalantercept, an activin receptor-like kinase-1 ligand trap, in patients with advanced cancer. Clin Cancer Res. 2014 Jan 15;20(2):480-9

Necchi A, Giannatempo P, Mariani L, Farè E, Raggi D, Pennati M, Zaffaroni N, Crippa F, Marchianò A, Nicolai N, Maffezzini M, Togliardi E, Daidone MG, Gianni AM, Salvioni R, De Braud F. PF-03446962, a fully-human monoclonal antibody against transforming growth-factor β (TGFβ) receptor ALK1, in pre-treated patients with urothelial cancer: an open label, single-group, phase 2 trial. Invest New Drugs. 2014 Jun;32(3):555-60

van den Bosch MH, Blom AB, van Lent PL, van Beuningen HM, Blaney Davidson EN, van der Kraan PM, van den Berg WB. Canonical Wnt signaling skews TGF-β signaling in chondrocytes towards signaling via ALK1 and Smad 1/5/8. Cell Signal. 2014 May;26(5):951-8

This article should be referenced as such:

Ornati F, Vecchia L, Scotti C, Plumitallo S, Olivieri C. ACVRL1 (activin A receptor type II-like 1). Atlas Genet Cytogenet Oncol Haematol. 2014; 18(11):789-796.