Hereditary Hemorrhagic Telangiectasia
Clinical and Molecular Genetics

Tom G.W. Letteboer

ISBN: 978-90-5335-333-2

Author: Tom G.W. Letteboer

Cover design: Wilmy Swaerdens, using painting from Nelleke Docter (Pimpelnell), called “The Family”

Lay-out and printing: Simone Vinke, Ridderprint B.V., Ridderkerk, the Netherlands

Hereditary Hemorrhagic Telangiectasia
Clinical and Molecular Genetics

Klinische en moleculair genetische aspecten van hereditaire hemorrhagische teleangiëctasieën

(met een samenvatting in het Nederlands)

Proefschrift
ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op woensdag 10 november 2010 des middags 12.45 uur

door
Thomas Gerrit Wim Letteboer
geboren op 9 februari 1967 te Zwolle

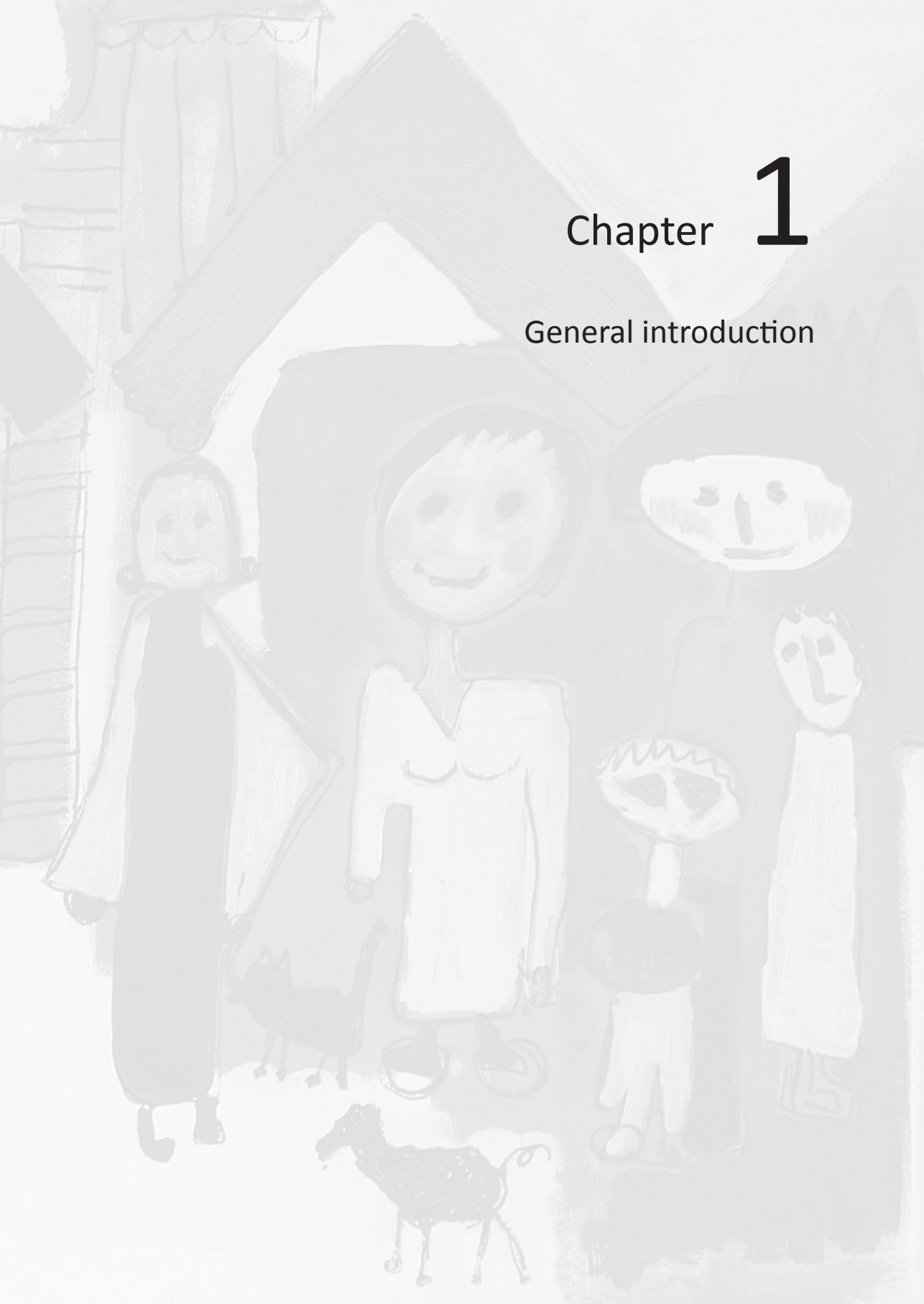
Promotor:	Prof. dr. D. Lindhout
Co-promotoren:	Dr. C.J.J. Westermann
	Dr. J.K. Ploos van Amstel

The author gratefully acknowledges financial support from the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences the Netherlands, for supporting collaboration in HHT research with the University of California, San Francisco, the Helen Diller Family Comprehensive Cancer Center

Abbreviations

ACVRL1	=	actvin A receptor type II-like 1 gene (*ALK1*)
AVF	=	arteriovenous fistula
AT	=	ataxia telangiectasia
AVM	=	arteriovenous malformation
BMP	=	bone-morphogenic-protein
CAVM	=	cerebral arteriovenous malformation
CM	=	capillary malformation
CM-AVM	=	capillary malformation-arteriovenous malformation
EC	=	endothelial cell
ENG	=	endoglin
GI	=	gastrointestinal
HAVM	=	hepatic arteriovenous malformation
HBT	=	hereditary benign telangiectasia
HHT	=	hereditary hemorrhagic telangiectasia
HHT1	=	hereditary hemorrhagic telangiectasia type 1 (*ENG*)
HHT2	=	hereditary hemorrhagic telangiectasia type 2 (*ACVRL1*)
HHT?	=	hereditary hemorrhagic telangiectasia, unknown genetic cause
JP-HHT	=	juvenile polyposis and hereditary hemorrhagic telangiectasia
MLPA	=	multiplex ligation-dependent probe amplification
MNC	=	mononuclear cells
MRI	=	magnetic resonance imaging
PAH	=	pulmonary arterial hypertension
PFO	=	patent foramen ovale
PAVM	=	pulmonary arteriovenous malformation
PH	=	pulmonary hypertension
RLS	=	right-left shunt
ROW	=	Rendu-Osler-Weber disease
SMAD4	=	mothers against decapentaplegic, drosophila, homolog of, 4 (MADH4)
SMC	=	smooth muscle cells
TGFβ	=	transforming growth factor beta
TIA	=	transient ischemic attack

Contents



Chapter 1

General introduction

History

Hereditary hemorrhagic telangiectasia (HHT) is also known by its eponym Rendu-Osler-Weber disease (ROW) after the names of the first physicians that described the disease. In 1896 Henry Jules Louis Marie Rendu published an article on recurrent epistaxis in a patient with "petits angiomes cutanés et muqueux". This was the first complete description of this disease. He was aware of its familial nature, its association with bleeding secondary to telangiectases, and its being an entity separate from hemophilia [1]. His paper was followed in 1901 by a report from William Osler and later in 1907 by one from Frederick Parkes Weber establishing the disease as an inherited disorder [2,3]. The term hereditary hemorrhagic telangiectasia was in 1909 proposed by Frank Hanes [4]. He advocated the name to be more in conformity with the medical nomenclature by referring to the three major clinical features. Despite the appropriateness and general usage of this descriptive name, the eponym Rendu-Osler-Weber is still widely used with the order of the names sometimes adapted, depending on the country of origin of the authors or on the appreciation of the contribution of the first physicians.

Hemorrhage of telangiectatic origin

HHT was established as a disorder that primarily results in bleeding (e.g. nosebleeds or epistaxis) due to "inadequate vessels", in which visceral involvement could occur and present in multiple subjects in a family, in men as well as in women [2].

HHT is an autosomal dominant vascular disorder characterized by the presence of multiple arteriovenous malformations (AVM). These AVMs are direct connections between arteries and veins, thereby lacking the (normally) intervening capillary bed. Telangiectases are in fact small AVMs, which can present on the face, lips, tongue, fingers and in the nasal, oral and gastrointestinal mucosa. AVMs in HHT usually refer to the larger arteriovenous connections, commonly occurring in the lung (pulmonary arteriovenous malformations or PAVM), the brain (cerebral arteriovenous malformations or CAVM) and/or the liver (hepatic arteriovenous malformations or HAVM). These AVMs cause direct shunting of blood, resulting in the absence of normal capillary exchange and the absence of a normal capillary filter function [5].

Prevalence

The prevalence of HHT varies considerably between countries and even between regions within a country. In part, this may be due to differences in ascertainment and diagnosis, but differences between populations truly exist, thought to be due to founder effects. As an example, in the Netherlands the frequency is estimated to be 1-2:10,000, whereas in the Dutch Antilles the prevalence is estimated to be as high as 1:1,330 [6]. Also within-country

regional variability has been documented: in some regions in France prevalence nears 1:3,500 [7].

Genetics of HHT

Linkage studies on HHT families identified two major gene loci associated with HHT. In 1994, the *ENG* gene encoding endoglin was identified on chromosome 9q34 as the gene associated with HHT1 (MIM 187300) [8]. However, locus heterogeneity was obvious, since a number of families were not linked to chromosome 9 [9].

Two years later, in 1996, mutations in *ACVRL1* (activin-like receptor kinase: *ACVRL1* or *ALK1*) located on chromosome 12q13 were found to cause HHT2 (MIM 600376) [10]. Both genes encode proteins involved in the TGFβ pathway. The spectrum of mutations for both the *ENG* gene and the *ACVRL1* gene consists of all kinds of mutations: missense, nonsense, frame shift, splice site etc. These mutations are typical for a loss of function. Mutations in HHT are collected in two major mutation databases (http://www.hhtmutation.org/ ; http://www.hgmd.cf.ac.uk/ac/index.php).

In January 2010, the Human Gene Mutation Database reported 316 mutations for the *ENG* gene; 100 missense/nonsense mutations, 134 insertions/deletions, 51 splice site mutations, 30 large deletions or duplications and 1 regulatory region mutation. For the *ACVRL1* gene 273 different mutations are reported; 160 missense/nonsense mutations, 84 insertions/deletions, 20 splice site mutations and 9 large deletions/duplications. Protein expression studies in human umbilical vein endothelial cells and peripheral blood monocytes have confirmed haploinsufficiency, mutations that cause loss of function of one allele, as the main model in HHT1 and HHT2 [11-13]. *SMAD4* has been implicated as a third gene involved in HHT.

In 2004 Gallione *et al.*[14] reported mutations in the *SMAD4* gene in a rare group of patients that show clinical features of two diseases: juvenile polyposis and HHT or JP-HHT (MIM 175050). The *SMAD4* gene is located on chromosome 18q21.1, codes for the protein *SMAD4* and is expressed in a variety of cell types. The protein, like *ENG* and *ACVRL*1, has a role in the TGFβ pathway as well as in the bone-morphogenic-protein (BMP) pathway. HHT as part of the JP-HHT syndrome can thus also be explained by *SMAD4* mutations.

Other candidate loci for HHT are linked to chromosome 5 and chromosome 7 [15,16]. These loci are now described as being responsible for HHT3 and HHT4 respectively. Cole *et al.*[16] described linkage to chromosome 5q31 in a family, in which linkage with *ENG*, *ACVRL1* and *SMAD4* was excluded and without mutations in any of these genes. They narrowed the linkage region on the chromosome down to 6 Mb with a maximum two point LOD score of 3.45. Bayrak-Toydemir *et al.*[15] excluded linkage to the HHT causing genes and detected a maximum two point LOD score of 3.6 with an STR marker on chromosome 7p14, in one family. They narrowed the region to 7 Mb. To date, no causative genes have been found in these two regions.

The HHT1 gene: ENG

The *ENG* gene (depicted in figure 1) is located on 9q33-q34.1 and encompasses approximately 40 kb of genomic DNA. The gene consists of 14 exons or 15 exons if the alternative splicing within the most distal exon is taken into consideration. They respectively encode a longest mRNA of 3196 nt (NM_000118) and a shorter one with a length of 3072 nt (NM-01114753). The longer *ENG* mRNA encodes the protein endoglin (P17813-1), a 658 amino acid homodimeric transmembrane protein which is a major glycoprotein of the vascular endothelium. This protein is a component of the transforming growth factor beta receptor complex and it binds TGFβ1 and TGFβ3 as well as some BMP isoforms with high affinity. It is thought to sequester the ligand at the cell surface and deliver to the signaling receptor complex, although it might have some function of TGFβ signaling. The alternatively spliced transcript variant of *ENG* encoding endoglin (P17813-2) is also identified. The variant has an additional segment in the 3' coding region. However, it includes a more proximal stop codon.

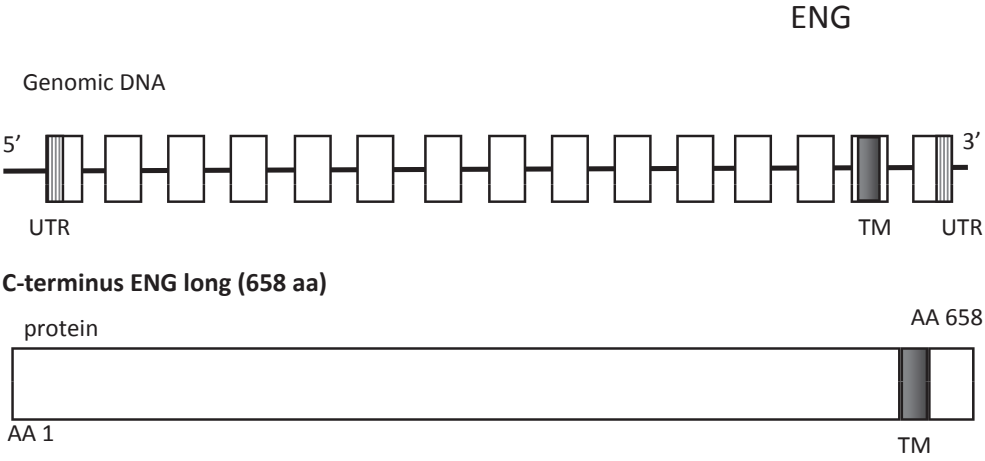


Figure 1 - *ENG* gene, TM = transmembrane region, UTR = untranslated region

The resulting isoform (also known as S-endoglin) has a shorter and distinct C-terminus, and a length of 625 amino acids: the long and predominant variant (known as L-endoglin) has a 47-amino acid cytoplasmic tail while the tail of the short variant contains only 14 residues. In addition an extracellular soluble form of endoglin has been found, that has been implicated in cancer and pre-eclampsia. Soluble endoglin results from the shedding of endoglin by MMP-14 mediated proteolytic cleavage at a site close to the transmembrane domain on the cell surface (Entrez gene; http://www.ncbi.nlm.nih.gov/gene/2022) [17].

The HHT2 gene: ACVRL1

The *ACVRL1* gene (see figure 2) is located on 12q13 and encompasses approximately 15 kb of genomic DNA. The gene consists of 10 exons, the starting codon for the initiation of the translation is in exon 2 and the termination codon in exon 10. *ACVRL1* encodes an mRNA of 4263 nt (NM_000020). This *ACVRL1* mRNA encodes a protein (P37023) with a length of 503 amino acids, a type I cell-surface receptor for the TGFβ superfamily of ligands. It shares with other type I receptors a high degree of similarity in serine-threonine kinase subdomains, a glycine- and serine-rich region (called the GS domain) preceding the kinase domain, and a short C-terminal tail. The encoded protein, often termed *ALK1*, shares similar domain structures with other closely related activin receptor-like kinase proteins that form a subfamily of receptor serine/threonine kinases, that signal for TGFβ superfamily ligands, BMPs, activins and TGFβ1 (Entrez gene; http://www.ncbi.nlm.nih.gov/gene/94).

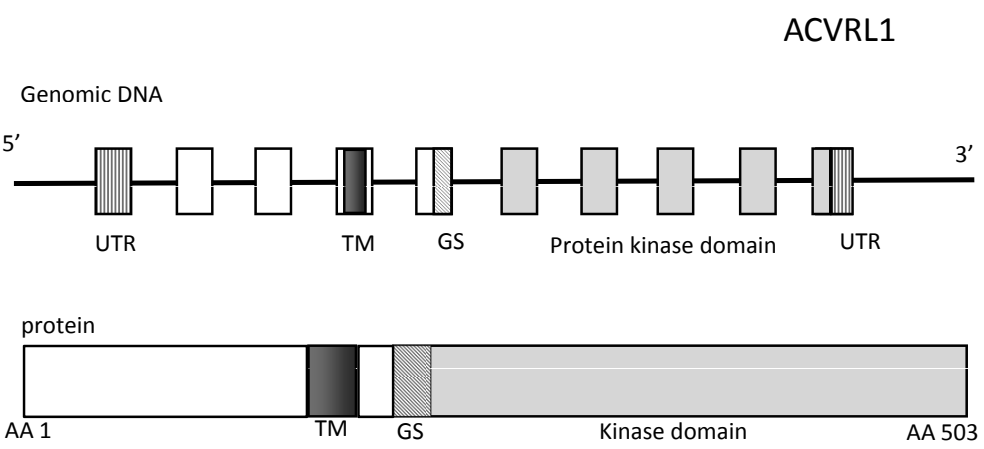


Figure 2 - *ACVRL1* gene, TM = transmembrane region, UTR = untranslated region, GS = GS domain

The JP-HHT gene: SMAD4

The *SMAD4* gene (see figure 3) is located on 18q21.1 and encompasses approximately 55 kb of genomic DNA. The gene consists of 11 exons that together encode an mRNA of 8789 nt (NM_005359). This *SMAD4* mRNA encodes a protein (Q13485) with a length of 552 amino acids: the protein is a member of the SMAD family of signal transduction proteins. The *SMAD4* gene product encodes a nuclear shuttling SMAD, which unlike the other SMAD family members, does not interact with the cell surface TGFβ receptors. *SMAD4* exists as a homomeric complex until TGFβ signaling is activated. On activation of the TGFβ or BMP signaling pathways, receptor-associated SMADs are phosphorylated by the appropriate type I receptor, which then form heterohexameric complexes with the nuclear-shuttling *SMAD4*,

resulting in transfer of the entire complex to the nucleus, chromatin binding and consequent transcriptional regulation of target genes. *SMAD4* binds to DNA and recognizes an 8-bp palindromic sequence (GTCTAGAC) called the SMAD-binding element (SBE). Depending on which co-transcriptional partners associate with the SMAD hexameric complex, the outcome may be transcriptional activation or repression (http://www.ncbi.nlm.nih.gov/gene/4089).

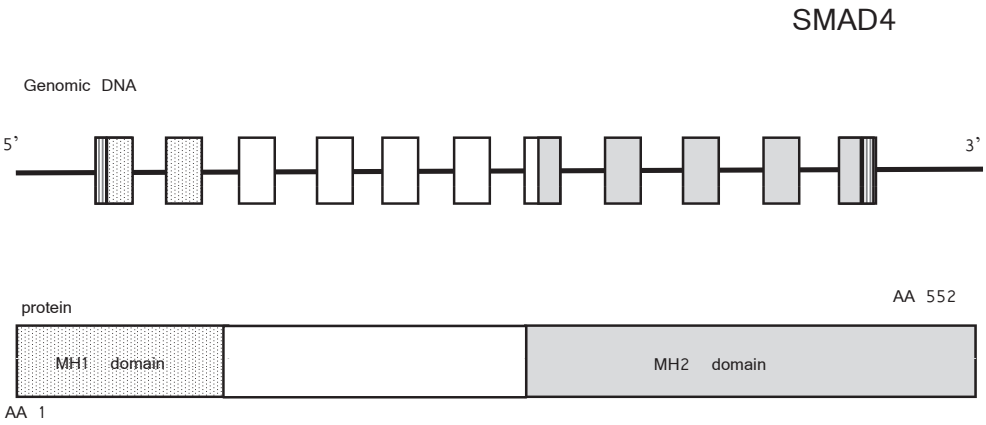


Figure 3 - *SMAD4* gene

ENG, ACVRL1 and SMAD4: components of the TGFβ/BMP signaling pathway

ENG and *ACVRL1* are both expressed in endothelial cells and encode different receptor subtypes of the TGFβ/BMP signaling pathway. The TGFβ signaling pathway regulates different cellular processes including cell growth, cell differentiation, proliferation, migration and apoptosis (see also figure 4).

Members of the TGFβ/BMP superfamily function by binding to a heterotetrameric complex of type I and type II transmembrane serine/threonine kinase receptors. In endothelial cells (ECs), TGFβ can signal via two distinct type I receptors, via *ACVRL1* and *ALK5*, through SMAD dependent pathways. TGFβ activates TβRII, which then phosphorylates and activates the broadly expressed *ALK5*, which in turn phosphorylates SMAD2 and SMAD3 downstream. The type I receptor *ACVRL1* is EC specific and mediates phosphorylation of SMAD1/5/8. Activated SMADs form complexes with *SMAD4* and translocate into the nucleus, where they can regulate transcription of genes, together with co-activators and co-repressors. ACVRL1 and ALK5 appear to have opposite effects on the EC and the balance between the two pathways is probably important for regulating the state of the endothelium from proliferation to quiescence. TGFβ/ALK5 pathway leads to inhibition of cell proliferation and migration, whereas TGFβ/ACVRL1 induces these processes. Endoglin plays an important role in both of these arms of the signaling pathway. It is essential for efficient TGFβ/ALK1 signaling

and indirectly inhibits TGFβ/ALK5 signaling. Endoglin is highly expressed in endothelial cells undergoing activated angiogenesis, in monocytes and in syncytiotrophoblast. Angiogenesis is the process in which new blood vessels are formed from preexisting ones. Angiogenesis is important in the developing embryo, but also plays an important role in revascularization of ischemic and injured/damaged organs in adults.

Mouse model

Mice lacking *Eng* die mid gestation from cardiovascular defects and show obvious vascular problems in the yolk sac. Mice heterozygous for *Eng* or *Acvrl1* can demonstrate the clinical phenotype of HHT. This phenotype seems to be dependent on the genetic background of the mice [18,19]. Furthermore, there is considerable phenotypic resemblance between the prenatal vascular abnormalities of *Acvrl1, Tgfbr1, Tgfbr2, Eng* and *Tgfb1* homozygous knockout mice. Apparently, deficiency of these TGFβ superfamily members has similar effects on angiogenesis.

In *Eng* heterozygous mice it was shown that after an induced myocardial infarction, angiogenesis was impaired as a result of reduced endoglin levels, *Eng*+/- mice showed markedly less recovery compared to controls [20]. To investigate the role of mononuclear cells (MNCs: endothelial progenitor cells, endothelial cells, bone marrow monocytic cells), mice with an induced infarction were injected with MNC of HHT1 patients and compared to mice injected with MNC of healthy controls. MNC from healthy controls significantly improved heart function (recovery) in endoglin deficient mice whereas mice injected with MNC of HHT1 patients showed no improvement. Although the cell population inducing this effect is unknown, it was proposed by the authors that activated monocytes with reduced endoglin expression might be defective in HHT1 [21].

Reduced endoglin levels influence TGFβ signaling [22], which also plays an important role in EC-SMC interaction. Smooth muscle cells or pericytes are necessary for giving the vessel support to create a stable, strong vascular wall. Reduced TGFβ secretion might therefore also explain the formation of fragile vessels in HHT patients [23].

Clinical diagnosis

In 2000, consensus clinical diagnostic criteria were established, called the Curaçao criteria [24]. These criteria are widely used and an aid in establishing a consistent and reproducible clinical diagnosis. For a definite clinical diagnosis three or more of these criteria are required. When two of four are present, the diagnosis of HHT is regarded “possible or suspected”. The criteria are:

- spontaneous recurrent epistaxis
- mucocutaneous telangiectases at characteristic sites (lips, finger tips, oral mucosa or nose)

- visceral involvement such as gastrointestinal telangiectases, pulmonary arteriovenous malformation (PAVM), cerebral arteriovenous malformation (CAVM) or hepatic arteriovenous malformation (HAVM)
- a family history; a first degree relative affected according to these criteria

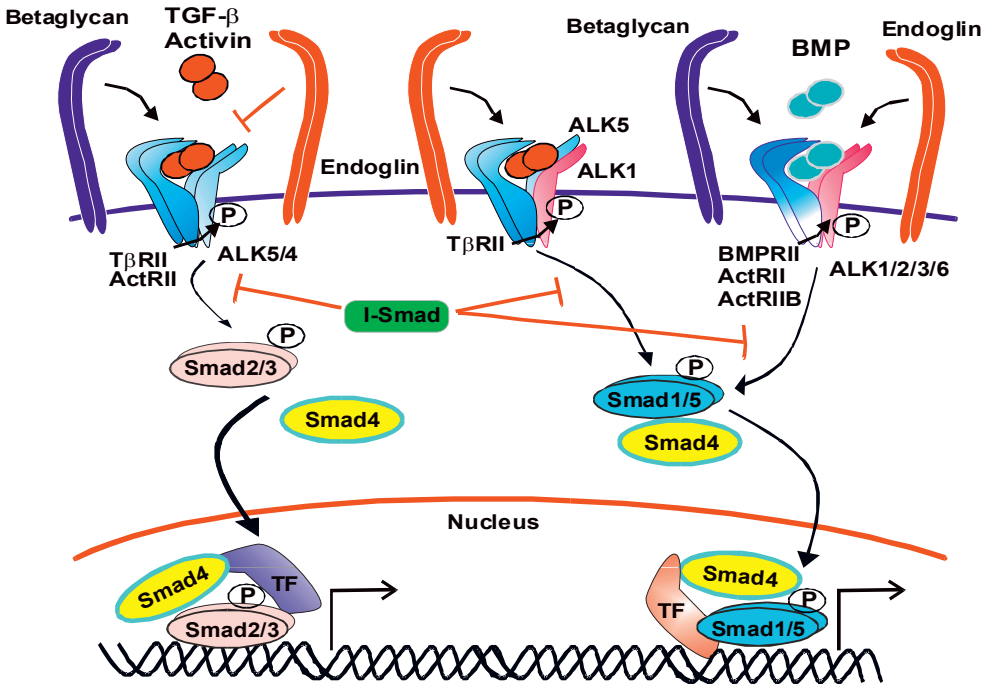


Figure 4 - The TGFβ pathway Signal transduction by TGFβ family members is mediated via specific heteromeric complexes of type I and type II serine/threonine kinase receptors. In most cells TGFβ interacts with TβRII and ALK5, but in endothelial cells it can also signal via ALK1/ACVRL1. BMPs signal via BMPRII, ActRIIA and ActRIIB, and type I receptors ALK1, 2, 3 and 6. Co-receptors betaglycan and endoglin can facilitate TβRII/ALK5 and TβRII/ALK1 signaling. Intracellular signaling can be divided into two main Smad signaling pathways: ALK5 induces phosphorylation of Smad2 and Smad3, and ALK1, 2, 3 and 6 mediate phosphorylation of Smad1, 5 and 8. Activated R-Smads form heteromeric complexes with common mediator Smad4, which accumulate in the nucleus, where they can act as transcription factor complexes and regulate the expression of specific target genes. *(Reprinted by permission from Macmillan Publishers Ltd: Cell Research, copyright 2008; Goumans et al. Cell Res. 2009)*

Epistaxis

Spontaneous and recurrent epistaxis is the most common and often the presenting symptom in HHT. These nosebleeds can appear early in life, can commence in childhood, and can vary between mild and very severe. Almost all HHT patients will eventually develop epistaxis. The frequent nosebleeds can lead to iron deficiency and may require blood transfusion.

Telangiectases

In HHT, multiple telangiectases occur particularly on the lip, tongue, nose, conjunctivae and on the finger tips (Figure 5 a+b). Telangiectases in the nose may result in epistaxis early in life and could require treatment. Telangiectases of the skin and buccal mucosa are present in about 75% of individuals, typically presenting from about the third decade of life [10].

Gastrointestinal telangiectases are likely to be present in the majority of patients, but only a fraction of the patients will develop symptomatic telangiectases and will develop GI bleeding. GI bleeding usually presents at an older age as anemia that cannot be explained by epistaxis or manifested by melena.

Arteriovenous malformations

PAVMs are direct right-to-left shunts without the intervening pulmonary capillary bed (see figure 5c). Because of the lack of a capillary bed, the filtering function and the exchange of oxygen may be hampered. This can lead to serious and life threatening complications. PAVMs can cause hypoxemia, bleeding (hemothorax, hemoptysis) and bypass of emboli or septic material, which may lead to serious systemic complications such as cerebral abscess and infarction [5,26,27]. Furthermore they are associated with migraine with aura [5]. Retrospectively, it was estimated that, on presentation, 24% of patients with PAVM experienced TIA or stroke and 9% a cerebral abscess as a symptom of PAVM. Furthermore, dyspnoea was common (47%) [5]. Because of these serious complications screening for PAVMs is advised and treatment of PAVMs is justified, even when asymptomatic [5,28].

Cerebral arteriovenous malformations (**CAVMs**) are less common than PAVMs. Although they are often silent, they can cause local hypoxia resulting in headache, seizures, ischemic complications and bleeding [29,30]. The bleeding risk has been estimated to range from 0.5% to 1.5-2% per annum per patient [29,31].

The reported frequency of hepatic involvement (**HAVMs**) in HHT varies considerably, mainly because HAVM has not been screened for in HHT patients regularly. Liver involvement predominantly concerns shunts between hepatic artery and hepatic veins. HAVMs are often asymptomatic, but in a minority of cases may lead to a high cardiac output with heart failure and lead to portal hypertension and biliary disease. In rare cases HAVMs can lead to end stage liver disease.

Family history: phenotypic variability

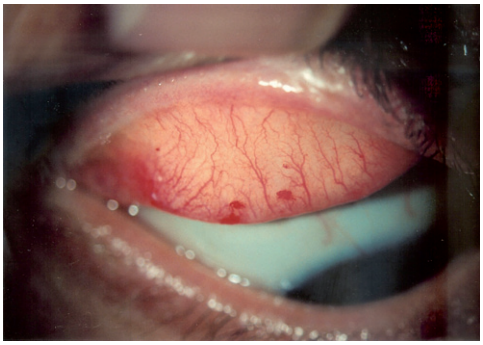
It should be kept in mind that in HHT the phenotype shows considerable inter- and intrafamilial variability for both age-related penetrance and for the clinical expression [5,10]. At least in adult patients the Curaçao criteria are very helpful in distinguishing affected from non-affected family members. However, in children and young adults, due to the age-dependent penetrance of the different symptoms, the absence of HHT manifestations cannot exclude carriership. It remains to be established how often a diagnosis of HHT will be missed using these criteria, especially in the young. In families without a known causative defect attributed to either the *ENG*, *ACVRL1* or *SMAD4* gene (HHT?), these clinical criteria are very useful to distinguish affected family members from the family members without the disease. Of course the Curaçao criteria are also helpful when DNA analysis is not desired by the patient for whatever (personal) reason. The clinical criteria can be used for all types of HHT, since there is considerable overlap between the different forms of HHT, with the manifestations observed in the same organs. For JP-HHT, no separate diagnostic criteria exist; the diagnostic criteria for both HHT and JP can be used to establish the clinical diagnosis JP-HHT.

Differential diagnosis

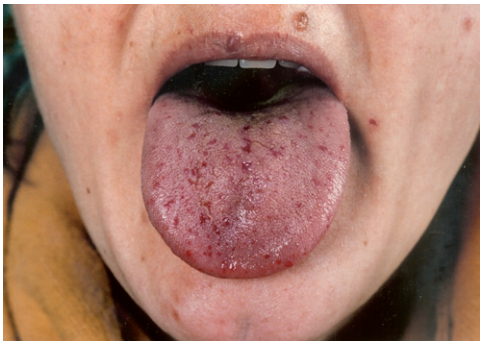
When encountering a patient with clinical manifestations of HHT, other diseases should be considered and, when possible, excluded.

Ataxia telangiectasia (*AT*, MIM 607585) can present with telangiectases, mostly of the conjunctivae. AT is characterized by progressive cerebellar ataxia, usually beginning at a young age. Furthermore AT patients develop oculomotor apraxia and have recurrent infections. Because of the autosomal recessive inheritance of AT, patients usually lack a family history. Ataxia telangiectasia is caused by mutations in the *AT* gene.

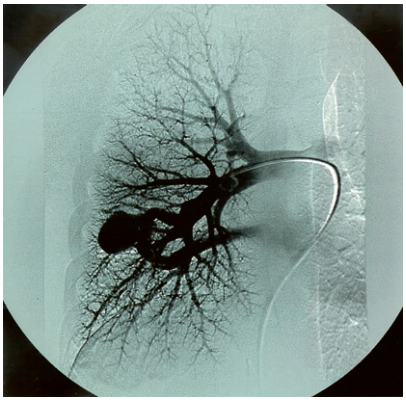
Hereditary benign telangiectasia (HBT, MIM 608354) is a rare genetic skin disorder with telangiectases occurring at a young age, usually in early childhood. The lesions appear in a random distribution and are asymptomatic. Usually, there is no history of bleeding like epistaxis and no systemic lesions, distinguishing HBT from HHT. Several families have been reported, with an autosomal dominant inheritance. Brancati *et al.*[32] showed linkage in a HBT family with the CMC1 locus. This locus had been associated with capillary malformations (CM), “port wine stain”, which can also inherit as an autosomal dominant trait. Years later, this specific family turned out to have a *RASA1* mutation. *RASA1* has been found to cause autosomal dominant capillary malformation-arteriovenous malformation (CM-AVM). The hallmark of this disorder is multifocal CM, but some patients can have AVM or an arteriovenous fistula (intracranial or peripheral). Most of the intracranial AVMs/AVFs cause symptoms at birth or in the first year [33]. The absence of epistaxis or telangiectases and the involvement of liver and lung in HHT can differentiate CM-AVM from HHT.



A



B



C

Figure 5 - Telangiectases A+B: Multiple telangiectases occur in HHT, particularly on the lip, tongue, nose, conjunctivae and on the finger tips. C: Angiogram showing PAVM

The term scleroderma is used to describe the presence of thickened, hardened skin. It is the main feature in a heterogeneous group of conditions, with a complex classification, starting with “localized” and “systemic” forms and thereafter subdivision. In limited systemic cutaneous sclerosis many patients have symptoms of the CREST syndrome (MIM 181750, calcinosis, Raynaud’s phenomenon, esophageal motility dysfunction, sclerodactyly and telangiectasia). These patients have prominent vascular malformations, with as the most prominent sign Raynaud’s phenomenon and telangiectases. The mucosal and cutaneous telangiectases can mimic telangiectases in HHT. Furthermore patients can have systemic involvement (GI tract, lung, kidney, and hearth). Scleroderma is a multifactorial disease; involvement of genetic factors has been shown by familial clustering of the disease and differences among race and ethnic groups. Since CREST syndrome can be familial, family history cannot always distinguish between CREST syndrome and HHT

For epistaxis at a young age one should always consider bleeding disorders such as complement factors deficiencies, von Willebrand disease or thrombocytopathy as the cause.

Aims of this study

Since the establishment of *ENG* and *ACVRL1* as the major genes responsible for HHT, it has been possible to perform DNA analysis in families with HHT. The HHT centre in Nieuwegein, in the Netherlands, has been a centre of excellence for a long time and has phenotypic data on HHT families: on probands, affected family members and their unaffected relatives. Currently, this cohort is one of the largest, uniformly described cohorts of HHT patients worldwide. This offers the unique opportunity to study the genotype-relationship of HHT in a well established and large panel of HHT patients.

The major aims of this thesis are to get insight into the molecular genetic basis of HTT (especially in the Netherlands) and use this knowledge to secondly get insight into the genotype-phenotype relationship of HHT. The genotype will be instrumental in the unequivocal identification of family members at risk for the HHT manifestations and on establishing the risk of transmitting the gene defect to offspring. Insight in the genotype-phenotype relationship will be helpful to anticipate on the course of the disease, and consequently will give the opportunity to a timely diagnosis and potential treatment. Genotype-phenotype analysis is of great importance for family members and proband counseling. It creates the opportunity to determine whether there are gene specific features or frequencies of the different manifestations, possibly enabling the creation of gene specific screening advices and management of the disease.

Outline of this thesis

In chapter 2 the genetic and molecular heterogeneity in HHT especially in Dutch patients, is established. Chapter 2.1 describes the genetic and molecular heterogeneity of HHT using PCR based sequencing of the *ENG* and *ACVRL1* gene. Chapter 2.2 reports on the identification of large deletions and duplications in these genes. Because the sequencing approach will miss large deletions and duplications, probands that show no mutation are subjected to Multiplex Ligation-dependent Probe Amplification analysis. Chapter 2.3 describes the frequency of *SMAD4* mutations in unselected HHT probands, to determine the frequency of *SMAD4* mutations in HHT patients without apparent symptoms of juvenile polyposis.

Once the molecular cause of HHT in a family is established, molecular analysis was used to identify symptomatic and asymptomatic carriers in families. The main clinical manifestations in HHT are arterial venous malformations (in the lung, cerebrum and liver), telangiectases, epistaxis and intestinal vascular malformations.

The phenotype in relationship to the genotype is the focus of chapter 3 of this thesis. Chapter 3.1 describes the occurrence of mainly PAVMs, CAVMs and HAVMs in relationship to the genotype and describes the frequencies of these manifestations for HHT1 and HHT2, also stratified for gender. Chapter 3.2 focuses on the *where* and *when* do telangiectases occur in relationship to the genotype. Chapter 3.3 describes the onset and severity of nosebleeds in patients with mutations in either the *ENG* or *ACVRL1* gene. Chapter 3.4 gives the assessment of intestinal vascular malformations in HHT1 and HHT2 patients with emphasis on the small intestine using videocapsule endoscopy.

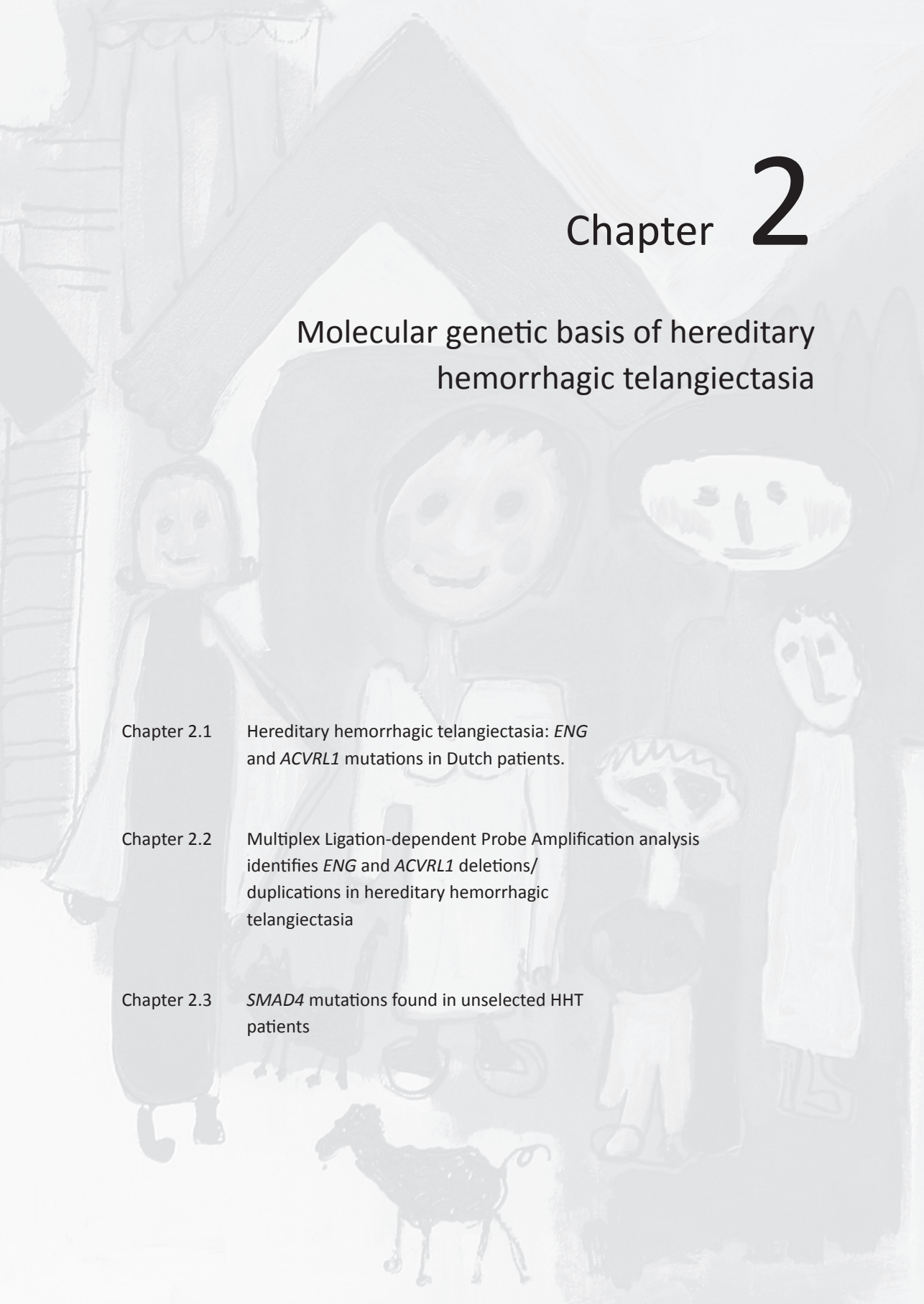
References

1. Rendu M. Epistaxis répétes chez un sujet porteur de petits angiomes cutanés et muqueux. Gaz H6p (Paris) 1896; 68: 1322-1323.
2. Osler W. On a family form of recurring epistaxis, associated with multiple telangiectases of the skin and mucous membranes. Johns Hopk Hosp Bull 1901; 12: 333-337.
3. Weber FP. Multiple hereditary developmental angiomata (telangiectases) of the skin and mucous membranes associated with recurring haemorrhages. Lancet 1907; ii: 160-162.
4. Hanes FM. Multiple hereditary telangiectases causing haemorrhage. (Hereditary haemorrhagic telangiectasia). Johns Hopk Hosp Bull 1909; 20: 63-73.
5. Shovlin CL, Letarte M. Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax.1999; 54: 714-729.
6. 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; 116: 324-328.
7. 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.
8. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, McCormick MK.; Pericak-Vance MA, Heutink P, Oostra BA, Haitjema T, Westerman CJJ, Porteous ME, Guttmacher AE, Letarte M, Marchuk DA. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 1994: 345-351.
9. Heutink P, Haitjema T, Breedveld GJ, Janssen B, Sandkuijl LA, Bontekoe CJ, Westerman CJ, Oostra BA. Linkage of hereditary haemorrhagic telangiectasia to chromosome 9q34 and evidence for locus heterogeneity. J Med Genet. 1994; 31:933-936.
10. 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, 13: 189-195.
11. Van den Driesche S, Mummery CL, Westermann CJ. Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. Cardiovasc Res 2003; 58: 20-31.
12. Bourdeau A, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M. Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. Am J Pathol 2000; 156: 911-923.
13. 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.
14. 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). The Lancet 2004; 363: 852-859.
15. 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 Part A 2006; 140: 2155-2162.
16. Cole SG, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet 2005;.42: 577-582.
17. Hawinkels LJ, Kuiper P, Wiercinska E, Verspaget HW, Liu Z, Pardali E, Sier CF, ten Dijke P. Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. Cancer Res. 2010; 70: 4141-4150
18. Bourdeau A, Faughnan ME, Letarte M. Endoglin-deficient mice, a unique model to study hereditary hemorrhagic telangiectasia. Trends Cardiovasc Med. 2000; 10: 279-285
19. Torsney E, Charlton R, Diamond AG, Burn J, Soames JV, Arthur HM. Mouse model for hereditary hemorrhagic telangiectasia has a generalized vascular abnormality. Circulation 2003; 107: 1653-1657.
20. van Laake LW, van den Driesche S, Post S, Feijen A, Jansen MA, Driessens MH, Mager JJ, Snijder RJ, Westermann CJ, Doevendans PA, van Echteld CJ, ten Dijke P, Arthur HM, Goumans MJ, Lebrin F, Mummery CL. Endoglin has a crucial role in blood cell-mediated vascular repair. Circulation. 2006; 114: 2288-2297.

21. Lebrin F, Mummery CL Endoglin-mediated vascular remodeling: mechanisms underlying hereditary hemorrhagic telangiectasia. Trends Cardiovasc Med. 2008; 18: 25-32.
22. ten Dijke P, Goumans MJ, Pardali E. Endoglin in angiogenesis and vascular diseases. Angiogenesis. 2008; 11:79-89.
23. Goumans MJ, Liu Z, ten Dijke P. TGF-beta signaling in vascular biology and dysfunction. Cell Res. 2009; 19: 116-127.
24. 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.
25. Plauchu H, de Chadarevian JP, Bideau A, Robert JM. Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. Am J Med Genet 1989; 32: 291-297.
26. Guttmacher AE, Marchuk DA, White RI Jr. Hereditary hemorrhagic telangiectasia. N.Engl J Med 1995; 333: 918-924.
27. Khurshid I, Downie GH. Pulmonary arteriovenous malformation. Postgrad Med J. 2002; 78; 191-197
28. Mager JJ, Overtoom TT, Blauw H, Lammers JW, Westermann CJ. Embolotherapy of pulmonary arteriovenous malformations: long-term results in 112 patients. J Vasc Interv Radiol 2004; 15: 451-456.
29. Willemse RB, Mager JJ, Westermann CJ, Overtoom TT, Mauser H, Wolbers JG. Bleeding risk of cerebrovascular malformations in hereditary hemorrhagic telangiectasia. Neurosurg. 2000; 92: 779-784.
30. Haitjema T, Disch F, Overtoom TT, Westermann CJ, Lammers JW. Screening family members of patients with hereditary hemorrhagic telangiectasia. Am J Med 1995; 99: 519-524.
31. Easey AJ, Wallace GM, Hughes JM, Jackson JE, Taylor WJ, Shovlin CL. Should asymptomatic patients with hereditary haemorrhagic telangiectasia (HHT) be screened for cerebral vascular malformations? Data from 22,061 years of HHT patient life. J Neurol Neurosurg Psychiatry. 2003; 74: 743-748.
32. Brancati F, Valente EM, Tadini G, Caputo V, Di Benedetto A, Gelmetti C, Dallapiccola B. Autosomal dominant hereditary benign telangiectasia maps to the CMC1 locus for capillary malformation on chromosome 5q14. J Med Genet. 2003; 40: 849-853.
33. Revencu N, Boon LM, Mulliken JB, Enjolras O, Cordisco MR, Burrows PE, Clapuyt P, Hammer F, Dubois J, Baselga E, Brancati F, Carder R, Quintal JM, Dallapiccola B, Fischer G, Frieden IJ, Garzon M, Harper J, Johnson-Patel J, Labrèze C, Martorell L, Paltiel HJ, Pohl A, Prendiville J, Quere I, Siegel DH, Valente EM, Van Hagen A, Van Hest L, Vaux KK, Vicente A, Weibel L, Chitayat D, Vikkula M. Parkes Weber syndrome, vein of Galen aneurysmal malformation, and other fast-flow vascular anomalies are caused by RASA1 mutations. Hum Mutat. 2008; 29: 959-965.

Chapter 2

Molecular genetic basis of hereditary hemorrhagic telangiectasia



Chapter 2.1

Hereditary hemorrhagic telangiectasia: *ENG* and *ACVRL1* mutations in Dutch patients

Letteboer TG, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK.

Hum Genet. 2005; 116: 8-16.

Abstract

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber disease is an autosomal dominant disorder characterized by an aberrant vascular development. The resulting vascular lesions range from smaller mucocutaneous telangiectases to large visceral arteriovenous malformations, especially in the skin, lung, gastrointestinal tract and the brain.

Mutations in endoglin (*ENG*, chromosome 9q34) and *ACVRL1* (*ALK1*, chromosome 12q13) are associated with HHT1 and HHT2, respectively. We report here on the genetic and molecular heterogeneity found in the HHT population in the Netherlands. Probands of 104 apparently unrelated families were studied and we performed sequence analysis on both the *ENG* gene and *ACVRL1* gene. In most of the probands, we found a mutation in one of the two genes: 53% in the *ENG* gene and 40% in the *ACVRL1* gene. In 7% of the families no *ENG* or *ACVRL1* mutation was found. The mutations detected were deletions, insertions, nonsense, missense and splice site mutations. The majority were novel mutations.

Introduction

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber (ROW) syndrome is an autosomal dominant disease characterized by vascular malformations in multiple organ systems. The clinical manifestations are caused by direct arteriovenous connections without an intervening capillary bed. This can result in a range of malformations from smaller mucocutaneous telangiectases to large visceral arteriovenous malformations (AVM). The clinical manifestations of HHT include recurrent epistaxis, multiple telangiectases, at characteristic sites (lips, oral cavity, nose, fingers), visceral lesions, such as gastrointestinal telangiectases, pulmonary arteriovenous malformations (PAVM), cerebral arteriovenous malformations (CAVM) and hepatic arteriovenous malformations (HAVM) [1,2].

Recurrent epistaxis is usually the first symptom and present in more then 90% of patients with HHT [2,3]. Telangiectases usually start to appear in the third decade of life and can be detected in more than 80% of the HHT patients [1,2,4]. Gastrointestinal bleeding from intestinal telangiectatic lesions usually does not start before fifth decade and may cause severe anemia [1,4].

The most common site of AVM is the lung. Pulmonary arteriovenous malformations are estimated to develop in more then 20% of the patients [2]. PAVMs result in a right to left shunt and thus hypoxemia and may cause serious complications such as, bleeding (hemothorax), bypass of emboli or bacteria causing serious systemic complications (stroke, cerebral abscess) [1,2]. Because of these serious complications, treatment of PAVMs is indicated, even when asymptomatic.

Cerebral arteriovenous malformations (CAVMs) are less common, but are probably under-recognized, and present in 10-15% of the patients [5]. Although they are often silent, they can cause headache, seizures, ischemia and bleeding [6].

Mutations in the endoglin (*ENG*, OMIM 131195) and activin A receptor type-like kinase 1 (*ACVRL-1*, *ALK-1*, OMIM 601284) genes are associated with HHT. In 1994 the endoglin gene was identified on chromosome 9q34 as the gene, when mutated, responsible for HHT1 (OMIM 187300) [7]. In 1996 mutations in *ACVRL1* (activin-like receptor kinase: *ACVRL1* or *ALK-1*) located on chromosome 12q13 were found to cause HHT2 (OMIM 600376) [8]. Both genes are expressed predominantly in endothelial cells. The proteins mediate binding and signaling of TGFβ, which is important in blood vessel growth and repair [9]. Protein expression studies in human umbilical vein endothelial cells and peripheral blood monocytes have confirmed haploinsufficiency as the model in HHT1 and HHT2 [9-11]. Mutations in *ACVRL1* and *ENG* both result in epistaxis and telangiectases, but a higher prevalence of PAVMs and CAVMs has been reported in HHT1. Families with HHT2 generally also show a later onset of the symptoms [2,12,13]. However, within families there is considerable inter- and intra-familial variability with respect to age-related penetrance and pattern of clinical expression of the disease.

Recently, Gallione *et al.*[14] reported mutations in the MADH4 gene in patients that show

clinical features of both HHT and juvenile polyposis. MADH4 codes for the protein *SMAD4* and is expressed in a variety of cell types. The protein has a role in the TGFβ pathway, like *ENG* and *ACVRL1* as well as in the bone-morphogenic-protein pathway. HHT as part of this syndrome can thus be explained by MADH4 mutations.

To date, more than 111 different mutations have been identified in the *ENG* gene [15-17], whereas the number of different mutations reported in the *ACVRL1* gene is 77 [9,17]. Almost all the mutations reported are unique for a particular family.

In this study we report on the genetic molecular analysis of the probands of 104 families from the Netherlands with the clinical diagnosis HHT, to provide insight into the genetic and molecular heterogeneity of patients living in the Netherlands.

Materials and methods

Patients

All probands were from a single tertiary referral center in the Netherlands, specializing in the diagnosis and treatment of HHT. All but eight families were of Dutch origin; two families were from Belgium (F112, F113), one came from former Yugoslavia (F26), one from Turkey (F37), one from Iran (F36), one from Lithuania (F83), one from the Netherlands Antilles (F153) and one family from Surinam (F25).

The medical and family history of each patient was recorded. The diagnosis was established according to the Curaçao criteria [18]. At least three of the following four criteria were required for a clinical diagnosis: spontaneous and recurrent epistaxis, telangiectases at characteristic sites, visceral manifestations (PAVM, CAVM, HAVM) and a first-degree relative with HHT.

Amplification and nucleotide sequence determination

High molecular weight DNA of the probands was isolated from peripheral blood leukocytes according to established procedures. The exons 1-14 of the *ENG* gene and exons 1-10 of the *ACVRL1* gene and their flanking sequences were amplified using PCR (polymerase chain reaction). The amplification primers were derived from the *ENG* genomic sequence (GenBank BC014271.2) and *ACVRL1* genomic sequence (GenBank: NM_000020.1) (see table 1). The PCR was carried out essentially as described by Bergman *et al.* [19]. For exons 7 and 8 of the *ACVRL1* gene, the GeneAmp 10X PCR buffer II (Applied Biosystems, Foster City, CA) was used. Amplification was performed on a Gene Amp 9700 thermal cycler (Applied Biosystems) using an initial denaturation step at 94ºC for 4 min. followed by 33 cycles at 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min for the exons. For *ENG* exons 5 and 14 an annealing temperature of 58ºC was used.

The amplified fragments were first analyzed by agarose-gel electrophoresis. Next,

the PCR products were purified using the QIAquick 96 PCR BioRobot Kit (Qiagen, Venlo, the Netherlands). The purified fragments were sequenced, using the ABI Prism Big Dye terminator Cycle Sequencing ready Reaction KIT (Applied Biosystems) on an ABI-PRISM 3700 DNA analyzer (Applied Biosystems). The sequences were compared to the reported gene sequence using the Seqscape program (Applied Biosystems).

Table 1 - Nucleotide sequence of the amplification primers of *ENG* and *ACVRL1*

	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon (bp)
ENG			
exon 1	ACTGGACACAGGATAAGGCCCAG	AATACTTGGGGCCTGGTCCGTG	214
exon 2	CACCTTATTCTCACCTGGCCTCTT	CTGCCTTGGAGCTTCCTCTGAG	282
exon 3	GGGTGGCACAACCTATACAAAT	CAGAGATGGACAGTAGGGACCT	294
exon 4	TTCCTGACCTCCTACATGGG	TTCAGCTCAGCAGCAGAGG	310
exon 5	TGAGGGAAGGGACTGAGGTG	GTGGGGACTAGTGTCAGGGGC	272
exon 6	GGCCTGTCCGCTTCAGTGTT	GTTTTGTGTCCCGGGAGCTG	237
exon 7	CCCCCTGTTCTGCCTCTCTC	CTGATCCAAGGGAGGGGAAG	292
exon 8	ACACATATCACACAGTGACCAGC	CTAGGGGAGGAACCAGATGTC	286
exon 9a	CTCCTGATGGTGCCCCTCTCTT	TTGTCTTGTGTTCTGAGCCCCTG	330
exon 9b	ATTGGGTGGGATACCCTCTGGG	GGGTTAAGCACGTGACTGTCC	247
exon 10	ATTGACCAAGTCTCCCTCCC	GAAAGGCGGAGAGGAAGTTC	301
exon 11	GGTGGGGTGAAGAGCAGCTG	GACCTGGAAGCTCCCACTTGAA	390
exon 12	GAGTAAACCTGGAAGCCGC	GCCACTAGAACAAACCCGAG	188
exon 13	GACTGAGGTTCAGAGAAGTCGAG	CTCAGAGGCTTCACCTGGGCTCC	289
exon 14	AGGACCCTGACCTCCGCC	CTCTCCTGCTGGGCGAGC	232
ACVRL1			
exon 1	AGTCGGCGAGCTGGGCAATA	ACTGAGCCTCCCGCGACCAC	375
exon 2	CTCTGTGATTTCCTCTGGGCA	TACATTCTCCCCAGCTTCTCAA	300
exon 3	AGCTGGGACCACAGTGGCTGA	GGAGGCAGGGGCCAAGAAGAT	380
exon 4	AGCTGACCTAGTGGAAGCTGA	CTGATTCTGCAGTTCCTATCTG	352
exon 5	AGGAGCTTGCAGTGACCCAGCA	ATGAGAGCCCTTGGTCCTCATCCA	276
exon 6	AGGCAGCGCAGCATCAAGAT	AAACTTGAGCCCTGAGTGCAG	330
exon 7	TGACGACTCCAGCCTCCCTTAG	CAAGCTCCGCCCACCTGTGAA	423
exon 8	AGGTTTGGGAGAGGGGCAGGAGT	GGCTCCACAGGCTGATTCCCCTT	327
exon 9	TCCTCTGGGTGGTATTGGGCCTC	CAGAAATCCCAGCCGTGAGCCAC	290
exon 10	TCTCCTCTGCACCTCTCTCCCAA	CTACCTCTACCCAGATAGGG	283

Results

The 104 HHT probands were screened for genetic changes in the protein encoding exons and their flanking sequences of both the *ENG* and *ACVRL1* gene. Exon 1 of the *ACVRL1* was analyzed only for those patients who did not show a mutation in either *ACVRL1* or *ENG* protein encoding exons, as it encodes the 5' untranslated region of the mRNA only. Sequence alterations in the *ENG* and *ACVRL1* gene were detected in 97 probands. *ENG* mutations were found in 55 patients (summarized in table 2) and

Table 2 - Summary of the identified *ENG* mutations

mutation type	Proband	Exon	Mutations cDNA	Protein	Reference
nonsense	F112	2	c.142C>T	p.Q48X	
	F75	2	c.157C>A	p.C53X	
	F2, F19, F49	3	c.247C>T	p.Q83X	
	F15	3	c.277C>T	p.R93X	Cymerman et al. [2000]
	F10	8	c.1078C>T	p.Q360X	
	F18	11	c.1611C>A	p.Y537X	
	F54	11	c.1778C>T	p.Q560X	
	F144	11	c.1684C>T	p.Q562X	
deletion /	F3	3	c.263delA	p.N88fs	
insertion	F183	3	c.332_338delCCCTGGG	p.A111fs	
	F11	4	c.497_498insC	p.Q166fs	
	F4	5	c.577_578insGC	p.T193fs	
	F150	6	c.701_702insACGG	p.V234fs	
	F78	6	c.733delG	p.G245fs	
	F113	6	c.766_767insC	p.P256fs	
	F39, F131	6	c.787_789delATC	p.Idel263	Lesca et al. [2004]
	F9, F149	7	c.887_918del;c919_920insCAAGCTCCCAG	Del32bp,ins11bp	McAllistar et al. [1994]
	F36	8	c.995delG	p.G332fs	
	F55	8	c.1117_1118insT	p.K373fs	
	F77	9	c.1142_1143insT	p.K381fs	
	F28	9	c.1255delA	p.S419fs	
	F30	10	c.1317_1318insA	p.V440fs	
	F12	10	c.1346_1347delCT	p.S449fs	Lesca et al. [2004]
	F26	11	c.1437_1438delGT	p.V479fs	

mutation type	Proband	Exon	Mutations cDNA	Protein	Reference
splice site	F153	1	c.67+1g>a		Gallione et al [2000]
	F37	3	c.360+1g>a		Pece et al.[1997], Cymerman et al. [2003]
	F151	3	c.360+5g>a		
	F6	5	c.689+2t>c		Lesca et al. [2004]
	F45	8	c.1134G>A TGCGgtaa	p.A378A	
	F16	9b	c.1310delG		
	F43, F48, F56, F73, F142	9b	c.1311G>A AGCGGtgag	p.R437R	
missense	F7	1	c.1A>G	p.M1V	
	F25	4	c.392C>T	p.P131L	Cymerman et al. [2003]
	F81	5	c.572G>A	p.G191D	Lesca et al. [2004], polymorphism?
	F8, F23, F41, F59, F62, F68, F74, F176	6	c.781T>C	p.W261R	
	F145	6	c.791A>G	p.D264N	
	F14	7	c.917T>C	p.L306P	Gallioni et al. [1998]
	F186	7	c.991G>A	p.G331S	
	F154	8	c.1121_1122 AA>GC	p.K374S	
	F13	9a	c.1238G>T	p.G413V	Lux et al. [2000]

ACVRL1 mutations in 42 patients (summarized in table 3). One proband showed mutations in both the *ENG* and *ACVRL1* gene and one proband had two mutations in *ACVRL1*. Seven probands had no mutation in either the *ENG* gene or the *ACVRL1* gene.

Mutations in the *ENG* gene

Forty different mutations were identified, 29 of these have not previously been reported.

Eight different **nonsense mutations** were found. One new nonsense mutation was found in three probands (F2, F19, F49): a c.247C>T transition that results in a premature stop at codon 83 (p.Q83X). The other seven nonsense mutations were unique, six of which were novel (p.Q48X, p.C53X, p.Q360X, p.Y537X, p.Q560X, and p.Q562X). The p.R93X of F15 was

reported by Cymerman *et al.* [20].

Nine different **missense mutations** were found in 16 probands. Of these, the amino acid change p.W261R caused by c.781T>C was found in eight probands. In one of these probands (F68) a mutation was detected also in the *ACVRL1* gene, p.A482V (c.1445C>T). The substitutions p.L306P, p.G413V, p.G191D and p.P131L have already been published [15,17,21,22]. F7 showed an alteration of the very first base pair of the first codon. This alteration causes a p.M1V missense mutation and destroys the AUG initiation site of translation. In F186, the last base pair in exon 7 is changed (c.991G>A), thereby disrupting the donor splice site consensus as well. The p.K374S (F154) is caused by a substitution of two base pairs. The mutation in F145 (p.D264N) changes aspartic acid into asparagine, amino acids with different polarities. In F81 a c.572G>A resulted in a p.G191D substitution. This mutation has also been identified in a panel of normal individuals, albeit at a low frequency. It therefore suggests that the p.G191D substitution is not a disease-causing mutation.

Eight **deletions,** seven **insertions and** one **complex rearrangement** were identified in 18 patients. The inframe deletion (c.787_789delATC) was found in two families and reported earlier in a French patient [17]. The c.1346_1347delCT was published earlier [17] as well. A complex inframe rearrangement occurred in F9 and F149, which has been reported as a deletion of 21 base pairs by others [7]. The rearrangement involves a 32 base pair deletion and an insertion-duplication of 11 base pairs (see Figure 1).

ENG Normal

```
851 AGAAAAACATTCGTGGCTT**CAAGCTCCCAG**ACACAC*CTCAAGGCCTCCTGGGGGAGGCCC*
284 E--K--N--I--R--G--F--K--L--P--D--T--P--Q--G--L--L--G--E--A--

911 *GGATGCTC***A**ATGCCAGCATTGTGGCATCCTTCGTGGAGCTACCGCTGGCCAGCATTGTCT
304 R--M--L--N--A--S--I--V--A--S--F--V--E--L--P--L--A--S--I--V--
```

ENG Mutation F9: c.887_918del;c919_920insCAAGCTCCCAG

```
851 AGAAAAACATTCGTGGCTT**CAAGCTCCCAG**ACACAC**ACAAGCTCCCAGAT**----------
284 E--K--N--I--R--G--F--K--L--P--D--T--H--K--L--P--D-

911 ----------GCCAGCATTGTGGCATCCTTCGTGGAGCTACCGCTGGCCAGCATTGTCT
304 ----------A--S--I--V--A--S--F--V--E--L--P--L--A--S--I--V--
```

Figure 1 - The complex alteration in exon 7 of the *ENG* gene in family F9. The deleted sequence is depicted in Italic, underlined in bold is the duplicated sequence.

Seven different **splice site mutations** were found in 11 families. Three mutations (c.360+1g>a, c.689+2t>c and c.67+1g>a) have been found also by others [15,17,23,24]. In five probands (F43, F48, F56, F73, and F142), a p.R437R silent mutation due to a c.1311G>A transition was

identified, in one proband (F45) a p.A378A due to c.1134G>A. However, both transitions occur at the last position of the exons (exon 9b and exon 8). These positions are part of the consensus sequence of the donor splice site and are highly conserved. The c.1310delG mutation identified in F16 is in the last triplet of exon 9 and will disrupt the reading frame but might also influence the consensus sequence of the donor splice site. Finally, the mutation in F151 (c.360+5g>a) also changes the donor splice site creating a less favorable splice site.

Mutations in the ACVRL1 gene

Thirty-one different mutations were detected in the *ACVRL1* gene, 25 of which were novel.

Seven **nonsense mutations** were found, five of which (p.W50X, p.Q118X, p.Q147X, p.Q321X, p.E470X) were novel. The c.858C>A change (F24) resulting in a p.Y286X and the c.1435C>T resulting in a p.R479X were reported earlier [17,25].

Nine different **deletions** and two **insertions** were found in 16 patients. The c.1042delG deletion was identified in seven families (F33, F44, F46, F82, F95, F123 and 152). The mutation in F42 deletes 18 base pairs, leaving the reading frame in tact, but deleting six amino acids at the protein level. One family carried a c.625_626insTG insertion of 2 base pairs next to the last coding base pair of exon 5. This mutation disrupts the reading frame but might also influence the donor splice site. In F108 a 14-base pair deletion was identified that encompasses the acceptor splice site and the first nucleotides of exon 6.

Thirteen different **missense mutations** were detected in 17 patients. We identified the c.1120C>T that causes a p.R374W substitution, also reported by others [11,26,27], in three patients (F58, F83, F85). The c.1121G>A mutation (p.R374Q) [11,17], the c.1231C>T (p.R411W) [11,17,28] and the c.1450C>T (p.R484W) [17,28] were reported earlier. The p.G309S, p.V380G, p.W406C, p.P433S, p.M438T, p.P424L, p.D330N and p.D397N mutations were all novel. Three amino acid substitutions in the same codons were reported by others (p.D397G by Lesca *et al.*[17]; p.P424T by Berg *et al.*[26] and p.D330Y by Olivieri *et al.*[25]).The novel c.1048G>C (p.G350R) mutation involves the last base pair of exon 7, changing the donor splice site as well. In F181 two mutations were present (p.P424L and p.A482V).

Table 3 - Summary of the identified ACVRL1 mutations.

Mutation type	Patient	Exon	Mutation cDNA	*Protein*	Reference
nonsense	F139	3	c.150G>A	p.W50X	
	F51	4	c.352C>T	p.Q118X	
	F50	4	c.439C>T	p.Q147X	
	F24	7	c.858C>A	p.Y286X	Olivieri et al. [2002]
	F34	7	c.961C>T	p.Q321X	
	F22, F141	10	c.1408G>T	p.E470X	

Mutation type	Patient	Exon	Mutation cDNA	*Protein*	Reference
nonsense	F1	10	c.1435C>T	p.R479X	Lesca et al. [2004]
deletion /	F96	3	c.83delG	p.R28fs	
insertions	F107	3	c.190delC	p.Q64fs	
	F71	3	c.203delG	p.G68fs	
	F32	4	c.372_373insCC	p.G124fs	
	F57	5	c.625_626insTG	p.G209fs	
	F123, F44, F95, F82, F46, F33, F152	7	c.1042delG	p.D348fs	
	F137	8	c.1061_1068del8bp	p.M354fs	
	F79	8	c.1071delG	p.Q357fs	
	F5	8	c.1107_1108delAG	p.R369fs	
	F42	8	c.1120_1137del18bp	p.R374_E379del	
splice site	F108		c.626-6del14bp		
missense	F116	7	c.925G>A	p.G309S	
	F67, F69	7	c.988G>A	p.D330N	
	F105	7	c.1048G>C	p.G350R	
	F58, F83, F85	8	c.1120C>T	p.R374W	Berg et al. [1997], Kjeldsen et al. [2001], Abdalla et al. [2003]
	F35	8	c.1121G>A	p.R374Q	Abdalla et al [2003], Lesca et al. [2004]
	F61	8	c.1139T>G	p.V380G	
	F53	8	c.1189G>A	p.D397N	
	F109	8	c.1218G>C	p.W406C	
	F84, F80	8	c.1231C>T	p.R411W	Trembath et al. [2001], Abdalla et al. [2003], Lesca et al. [2004]
	F181	9	c.1271C>T	p.P424L	
	F86	9	c.1297C>T	p.P433S	
	F187	9	c.1313T>C	p.M438T	
	F20	10	c.1450C>T	p.R484W	Trembath et al. [2001], Lesca et al. [2004]

Discussion

We describe here mutations in *ENG* and *ACVRL1* in a large group of HHT patients living in the Netherlands. The mutations are distributed along both genes and comprise all types of mutations, e.g. affecting splice sites, missense mutations, nonsense mutations, insertions and deletions. The majority of the mutations detected were novel since only 17 of the 71 mutations had been published earlier. Our study therefore adds 54 novel mutations to the HHT mutation database (http://genetics.mc.duke.edu/hht/). To date, 188 different HHT mutations have been described.

In the HHT families, we identified more defects in the *ENG* gene (40 different mutations, 53% of probands) than in the *ACVRL1* gene (23 different mutations, 40% of probands). Ten mutations were found in more than one proband. The families were first thought to be unrelated but recent genealogical analysis has provided insight into the possibility of shared ancestry. Families with the *ENG* mutation p.W261R had a common ancestor dating back to 1765. The *ACVRL1* mutation c.1042delG appears to be a founder mutation, originating from a common ancestor in 1722. Finally, all five families with the p.R437R *ENG* mutation could be traced back to a single founder in 1745.

Two probands had two mutations. Proband F181 showed two mutations in the *ACVRL1* gene, a p.P424L and a p.A482V (c.1445C>T) missense mutation. It still has to be established whether they are in cis or in trans. The p.A482V was also found in proband F68 with the deleterious *ENG* mutation p.W261R, while p.A482V was also reported by D'Abronzo *et al.*[29]. This mutation was found in just one of 64 patients screened for *ACVRL1* mutations with a pituitary tumor. The patient and his family showed no symptoms of HHT, which therefore suggests that this mutation is not the cause of HHT. However, Lesca *et al.*[17] did identify this mutation in a patient with confirmed HHT. Furthermore, the alanine in this position is a highly conserved (see figure 2). Apparently the mutation does not always result in HHT symptoms. Functional studies should elucidate whether this mutation has a deleterious effect.

In the *ENG* gene we detected 8 nonsense, 8 deletions, 7 insertions, 1 complex rearrangement, 7 splice site mutations and 9 missense mutations. All the insertions and all but one deletion lead to a shift in the reading frame and will result in a truncated protein that is deleterious. In addition, it has been well established that mutations involving a premature translation stop also may cause a nonsense-mediated decay of mRNA. The c.360+1g>a donor splice site mutation seems to be a recurrent mutation. We found it in one family, others have found the mutation in different, independent families [15,23]. It has also been found as a *de novo* mutation in one family [23]. The p.R437R silent mutation found in F43, F48, F56, F142 and F73 is due to a c.1311G>A transversion at the last nucleotide of exon 9b. It creates a less favorable splice donor site, as confirmed by the Berkeley Drosophila Genome Project Splice Site Prediction Program. According to this program, the donor-motif probability decreased from 0.6 to undetectable. Consequently, it will hamper the proper maturation

of the pre-mRNA by intron retention and/or exon skipping. Gallione *et al.*[21] described a c.1311G>C change at the same position; this mutation was seen to co-segregate with the disease in the family. The donor-motif probabilities for the other splice site mutations were also unfavorable according to the prediction program.

Nine different *ENG* missense mutations were detected, one (p.W261R) was found in eight probands. The p.L306P was published by Gallione *et al.*[21] as completely disrupting the helix structure. The p.G413V was described as a mutation originating from the Netherlands and present in the Dutch Antilles [22]. The p.P131L was also reported by Cymerman *et al.*[15]. It is located in a sequence that is strongly conserved between man, mouse and pig. The mutation associates with a significantly reduced level of endoglin. The p.M1V mutation is a mutation at the very first nucleotide changing an A>G, changing the AUG start codon. A similar mutation (p.M1Y) was described by Gallione *et al.*[21]. These mutations are considered as disease causing by eliminating the proper start site of translation. The mutation in F186 (c.991G>A) changes the last base pair of exon 7, disrupting the donor splice site as well. The c.791A>G (p.D264N) and c.1121_1122AA>GC (p.K374S) involve amino acids that are conserved between man, mouse and pig.

The status of the p.G191D missense mutation is not obvious. The p.G191D mutation was found in two families. In one of our largest HHT families (F49), this mutation was detected in a branch of the family where the diagnosis of HHT was questionable. In another branch of this family, HHT segregated with the *ENG* nonsense mutation p.Q83X. Furthermore, the p.G191D mutation was also present at low frequency (1-5%) in a panel of normal individuals (presented at the Bonaire Annual HHT Meeting). And recently, this mutation was described in a French patient as a polymorphism [17], but not detected in 188 French control individuals. Still, our F81 showed no other abnormality than p.G191D. This could indicate that the disease-causing mutation has not been identified so far or that p.G191D is a mutation with a reduced risk or interacts with another gene mutation not yet discovered. Functional analysis should shed light on the effect of p.G191D and other mutations.

In *ACVRL1* we found 7 nonsense, 8 deletions, 2 insertion, 1 splice site mutation and 13 missense mutations. Exon 1 was not included in the analysis of all patients, but limited to those who did not show a mutation. Exon 1 contains no protein coding information, a deleterious mutation like a donor splice site mutation however, might be present. To date, no mutations in exon 1 of the *ACVRL1* have been reported. We did not identify any mutations in exon 1 of the *ACVRL1* gene in our probands.

All thirteen missense mutations that we identified were located in exons 7, 8, 9 and 10. The substitutions cause either an alteration in polarity, hydrophobicity or side chain-length. Four of these missense mutations (p.R374Q, p.R374W, p.R411W and p.R484W) have already been published. Abdalla *et al.*[11] reported several missense mutations in the *ACVRL1* cytoplasmatic domain. The amino acid changes occurred at positions that are highly conserved between *ACVRL1* and *ALK-5*, but also between *ACVRL1* and other

serine-threonine protein kinases. In addition, the missense mutations p.G309S, p.P424L, p. D330N, p.G350R, p.V380G, p.D397N, p.W406C, p.P433S, and p.M438T are located in the cytoplasmatic kinase domains and all involve residues conserved between *ACVRL1* and *ALK-5*. Finally, all missense mutations occurred at highly conserved residues between species (Rattus norvegicus, Musculus musculus, Gallus gallus and Drosophila melanogaster) as shown in figure 2. The conservation of amino acids between orthologous and paralogous sequences was analyzed using a web-based program (http//blocks.thcrc.org/sift) which predicts the effect of a missense mutation. All the codons containing missense mutations were indeed highly conserved and the mutations were predicted to disrupt the protein function. We therefore consider them as deleterious.

```
(1) 301 RLAVSAACGL AHLHVEIFGT QGKPAIAHRD 330
(2) 302 RLAVSAACGL AHLHVEIFGT QGKPAIAHRD 331
(3) 300 RLAVSPACGL AHLHVEIFGT QGKPAIAHRD 329
(4) 302 RIVLSIASGL AHLHIEIFGT QGKPAISHRD 331
(5) 363 WICLSIANGL VHLHTEIFGK QGKPAMAHRD 392

(1) 371 GTKRYMAPEV LDEQIRTDCF ESYKWTDIWA FGLVLWEIAR RTIVNGIVED 420
(2) 372 GTKRYMAPEV LDEQIRTDCF ESYKWTDIWA FGLVLWEIAR RTIINGIVED 421
(3) 370 GTKRYMAPEV LDEHIRTDCF ESYKWTDIWA FGLVLWEIAR RTIINGIVED 419
(4) 372 GTKRYMAPEV LDETIQADCF DSYKRVDIWA FGLVLWEVAR RMVSNGIVED 421
(5) 433 GTKRYMAPEV LDESIDLECF EALRRTDIYA FGLVLWEVCR RTISCGIAEE 482

(1) 421 YRPPFYDVVP NDPSFEDMKK VVCVDQQTPT 450  481 TALRIKKTLQ 490
(2) 422 YRPPFYDMVP NDPSFEDMKK VVCVDQQTPT 451  482 TALRIKKTLQ 491
(3) 420 YRPPFYDMVP NDPSFEDMKK VVCVDQQTPT 449  480 TALRIKKTLQ 489
(4) 422 YKPPFYDLVP NDPSFEDMRK VVCVDQQRPN 451  482 TALRIKKTLT 491
(5) 483 YKVPFYDVVP MDPSFEDMRK VVCIDNYRPS 512  544 PALRIKKTIH 553
```

(1) Homo sapiens, (2) Rattus norvegicus, (3) Musculus musculus, (4) Gallus gallus, (5) Drosophila melanogaster. Aminoacid changes reported in table 3 are given in bold.

Figure 2 - Alignment of amino acid sequence for genes orthologous to *ACVRL1*

Before this study, no mutations had been detected in exon 5 of the *ACVRL1* gene. This led to the speculation by Lesca's group that some missense mutations in exon 5 lead to a more severe phenotype, different to HHT and probably lethal. We report a frameshift mutation in exon 5 of the *ACVRL1* gene, a two base-pair deletion, proving that mutations in exon 5 do occur.

Our PCR and sequence-based approach enabled the identification of more than 90% of the disease-causing mutations in a panel of 104 HHT families. We did not screen for large deletions or insertions in *ACVRL1* or *ENG*, nor did we search for mutations in the promoter region or in intronic sequences apart from the splice sites. In general, large deletions may account for 5-10% of the genetic defects of monogenic disorders. We therefore plan to screen the remaining seven probands for large deletions by employing Multiplex Ligation-

dependent Probe Amplification (MLPA). Alternatively, more extensive locus heterogeneity may play a role. A few families have been described that show no significant linkage to either *ENG* or *ACVRL1* [30-32]. After re-evaluating all the members in the family studied by Piantanida and Buscarini [30,31], the evidence for exclusion of chromosome 12 was no longer considered significant and analysis revealed an *ACVRL1* mutation (p.R67W) [25]. However, the presence of a third locus cannot be ruled out. In this respect, the finding of MADH4 mutations in families with both juvenile polyposis and HHT renders it a good candidate gene.

Our observation that in an uniformly and well-classified panel of HHT families, disease-causing mutations in *ENG* or *ACVRL1* were identified in more than 90% of the HHT families, taken together with the known short comings of our approach, make the existence of a prominent third HHT locus in our population less likely. We expect to be able to increase the detection rate by applying MLPA. The third HHT gene will account for only a small minority of our HHT population.

Recently, Lesca's group published molecular analyses of 160 French HHT patients and found germline mutations in 100 patients (62.5%). In a subgroup of their probands (only confirmed diagnosis), the mutation rate was 68%. They used a different DNA analysis technique in 133 patients of the 160 patients, based on heteroduplex analysis of coding regions and intronic borders after PCR amplification and electrophoresis. Because of the reduced sensitivity of the heteroduplex analysis, this might in part explain the difference in mutation detection percentage. An additional explanation is that not all their probands had a definite diagnosis of HHT.

Many mutations have been described in families with HHT. The majority of the mutations are unique; only a few mutations occur more than once. As far as has been investigated in the Dutch population (three mutations: in the c.1042delG five of the seven families had a common ancestor, in the p.W261R three families and in the p.R437R all families had a common ancestor), the mutations originate from a common ancestor. Mutations in our HHT population already published by others could also be due to these old ancestral mutations.

In our HHT panel we found more different mutations in the *ENG* gene (40) than in the *ACVRL1* gene (31). The most obvious explanation is that the *ENG* gene has a correspondingly higher content of coding information (658 amino acids, 15 exons) than the *ACVRL1* gene (504 amino acids, 10 exons), and consequently has more positions prone to mutation. Indeed the relative content of the coding information between *ENG* and *ACVRL1* is in good concordance with the number of mutations found in the *ENG* and *ACVRL1* gene.

Another explanation from a clinical point of view might be that the HHT2 phenotype is milder compared to HHT1, as suggested by Shovlin *et al.*[2] and Berg et al.[13]. In HHT2, symptoms are reported to arise later and the PAVM and CAVM are less frequent. HHT1 patients are therefore likely to be more easily diagnosed and thus over-represented in the patient population we have studied.

In the French population, *ENG* mutations were found in 36 probands, and *ACVRL1*

mutations in 64 probands. The higher proportion of *ACVRL1* mutations was partly explained by the finding of a recurrent mutation in the *ACVRL1* gene, the frameshift mutation c.1112_1113dupG was found in 17 probands with a common haplotype, suggesting a founder mutation. Still, more mutations were discovered in the *ACVRL1* gene than in the *ENG* gene (36 versus 34). Whether this is a population effect or due to a patient selection bias remains to be elucidated.

To conclude, our findings raise the total number of mutations in the *ENG* and the *ACVRL1* genes associated with HHT significantly. The total number of published *ENG* mutations (excluding the p.G191D) is now 140 and for *ACVRL1* it is 102. The mutations detected in our cohort of HHT patients offer an additional means of identifying carriers in HHT families unambiguously. In this way, appropriate medical attention can be given to only those carrying a mutation and we can prevent unnecessary screening of non-carrier children in the family.

The identification of the disease-causing mutations in a large panel of HHT families provides us with a good basis for studying the phenotype-genotype relationship for both *ENG* and *ACVRL1* within and between families in detail. In this way we hope to gain more insight into the variability of clinical expression and the factors contributing to HHT.

References

1. Guttmacher AE, Marchuk DA, White RI Jr. Hereditary hemorrhagic telangiectasia. N Engl J Med 1995; 333: 918-924.
2. Shovlin CL, Letarte M. Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax 1999; 54: 714-729.
3. Haitjema T, Balder W, Disch FJ, Westermann CJ. Epistaxis in hereditary haemorrhagic telangiectasia. Rhinology 1996; 34: 176-178.
4. Haitjema T, Westermann CJ, Overtoom TT, Timmer R, Disch F, Mauser H, Lammers JW. Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu disease): new insights in pathogenesis, complications, and treatment. Arch Intern Med 1996; 156: 714-719.
5. 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.
6. Haitjema T, Disch F, Overtoom TT, Westermann CJ, Lammers JW. Screening family members of patients with hereditary hemorrhagic telangiectasia. Am J Med 1995; 99: 519-524.
7. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, et al. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 1994; 8: 345-351.
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; 13: 189-195.
9. Van den Driesche S, Mummery CL, Westermann CJ. Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. Cardiovasc Res 2003; 58: 20-31.
10. Bourdeau A, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M. Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. Am J Pathol 2000: 156: 911-923.
11. 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.
12. Berg JN, Guttmacher AE, Marchuk DA, Porteous ME. Clinical heterogeneity in hereditary haemorrhagic telangiectasia: are pulmonary arteriovenous malformations more common in families linked to endoglin? J Med Genet 1996: 33: 256-257.
13. 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 ALK-1 mutations. J Med Genet 2003; 40: 585-590.
14. 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). The Lancet 2004: 363: 852-859.
15. Cymerman U, Vera S, Karabegovic A, Abdalla S, Letarte M. Characterization of 17 novel endoglin mutations associated with hereditary hemorrhagic telangiectasia. Hum Mutat 2003; 21: 482-492.
16. Lastella P, Sabba C, Lenato GM, Resta N, Lattanzi W, Gallitelli M, Cirulli A, Guanti G. Endoglin gene mutations and polymorphisms in Italian patients with hereditary haemorrhagic telangiectasia. Clin Genet 2003; 63: 536-540.
17. Lesca G, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Riviere 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.
18. 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.
19. Bergman AJ, van den Berg IE, Brink W, Poll-The BT, Ploos van Amstel JK, Berger R. Spectrum of mutations in the fumarylacetoacetate hydrolase gene of tyrosinemia type 1 patients in northwestern Europe and Mediterranean countries. Hum Mutat 1998; 12: 19-26.

20. Cymerman U, Vera S, Pece-Barbara N, Bourdeau A, White RI Jr, Dunn J, Letarte M. Identification of hereditary hemorrhagic telangiectasia type 1 in newborns by protein expression and mutation analysis of endoglin. Pediatr Res 2000: 47: 24-35.
21. Gallione CJ, Klaus DJ, Yeh EY, Stenzel TT, Xue Y, Anthony KB, McAllister KA, Baldwin MA, Berg JN, Lux A, Smith JD, Vary CP, Craigen WJ, Westermann CJ, Warner ML, Miller YE, Jackson CE, Guttmacher AE, Marchuk DA. Mutation and expression analysis of the endoglin gene in hereditary hemorrhagic telangiectasia reveals null alleles. Hum Mutat 1998; 11: 286-294.
22. Lux A, Gallione CJ, Marchuk DA. Expression analysis of endoglin missense and truncation mutations: insights into protein structure and disease mechanisms. Hum Mol Genet 2000; 9: 745-755.
23. Pece N, Vera S, Cymerman U, White RI Jr, 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.
24. Gallione CJ, Scheessele EA, Reinhardt D, Duits AJ, Berg JN, Westermann CJ, 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.
25. Olivieri C, Mira E, Delu G, Pagella F, Zambelli A, Malvezzi L, Buscarini E, Danesino C. Identification of 13 new mutations in the ACVRL1 gene in a group of 52 unselected Italian patients affected by hereditary haemorrhagic telangiectasia. J Med Genet 2002; 39: E39.
26. 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: 60-67.
27. Kjeldsen AD, Brusgaard K, Poulsen L, Kruse T, Rasmussen K, Green A, Vase P. Mutations in the ALK-1 gene and the phenotype of hereditary hemorrhagic telangiectasia in two large Danish families. Am J Med Genet 2001; 98: 298-302.
28. 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; 345: 325-334.
29. 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; 84: 1716-1721
30. Piantanida M, Buscarini E, Dellavecchia C, Minelli A, Rossi A, Buscarini L, Danesino C. Hereditary haemorrhagic telangiectasia with extensive liver involvement is not caused by either HHT1 or HHT2. J Med Genet 1996; 33: 441-443.
31. Buscarini E, Buscarini L, Danesino C, Piantanida M, Civardi G, Quaretti P, Rossi S, Di Stasi M, Silva M. Hepatic vascular malformations in hereditary hemorrhagic telangiectasia: Doppler sonographic screening in a large family. J Hepatol 1997: 26: 111-118.
32. Wallace GM, Shovlin CL. A hereditary haemorrhagic telangiectasia family with pulmonary involvement is unlinked to the known HHT genes, endoglin and ALK-1. Thorax 2000; 55: 685-690.

Chapter 2.2

Multiplex Ligation-dependent Probe Amplification analysis identifies *ENG* and *ACVRL1* deletions/duplications in hereditary hemorrhagic telangiectasia

T.G.W. Letteboer, J.J. Mager, R.J. Snijder, D. Lindhout, C.J. Gallione, J.J van de Ende, M. Kerstjens, J.W. Dixons, C.J.J. Westermann, J.K. Ploos van Amstel

submitted

Abstract

Hereditary hemorrhagic telangiectasia is an autosomal dominant disease characterized by epistaxis, cutaneous and mucosal telangiectases and visceral arteriovenous malformations. Mutations in *ENG* and *ACVRL1* have been implicated in the majority of patients with HHT. The aim of this study was to further improve the molecular diagnosis of HHT by also identifying large genomic rearrangements in HHT using Multiplex Ligation-dependent Probe Amplification (MLPA). In 123 probands, who were without a pathogenic mutation using direct sequencing of the *ENG* and *ACVRL1* genes, MLPA analysis was performed. In five probands (4%), MLPA analysis identified four different pathogenic mutations; two deletions and one duplication in *ENG* and one deletion in *ACVRL1*. These results show that MLPA analysis improves the mutation detection in HHT and should be performed in HHT. Large deletions and duplications account for a small but significant minority of the pathogenic mutations involved in HHT.

Introduction

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber (ROW) syndrome is an autosomal dominant disease characterized by vascular malformations in multiple organ systems. The clinical manifestations are caused by direct arteriovenous connections without an intervening capillary bed resulting in a range of malformations from smaller mucocutaneous telangiectases to large visceral arteriovenous malformations (AVM). The clinical diagnosis is established using the Curaçao criteria based on the manifestations in HHT (recurrent epistaxis; multiple telangiectases at characteristic sites; visceral lesions i.e. pulmonary, cerebral and hepatic AVMs or gastrointestinal bleeding; a first degree relative with HHT) [1].

Loss of function mutations in the endoglin (*ENG*, OMIM 131195) and activin A receptor type-like kinase 1 (*ACVRL1, ALK1*, OMIM 601284) genes are associated with HHT [2, 3]. Protein expression studies in human umbilical vein endothelial cells and peripheral blood monocytes have confirmed haploinsufficiency as the model in HHT1 (*ENG* mutations) and HHT2 (*ACVRL1* mutations) [4-6]. Gallione *et al.*[7] reported mutations in the *SMAD4* gene in patients that show clinical features of both HHT and juvenile polyposis. A few other loci for HHT were implicated in HHT, linked to chromosome 5 and chromosome 7 [8, 9].

To date, mutation analysis in the Dutch population revealed mutations in more than 92% of the probands (families). This relatively high yield could be explained by the inclusion of only clinically affected probands that fulfil the Curaçao criteria [10]. In the literature the mutation detection rates differ between different groups and different populations [11,12]. This might be caused by regional differences in populations, by different application of the diagnostic criteria, but also by different mutation analysis techniques.

Although in more than 90% of the Dutch HHT patients a mutation in either *ENG* or *ACVRL1* was identified, the molecular cause of the remaining patients is unknown. In this study we used the Multiplex Ligation-dependent Probe Amplification technique to screen for deletions and duplications. The tested probands (in a total of 123 patients) did not show a pathogenic abnormality upon sequencing of the *ENG* and *ACVRL1* gene. The yield of mutation detection also depends on the degree of certainty of the clinical diagnosis, therefore the probands were also stratified for referral (by HHT centre or by other hospitals) and for completeness of the clinical data (with or without known medical history). The results of these analyses will give insight in the proportion of families carrying large deletions or duplications as a cause of HHT and in the added value of screening for deletions and duplications in DNA-diagnostics of HHT.

Materials and methods

Patients

Since the start of the diagnostic testing, all probands referred to our genome diagnostics laboratory were analysed for mutations in *ENG* and *ACVRL1*. The patients can be divided into two groups (see table 1). The first group consists of patients referred by a single tertiary referral centre in the Netherlands, specialized in the diagnosis and treatment of HHT. Most of these patients were referred with a confirmed diagnosis fulfilling three or more of the Curaçao criteria. The majority of these families were of Dutch origin; only few families were from abroad (Belgium, former Yugoslavia, Turkey, Iran, the Netherlands Antilles and Surinam). The second group of patients was referred for diagnostic testing, but without the provision of detailed clinical data. Of these patients it is unknown whether or not the clinical diagnosis is established according to the Curaçao criteria or whether DNA mutation analysis was asked to further exclude HHT in a patient with doubtful diagnosis. These probands are mainly from Europe, but some were also referred from other continents.

Amplification, nucleotide sequence determination, Multiplex Ligation-dependent Probe Amplification (MLPA)

High molecular weight DNA of the probands was isolated from peripheral blood leukocytes according to established procedures. Sequencing for the exons 1-14 of the *ENG* gene and exons 1-10 of the *ACVRL1* gene and their flanking sequences was performed as described earlier [10]. In patients without a pathogenic mutation after sequencing, subsequent MLPA analysis was performed on genomic DNA. Probes for MLPA analysis (P093 Salsa MLPA probe set) were purchased from MRC Holland (Amsterdam, The Netherlands). Amplification products were run on ABI 3130 DNA sequencer (Applied Biosystems) MLPA peak plots were visualized and normalized and dosage ratio's were derived with the use of Genemarker software (Soft genetics LLC, State College, PA). Ratio's $\leq$0.7 and $\geq$1.3 were considered pathogenic. When a deletion was detected, the earlier derived sequence data were used to check for polymorphisms that may interfere with the attachment of the MLPA probe and consequently lead to a false positive result. Indeed, in our analysis the MLPA data suggested a common deletion in exon 8 of the *ENG* gene (data not shown) and the sequence data confirmed this was caused by a common polymorphism interfering with the MLPA probes.

Results

MLPA analysis was performed in the two predefined groups of patients, one referred by the HHT centre (subdivided in $\geq$ 3 criteria present or less criteria present) and one referred by other clinics (without adequate clinical information). The ascertainment of the patients and the results of DNA analysis are depicted in table 1.

Before searching for large rearrangements, sequence analysis was performed in 330 apparently unrelated patients. 177 (54%) of these probands were referred by the specialized HHT centre, 153 patients came from elsewhere. Sequencing revealed 70 different *ENG* mutations in 115 probands. The mutations detected were 27 different frame shift mutations, 17 nonsense mutations, 12 missense mutations, 9 splice site mutations and 5 uncertain variants. In *ACVRL1* 57 different mutations were detected in 92 probands. The mutations in *ACVRL1* were 22 missense mutations, 16 frame shift mutations, 13 nonsense mutations, 4 splice site mutations and 2 uncertain variants.

Table 1 - Characteristics of the population referred for DNA analysis. The probands are divided in different ascertainment groups. When possible the probands were subdivided in patients with (> 3) or without (< 3) a confirmed clinical diagnosis.

	Referral from				
	Specialised HHT centre			other clinics	total
			total	total	
Curacao criteria	≥ 3	< 3		**?**	
number of patients	124	53	**177**	**153**	**330**
pts with ENG point mutation	68	7	**75**	**40**	**115**
pts with ACVRL1 point mutation	45	10	**55**	**37**	**92**
pts remaining for MLPA analysis	11	36	**47**	**76**	**123**
pts with deletions/duplications	2		**2**	**3**	**5**

Subsequent MLPA analysis was performed in the remaining 123 probands. MLPA analysis revealed 4 large genomic rearrangements in 5 patients (table 1 and table 2). In the *ENG* gene two deletions and one duplication were detected. One deletion found in a proband from New Zealand, contained (at least part of) exon 3 to (at least part of) exon 14 (c.220-?1977+?del), shown by lack of amplification of 8 contiguous MLPA probes. The second *ENG* deletion was found in two probands (both originating from the Dutch Antilles); a deletion of (at least part of) exon 3 (c.220-?_360+?del). Because the latter deletion showed the loss of only one exon and involved only a single probe, an examination of the sequence data was performed. This confirmed that the attachment of this probe was not hampered by a sequence variant in the patients DNA thereby preventing annealing and amplification of the probes. Furthermore, family members were examined which demonstrated that the deletion segregated with the disease (data not shown). An *ENG* duplication was found in a family from the Netherlands with duplication of genomic DNA detected by the probes for exons 4-11 (c.361-?_1686+?dup).

In *ACVRL1* a single genomic rearrangement was detected. A Belgian proband showed a deletion that spans at least a part of exon 2 to exon 8 of the *ACVRL1* gene (c.1-?_1246+?del).

Table 2 - The large deletions (4) and the insertion (1) are depicted, combined with the clinical characteristics of the probands in which the mutation was found. Furthermore the presence (+) or absence (-) of HHT manifestations in the patients with a confirmed clinical diagnosis, but without a mutation using MLPA are given.

mutation	gene	gender	telangiectases	epistaxis	AVM / GI	family	Curacao
c.1-?_1246+?del (exon 2-8)	ACVRL1	female	+	+	-	+	3
c.361-?_1686+?dup (exon 4-11)	ENG	female	+	+	PAVM/CAVM	+	4
c.220-?_360+?del (exon 3)	ENG	male	+	+	-	+	3
c.220-?_360+?del (exon 3)	ENG	female	+	+	PAVM	+	4
c.220-?_1977+?del (exon 3-14)	ENG	female	-	+	PAVM	+	3
families referred by HHT centre, without mutation							
38	?	female	+	+	HAVM / GI	+	4
52	?	female	+	+	PAVM	+	4
66	?	male	+	+	PAVM	+	4
70	?	female	-	+	PAVM / CAVM	+	3
93	?	female	+	+	-	+	3
142	?	female	+	+	-	+	3
157	?	female	+	+	PAVM	+	4
64	?	male	+	+	-	+	3
1027	?	female	+	-	PAVM	+	3

Discussion

In this study, MLPA analysis was performed to establish how often large rearrangements in *ENG* or *ACVRL1* are the cause of HHT and to increase the number of HHT patients with a molecular diagnosis and the possibilities for genetic counselling. The probands were divided into two groups, the first group, referred by the specialized HHT centre in Nieuwegein, was also stratified for a confirmed clinical diagnosis (≥ 3 criteria present) or possible HHT (< 3 present). The results are depicted in table 1.

Sequencing that was based on PCR resulted in a mutation detection rate of 91% (113 probands out of 124) (table 1) in patients that have three or more of the diagnostic criteria. In the remaining 11 probands that showed no mutation, MLPA analysis revealed two additional mutations, improving the detection rate by 1.6% (2/124). No MLPA mutations were detected in the group of patients with less than 3 of the Curaçao criteria present. Testing the group of patients referred by other clinics, the percentage of patients with a large deletion/duplication is similar to the group of patients from the HHT centre (3/153). In both groups combined, MLPA analysis increased the overall mutation detection rate from 62.7% to 64.2%. This implies that genomic rearrangements form only a small proportion of mutations detected in HHT.

Of the mutations detected using sequencing and MLPA, 4.1% are large deletions or duplications in *ENG* (3/73), whereas in the *ACVRL1* gene this figure is 1.7% (1/58). Three of the four different deletion/duplication mutations were novel.

The deletion of exon 3 in the *ENG* gene has been reported before [12]. This deletion was found in two probands (one from the Dutch Antilles and one from the Netherlands, but with forefathers from the Dutch Antilles). These probands are likely related, but this could not be confirmed. Gallione *et al.* reported on the result of mutation analysis in the Dutch Antilles, with the highest prevalence of HHT to date [13]. They found the cause of HHT in the Dutch Antilles to be limited to the *ENG* gene and associated with three different haplotypes. Two haplotypes were elucidated i.e. two mutations were described: c.67+1G>A (IVS1+1(G>A) and the c.1238G>T (p.Gly413Val). For the third haplotype no mutation had been found. With the current study, using MLPA analysis in the same family, we can conclude that the third mutation causing HHT in the Dutch Antilles is the deletion of exon 3. Families with HHT and originating from the Dutch Antilles therefore are to be tested for these three mutations in the *ENG* gene first.

The clinical data of the probands with a deletion or duplication are depicted (table 2): all five patients had a confirmed clinical diagnosis. We conclude that as expected the manifestations of HHT in the latter group are not different from patients with a 'small' mutation that could be identified by sequencing. Furthermore, there is no obvious clinical difference between patients with a confirmed diagnosis and a large rearrangement compared to HHT probands without a mutation (table 2).

When comparing patients with certain clinical diagnosis (≥ 3) to uncertain (< 3), the

difference in causative gene distribution is striking. When a clinical diagnosis is confirmed (≥ 3), *ENG* mutations are the cause of HHT in the majority of the patients. In the other HHT group (<3), *ACVRL1* mutations are found more often than *ENG*. It is tentative to speculate that the observed difference of involvement of the respective genes is a reflection of in general a milder phenotype in HHT2. Consequently, this might implicate there is a risk that strict application of the Curaçao criteria is too stringent for identifying HHT2 patients.

To date, only a few publications on large rearrangement in HHT have appeared. Lesca *et al.*[11] found large rearrangements in *ENG* only, accounting for 2.5% of all mutations (*ENG* and *ACVRL1*) and 7.5% of *ENG* mutations alone. They found large rearrangements in three out of 136 clinically confirmed patients (2.2%) and 15% (3/20) of the clinically affected patients without a mutation using sequencing. This is very similar to our findings.

Shoukier *et al.*[14] described five large rearrangements in 45 HHT families (11%) with three or more Curaçao criteria present but without a pathogenic mutation detected using sequencing. However, they did not describe the total number of families that were screened and were found to have small mutations. Therefore, the contribution of their five mutations to overall mutations cannot be determined.

Bossler *et al.*[12] screened 48 patients without a detectable small mutation and found 10 deletions (21%). They started with 200 probands (119 with a clinically confirmed diagnosis), thus large genomic rearrangements accounted for 5% of their population.

The mutation detection rate is heavily dependent on the ascertainment of the patients. Mutation detection rates reach 91% when strict clinical criteria are applied (table 1) [10, 11]. The detection rate decreases significantly when no clinical data are available.

The combined data show that of all mutations in the *ENG* and *ACVRL1* genes in HHT the percentage of large rearrangements is approximately 2-3%. Although screening for large deletions in HHT is helpful and will improve the molecular diagnosis, they account for a small part of the mutation spectrum.

References

1. 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.
2. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, et al. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 1994; 8: 345-351.
3. 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;13: 189-195.
4. Bourdeau A, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M. Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. Am J Pathol 2000; 156: 911-923.
5. 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: 279-87.
6. Van den Driesche S, Mummery CL, Westermann CJ. Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. Cardiovasc Res 2003; 58: 20-31.
7. 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). The Lancet 2004; 363: 852-859.
8. 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; 15;140: 2155-2162.
9. Cole SG, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet. 2005;42:577-82.
10. Letteboer TG, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum Genet. 2005; 116(1-2):8-16.
11. 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 Jun;27(6):598.
12. Bossler AD, Richards J, George C, Godmilow L, Ganguly. Novel mutations in ENG and ACVRL1 identified in a series of 200 individuals undergoing clinical genetic testing for hereditary hemorrhagic telangiectasia (HHT): correlation of genotype with phenotype. Hum Mutat. 2006 Jul;27(7):667-75
13. Gallione CJ, Scheessele EA, Reinhardt D, Duits AJ, Berg JN, Westermann CJ, 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.
14. Shoukier M, Teske U, Weise A, Engel W, Argyriou L. Characterization of five novel large deletions causing hereditary haemorrhagic telangiectasia. Clin Genet. 2008 ;73 :320-30.

Chapter 2.3

SMAD4 mutations found in unselected HHT patients

Gallione CJ, Richards JA, Letteboer TG, Rushlow D, Prigoda NL, Leedom TP, Ganguly A, Castells A, Ploos van Amstel JK, Westermann CJ, Pyeritz RE, Marchuk DA.

J Med Genet. 2006; 43: 793-797

Abstract

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disease exhibiting multifocal vascular telangiectases and arteriovenous malformations. The majority of cases are caused by mutations in either the endoglin (*ENG*) or activin receptor-like kinase 1 (*ALK1*, *ACVRL1*) genes; both members of the transforming growth factor (TGF)-ß pathway. Mutations in *SMAD4*, another TGF-ß pathway member, are seen in patients with the combined syndrome of juvenile polyposis (JP) and HHT (JP-HHT).

We sought to determine if HHT patients without any apparent history of JP, who were undergoing routine diagnostic testing, would have mutations in *SMAD4*. We tested 30 unrelated HHT patients, all of whom had been referred for DNA based testing for HHT and were found to be negative for mutations in *ENG* and *ACVRL1*.

Three of these people harboured mutations in *SMAD4*, a rate of 10% (3/30). The *SMAD4* mutations were similar to those found in other patients with the JP-HHT syndrome.

The identification of *SMAD4* mutations in HHT patients without prior diagnosis of JP has significant and immediate clinical implications, as these people are likely to be at risk of having JP-HHT with the associated increased risk of gastrointestinal cancer. We propose that routine DNA based testing for HHT should include *SMAD4* for samples in which mutations in neither *ENG* nor *ACVRL1* are identified. HHT patients with *SMAD4* mutations should be screened for colonic and gastric polyps associated with JP.

Introduction

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disease of vascular dysplasia. The symptoms of HHT include epistaxis, telangiectases, and arteriovenous malformations (AVMs), which are most often found in the lungs, brain, liver, and gastrointestinal tract. There is wide variation in penetrance and severity of these symptoms in patients even within the same family [1], suggesting that environmental or other genetic factors influence the phenotype.

Mutations in either one of two genes cause HHT. Mutations in the endoglin (*ENG*) gene are responsible for HHT1 and mutations in the activin receptor-like kinase 1 (*ACVRL1*) gene for HHT2 [2,3]. Both of these genes encode TGF-ß binding proteins and play important roles in regulating endothelial cell growth [4,5].

Screening for mutations in either *ENG* or *ACVRL1* in HHT patient cohorts yields mutation detection rates of between 62% and 93% [6-14]. The failure to achieve a 100% detection rate is not unique to HHT. This range of mutation detection rates is similar to that found for many other mendelian disorders [15], implying that most, if not all, mutation scans share common detection limitations. In the case of HHT, people may have large deletions or insertions, deep intronic mutations affecting splicing, or regulatory mutations in either *ENG* or *ACVRL1*. A subset of the mutation negative samples may instead have a mutation in the as yet undiscovered HHT3 gene recently linked to chromosome 5 [16]. Because the majority of HHT patients have been found to have mutations in *ENG* or *ACVRL1*, it is probable that only a small fraction of the remaining cases will be caused by mutations in other genes.

SMAD4 is a key downstream effector of transforming growth factor (TGF)-ß signalling. Mutations in *SMAD4* and *BMPR1A* are known to be causative for juvenile polyposis (JP) [17,18]. JP is characterised by the presence of five or more hamartomatous gastrointestinal polyps, or any number of polyps in addition to a family history of polyposis [19]. There is an increased risk of gastrointestinal cancers associated with these polyps. Recently, *SMAD4* was identified as mutated in a subset of HHT patients with JP, a condition termed JP-HHT syndrome [20], in which juvenile polyps and anemia are the predominant clinical features. HHT symptoms vary among patients, but all meet the Curaçao criteria for being either definitely or possibly affected [21]. There is a high penetrance of AVMs in this JP-HHT cohort, particularly in young patients. Importantly, each of these patients has symptoms of both JP and HHT.

Both JP and HHT are diseases known to have a range of clinical presentations and to be variably penetrant [1,22]. One feature common to both disorders is gastrointestinal (GI) bleeding, potentially confounding the correct diagnosis. Because of this potential for uncertainty in diagnoses, we questioned whether, among a cohort of *ENG* and *ACVRL1* mutation negative HHT cases, there were occult cases of JP-HHT, diagnosed as only having the HHT component of the combined phenotype. Using sequence analysis for *SMAD4* mutations, we screened 30 people from an unselected group of HHT patients, who had been referred for DNA based testing for HHT and found to be negative for mutations in either *ENG* or *ACVRL1*.

Methods

Subjects

Of the subjects in this cohort, 20 were referred to the genetic diagnostic laboratory at the University of Pennsylvania, five were enrolled through the Dutch HHT Center in the Netherlands, and four were referred through HHT Solutions in Toronto, Canada. Individuals were referred to these centres of DNA based diagnostics because of a diagnosis or suspicion of HHT (table 1). One patient with HHT was independently assessed at the hospital clinic in Barcelona, Spain, and referred to Duke University for this study after being diagnosed with colon cancer. All participants in this study were enrolled with their informed consent. Clinical subjects also gave consent for their DNA to be used in further research. Enrolment of participants through Duke University and the Dutch HHT Center was given approval by the institutional review boards of the participating institutions (Duke University Health System Institution Review Board Committee, and METC, Utrecht).

Our cohort comprised 30 subjects, of whom 24 (80%) had definite HHT and 6 (20%) had possible HHT, according to the Curaçao diagnostic criteria [21]. Ages of the participants ranged from 18 to 87 years (mean 51.3, median 49). The majority of the subjects reported having a positive family history for HHT (19/30, 63%) while four people reported a negative family history. The remaining seven did not respond to the query on their family history. Visceral lesions were present in 25 people, absent in 3, and not reported in 2. Five different participants noted GI bleeding, and individual subjects report having stroke, migraines, gastric polyps, and liver shunts. One individual had both anemia and colon cancer, and another had GI bleeding and colonic polyps.

The samples included in this study are a subset of 102 *ENG*/*ACVRL1* mutation negative samples from all of the institutions. The 30 participants comprising this cohort had granted consent for additional research using their DNA. They were not selected for *SMAD4* testing based on any reported clinical symptoms.

Diagnostic testing and genetic screening

All 30 subjects were screened by direct DNA sequencing of PCR products consisting of the coding exons and adjacent intronic regions for *ENG*, *ACVRL1* and *SMAD4* from genomic DNA. In all cases, any sequence variations found were reamplified and resequenced to confirm the observed changes. The sequences generated were compared with wild type *ENG* (GenBank accession no. NT_008470 [GenBank] or BC014271 [GenBank]), *ACVRL1* (GenBank NT_029419 [GenBank] or NM_000020 [GenBank]), and *SMAD4* (GenBank NM_005359 [GenBank]). There were 113 control individuals screened for the presence of the *SMAD4* missense mutation by using a Sau3A restriction digest assay. The mutation destroys the single Sau3A site in the exon 8 amplimer. In all cases, nucleotides were numbered starting

with the A in the initial ATG of the cDNA sequence as c.1, and the starting methionine in the protein sequence as p.1 (http://www.hgvs.org/mutnomen/).

Although all centres used direct DNA sequence analysis to identify point mutations, each diagnostic centre employed a different technique to further examine the DNAs for large scale rearrangements. The 20 samples from the genetic diagnostic laboratory at University of Pennsylvania were tested by quantitative Southern blots. Genomic DNA was digested with RsaI, because most fragments represent individual exons. cDNA probes covering exons 2-14 for *ENG* and 2-10 for *ACVRL1* were used for the initial analysis. If a change in dosage for any one or more exons was detected, probing with specific individual exonic clones was performed. In addition, genomic probes for exons 1 of both *ENG* and *ACVRL1* were tested separately. Whole gene deletions were detected by comparison with an internal control (ß-globin gene) run on the same filter.

The four samples from HHT Solutions were tested for changes in exon size and copy number using quantitative multiplex PCR with intronic primers for all 15 *ENG* exons and 9 *ACVRL1* exons. The fluorescent fragments were analysed using Gene Objects software (version 3.1; Bayer). RNA analysis was used in some cases as an additional test where no mutation was found by sequencing or QM-PCR.

The five samples from the Dutch HHT Center were probed for exonic deletions or duplications in *ENG* and *ACVRL1* by MLPA. The DNA from patient 3892 was not examined for genomic rearrangements; however, the coding regions and surrounding intronic regions of *BMPR1A* were sequenced and compared with a reference sequence (GenBank accession number NM_004329).

Results

Subjects were referred to four different institutions for DNA based diagnostic testing for HHT based on the clinical diagnosis or suspicion of HHT. Of 374 people sent for DNA testing for HHT, 30 had also consented to allow their DNA to be included in research studies. Of these 30, none was known to have JP, although GI bleeding, a feature in common with HHT and JP, was reported for five of the individuals. All 30 of these subjects had been screened for *ENG* and *ACVRL1* mutations by direct DNA sequencing but no mutations had been found. As part of the attempted molecular diagnosis for HHT, 29 of these people had also been screened for genomic rearrangements of the *ENG* and *ACVRL1* genes, but no alterations had been uncovered.

We then sequenced the coding exons and adjacent intronic sequences of *SMAD4* and identified mutations in three subjects. Individual UP015 was referred to the genetic diagnostic laboratory at the University of Pennsylvania. This 37 year old has telangiectases, epistaxis, and AVMs in the lung and gastrointestinal tract. Although no family history of HHT was noted, this patient fulfils the Curaçao criteria for definite HHT [21]. This person was found to have a frameshift mutation in exon 11 of *SMAD4*, c.1596_97delCCinsT. After this

molecular finding, inspection of patient charts revealed that this patient had colonic polyps that had been discovered during endoscopy.

Individual 5068 (family 530) was referred to HHT Solutions in Toronto. This 39 year old has telangiectases, epistaxis, pulmonary AVMs, and liver shunts, and therefore meets diagnostic criteria as definitely affected with HHT. Although there was no reported past family history of HHT, the subject's children show some presumptive signs of HHT (epistaxis and telangiectases). A missense mutation in exon 8 of *SMAD4*, c.1081C>T, p.R361C, was found.

Individual 3892, 47 years old, was diagnosed at 13 years of age with HHT, and had telangiectases of the lips and tongue, epistaxis, and a hepatic AVM. This person has had iron deficiency anemia for more than 15 years, requiring blood transfusions on multiple occasions, and is on regular iron supplementation. Signet ring cell type colorectal cancer located in the caecum was diagnosed in June 2004. Upon examination, seven hamartomatous polyps were found in the ascending colon and three in the duodenum. There is no history of either HHT or gastrointestinal cancer in the family. This person harboured the same *SMAD4* exon 8 missense mutation (c.1081C>T, p.R361C) seen in patient 5068.

All three mutations identified were found within in the COOH terminus of *SMAD4*, where all of the mutations in previously reported JP-HHT cases have been identified (fig 1). One mutation, c.1081C>T (p.R361C) in exon 8, was found in two unrelated members of our study cohort. This same mutation has been reported in a number of people with JP [23-25], and the same base is mutated in a previously reported case of JP-HHT (c.1081C>G, p.R361G) [20]. The missense mutation at codon 361 was not identified by us in 113 unaffected population controls (226 alleles) or by Houlston *et al.* in the 50 controls (100 alleles) tested in the case of a JP patient [23]. Using two different programs that use evolutionary conservation to investigate the effects of putative missense mutations (SIFT (http://blocks.fhcrc.org/sift/SIFT.html) and PolyPhen (http://genetics.bwh.harvard.edu/cgi-bin/pph/polyphen.cgi)), the R361C amino acid change is predicted to negatively affect protein function. The third mutation found in this study constitutes a frameshift in exon 11 (c.1596_97delCCinsT). This is a novel change, although frameshift mutations in exon 11 of *SMAD4* have also been identified in JP-HHT patients [20].

Table 1 - Clinical Characteristics of the testing cohort

Sample	Age (years)	Family history	Visceral lesions	Telangiectasia	Epistaxis	Other	HHT status
UP001	18	Yes	Yes	N/A	N/A		Possible
UP002	61	Yes	Yes	N/A	Yes	GI bleeding	Definite
UP003	73	Yes	Yes	Yes	N/A		Definite
UP004	77	Yes	Yes	Yes	Yes		Definite
UP005	21	N/A	Yes	N/A	Yes		Possible
UP006	37	N/A	N/A	Yes, (rectal)	Yes		Possible
UP007	73	Yes	Yes	N/A	Yes	GI bleeding	Definite
UP008	54	Yes	Yes	Yes	Yes		Definite
UP009	23	N/A	Yes	Yes	Yes		Definite
UP010	38	N/A	Yes (lung)	Yes	N/A	Stroke	Possible
UP011	78	N/A	Yes	N/A	Yes	GI bleeding	Possible
UP012	74	Yes	N/A	Yes	Yes		Definite
UP013	52	Yes	Yes	Yes	Yes		Definite
UP014	69	N/A	Yes	Yes	Yes		Definite
UP015*	37	No	Yes (lung, GI)	Yes	Yes	GI bleeding, colonic polyps	Definite
UP016	36	Yes	Yes	Yes	Yes		Definite
UP017	69	Yes	Yes	Yes	Yes		Definite
UP018	32	Yes	Yes	Yes	N/A		Definite
UP019	87	Yes	Yes	Yes	Yes		Definite
UP020	56	N/A	Yes (stomach)	Yes	Yes		Definite
D1827	47	Yes	Yes (liver)	Yes (inc. GI)	Yes	Confirmed HHT	Definite
DKCL331	44	Yes	Yes (lung)	Yes	Yes	Confirmed HHT	Definite
D0883	63	Yes	No	Yes	Yes	Confirmed HHT	Definite

Sample	Age (years)	Family history	Visceral lesions	Telangiectasia	Epistaxis	Other	HHT status
D1488	44	Yes	Yes (lung)	Yes	Yes	Confirmed HHT	Definite
D3748	51	Yes	No	Yes	Yes	Confirmed HHT	Definite
5001	56	Yes	Yes (hip)	Yes	Yes	Confirmed HHT, GI bleeding, gastric polyps	Definite
5031	37	No	Yes (lung, multiple)	No	Yes	Confirmed HHT, migraines	Possible
5051	46	Yes	No	Yes (chest)	Yes	Confirmed HHT	Definite
5068*	39	No	Yes (lung)	Yes	Yes	Confirmed HHT, liver shunts	Definite
		Yes (in children)					
3892*	47	No	Yes (liver)	Yes (lips, tongue)	Yes	Confirmed HHT, anaemia, colon cancer	Definite

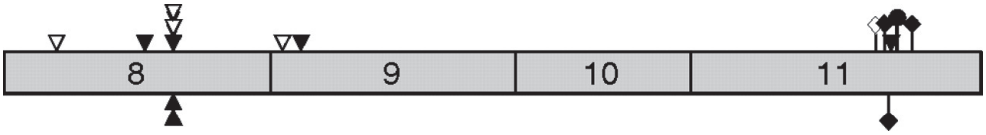


Figure 1 - Schematic diagram of exons 8-11 of *SMAD4* showing HHT associated mutations. These four exons encode the MH2 domain of the protein. In confirmed JP-HHT syndrome patients, no mutations have been identified in the first seven exons of the gene. Shaded boxes, exons; triangles, missense mutations; diamonds, frameshift mutation; circles, nonsense mutations; filled symbols above the shaded exons, previously reported mutations in JP-HHT patients;20 open symbols, from our unpublished data; marks below the exons, mutations reported here from people exhibiting symptoms of HHT.

The total diagnostic testing cohort from the four centres was 374 HHT patients. *ENG* or *ACVRL1* mutations were found in 272 people, a detection rate of 72.7%. Thus, 102 subjects (27.3%) were considered *ENG*/*ACVRL1* mutation negative for this study. The 30 people available to us for research represent approximately 30% of the *ENG*/*ACVRL1* mutation negative group and 8% of the total HHT cohort. *SMAD4* mutations were found in three of the 30, a *SMAD4* mutation rate of 10%. Extrapolating to the larger *ENG*/*ACVRL1* mutation negative group, up to 10 presumptive HHT patients might harbour *SMAD4* mutations. This would represent 2-3% (10/374) of the original cohort referred for HHT molecular testing.

Discussion

DNA sequencing performed by numerous groups around the world shows that the majority of HHT patients have mutations in either *ENG* or *ACVRL1* [6-14].The mutation detection rates in the scans range from 62% to 93%, a range that matches the detection rates found in scans of other genes in mendelian diseases [15].

There are several explanations why a patient might appear mutation negative in a mutation screen. For diseases such as HHT that have highly variable clinical presentations, it is possible that some of the referred patients may not actually have the disease in question. In the present study, presuming that all members of the cohort are affected HHT patients, another explanation could be that these patients may harbour non-coding mutations, such as alterations in poorly characterised regulatory regions of the gene, including the promoter, introns, and 5' and 3' untranslated regions.

Additionally, large scale genomic rearrangements involving whole exons, partial gene loss or duplication, and intrachromosomal or interchromosomal rearrangements will bypass detection by DNA sequencing. It is also possible that some patients will have mutations in other genes that lead to the same disease phenotype. A recent report of two unrelated HHT families that exhibit linkage to a region on chromosome 5 demonstrates that at least one other gene is involved in HHT [16]. Non-*ENG*, non-*ACVRL1* HHT patients may harbour a mutation in this as yet unidentified gene.

A final explanation for these *ENG*/*ACVRL1* mutation negative HHT cases might be that some of these patients harbour mutations in *SMAD4*. Based on a molecular diagnosis, these patients would have the JP-HHT syndrome, but they may have been diagnosed with HHT due to occult or unrecognised manifestations of JP. In this study we queried if *SMAD4* mutations were found in the general HHT population. To determine the answer, we sequenced the coding exons and adjacent flanking intronic regions of *SMAD4* in a cohort of 30 unrelated people with presumptive HHT. These people had been referred for DNA based diagnostic testing for HHT and all were negative for mutations in *ENG* and *ACVRL1*. One of them had colon cancer, which is sometimes the first symptomatic manifestation of JP, but none of the others was known or reported to have JP. Although GI bleeding was reported in five of these subjects, this was interpreted as a symptom of HHT.

All of the *SMAD4* mutations identified in JP-HHT patients to date are found in the COOH terminus of the protein. The three mutations identified in the subjects here are in the COOH terminus of *SMAD4* and are nearly identical in location and type of mutation to those seen in JP-HHT patients (figure 1). This strongly suggests that the three HHT subjects with *SMAD4* mutations in this cohort are affected with JP-HHT syndrome. Two of these three *SMAD4*-HHT subjects are known to have colonic polyps, and one of these two has colorectal cancer. The status of the third individual regarding JP symptoms is unknown.

There is growing evidence of distinct phenotypic differences between patients with HHT1 and HHT2 [26-28]. Similarly, other studies have demonstrated phenotypic differences between JP patients with *SMAD4* mutations compared with patients with mutations in *BMPR1A* [29,30], the second gene known to be involved in JP. JP is also known to be variably penetrant and is associated with an increased risk of gastrointestinal cancer [22,31]. Knowing which gene is responsible for the disease in an individual HHT patient will aid in the proper management and care of the patient and, significantly, of related family members.

We recommend that when genetic testing is advised for HHT patients, *SMAD4* should be screened if no mutations are found in either *ENG* or *ACVRL1*. We also recommend that screening for colonic and gastric polyps be considered in people in whom neither *ENG* nor *ACVRL1* mutations have been found, in whom *SMAD4* mutations have been uncovered, or in anyone with anemia that cannot be completely explained by epistaxis or some other cause. This screening will identify those HHT patients with occult polyposis. Early detection of colonic polyps in any patient, but in particular in JP-HHT patients, could prevent the development of colorectal cancer by finding and removing precancerous polyps.

There appears to be a high rate of de novo cases of JP-HHT [20], and all three of the *SMAD4*-HHT patients in this study reported no family history of HHT. Although a positive family history is one of the criteria for HHT diagnosis [21], *SMAD4* mutation carriers may often lack this key diagnostic feature, potentially making the clinical diagnosis more difficult. In the cases of presumed HHT without any apparent family history, an argument might be made that *SMAD4* mutation analysis should precede analysis of *ENG* or *ACVRL1*, as de novo

mutations in these other HHT genes appear to be rare [8,13,14].

An HHT patient found to harbour a *SMAD4* mutation would be a prime situation where genetic testing can be used to guide clinical management. Because of the increased risk of gastrointestinal cancer associated with JP, it is critically important for a patient with a *SMAD4* mutation to be screened for JP. We suggest that HHT patients harbouring a *SMAD4* mutation should be considered at high risk of JP-HHT, requiring more intensive colorectal cancer screening strategies than those recommended for the average risk population [32]. Correspondingly, JP patients with *SMAD4* mutations similar to those seen in JP-HHT patients should be examined for the visceral manifestations of HHT which can present suddenly and catastrophically.

SMAD4, *ENG*, and *ACVRL1* are all members of the TGF-ß signalling pathway, and mutations in the genes encoding them can cause a broad constellation of phenotypes with both distinct and overlapping clinical features. It has been known for a number of years that mutations in *SMAD4* cause JP [17], and we have recently shown that certain types of mutations in *SMAD4* cause JP-HHT [20]. Here we report that unselected HHT patients have *SMAD4* mutations that are strikingly similar to those seen in JP-HHT patients. In JP cases with the same types of *SMAD4* mutations, it remains to be seen if these JP patients exhibit symptoms of HHT due to the paucity of clinical descriptions in the literature. Mutations in *ENG* have recently been reported in patients with JP [33], and mutations in *ACVRL1* cause some cases of primary pulmonary hypertension [34-36]. Molecular dissection of the interconnections between these genes and the effects of aberrant signalling through the TGF-ß and other alternative signalling pathways, will help to further elucidate the pathophysiology of these inherited diseases.

References

1. Begbie ME, Wallace GM, Shovlin CL. Hereditary haemorrhagic telangiectasia (Osler-Weber-Rendu syndrome): a view from the 21st century. Postgrad Med J 2003; 79: 18-24.
2. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J , McCornlich MK, Perieak-Vanee MA, Heutink P , Oostra BA, Haitjema T , Westermann CJJ, Porteous ME, Guttmacher AE, Letarte M , Marchuk DA. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 1994; 8: 345-351.
3. 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; 13: 189-195.
4. Lebrin F, Deckers M , Bertolino P, Ten Dijke P. TGF-beta receptor function in the endothelium. Cardiovasc Res 2005; 65: 599-608.
5. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. Trends Biochem Sci 2004; 29: 265-273.
6. Letteboer TG, Zewald RA, Kamping EJ, de Haas G , Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum Genet 2005; 116: 8-16.
7. Schulte C , Geisthoff U, Lux A , Kupka S, Zenner HP, Blin N, Pfister M. High frequency of ENG and ALK1/ACVRL1 mutations in German HHT patients. Hum Mutat 2005; 25: 595.
8. Abdalla SA, Cymerman U, Rushlow D, Chen N, Stoeber GP, Lemire EG, Letarte M. Novel mutations and polymorphisms in genes causing hereditary hemorrhagic telangiectasia. Hum Mutat 2005; 25: 320-1.
9. Kuehl HK, Caselitz M, Hasenkamp S, Wagner S, El-Harith el HA, Manns MP, Stuhrmann M. Hepatic manifestation is associated with ALK1 in hereditary hemorrhagic telangiectasia: identification of five novel ALK1 and one novel ENG mutations. Hum Mutat 2005; 25: 320.
10. Draghi F, Precerutti M, Danesino GM, Olivieri C, Valacca C, Danesino C, Pagella F , Semino L, Lanzarini L, Buscarini E, Danesino C. Vascular abnormalities in the fingers of patients affected with hereditary hemorrhagic telangiectasia (HHT) as assessed by color doppler sonography. Am J Med Genet A 2005; 135: 106-9.
11. Lesca G, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Riviere 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.
12. Brusgaard K, Kjeldsen AD, Poulsen L, Moss H, Vase P, Rasmussen K, Kruse TA, Horder M. Mutations in endoglin and in activin receptor-like kinase 1 among Danish patients with hereditary haemorrhagic telangiectasia. Clin Genet 2004; 66: 556-561.
13. Lastella P, Sabba C, Lenato GM, Resta N, Lattanzi W, Gallitelli M, Cirulli A, Guanti G. Endoglin gene mutations and polymorphisms in Italian patients with hereditary haemorrhagic telangiectasia. Clin Genet 2003; 63: 536-540.
14. Cymerman U, Vera S, Karabegovic A, Abdalla S, Letarte M. Characterization of 17 novel endoglin mutations associated with hereditary hemorrhagic telangiectasia. Hum Mutat 2003; 21: 482-92.
15. van Ommen GJ, Bakker E, den Dunnen JT. The human genome project and the future of diagnostics, treatment, and prevention. Lancet 1999; 354 (Suppl 1) :SI5-10.
16. Cole SG, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet 2005; 42: 577-582.
17. Howe JR, Roth S, Ringold JC, Summers RW, Jarvinen HJ, Sistonen P, Tomlinson IP, Houlston RS, Bevan S, Mitros FA, Stone EM, Aaltonen LA. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science 1998; 280: 1086-8.
18. Howe JR, Bair JL, Sayed MG, Anderson ME, Mitros FA, Petersen GM, Velculescu VE, Traverso G, Vogelstein B. Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat Genet 2001; 28: 184-7.
19. Jass JR, Williams CB, Bussey HJ, Morson BC. Juvenile polyposis--a precancerous condition. Histopathology 1988; 13: 619-630.
20. 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; 363: 852-859.

21. 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.

22. Stemper TJ, Kent TH, Summers RW. Juvenile polyposis and gastrointestinal carcinoma. A study of a kindred. Ann Intern Med 1975; 83: 639-646.

23. Houlston R, Bevan S, Williams A, Young J, Dunlop M, Rozen P, Eng C, Markie D , Woodford-Richens K, Rodriguez-Bigas MA, Leggett B, Neale K, Phillips R, Sheridan E, Hodgson S, Iwama T, Eccles D, Bodmer W, Tomlinson I. Mutations in DPC4 (SMAD4) cause juvenile polyposis syndrome, but only account for a minority of cases. Hum Mol Genet 1998; 7: 1907-1912.

24. Woodford-Richens K, Bevan S, Churchman M, Dowling B, Jones D, Norbury CG, Hodgson SV, Desai D, Neale K, Phillips RK, Young J, Leggett B, Dunlop M, Rozen P, Eng C, Markie D, Rodriguez-Bigas MA, Sheridan E, Iwama T, Eccles D, Smith GT, Kim JC, Kim KM, Sampson JR, Evans G, Tejpar S, Bodmer WF, Tomlinson IP, Houlston RS. Analysis of genetic and phenotypic heterogeneity in juvenile polyposis. Gut 2000; 46: 656-660.

25. Howe JR, Sayed MG, Ahmed AF, Ringold J , Larsen-Haidle J , Merg A , Mitros FA, Vaccaro CA, Petersen GM, Giardiello FM, Tinley ST, Aaltonen LA, Lynch HT. The prevalence of MADH4 and BMPR1A mutations in juvenile polyposis and absence of BMPR2, BMPR1B, and ACVR1 mutations. J Med Genet 2004; 41: 484-491.

26. 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: 585-590.

27. Letteboer TG, Mager HJ, Snijder RJ, Koeleman BP, Lindhout D, Ploos van Amstel HK, Westermann KJ. Genotype - phenotype relationship in Hereditary Hemorrhagic Telangiectasia. J Med Genet 2006; 43: 371-377.

28. Kjeldsen AD, Moller 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.

29. Friedl W, Uhlhaas S, Schulmann K, Stolte M, Loff S, Back W, Mangold E, Stern M, Knaebel HP, Sutter C, Weber RG, Pistorius S, Burger B, Propping P. Juvenile polyposis: massive gastric polyposis is more common in MADH4 mutation carriers than in BMPR1A mutation carriers. Hum Genet 2002; 111: 108-111.

30. Sayed MG, Ahmed AF, Ringold JR, Anderson ME, Bair JL, Mitros FA, Lynch HT, Tinley ST, Petersen GM, Giardiello FM, Vogelstein B, Howe JR. Germline SMAD4 or BMPR1A mutations and phenotype of juvenile polyposis. Ann Surg Oncol 2002; 9: 901-906.

31. Merg A, Howe JR. Genetic conditions associated with intestinal juvenile polyps. Am J Med Genet C Semin Med Genet 2004;129: 44-55.

32. Gazelle GS, McMahon PM, Scholz FJ. Screening for colorectal cancer. Radiology 2000; 215: 327-335.

33. Sweet K, Willis J, Zhou XP, Gallione C, Sawada T, Alhopuro P, Khoo SK, Patocs A, Martin C, Bridgeman S, Heinz J, Pilarski R, Lehtonen R, Prior TW, Frebourg T, Teh BT, Marchuk DA, Aaltonen LA, Eng C. Molecular classification of patients with unexplained hamartomatous and hyperplastic polyposis. JAMA 2005; 294: 2465-2473.

34. 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, Raisanen-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; 40: 865-71.

35. Abdalla SA, Gallione CJ, Barst RJ, Horn EM, Knowles JA, Marchuk DA, Letarte M, Morse JH. Primary pulmonary hypertension in families with hereditary haemorrhagic telangiectasia. Eur Respir J 2004; 23: 373-377.

36. Harrison RE, Berger R, Haworth SG, Tulloh R, Mache CJ, Morrell NW, Aldred MA, Trembath RC. Transforming growth factor-beta receptor mutations and pulmonary arterial hypertension in childhood. Circulation 2005; 111: 435-41.

Chapter 3

Genotype – phenotype relationship in hereditary hemorrhagic telangiectasia

Chapter 3.1

Genotype-phenotype relationship in hereditary hemorrhagic telangiectasia.

Letteboer TG, Mager JJ, Snijder RJ, Koeleman BP, Lindhout D,
Ploos van Amstel JK, Westermann CJ.

J Med Genet. 2006; 43: 371-377

Abstract

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder characterised by vascular malformations in multiple organ systems, resulting in mucocutaneous telangiectases and arteriovenous malformations predominantly in the lungs (pulmonary arteriovenous malformation; PAVM), brain (cerebral arteriovenous malformation; CAVM), and liver (hepatic arteriovenous malformation; HAVM). Mutations in the *ENG* and *ACVRL1* genes lead to HHT1 and HHT2 respectively.

In this study, a genotype-phenotype analysis was performed. A uniform and well classified large group of HHT patients and their family members were screened for HHT manifestations. Groups of patients with a clinically confirmed diagnosis and/or genetically established diagnosis (HHT1 or HHT2) were compared. The frequency of PAVM, CAVM, HAVM, and gastrointestinal telangiectases were determined to establish the genotype-phenotype relationship.

The analysis revealed differences between HHT1 and HHT2 and within HHT1 and HHT2 between men and women. PAVMs and CAVMs occur more often in HHT1, whereas HAVMs are more frequent in HHT2. Furthermore, there is a higher prevalence of PAVM in women compared with men in HHT1. In HHT1 and HHT2, there is a higher frequency of HAVM in women. HHT1 has a distinct, more severe phenotype than HHT2. There is a difference in the presence of symptoms between men and women. With these data, genetic counseling can be given more accurately when the family mutation is known.

Introduction

Hereditary hemorrhagic telangiectasia (HHT or Rendu-Osler-Weber disease) is an autosomal dominant disorder characterised by vascular malformations in multiple organ systems. Estimates of the frequency of the disease vary widely, the prevalence in the Netherlands is estimated 1 in 10 000, while in the Dutch Antilles the prevalence is at least 1 in 1330 [1].

The clinical symptoms of HHT are caused by direct arteriovenous connections without an intervening capillary bed. The resulting mucocutaneous telangiectases can occur anywhere, but particularly in the oral cavity (lips, tongue), nose, and conjunctivae, and on the fingertips. Telangiectases in the nasal mucosa can result in epistaxis, usually the first and most common symptom, present in more than 90% of patients with HHT [2–4].

Larger arteriovenous malformations (AVM) are mostly located in the lung (pulmonary arteriovenous malformations; PAVM), brain (cerebral arteriovenous malformations; CAVM) and liver (hepatic arteriovenous malformations; HAVM)[3-7]. PAVMs are estimated to develop in 15–35% of patients [3,8], resulting in a right to left shunt. PAVMs can cause hypoxemia, bleeding (hemothorax), and bypass of emboli or septic material, which can lead to serious systemic complications such as cerebral abscess and infarction [3,5,9]. Screening for PAVMs is therefore advised, and treatment of PAVMs is justified even when asymptomatic.

Cerebral arteriovenous malformations (CAVMs) are less common (5–13% of patients), but are probably under recognised [6,10-12]. Although they are often silent, they can cause headache, seizures, ischemia, and bleeding [6,10]. The bleeding risk ranges from <1% to 1.5–2% per year per patient [10,13].

The frequency of hepatic involvement in HHT varies considerably, mainly because HHT patients have not been routinely screened for HAVM. The frequency is estimated to be up to 32% [8,14-17]. Liver involvement predominantly concerns shunts between the hepatic artery and hepatic veins. HAVMs are often asymptomatic, but may lead to a high cardiac output with heart failure and eventually to portal hypertension and biliary disease.

Gastrointestinal (GI) bleeding is usually present at an older age, due to telangiectases in the GI tract, which can cause severe anemia. The estimated prevalence is 15–45% [4,6,8,18].

It should be emphasized that there is considerable interfamilial and intrafamilial variability with respect to age related penetrance and pattern of clinical expression.

Mutations in the endoglin (*ENG*; OMIM #131195) or activin A receptor type-like kinase 1 (*ACVRL-1*, *ALK-1*; OMIM #601284) genes cause HHT. Expression studies in human umbilical vein endothelial cells and peripheral blood monocytes have confirmed haploinsufficiency as the causative mechanism in both forms of HHT [8,19-21]. In 2004, patients with clinical features of both HHT and juvenile polyposis were shown to carry mutations in the *MADH4* gene [22]. To date, mutations in the *MADH4* gene have not been reported in patients with HHT without juvenile polyposis. Recently, a new locus has been mapped to chromosome 5, associated with classical HHT [23].

Mutations in *ENG* and *ACVRL1* result in HHT1 and HHT2 respectively. The identification and characterisation of mutations in HHT patients revealed extensive molecular heterogeneity [20,24,25]. As a result of different selection criteria, populations, and detection methods for the mutation analysis, different groups report different mutation detection rates. In a national study of Dutch HHT patients, pathogenic mutations were detected in 93% of the families, of which 53% were in the *ENG* gene and 40% in the *ACVRL1* gene [24].

The genetic heterogeneity in HHT explains part of the phenotypic variability. A higher prevalence of PAVMs and CAVMs was suggested in HHT1, while families with HHT2 generally tend to show a later onset of the symptoms and a milder phenotype [3,4,7,8]. Accurate data on the prevalence of the symptoms in HHT1 and HHT2 are, however, scarce. No extensive studies on the clinical manifestations in relation to the gene involved and the type of pathogenic mutation have been reported. Although Berg *et al.*[4] were the first to compare patients with HHT1 and HHT2 and to report differences in clinical features between the two groups, the clinical data were obtained using a questionnaire. The presence of symptoms was provided by the participants, and data were not cross checked with a review of the medical records. The participants were from the UK and the USA, areas with different screening protocols and population backgrounds. In 83 participants, this group found a PAVM significantly more often in patients with HHT1 (35%) compared with HHT2 (0%).

Abdalla *et al.*[8] reported the analysis of patients with *ACVRL1* documented in the literature (281). They found PAVM in only 5% of patients, CAVM in 2%, HAVM in 13%, and GI manifestations in 12%. Although the visceral manifestations are reported more frequently in HHT1, publications on this subject are limited.

We report on the frequencies of the visceral manifestations in HHT1 and HHT2 in a large Dutch cohort. This is the second study to compare the clinical data of patients from families with *ENG* (HHT1) and *ACVRL1* (HHT2) mutations. This is the first study to use clinical data obtained from one national HHT centre covering a circumscribed region in north western Europe with equal access to healthcare facilities.

Materials and methods

Patients

The patients were selected from a panel consisting of all probands and family members screened for HHT. Family members of index cases were advised to attend the hospital. Subjects referred until August 2004 were included in a database. This date was also used to calculate the age of each person; the ages depicted are not the ages at diagnosis, but the age at the time of the analysis. Most probands and family members were screened for visceral manifestations at St. Antonius Hospital, which specialises in the diagnosis and treatment of HHT. Clinical data of a minority of the patients (n = 57) were obtained through medical records from elsewhere; these patients were included after re-evaluation of the medical

records. All manifestations of HHT were recorded in the database, for both probands and family members. At the time of analysis, the database consisted of 1291 people screened for the presence of HHT.

The clinical diagnosis HHT was established according to the Curaçao criteria [26]. At least three of the following four criteria were required for a clinical diagnosis: spontaneous and recurrent epistaxis, telangiectases at characteristic sites, visceral manifestations (PAVM, CAVM, HAVM, or GI telangiectases) and a first degree relative with HHT. In the presence of two criteria, the diagnosis was considered possible.

Molecular analysis

Mutation analysis was performed as reported [24]. In short, DNA was isolated from each of the probands, and exons 1–14 of *ENG* and exons 1–10 *ACVRL1* and their flanking intronic sequences were amplified using PCR. The PCR products were purified and sequenced. Once the mutation was identified, relatives were tested for the disease causing mutation.

A genetic diagnosis was considered to be positive when the family mutation was present or when the patient was an obligate carrier of the mutation. All patients with a clinically and/or genetically confirmed diagnosis and who were older than 16 years at the time of the screening were included in the study.

When a pathogenic mutation was found in the proband, apparently affected and unaffected relatives were offered genetic counseling and DNA analysis, which was performed for most relatives but not all. Patients not tested but with a proven HHT and with one or more affected family members with a known pathogenic mutation were considered to have the same mutation. The affected patients were divided in three groups, HHT1, HHT2, or HHT? on the basis of the mutation findings. The group HHT? consisted of probands and their relatives for whom DNA was either not available (66 patients from 37 families) or in whom a pathogenic mutation was not found (10 patients from 7 HHT? families).

Screening for visceral manifestations

Screening for the presence of a PAVM was performed routinely by chest radiography and by measuring partial oxygen pressure in arterial blood and, if abnormal, followed by the 100% oxygen right to left shunt test [27]. A normal result excluded a PAVM. Patients with a suspected PAVM were offered subsequently a conventional angiography, a digital subtraction angiography of the pulmonary arteries, or computed tomography (CT) of the chest. When an abnormal chest radiography and or a pathological right to left shunt (>5%) was found, but confirmation through subsequent angiography or CT analysis was not performed, the PAVM was classified as doubtful.

Until 2001, screening for CAVMs was performed using intravenous digital subtraction angiography; when a CAVM was suspected, conventional cerebral angiography was also

performed. Since 2001, this screening was performed with CT or magnetic resonance imaging (MRI) only when, after counseling, the patient requested it or because of symptoms. The screening for HAVM was not done routinely. Ultrasonography, CT or MRI was performed in cases of elevated alkaline phosphatase level or gamma glutamyl transpeptidase, or the presence of a murmur over the liver, heart failure, or abdominal pain. Between 1996 and 2003, screening for an HAVM was also performed before embolisation of a PAVM, in order to avoid embolisation complications [28].

The search for GI involvement was only performed (by means of a regular diagnostic endoscopy or videocapsule endoscopy) when unexplained iron deficiency anemia was detected or in cases of overt bleeding. Only when multiple GI telangiectases were detected was GI involvement considered confirmed.

Statistical analysis

The proportion of subjects in each group with visceral manifestations was calculated. The statistical analysis was performed using 2×2 table analysis with the $\chi 2$ test. To compensate for multiple testing, the p value for individual tests was multiplied by the number of comparisons made (Bonferroni correction, p_c).

Results

In total, 1291 people (558 men, 733 women) were included in the database containing patients and their family members screened for HHT symptoms. Of these, 1130 were older than 16 years at the time of the screening. Four people were excluded from the analysis because *MADH4* mutations were detected as a cause of HHT and juvenile polyposis. The 1126 people were 100 probands (32 men and 68 women), 484 affected family members, and 542 family members in whom the diagnosis could not be established or was excluded. Thus, 584 (242 men and 342 women) were older than 16 years and had been diagnosed clinically or genetically with HHT (table 1). Telangiectases detected on physical examination or in the nose (rhinoscopy) were present in 98.4% of patients, and 97.2% of patients were known to have epistaxis (data not shown).

A clinically doubtful but genetically confirmed diagnosis was found in 19 patients in HHT1 (5%) and in 11 patients with HHT2 (8.6%). Numbers, ages, sex, and mean ages of different groups are depicted in table 1. There was a preponderance of women in the database, both as affected and unaffected family members. There was no significant difference in sex ratio between the three groups. The frequency of visceral manifestations found in HHT1, HHT2, and HHT? is depicted (tables 2 and 3).

Table 1 - Proportion of male (M) and female (F) subjects for HHT1, HHT2 and HHT?, with mean ages (SD). Family members with a clinical and or genetic certain diagnosis, ascertained from the database, are shown. The 'HHT present' group consists of patients with a clinically and or genetically confirmed diagnosis. Patients were deemed "possible" when two of the four criteria are present. Patients with HHT in combination with juvenile polyposis were excluded. (M/F ratio) indicates the proportion of males, (SD) gives the standard deviation

	HHT1	HHT2	HHT?
Probands and family			
No of subjects (m/f ratio)	735 (0.74)	216 (0.77)	175 (0.68)
mean age (SD)	44.8 yr (17.2)	46.2 yr (16.6)	47.8 yr (18.1)
HHT present (m/f ratio)	380 (0.74)	128 (0.71)	76 (0.58)
mean age (SD)	48,4 yr (18.2)	51,2 yr (16.2)	53,7 yr (15.5)
HHT possible (m/f ratio)	84 (0.83)	25 (1.27)	60 (0.67)
mean age (SD)	40.0 yr (16.8)	38.0 (14.8)	43.8 (20.9)
HHT absent (m/f ratio)	271 (0.73)	63 (0.75)	39 (0.95)
mean age (SD)	41.2 yr (14.7)	39.1 yr (16.8)	42.6 (15.2)
HHT present			
no of subjects	380	128	76
no of families	63	40	44
members per family	6	3,2	1,7
men	161 (42,4%)	53 (41,4%)	28 (36,8%)
women	219 (57,6%)	75 (58,6%)	48 (63,1%)
mean age (SD)	48,4 yr (18.2)	51,2 yr (16.2)	53,7 yr (15.5)
mean age M (SD)	47,9 (18,8)	53,5 (13,6)	51,9 (13,6)
mean age F (SD)	48,8 (17,7)	49,7 (17,7)	54,8 (16,5)

PAVM

In the HHT1 group, 359 patients were examined clinically. Of these, 16 patients (4.4%) had a doubtful result. Of the remaining 343 patients, 167 (48.7%) were diagnosed clinically with a PAVM. In the HHT2 group, 12 of the 126 examined patients (9.5%) had a doubtful result. The frequency of PAVM in the HHT2 group was 5.3% (6/114 patients).

In total, 151 men with HHT1 were screened for PAVM, of whom eight had a doubtful result. Of the remaining 143 men, 58 men had a PAVM (40.6%). Of the 208 women screened for a PAVM, eight had an uncertain result. Of the remaining 200 women, 109 had a PAVM (54.5%). Therefore, PAVM occurred not only significantly more often in HHT1 than in HHT2 ($p = 6\times10^{-16}$), but also occurred more often in HHT1 women than HHT1 men, although this difference was not significant after correction for multiple testing.

In the HHT? group, 71 patients were examined for the presence of a PAVM, of whom 11

had a doubtful result. A PAVM was detected in 27 out of the remaining 60 patients (45%); 10 of 20 men and 17 of 40 women.

Table 2 - Prevalence of visceral manifestations in HHT1, HHT2 and HHT?.
Patients with a doubtful result are not included in the analysis. Denominators vary between categories since not all patients underwent all examinations for all visceral organs. The statistical analysis is performed comparing HHT1 and HHT2. The p values are shown with the p values after correction for multiple testing in brackets.

	HHT1	HHT2	HHT?	p value
PAVM	167/343 (48.7%)	6 /114 (5.3%)	27/60 (45%)	$1.2x10^{-16}$ ($p_c = 6x10^{-16}$)
CAVM	38/260 (14.6%)	1/76 (1.3%)	4/42 (9.5%)	0.0015 ($p_c = 0.007$)
CAVM + PAVM	22/253 (8.7%)	0/67 (0%)	1/38 (2.6%)	0.012 ($p_c = 0.062$)
HAVM	11/144 (7.6%)	13/32 (40.6%)	7/33 (21.2%)	8.7×10^{-7} ($p_c = 4.4 \times 10^{-6}$)
GI telangiectasia	56/78 (71.8%)	19/29 (65.5%)	11/16 (68.8%)	ns (ns)

CAVM

In total, 268 HHT1 patients were investigated for a CAVM. In eight patients (four men, four women) the presence of a CAVM could not be definitely determined. The frequency of CAVM in HHT1 was 14.6% (38/260). Of 76 HHT2 patients (28 men, 48 women) screened for CAVM, none had a doubtful result and only one woman had a CAVM (1.3%).

In the HHT1 group, of the 109 men 14 had a CAVM (12.8%) compared with 24/151 of the women (15.9%). In the HHT? group, two patients had a dubious result. Of the remaining 42 patients (16 men, 26 women), four had a CAVM (9.5%).

The combination of PAVM and CAVM in the same patient was found only in the HHT1 group. Patients were included who were screened for both manifestations, that is had screening performed for PAVM and CAVM and had definite absence or presence of the manifestations. In patients with only one manifestation, the other one excluded, the combination PAVM/CAVM was considered absent. In HHT1, the combination was present in 22 (6 men and 16 women) of 253 patients (8.7%). Of the 231 patients without the combination, 120 had only PAVM, 13 had CAVM only, and 98 had neither PAVM nor CAVM. In the HHT2 patients, the combination of CAVM and PAVM was not detected in any of the 67 patients. Six of the 67 patients had a PAVM, 1 a CAVM and 60 had no manifestation. In the HHT? group, the combination was detected in one of 38 patients (2.6%).

Table 3 - Prevalence of visceral manifestations in HHT1 and HHT2, for men and women.
Patients with a doubtful result are not included in the analysis. Denominators vary between categories because not all patients underwent all examination for all visceral organs. The statistical analysis is performed comparing men and women. The p values are shown, with p values after correction for multiple testing in brackets.

HHT1	males	females	p value
PAVM	58/143 (40.6%)	109/200 (54.5%)	0.011 (p_c = 0.054)
CAVM	14/109 (12.8%)	24/151 (15.9%)	0.49 (ns)
PAVM + CAVM	6/105 (5.7%)	16/148 (10.8%)	0.16 (ns)
HAVM	1/56 (1.8%)	10/88 (11.4%)	0.035 (p_c = 0.174)
GI telangiectases	23/33 (69.7%)	33/45 (73.3%)	ns (ns)

HHT2	males	females	p value
PAVM	2/50 (4%)	4/64 (6.3%)	0.593 (ns)
CAVM	0/28 (0%)	1/48 (2.1%)	0.442 (ns)
PAVM + CAVM	0/27 (0%)	0/40 (0%)	ns (ns)
HAVM	2/12 (16.7%)	11/20 (55%)	0.033 (p_c = 0.162)
GI telangiectases	11/16 (68.8%)	8/13 (61.5%)	ns (ns)

HAVM

In the HHT1 group, 162 patients (61 men, 101 women) were screened for HAVM, of whom 18 had a doubtful result. Of the remaining 144 patients (56 men, 88 women) 1 man and 10 women were diagnosed with an HAVM. In the HHT2 group, the liver was examined in 38 patients, of whom six had a doubtful result. An HAVM was detected in 13 (2 men, 11 women) of the remaining 32 patients (12 men, 20 women). Significantly more HAVMs were detected in HHT2 (40.6% versus 7.6%, p = 0.0004). Furthermore, in both groups HAVMs were present more often in women than in men. However, this difference was not significant after Bonferroni correction. In the HHT? group, there was a frequency of 21.2% (7/33) for HAVM.

GI localisation of HHT (telangiectases) was investigated in 78 HHT1 patients. In 56 (23 men, 33 women) multiple telangiectases were detected. Screening of the GI tract was undertaken in 29 HHT2 patients, and in 19 GI telangiectases were found (11 men, 8 women). In the HHT? group, the intestines were investigated in 16 patients, of whom 11 were diagnosed with HHT of the bowels. In an attempt to correct for possible referral bias, we performed a second analysis, in which we excluded the proband of each of the families, the first of the family who was referred.

The results are given in table 4. The significant differences between HHT1 and HHT2 for PAVM and HAVM remained after exclusion of the probands. In HHT1, significantly more women have an HAVM (table 5). An increased frequency of CAVM was again observed in

HHT1 compared with HHT2, but this trend was not significant after Bonferroni correction, nor was the difference in PAVM in HHT1 between men and women significant.

Table 4 - Prevalence of visceral manifestations in HHT1, HHT2 and HHT? after exclusion of the proband of each family (first patient referred and ascertained) in order correct for possible referral bias. Patients with a doubtful result were not included in the analysis. Denominators vary between categories because not all patients underwent all examinations for all visceral organs. The statistical analysis was performed comparing HHT1 and HHT2. The p values are shown, with the p values after correction for multiple testing in brackets

	HHT1	HHT2	HHT?	p value
PAVM	133/300 (44.3%)	3/88 (3.4%)	16/42 (38.1%)	$1.5x10^{-12}$ ($p_c = 6x10^{-12}$)
CAVM	31/225 (13.8%)	1/50 (2.0%)	2/27 (7.4%)	0.019 (p_c = 0.094)
CAVM + PAVM	16/218 (7.3%)	0/45 (0%)	0/27 (0%)	0.06 (ns)
HAVM	9/119 (7.6%)	8/21 (38.1%)	6/19 (31.6%)	7.8x10-5 (p_c = 0.0004)
GI telangiectasia	44/62 (71%)	12/18 (66.7%)	7/11 (63.3%)	ns (ns)

Table 5 - Prevalence of visceral manifestations in men and women after exclusion of the proband of each family (first patient referred and ascertained) in order correct for possible referral bias. Patients with a doubtful result were not included. Denominators vary between categories, because not all patients underwent all examinations of all visceral organs. The statistical analysis was performed comparing men and women. The p values are shown, with p values after correction for multiple testing in brackets.

HHT1	men	women	p value
PAVM	50/132 (37.9%)	83/168 (49.4%)	0.046 (p_c = 0.23)
CAVM	13/101 (12.9%)	18/124 (14.5%)	0.72 (ns)
CAVM + PAVM	5/97 (5.2%)	11/121 (9.1%)	0.35 (ns)
HAVM	0/50 (0%)	9/69 (13.0%)	0.008 (p_c = 0.04)
GI telangiectasia	20/29 (69%)	24/33 (72.7%)	ns (ns)

HHT2	men	women	p
PAVM	1/39 (2.6%)	2/49 (4.1%)	0.70 (ns)
CAVM	0/20 (0%)	1/30 (3.3%)	0.41 (ns)
CAVM + PAVM	0/19	0/26	ns (ns)
HAVM	2/9 (22.2%)	6/12 (50%)	0.19 (ns)
GI telangiectasia	8/11 (72.7%)	4/7 (57.1%)	Ns (ns)

Discussion

This study is the first analysis based on a national HHT population evaluated by use of a standard protocol applied within a single national HHT centre. In this study, we compared patients from families with *ENG* mutations with patients from families with *ACVRL1* mutations. We report on the frequencies of disease manifestations in HHT1, HHT2, and HHT?. The results reveal differences between HHT1 patients and HHT2 patients and between men and women.

The three patient groups, HHT1, HHT2, and HHT?, were comparable with respect to age and age distribution, which is important when comparing age dependent disease expressions. The groups are also large from the viewpoint of statistical power. The proportion of family members with a certain diagnosis in the database was slightly different for HHT1 and HHT2. For HHT1, 51.7% of the family members were diagnosed with HHT, while for HHT2 this was 59.3%. In the HHT2 group, obviously fewer unaffected family members older than 16 years are known in the clinic. This may be due to the fact that family members of HHT1 patients are more likely to attend hospital because of the more severe phenotype in their relatives, even when they themselves are asymptomatic. This may also explain the higher number of relatives referred or examined from families with HHT1 compared with HHT2.

In the HHT1 and HHT2 groups, the percentage of family members with possible diagnosis was 11.4% and 11.5% respectively. The mean ages of these groups were lower than the mean ages of the total group. This probably reflects the age related penetrance, but has no influence on the comparison of the two groups.

In all three HHT groups, there was a significant female preponderance. The female preponderance was uniformly present in the database of 1291 people (56.7% women), after selection for the family members above 16 years (57.5% women) and in the group with clinically or genetically confirmed HHT (58.6% women). A female preponderance was also found among unaffected family members and among the groups that were screened but with an uncertain diagnostic result. Only in the HHT2 “possibly affected” group was there a male preponderance, but this is a small group. This finding may reflect the notion, held by both families and physicians, that women have an AVM more often than men. Therefore, women in a family are more aware of HHT or are stimulated to have screening performed. Another explanation might be that a different attitude towards healthcare exists between men and women. This was suggested to be the cause for female preponderance in, for example, families with colon cancer.[29] The fact that there is a female preponderance in both the affected and unaffected cohorts also raises the question as to whether there is a difference in genetic fitness between men and women. When there is a disadvantage for male fetuses in the early embryonic period, more girls will be born, resulting in a female preponderance. Thorough family investigations will shed light on this aspect.

Phenotypic differences between HHT1 and HHT2

A PAVM was significantly more frequent in HHT1 (48.7%) than in HHT2 (5.3%). This concurs with earlier reports. Berg *et al.*[4] reported PAVM in 34.7% of HHT1 patients and no PAVM in HHT2 patients. The combined data published by Abdalla *et al.*[8] show a frequency of PAVMs in HHT2 patients of 5%, very similar to our findings. Our screening technique with chest radiography and arterial blood gas is not as sensitive in the detection of PAVM as the echo bubble technique [30], therefore, small PAVMs may have been missed in our study. As the same screening method was used in HHT1 and HHT2 and there is no evidence that patients with HHT2 have smaller PAVMs than patients with HHT1, our results probably reflect the proportional difference between HHT1 and HHT2, and provide a good estimate of the frequency of PAVM.

CAVM was detected in 14.6% of patients with HHT1 and 1.3% of the HHT2 patients (p_c = 0.007). Although the significance was lost after correction for referral bias, caused by the smaller number of patients, the difference remains striking. The gold standard for diagnosing CAVM is carotid angiography, but this technique is too invasive for screening asymptomatic patients. Therefore, small CAVMs could have remained undetected. The prevalence of CAVM in HHT1 and HHT2 in our study is comparable with other reports [4,8,11,12]. In the literature, very few reports found significant different frequencies for HHT1 and HHT2, but owing to low numbers, the power to detect significant differences was low. Two earlier reports found CAVMs in 8.2% of cases in HHT1[4] and 2–3% in HHT2 [4,8].

In this study, the combination of PAVM and CAVM in the same patient was found only in the HHT1 cohort, in 8.7% of patients. This is very similar to the expected frequency that can be calculated by multiplying the separate frequencies from PAVM and CAVM solely (7.1%). This suggests that PAVM and CAVM occur independently of each other, are not due to a common pathogenic factor such as specific HHT1 mutations and may be due to different interacting factors that are genetic, environmental, or both.

There is a highly significant difference in the prevalence of HAVM between HHT1 (7.6%) and HHT2 (40.6%). A potential source of bias is the fact that in HHT1 relatively more asymptomatic patients have been screened because of the high number of embolisations. When all patients with a PAVM were excluded, only 2.4% (1/41) of the HHT1 patients had an HAVM compared with 40.7% (11/27) of the HHT2 patients, which is still significantly different ($p_c = 2.5\times10^{-4}$). Telangiectases of the GI tract were found in similar proportions in HHT1 and HHT2. The high prevalence is probably the result of the fact that only patients with unexplained anemia or overt GI bleeding were examined. Therefore, the true prevalences are hard to estimate.

The group named HHT? comprised patients from families with an unknown genotype, either because DNA was unavailable (66 patients from 37 families) or no mutation could be detected (10 patients from 7 families). In these seven families, subsequent MLPA analysis revealed no large rearrangements, making HHT1 or HHT2 in these families unlikely. In the

10 patients, 3 of 8 patients had a PAVM, 0 of 6 a CAVM, 0 of 7 an HAVM, and 3 of 3 GI manifestations. We assume that most of the remaining HHT? patients have either HHT1 or HHT2. However, we cannot exclude the possibility of one or more alternative genes for HHT with a much lower frequency in this population. The relatively high prevalence of PAVM and CAVM in HHT? suggests a larger proportion of HHT1 in the HHT? panel. On the other hand, the presence of HAVMs is higher than would be expected in HHT1, suggesting that there is indeed a mix of both HHT1 and HHT2 in HHT?.

Differences between men and women

To our knowledge, systematic phenotype analysis in relation to sex has not been previously performed. There are publications suggesting that women are more prone to develop visceral manifestations, but significant differences have not been reported. We found a higher prevalence of PAVMs in women compared with men for HHT1, and more HAVMs were found in women for both HHT1 and HHT2. The differences were not significant after correction for multiple testing, but the prevalence of manifestations shows obvious differences between men and women. As women more frequently have a PAVM, and consequently more women underwent embolisations in our study, more asymptomatic women will have been screened for HAVM. Correction for patients with a PAVM resulted in very small groups; for HHT1 no men and 1/22 women (4.5%) had an HAVM, while for HHT2, there were 2/10 men (20%) and 9/17 women (53%).

Explanations for these sex related differences are still diffuse, such as environmental factors, modifier genes, or hormonal differences. Additionally, within families there is a wide variety of expression of symptoms. The six HHT2 patients with a PAVM did not cluster in a single family but were from six different families. The 167 PAVMs in HHT1 occurred in 51 (of 63) families, with some degree of familial clustering. For example, in one family 7 of the 8 affected family members had a PAVM, while in another family a low prevalence was detected (9 of 28 patients). The observed sex differences and the intrafamilial variability may provide an interesting clue for the search for (sex) related genetic and/or environmental factors interacting with the major gene mutations.

In order to correct for potential referral bias associated with features of the probands' phenotypes, we performed a second analysis excluding the probands (tables 4 and 5). After this correction, the difference between HHT1 and HHT2 for PAVM and HAVM remained statistically significant. The proportion of patients with a CAVM showed a minor change, and the statistical significance was lost after correction for multiple testing. The different frequency in PAVMs, CAVMs, and HAVMs between HHT1 men and HHT1 women showed only slight changes compared with the analysis of the whole group. Despite this correction, there may still be a referral bias left, owing to the effect of the severity of the phenotype in the family of the proband. This seems to be confirmed by the proportion of unaffected family members in HHT1 (36%) and HHT2 (29%). Apparently, fewer family members of probands with the less severe phenotype had screening performed.

Genetic Counseling

These data show that a significant phenotypic difference exists between HHT1 and HHT2. Genetic counseling of patients and family members can be given more accurately when the pathogenic gene mutation in the family is known. We intend to use the prevalence found before and after correction for referral bias. For HHT1, the chance of having a PAVM above the age of 16 years is 45–50%, and the risk of having a CAVM is 13–15%. For HHT2, PAVM is present in 3–5% and CAVM in 1–2%. Risk estimates for HAVM and GI involvement are difficult to give because most patients were symptomatic at the time of the screening. For HHT2, the frequency of HAVM appears to be between 38% and 41%, while for HHT1, it is between 2.5% and 8%.

It is our opinion that the differences between men and women should be confirmed by others, before adjusted percentages for sex difference can be used. For the time being, the significant difference between the sexes justifies mentioning that women with HHT1 are more likely to develop a PAVM or HAVM and women with HHT2 are more prone to develop HAVM. It should of course always be emphasized that there can be considerable intrafamilial and interfamilial variability and that the frequencies we calculated are averages and subject to potentially referral and selection bias. Family specific risk values may or may not vary but cannot be given, because the factors determining the clinical expression (genetic, environmental, or both) are still unknown.

Three out of four Curaçao criteria are required for a definite clinical diagnosis of HHT. Our data show that visceral involvement (PAVM and CAVM) is rare in HHT2 and will be of little value in the clinical diagnosis. Assuming similar degrees of clinical variability for the remaining three criteria, there may be a larger proportion of patients with HHT2 that remain undiagnosed than for HHT1. This raises the question as to how to apply the Curaçao criteria in HHT now that we are more aware of the fact that clinical expression shows consistent variability between sexes and is dependent on the type of gene involved. The prevalence of HAVM in HHT2 is high, and routine screening for HAVM with ultrasound Doppler might be indicated in members of HHT2 families as well as in new HHT patients and their relatives, for whom a molecular genetic diagnosis is not yet available, in order to arrive at the correct clinical diagnosis, despite the fact that the finding of HAVM usually has few therapeutic consequences.

References

1. 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; 1;116: 324-328.
2. Haitjema T, Balder W, Disch FJ, Westermann CJ. Epistaxis in hereditary haemorrhagic telangiectasia. Rhinology 1996; 34: 176-178.
3. Shovlin CL, Letarte M. Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax.1999; 54: 714-729.
4. 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: 585-590.
5. Guttmacher AE, Marchuk DA, White RI Jr. Hereditary hemorrhagic telangiectasia. N Engl J Med 1995; 333: 918-924.
6. Haitjema T, Disch F, Overtoom TT, Westermann CJ, Lammers JW. Screening family members of patients with hereditary hemorrhagic telangiectasia. Am J Med 1995; 99: 519-524.
7. Berg JN, Guttmacher AE, Marchuk DA, Porteous ME. Clinical heterogeneity in hereditary haemorrhagic telangiectasia: are pulmonary arteriovenous malformations more common in families linked to endoglin? J Med Genet. 1996 ;33: 256-257.
8. Abdalla SA, Geisthoff UW, Bonneau D, Plauchu H, McDonald J, Kennedy S, Faughnan ME, Letarte M. Visceral manifestations in hereditary haemorrhagic telangiectasia type 2. J Med Genet. 2003; 40: 494-502.
9. Khurshid I, Downie GH. Pulmonary arteriovenous malformation. Postgrad Med J. 2002; 78: 191-197
10. 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.
11. Fulbright RK, Chaloupka JC, Putman CM, Sze GK, Merriam MM, Lee GK, Fayad PB, Awad IA, White RI Jr. MR of hereditary hemorrhagic telangiectasia: prevalence and spectrum of cerebrovascular malformations. AJNR Am J Neuroradiol. 1998; 19: 477-484.
12. Moussouttas M, Fayad P, Rosenblatt M, Hashimoto M, Pollak J, Henderson K, Ma TY, White RI. Pulmonary arteriovenous malformations: cerebral ischemia and neurologic manifestations. Neurology. 2000; 10; 55: 959-964.
13. Easey AJ, Wallace GM, Hughes JM, Jackson JE, Taylor WJ, Shovlin CL. Should asymptomatic patients with hereditary haemorrhagic telangiectasia (HHT) be screened for cerebral vascular malformations? Data from 22,061 years of HHT patient life. J Neurol Neurosurg Psychiatry. 2003; 74:743-748.
14. Garcia-Tsao G, Korzenik JR, Young L, Henderson KJ, Jain D, Byrd B, Pollak JS, White RI Jr. Liver disease in patients with hereditary hemorrhagic telangiectasia. N Engl J Med. 2000; 28; 343: 931-936.
15. Buscarini E, Buscarini L, Danesino C, Piantanida M, Civardi G, Quaretti P, Rossi S, Di Stasi M, Silva M. Hepatic vascular malformations in hereditary hemorrhagic telangiectasia: Doppler sonographic screening in a large family. J Hepatol. 1997; 26: 111-118.
16. Sabba C, Pasculli G, Cirulli A, Gallitelli M, Virgilio G, Guastamacchia E, Resta F, Palasciano G. Rendu-Osler-Weber disease: experience with 56 patients. Ann Ital Med Int. 2002; 17: 173-179.
17. Kuehl HK, Caselitz M, Hasenkamp S, Wagner S, El-Harith el-HA, Manns MP, Stuhrmann M. Hepatic manifestation is associated with ALK1 in hereditary hemorrhagic telangiectasia: Identification of five novel ALK1 and one novel ENG mutations. Hum Mutat. 2005 ; 25: 320.
18. Kjeldsen AD, Oxhoj H, Andersen PE, Green A, Vase P. Prevalence of pulmonary arteriovenous malformations (PAVMs) and occurrence of neurological symptoms in patients with hereditary haemorrhagic telangiectasia (HHT). J Intern Med. 2000; 248: 255-262.
19. Bourdeau A, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M. Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. Am J Pathol 2000;156: 911-923.
20. Van den Driesche S, Mummery CL, Westermann CJ. Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. Cardiovasc Res 2003; 58: 20-31.
21. 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.

22. 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). The Lancet 2004; 363: 852-859.

23. Cole SG, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet. 2005 42: 577-582.

24. Letteboer TG, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum Genet. 2005; 116: 8-16.

25. Lesca G, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Riviere 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.

26. 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; 6; 91:66-67

27. Cotes JE, Leathart GL. Lung function: assessment and application in medicine. 5th ed. Blackwell Scientific Publications, Oxford 1993: 253-255

28. Haitjema T, ten Berg JM, Overtoom TT, Ernst JM, Westermann CJ. Unusual complications after embolization of a pulmonary arteriovenous malformation. Chest. 1996; 109:1401-1404.

29. Holloway S, Porteous M, Cetnarskyj R, Rush R, Appleton S, Gorman D, Campbell H. Referrals of patients to colorectal cancer genetics services in south-east Scotland. Fam Cancer. 2005; 4:151-161

30. Cottin V, Plauchu H, Bayle JY, Barthelet M, Revel D, Cordier JF. Pulmonary arteriovenous malformations in patients with hereditary hemorrhagic telangiectasia. Am J Respir Crit Care Med. 2004; 1;169: 994-1000

Chapter 3.2

Genotype-phenotype relationship for localization and age distribution of telangiectases in hereditary hemorrhagic telangiectasia

Letteboer TG, Mager HJ, Snijder RJ, Lindhout D,
Ploos van Amstel HK, Zanen P, Westermann KJ.

Am J Med Genet A. 2008; 146A: 2733-2739

Abstract

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disease characterized by arteriovenous malformations ranging from telangiectases to larger arteriovenous malformations. Mutations in two genes cause HHT; *ENG* (HHT1) and *ACVRL1* (HHT2). Although the hallmark for clinical diagnosis is the presence of telangiectases, there are few publications reporting the relative distribution and frequency of these features between HHT1 and HHT2. Here, the results of such analysis of telangiectases in 268 patients with HHT1 and 130 patients with HHT2 are described. Localization of the telangiectases is reported, and patients were clustered by age to estimate the site prevalence for different age categories. We show that telangiectases of the nasal mucosa are present at a higher prevalence and start to appear earlier in life than those of the oral mucosa or dermal sites in patients with either HHT1 or HHT2. Oral and nasal mucosal telangiectases are present earlier in life in patients with HHT1 compared to patients with HHT2, whereas dermal lesions are more frequent and appear earlier in life in patients with HHT2. In patients with either HHT1 or HHT2, the number of sites affected increases with age. In patients with HHT1, more women than men had skin telangiectases, particularly on the face. These results confirm that the frequency of arteriovenous malformations differ between patients with HHT1 and HHT2, and that these differences can be detected on physical examination.

Introduction

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber disease is inherited in an autosomal dominant pattern and is characterized by vascular dysplasia. Characteristic manifestations are epistaxis, telangiectases, and arteriovenous malformations. Telangiectases are found predominantly on the face, lips, tongue and fingers and in the nasal, oral, and gastrointestinal mucosa. Larger arteriovenous malformations (AVMs) occur mostly in the lungs (PAVM), brain (CAVM) and liver (HAVM) [1].

Mutations in endoglin (*ENG*, chromosome 9q34, OMIM 131195) or in the activin receptor like kinase 1 gene (*ACVRL-1, ALK1*, chromosome 12q13, OMIM 601284) cause HHT1 and HHT2, respectively [2,3]. The *ENG* and *ACVRL1* genes are predominantly expressed in vascular endothelium, and published data support haploinsufficiency as the main causative model of HHT. Mutations result in a reduction of the level of functional proteins and in dysregulation of the TGFβ pathway, which plays an important role in vascular homeostasis. It has been suggested that local processes (inflammation, cell injury) can act as trigger for the formation of arteriovenous malformations and that modifier genes might play a crucial role in the development of the manifestations [4,5,6].

Although the same clinical manifestations occur in patients with HHT1 and HHT2, the frequency of the visceral manifestations vary [7,8,9]. Furthermore, in HHT1 and HHT2 there is a considerable inter- and intra-familial variability for the visceral manifestations.

Little is known regarding the distribution and occurrence of telangiectases in patients with HHT1 and HHT2. In patients with HHT, telangiectases usually appear in the third decade of life and increase in size and in number [5,10,11]. Plauchu *et al*.[10] described the clinical manifestations in 324 patients with HHT and found telangiectases in 74% of the patients, with approximately half of the patients presenting before the age of 30 years. At that time it was not possible to distinguish between HHT1 and HHT2.

Berg *et al.*[12] was the first to report on differences between patients with HHT1 or HHT2 regarding the frequency of the clinical manifestations. The results were based on 83 patients from the USA and the UK. Both epistaxis and telangiectases were found earlier in patients with HHT1 compared to patients with HHT2. Folz *et al*.[5] described the telangiectases in 70 patients with HHT and found the highest frequency in the endonasal mucosa (88%) followed by the oral cavity (77%), facial skin (74%), and the hands (46%).

To date, no detailed age-related analysis has been performed and in most reports the age of appearance of the telangiectases is based on recollection of the patients. In this study we report on the presence and distribution of telangiectases in a large Dutch HHT cohort, based on the results of physical examination, performed by HHT specialists. This diminishes the inter-observer bias and avoids the problem of recall bias.

Materials and methods

The screening of family members of patients with HHT was performed from 1990 in the Dutch HHT center (St Antonius Hospital, Nieuwegein) [13]. Screening included an inspection for telangiectases on the first visit to the department. The skin (face, hands, legs, feet, thorax, abdomen and neck), conjunctiva and oral mucosa (tongue, lip and mouth) were examined using a 2X magnifying lens. Each patient was examined by one of three senior clinicians (RS, JM, CW). The nose was inspected by an otorhinolaryngologist (FD) with an endoscope (magnification x 10). The sites of the telangiectases were scored on a standardized form. These forms were studied retrospectively in all Caucasian patients with a definite genetic diagnosis or clinical diagnosis according to the Curaçao criteria[14]. In this study only Caucasian patients from families with *ENG* or *ACVRL1* mutations were included, because patients of African descent have less visible telangiectases [15].

Mutation analysis was performed to discriminate between HHT1 and HHT2 as described earlier [16]. When mutational analysis revealed a mutation in the proband, all clinically affected family members were considered to have the same mutation.

For each patient with HHT1 or HHT2, the location of telangiectases, the age at the time of examination, and the gender were scored. Dubious telangiectases were excluded. Telangiectases found using capillary microscopy of the nail fold were not included as telangiectases of the hands or arms, since this is not a widely used technique. Instead they were denoted as telangiectases “elsewhere”.

To gain insight into the relationship between telangiectases and age, patients were clustered into age groups, based on the age at the time of clinical investigation. The frequency of telangiectases in each group was calculated and studied in relation to HHT and gender. For each patient the number of sites involved was counted (including telangiectases elsewhere) and the average number of sites involved was calculated for each age group.

Statistical analysis was done using the $\chi 2$ test. Bonferroni correction for multiple-comparison was not used because the comparisons were not considered independent tests. To correct for multiple testing a more stringent p-value was used, p-values above 0.01 were considered to be statistically non-significant.

Results

Of the 315 patients with HHT1, 47 were excluded and of the 131 patients with HHT2 one was excluded because of insufficient examinations of several sites, typically because the diagnosis was made because of a PAVM and a positive family history. The remaining 268 patients with HHT1 were from 56 families and 130 patients with HHT2 were from 39 families (table 1).

The age distribution shows that patients with HHT1 presenting with telangiectases were significantly younger than similar patients with HHT2. There was a non-significant female

preponderance in both HHT1 (56.7%) and in HHT2 (60%). Men and women with HHT1 or HHT2 did not differ significantly regarding age (table 1).

The overall frequency of the HHT1 and HHT2 telangiectases at the various mucosal and dermal sites in both genders is shown in table 2. The nasal mucosa was only investigated in 219 patients with HHT1 and 108 with HHT2, because not all patients were seen by the otorhinolaryngologist. In the total group there was only a slight difference in the presence of nasal mucosal telangiectases; 95% in patients with HHT1 compared to 93% in patients with HHT2. Oral telangiectases (lip, tongue or mouth) were present in about 80% of all patients.

Table 1 - Proportion of males (M) and females (F) included and excluded in the analysis with their characteristics: mean age (SD, standard deviation), proportion of males (M/F ratio) and the number of families included

	HHT1 n	HHT2 n
patients investigated (M/F)	315 (0.78)	131 (0.66)
excluded (M/F)	47 (0.88)	1 (F)
mean age excluded (SD)	45,2 yr (22.3)	73 yr
oral mucosa and skin (M/F)	**268 (0.76)**	**130 (0.67)**
families	56	39
males, (mean age; SD)	116 (33.7 yr; 17.8)	52 (45.9 yr; 15.7)
females (mean age; SD)	152 (36.5 yr; 18.8)	78 (42.5 yr; 17.3)
nasal mucosa (M/F)	**219 (0.82)**	**108 (0.74)**
families	53	36
males, (mean age; SD)	99 (33.1 yr; 18.2)	46 (45.9 yr; 15.9)
females (mean age; SD)	120 (36.0 yr; 18.6)	62 (42.4 yr; 16.6)

The prevalence of dermal telangiectases is lower than the prevalence of nasal and oral lesions, in patients with either HHT1 or HHT2. Dermal lesions are found more often in patients with HHT2 (74%) compared to HHT1 (59%; p = 0.004), most significantly when comparing telangiectases on the hands (62% and 45%; p = 0.001), but also a suggestive difference in facial telangiectases (40% and 29%; p = 0.02).

The proportion of patients with conjunctiva involvement is similar for both HHT1 (13%) and HHT2 (15%). Telangiectases on the neck, feet and abdomen are found only in a minority of patients with HHT. The low prevalence on the feet compared to the hands has been observed before[5,10]. Significant differences between men and women were only found for dermal telangiectases in HHT1 with a higher prevalence in women (47% and 68%; p = 0.0008), particularly for the hands (31% and 56%; p = 4.9x10-5).

Table 2 - The presence of telangiectases for the entire HHT1 and HHT2 population. The number of patients is depicted for each group (n). The numbers represent the number of patients in which the telangiectases for the particular site were detected. Between brackets the percentage of patient affected for the site

	HHT1			HHT2		
	men	women	total	men	women	total
	n=99	**n=120**	**n=219**	**n=46**	**n=62**	**n=108**
nasal mucosa (%)	92 (92.9)	117 (97.5)	209 (95.4)	45 (97.8)	55 (88.7)	100 (92.6)
	n=116	**n=152**	**n=268**	**n=52**	**n=78**	**n=130**
oral mucosa (%)	**88 (75.9)**	**125 (82.2)**	**213 (79.5)**	**40 (76.9)**	**65 (83.3)**	**105 (80.8)**
tongue	*65 (56.0)*	*108 (71.1)*	*173 (64.6)*	*33 (63.5)*	*53 (67.9)*	*86 (66.1)*
lip	*70 (60.3)*	*101 (66.4)*	*171 (63.8)*	*32 (61.5)*	*47 (60.3)*	*79 (60.8)*
mouth	*36 (31.0)*	*63 (41.4)*	*99 (36.9)*	*16 (30.8)*	*27 (34.6)*	*43 (33.1)*
conjunctivae (%)	**18 (15.5)**	**18 (11.8)**	**36 (13.4)**	**10 (19.2)**	**9 (11.5)**	**19 (14.6)**
dermal total (%)	**55 (47.4)**	**103 (67.8)**	**158 (59)**	**39 (75.0)**	**57 (73.1)**	**96 (73.8)**
hands, arms	*36 (31.0)*	*85 (55.9)*	*121 (45.1)*	*36 (69.2)*	*45 (57.5)*	*81 (62.3)*
face	*34 (29.3)*	*43 (28.3)*	*77 (28.7)*	*22 (42.3)*	*30 (38.5)*	*52 (40.0)*
chest	*5 (4.3)*	*16 (10.5)*	*21 (7.8)*	*8 (15.4)*	*7 (9.0)*	*15 (11.5)*
neck	*9 (7.8)*	*14 (9.2)*	*23 (8.6)*	*6 (11.5)*	*2 (2.6)*	*8 (6.1)*
feet, legs	*3 (2.6)*	*1 (0.7)*	*4 (1.5)*	*2 (3.8)*	*1 (1.3)*	*3 (2.3)*
abdomen	*0 (0)*	*1 (0.7)*	*1 (0.4)*	*0 (0)*	*0*	*0 (0)*

The presence of telangiectases in the different age groups on the various mucosal and dermal sites for HHT1 and HHT2 are depicted in table 3 and figure 2. The frequency increases with age in both forms for all sites except the neck, feet and abdomen, which are rarely affected. A steeper curve was found in nasal and oral mucosa compared to the skin, indicating that mucosal lesions tend to appear earlier and in a higher proportion of the patients.

Nasal telangiectases are present at a younger age in patients with HHT1 compared to those with HHT2. Over 90% of the patients with HHT1 have nasal lesions in the age group 0-20 in HHT1, compared to 67% in the HHT2 group 0-20. Also, oral mucosal lesions in general occur at a younger age in patients with HHT1. In the age group 21-30 87% of the patients with HHT1 have oral lesions, whereas 88% of the patients with HHT2 have oral involvement ten years later, in the age group 31-40. Among the individual sites (tongue, lip, mouth) the difference is most striking on the lip.

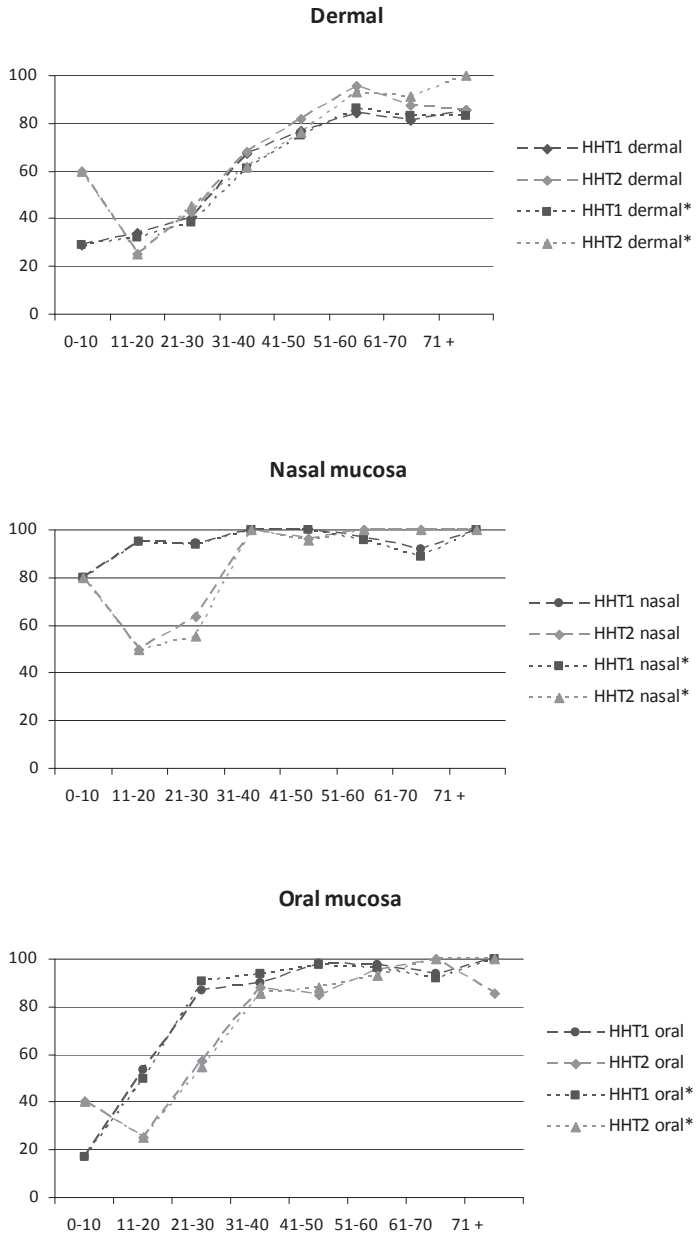


Figure 1 - Graphical view of the dermal (hands/arms, chest, feet/legs and abdomen), oral (tongue, lip and mouth) and nasal telangiectases. Age groups (years) are depicted on the X-axis, proportion of the patients with telangiectases for the site on the Y- axis. The different point prevalence's are connected. In an attempt to correct for possible referral bias, HHT* shows the results when the probands of the families were excluded.

Table 3 - The age at the time of examination determined the age group in which the patient is clustered. The presence of telangiectases per age group is given in numbers (n) and the percentage (between brackets) is the number of patient with telangiectases divided by the total of the corresponding age group (n).

	HHT1						
	0 - 10	11 - 20	21 - 30	31 - 40	41 - 50	51 - 60	61 - 70
number (M/F)	**n=20** (1.2)	**n=42** (0.75)	**n=36** (1.38)	**n=32** (0.39)	**n=41** (0.78)	**n=30** (0.76)	**n=12** (1.0)
mean age	5.9	15.9	25.6	35.4	45.6	55.2	65.5
nasal mucosa (%)	16 (80)	40 (95.2)	34 (94.4)	32 (100)	41 (100)	29 (96.7)	11 (91.7)
number (M/F)	**n=24** (1.0)	**n=47** (0.68)	**n=45** (1.5)	**n=40** (0.54)	**n=51** (0.70)	**n=38** (0.65)	**n=16** (0.78)
mean age	6.0	15.9	25.8	35.4	45.2	55.4	65.8
oral mucosa (%)	**4 (16.7)**	**25 (53.2)**	**39 (86.7)**	**36 (90.0)**	**50 (98.0)**	**37 (97.4)**	**15 (93.8)**
tongue	*2 (8.3)*	*16 (34.0)*	*28 (62.2)*	*28 (70.0)*	*42 (82.4)*	*36 (94.7)*	*14 (87.5)*
lip	*4 (16.7)*	*15 (31.9)*	*30 (66.7)*	*30 (75.0)*	*39 (76.5)*	*33 (86.8)*	*13 (81.3)*
mouth	*0 (0)*	*6 (12.8)*	*12 (26.7)*	*16 (40.0)*	*29 (56.9)*	*23 (60.5)*	*8 (50.0)*
conjunctivae (%)	**0 (0)**	**3 (6.4)**	**2 (4.4)**	**8 (20.0)**	**8 (15.7)**	**10 (26.3)**	**3 (18.8)**
dermal total (%)	**7 (29.2)**	**16 (34.0)**	**18 (40.0)**	**27 (67.5)**	**39 (76.5)**	**32 (84.2)**	**13 (81.3)**
hands, arms	*3 (12.5)*	*14 (29.8)*	*13 (28.9)*	*20 (50.0)*	*30 (58.8)*	*26 (68.4)*	*12 (75.0)*
face	*4 (16.7)*	*1 (2.1)*	*3 (6.7)*	*11 (27.5)*	*20 (39.2)*	*24 (63.2)*	*9 (56.3)*
chest	*0 (0)*	*2 (4.3)*	*3 (6.7)*	*1 (2.5)*	*7 (13.7)*	*6 (15.8)*	*1 (6.3)*
neck	*2 (8.3)*	*1 (2.1)*	*6 (13.3)*	*4 (10.0)*	*4 (7.8)*	*4 (10.5)*	*2 (12.5)*
feet, legs	*0 (0)*	*0 (0)*	*2 (4.4)*	*0 (0)*	*2 (3.9)*	*0 (0)*	*0 (0)*
abdomen	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*1 (2.0)*	*0 (0)*	*0 (0)*
mean localizations	1.7	2.6	3.3	4.0	4.5	5.3	4.7

In contrast, dermal telangiectases in general tend to be present at a younger age in patients with HHT2 than in patients with HHT1. The individual dermal sites also do not show a significant difference in the age related prevalence between HHT1 and HHT2. The point prevalence of telangiectases in the conjunctivae increases with age, but there is no difference between HHT1 and HHT2.

The mean number of sites with telangiectases also increases with increasing age (table 3). More sites were involved at a younger age in patients with HHT1 than in those with HHT2.

To correct for possible referral bias, the analyses per age group was also performed excluding the probands, the first patient of each family to attend the HHT centre. The results of this analysis show that excluding the probands resulted in negligible changes in the results (figure 1).

					HHT2			
71 +	**0 - 10**	**11 - 20**	**21 - 30**	**31 - 40**	**41 - 50**	**51 - 60**	**61 - 70**	**71 +**
n=6 (0.20)	**n=5** (0.67)	**n=4** (0.33)	**n=11** (0.83)	**n=23** (0.44)	**n=28** (0.87)	**n=19** (1.11)	**n=12** (0.71)	**n=6** (1.0)
74.0	7.6	15.5	26.4	36.1	44.6	55.1	64.7	74.0
6 (100)	4 (80.0)	2 (50)	7 (63.6)	23 (100)	27 (96.4)	19 (100)	12 (100)	6 (100)
n=7 (0.17)	**n=5 (0.67)**	**n=8** (0.14)	**n=14** (0.75)	**n=25** (0.47)	**n=33** (0.83)	**n=22** (1.0)	**n=16** (0.60)	**n=7** (0.75)
74.3	7.6	16.5	26.2	36.1	44.9	55.3	65.0	74.1
7 (100)	**2 (40.0)**	**2 (25.0)**	**8 (57.1)**	**22 (88.0)**	**28 (84.8)**	**21 (95.5)**	**16 (100)**	**6 (85.7)**
7 (100)	*1 (20.0)*	*1 (12.8)*	*5 (35.7)*	*18 (72.0)*	*22 (66.7)*	*18 (81.8)*	*15 (93.8)*	*6 (85.7)*
7 (100)	*0 (0)*	*1 (12.5)*	*4 (28.6)*	*17 (68.0)*	*22 (66.7)*	*16 (72.7)*	*15 (93.8)*	*4 (57.1)*
5 (71.4)	*1 (20.0)*	*0 (0)*	*1 (7.1)*	*8 (32.0)*	*11 (33.3)*	*10 (45.5)*	*9 (56.3)*	*3 (42.9)*
2 (28.6)	**0 (0)**	**0 (0)**	**2 (14.3)**	**3 (12.0)**	**2 (6.1)**	**6 (27.3)**	**4 (25.0)**	**2 (28.6)**
6 (85.7)	**3 (60.0)**	**2 (25.0)**	**6 (42.9)**	**17 (68.0)**	**27 (81.8)**	**21 (95.5)**	**14 (87.5)**	**6 (85.7)**
3 (42.9)	*2 (40.0)*	*1 (12.5)*	*6 (42.9)*	*14 (56.0)*	*22 (66.7)*	*17 (77.3)*	*13 (81.3)*	*6 (85.7)*
5 (71.4)	*1 (20.0)*	*2 (25.0)*	*1 (7.1)*	*7 (28.0)*	*12 (36.4)*	*17 (77.3)*	*7 (43.8)*	*5 (71.4)*
1 (14.3)	*0 (0)*	*0 (0)*	*1 (7.1)*	*4 (16.0)*	*7 (21.2)*	*1 (4.5)*	*0 (0)*	*2 (28.6)*
0 (0)	*0 (0)*	*0 (0)*	*1 (7.1)*	*0 (0)*	*3 (9.1)*	*2 (9.1)*	*0 (0)*	*2 (28.6)*
0 (0)	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*2 (6.1)*	*1 (4.5)*	*0 (0)*	*0 (0)*
0 (0)	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*
5.1	2	1.3	2.4	4.1	4.2	5.1	4.8	5.6

In both, the HHT1 and HHT2 groups, there were eight patients without telangiectases found on physical examination. In the HHT1 group, six of the eight patients were ≤ 10 years. Two of the six patients had a PAVM and three of the six had regular nosebleeds. Only two of the six patients had no epistaxis, no telangiectases and no visceral manifestations, but were diagnosed using DNA analysis. The two remaining patients with HHT1 (23 years and 53 years) had regular nosebleeds, but no other HHT manifestations.

Among patients with HHT2, three (7, 22 and 34 years) had no telangiectases or other HHT symptoms. One patient without telangiectases (27 years) had epistaxis, a PAVM, and a HAVM. Four patients (12, 20, 29 and 37 years) had nosebleeds regularly, without other manifestations of HHT.

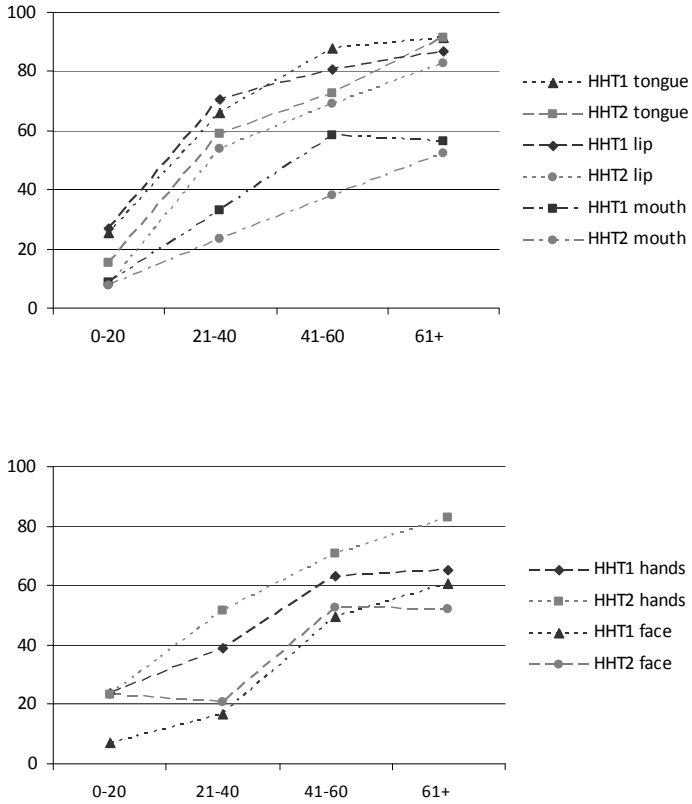


Figure 2 - Graph of separate oral localisations (lip, tongue mouth) and dermal localisations (hands and face) per age group. Age groups (years) are depicted on the X-axis, proportion of the patients with telangiectases for the particular site on the Y- axis. The different point prevalence's are connected.

Discussion

In this study, the sites of telangiectases on skin and mucosa were scored. The number of lesions per site was not considered, because telangiectases may be numerous and counting may be difficult and subject to observer bias. Moreover, the number does not add to diagnostic accuracy, as long as the typical telangiectases are present. The results of this analysis give a cross sectional view of the frequency and distribution of telangiectases in patients with HHT. Telangiectases were studied in families with confirmed HHT1 or HHT2 mutations. We observed that patients with HHT1 visited the outpatient department at a younger age than did patients with HHT2. This difference persisted after exclusion of the probands. This is not surprising, since more patients with HHT1 have PAVMs and CAVMs [7-9], and most probands are initially referred because of visceral manifestations, mostly PAVMs. This will bias towards screening of family members of patients with HHT1 at a younger age, as compared to those with HHT2. To circumvent this problem, analysis by age group was

performed.

A bias in the data for telangiectases is therefore not likely, but cannot be excluded. If the pathogenesis of the visceral manifestations and the telangiectases are overlapping, then referral with a PAVM might introduce bias. To correct for this, stratification by age was also performed discarding the initially referred proband of the family (figure 1). The graphs show only a slight change, indicating that the probands did not cause a major distortion in the analysis. In our HHT2 population, in the youngest group (0-10) more patients had telangiectases than in the next older group (11-20). Although the youngest group is small (n=5), the higher frequency might reflect an ascertainment bias. Parents who suspect telangiectases in their children at a younger age might attend the HHT center earlier. Another potential bias is the exclusion of 47 patients with HHT1, but only one with HHT2. These patients were referred for treatment of a PAVM and were therefore not thoroughly examined for telangiectases. Exclusion of probands (also referred with PAVM) did not change the results of the analysis; we conclude that the results did not change significantly because of exclusion of these patients.

The HHT phenotype has an age-dependent penetrance. The analysis by age group is therefore important and useful for clinicians. Age-dependent penetrance of telangiectases has been reported [5,10,12], based on the recollection of the patients. To study the relationship of age and penetrance of telangiectases, we performed physical examination. We show that with increasing age the frequency of telangiectases increases, for the nasal mucosa, the oral mucosa (tongue, lip, mouth), the conjunctiva and the skin of the hands and face. Furthermore, the nasal mucosa was affected significantly earlier in patients with HHT1 compared to those with HHT2. This confirms the findings of Berg *et al*.[12], who showed an earlier onset of epistaxis in patient with HHT1. A high prevalence of telangiectases in the nasal mucosa in patients below the age of 21 in those with HHT1 (90%) or HHT2 (67%) was found. In contrast, oral and dermal lesions are at this age present in a minority of the patients. This indicates that nasal endoscopy is a useful tool to establish a clinical diagnosis.

When comparing our results to earlier publications, we encountered three major problems. First, since there is an age-dependent penetrance for the symptoms, including telangiectases, the age of the population is crucial. In the reports by Plauchu *et al*.[10] and Folz *et al*.[5] the (mean) age of the investigated population is not specified. In both reports the population appeared to be younger, which explains the lower frequencies of telangiectases in those reports. Second, Plauchu *et al*.[10] and Folz *et al*.[5] report on the frequency of telangiectases in patients with HHT, comprising a mixture of patients with *ENG* mutations, *ACVRL1* mutations, and possibly other genes. Subsequent genetic analysis revealed that *ACVRL1* mutations are found more often in France than *ENG* mutations, whereas in the Dutch population *ENG* mutations are more frequent [16,17]. This apparent population difference between the French and the Dutch HHT populations can also explain part of the observed differences. Third, there is the problem of ascertainment bias. The way the patients were ascertained indicates what kind of referral bias one has to anticipate

and how to correct for it. The difference in telangiectases in the nose is striking. The nose was affected in the French population in 37% of the patients whereas epistaxis was present in 96% of the patients. Our panel showed nasal telangiectases in 92% of the patients with HHT2 and in 95% of those with HHT1. Since epistaxis in patients with HHT is usually caused by nasal telangiectases, a prevalence of nasal telangiectases of 37% seems surprisingly low. The strong concordance between epistaxis and nasal telangiectases in the Dutch population might be explained by the endoscopic examination used here.

In HHT1 more patients develop a PAVM and/or a CAVM compared to HHT2 [7-9]. HHT1 has also an earlier onset of telangiectases of the nasal and oral mucosa. The mechanisms leading to the inter- and intrafamilial variability in HHT are not obvious. Most likely these differences are caused, by a combination of multiple modifier genes interacting with several (maybe tissue specific) environmental factors in a complex manner. An important role for environmental factors in dermal telangiectases, is very likely, since the sun exposed skin (face and hands) are most often affected. In a normal population, telangiectases were associated with sun exposure, smoking, and increasing age [18]. Although these telangiectases are distinct from the telangiectases found in HHT, the association found in the healthy population might also hold true for the HHT population. The high frequency of telangiectases in the nasal and oral mucosa might be a reflection of the density and superficial localization of arteries in mucosal tissue in general. It is more likely that the high frequency is explained by a combination of the density and environmental factors; possible environmental factors being local stress, infection, minor trauma, exposure to toxic agents, and temperature changes.

Gender-associated differences in patients with HHT1 and HHT2 have been reported for the visceral manifestations [9]. The observed higher frequency of dermal telangiectases in women, significantly in those with HHT1, particularly on the hands, is in concordance with that observation. If this sex difference is confirmed, it will be worthwhile to devote research to its causal factors and mechanisms.

In conclusion, we show that in patients with HHT1 and patients with HHT2, telangiectases are identified in descending order in the nasal mucosa, oral mucosa, and skin. The number of affected sites increase with increasing age, in both HHT1 and HHT2. In general, oral and nasal lesions tend to appear earlier in patients with HHT1, whereas dermal lesions are observed earlier in patients with HHT2. This predilection of sites is likely to be the result of a combination of multiple modifier genes interacting with several (maybe tissue specific) environmental factors in a complex manner.

References

1. Guttmacher AE, Marchuk DA, White RI Jr. Hereditary hemorrhagic telangiectasia. N Engl J Med 1995; 333: 918-924.
2. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, McCormick MK, Pericak-Vance MA, Heutink P, Oostra BA, Haitjema T, Westerman CJJ, Porteous ME, Guttmacher AE, Letarte M, Marchuk DA. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 1994; 8: 345-351.
3. 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; 13: 189-195.
4. Van den Driesche S, Mummery CL, Westermann CJ. Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. Cardiovasc Res 2003; 58: 20-31.
5. Folz BJ, Lippert BM, Wollstein AC, Tennie J, Happle R, Werner JA. Mucocutaneous telangiectases of the head and neck in individuals with hereditary hemorrhagic telangiectasia analysis of distribution and symptoms. Eur J Dermatol 2004; 14: 407-411.
6. Abdalla SA, Geisthoff UW, Bonneau D, Plauchu H, McDonald J, Kennedy S, Faughnan ME, Letarte M. Visceral manifestations in hereditary haemorrhagic telangiectasia type 2. J Med Genet 2003; 40:494-502.
7. Bayrak-Toydemir P, McDonald J, Markewitz B, Lewin S, Miller F, Chou LS, Gedge F, Tang W, Coon H, Mao R. Genotype-phenotype correlation in hereditary hemorrhagic telangiectasia: mutations and manifestations. Am J Med Genet 2006; 140: 463-470.
8. Bossler AD, Richards J, George C, Godmilow L, Ganguly A. Novel mutations in ENG and ACVRL1 identified in a series of 200 individuals undergoing clinical genetic testing for hereditary hemorrhagic telangiectasia (HHT): correlation of genotype with phenotype. Hum Mutat 2006; 27: 667-675.
9. Letteboer TG, Mager HJ, Snijder RJ, Koeleman BP, Lindhout D, Ploos van Amstel HK, Westermann KJ. Genotype - phenotype relationship in Hereditary Hemorrhagic Telangiectasia. J Med Genet 2006; 43: 371-377.
10. Plauchu H, de Chadarevian JP, Bideau A, Robert JM. Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. Am J Med Genet 1989 ; 32: 291-297.
11. Shovlin CL, Letarte M. Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax 1999; 54: 714-729.
12. 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: 585-590.
13. Haitjema T, Disch F, Overtoom TT, Westermann CJ, Lammers JW. Screening family members of patients with hereditary hemorrhagic telangiectasia. Am J Med 1995; 99: 519-524.
14. 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.
15. 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 Part A 2003; 116: 324-328.
16. Letteboer TG, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum Genet 2005; 116: 8-16.
17. Lesca G, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Riviere 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.
18. Kennedy C, Bastiaens MT, Bajdik CD, Willemze R, Westendorp RG, Bouwes Bavinck JN; Leiden Skin Cancer Study. Effect of smoking and sun on the aging skin. J Invest Dermatol 2003; 120: 548-554.

Chapter 3.3

The onset and severity of epistaxis in patients with *ENG* or *ACVRL1* mutations

Letteboer TG, Mager HJ, Snijder RJ, S. Bulk, Lindhout D,
Ploos van Amstel HK, Zanen P, Westermann CJJ

submitted

Abstract

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber disease is an autosomal dominant disease with an age-related penetrance. Telangiectases are the hallmark of the clinical diagnosis for HHT. Bleeding from nasal telangiectases is often the presenting symptom, appearing early in life. Mutations in the *ENG* gene and *ACVRL1* cause HHT1 and HHT2 respectively. The present study was undertaken to evaluate the age of onset and the severity of epistaxis in HHT patients, comparing HHT1 with HHT2. A retrospective, questionnaire based, analysis was performed including 466 HHT patients, 294 HHT1 patients and 172 HHT2. Data were stratified for age, gender and ancestral background, to evaluate their influence on the severity of the phenotype. We found that in HHT1 epistaxis occurred significantly earlier in life than in HHT2, in both men and women and in both patients from the Netherlands and from the Dutch Antilles. The number of nosebleeds increases with increasing age, in HHT1 and HHT2, without gender differences. In our study, gender or ancestral background has no apparent influence on the occurrence or severity of epistaxis.

Introduction

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber disease is an autosomal dominant disease characterized by the presence of multiple direct arteriovenous connections (AVMs). Larger AVMs occur in the liver (HAVM), brain (CAVM) and lungs (PAVM). Complications of larger AVMs result from bleeding and shunting and warrant screening for these manifestations, especially for AVMs in the lung (PAVMs). Smaller lesions are called telangiectases and they present on the skin, mucosa of the mouth, digestive tract and particularly in the nose. Telangiectases can bleed easily and telangiectases of the nose can result in spontaneous epistaxis. Spontaneous and recurrent epistaxis is usually the presenting symptom in HHT [1-3], frequently leading to severe anemia requiring iron supplementation or blood transfusions. Patients identify epistaxis as a major health concern that affects every day life and is an important factor reducing quality of live [4].

Recurrent spontaneous epistaxis occurs in 85-95% of the patients with HHT and is therefore one of the main criteria for the clinical diagnosis. The clinical criteria to establish the clinical diagnosis are: recurrent and spontaneous epistaxis, multiple telangiectases at characteristic sites, visceral AVMs and a first degree relative with HHT. Three of the four criteria are required for a definite clinical diagnosis [5].

In the majority of cases, HHT is caused by mutations in one of two genes: in the gene coding for endoglin (*ENG*, OMIM 131195) or activin receptor-like kinase (*ACVRL1-ALK1*, OMIM 601284) [6,7]. *ENG* mutations result in HHT1, *ACVRL1* mutations cause HHT2. In 2004, patients with a combined phenotype of both HHT and juvenile polyposis were shown to carry *SMAD4* mutations (OMIM 600993) [8]. A few families with classical HHT, not linked to *ENG*, *ACVRL1* or *SMAD4* were linked to chromosome 5 and chromosome 7 [9,10].

DNA analysis in the Netherlands has shown *ENG* to be the cause in the majority of cases in this population; 53% of the families have *ENG* mutations whereas 40% show ACVRL1 mutations [7]. In the Dutch Antilles, only *ENG* mutations occur with three different haplotypes [11].

To distinguish between HHT1 and HHT2, mutation analysis is required. The clinical symptoms in HHT1 and HHT2 are similar, but the frequency of the different symptoms is different for *ENG* or *ACVRL1* mutations. There is a significantly higher prevalence of PAVMs and CAVMs in HHT1 and HAVM in HHT2 [12]. Dermal telangiectases tend to occur earlier in HHT2 than in HHT1, but mucosal lesions are present at a significantly earlier age in HHT1 [13]. Nasal telangiectases, the cause of nosebleeds, were found to be present in 92% of the Dutch HHT1 patients and in 67% of the HHT2 patients at the age of 20 years. A significantly earlier onset of epistaxis in HHT1 has been reported several times, but mostly based on limited number of patients [3, 14-19]. To our knowledge, an age-related analysis has only been performed once [17], but to date no distinction has been made between male and female patients for the frequency and severity of epistaxis. The present study was undertaken to evaluate the age of onset and the severity of epistaxis in a large group of patients with HHT1 and HHT2 from The Netherlands and the Dutch Antilles. Data were also stratified for age, gender and ancestral origin to establish whether these have an influence on the severity of the phenotype.

Patients and Methods

Data collection

We performed a retrospective cross-sectional study of patients registered at an HHT outpatient clinic (Antonius hospital, Nieuwegein). The patient population consisted of Dutch patients and Dutch Antillean patients because family screening has been carried out in The Netherlands and the Antillean Islands (Curaçao and Bonaire). Patients registered with the HHT clinic attend the clinic regularly for screening for HHT-associated pathology. As a part of the regular clinical care, probands and family members were screened for the presence of mutations in the *ACVRL1* gene and the *ENG* gene, as described previously [7]. For this study, patients with either HHT1 or HHT2 were included. All patients gave informed consent for this study.

A standard questionnaire has been used with queries concerning the age of onset of epistaxis and the frequency of epistaxis during the preceding year. Furthermore, the questionnaires contained queries about treatment of epistaxis and the results of treatment. As a measure of severity, we used the number of nosebleeds per month. The frequency of epistaxis was scored in grade 1 (no epistaxis to once a week), grade 2 (epistaxis 2 to 6 times a week) or grade 3 ($\geq$ daily epistaxis). Demographic variables such as gender, age and ethnicity were recorded. When necessary, incomplete questionnaires were completed by telephone interview.

The study population consisted of 466 Dutch and Dutch Antillean patients with HHT1 and Dutch patients with HHT2. Of the questionnaires, 27 were completed by telephone interview, 23 questionnaires from Dutch patients and 4 from patients originating from the Dutch Antilles. As described previously, family screening was carried out in The Netherlands and the Antillean Islands, Curaçao and Bonaire [20, 21]. Dutch patients are of Caucasian descent, whereas Antillean patients are of African descent. Only patients with a known family mutation and with a genetic diagnosis or a definite clinical diagnosis ($\geq$ 3 of the Curacao criteria) were included for this analysis.

Statistical analysis

Differences in number of patients with HHT1 or HHT2, gender of the patients and ethnicity were examined using $\chi 2$ statistics. Age at inclusion and age at first occurrence of epistaxis was investigated using analysis of variance. Differences between first occurrence of symptoms stratified by gender, ethnicity or HHT subtype were examined using Kaplan-Meier analysis. In order to relate the severity of epistaxis to age, we graded frequency of epistaxis (ranging from grade 0 to grade 3) and we divided age in 4 categories (i.e., below 21 years, 21-40, 41-60, and 61 years and over).

Results

We included a total of 466 patients, 294 patients (63.1%) diagnosed with HHT1: 105 (22.5%) from the Dutch Antilles and 189 (40.6%) from The Netherlands, consisting of 124 men and 170 women. Of our total cohort, 172 patients (36.9%) were diagnosed with HHT2, 72 men and 100 women of Dutch descent (Table 1).

The mean age at inclusion was significantly lower in HHT1 compared to HHT2, 38.0 versus 44.3 years respectively ($p < 0.001$), for both the Dutch HHT1 population (37.9 years, $p = 0.001$) and the Antillean population (38.2 years, $p = 0.007$), suggesting earlier clinic attendance of HHT1 patients than HHT2 patients. The age distribution was similar in men and women with HHT1 or with HHT2 (data not shown).

Table 1 - Ethnicity, age, and gender of study population

	HHT1			HHT2
	Dutch	Antillian	total	total
N (mean age ± SD)	189 (37.9 yr ± 18.5)	105 (38.2 yr ± 18.8)	294 (38.0 yr ± 18.6)	172 (44.3 yr ± 16.9)
gender (M/F)	0.67	0.84	0.73	0.72
male (mean age; SD)	76 (35.5 yr ± 18.8)	48 (38.2 yr ± 19.5)	124 (36.5 yr ± 19.1)	72 (44.8 yr ± 16.5)
female (mean age; SD)	113 (39.5 yr ± 18.1)	57 (38.2 yr ± 18.3)	170 (39.1 yr ± 18.1)	100 (44.0 yr ± 17.3)
number of families	27	8	35	39
number of mutations	20	3	23	33
epistaxis (%)	182 (96.3%)	97 (92.4%)	279 (94.9%)	156 (90.7%)
male (%)	75 (98.7%)	45 (93.7%)	120 (96.8%)	67 (93.1%)
female (%)	107 (94.7%)	52 (91.2%)	159 (93.5%)	89 (89.0%)

Prevalence of epistaxis

In the total population, spontaneous and recurrent epistaxis was not significantly different between HHT1 (94.9%) and HHT2 (90.7%) ($p = 0.128$), in men or in women, despite a younger mean age in HHT1 (Table 1). More men than women tended to have epistaxis in HHT1 (in both Dutch and Antillean patients) and HHT2, but the difference was not statistically significant (data not shown).

31 patients did not have epistaxis, 15/294 patients with HHT1 and 16/172 with HHT2 ($p = 0.079$). Gender did not influence the absence of epistaxis ($p = 0.128$; 22/270 women and 9/196 men). The 31 patients without epistaxis (33.7 years) were significantly younger compared to the patients with epistaxis (40.8 years; $p = 0.035$). For 3 patients, the age of

onset of epistaxis was not known (Table 2a).

The onset of epistaxis is shown in figure 1 and tables 2a and 2b stratified for ethnicity and gender. Epistaxis occurred significantly earlier in patients with HHT1 compared to patients with HHT2 ($p < 0.001$) for both Dutch and Antillean patients. There were no differences in the age of onset of epistaxis between men and women, with either HHT1 (Antillean or Dutch; $p = 0.458$ or 0.164, respectively) or HHT2 ($p = 0.903$) (data not shown).

Table 2a - First occurrence of epistaxis in age categories, stratified for gender, in patients with HHT1 and HHT2

	HHT1 total			HHT2 total		
age category, N (%)	**males**	**females**	**total**	**males**	**females**	**total**
0-10 yrs	81 (65.3%)	110 (64.7%)	191 (65%)	25 (34.7%)	42 (42%)	67 (39%)
11-20 yrs	27 (21.8%)	27 (15.9%)	54 (18.4%)	11 (15.3%)	20 (20%)	31 (18%)
21-30 yrs	3 (2.4%)	12 (7.1%)	15 (5.1%)	13 (18.1%)	10 (10%)	23 (13.4%)
31-40 yrs	4 (3.2%)	7 (4.1%)	11 (3.7%)	12 (16.7%)	9 (9%)	21 (12.2%)
41-50 yrs	5 (4.0%)	0 (0%)	5 (1.7%)	4 (5.5%)	2 (2%)	6 (3.5%)
51-60 yrs	0 (0%)	1 (0.6%)	1 (0.3%)	1 (1.4%)	2 (2%)	3 (1.7%)
61-70 yrs	0 (0%)	1 (0.6%)	1 (0.3%)	0 (0%)	3 (3%)	3 (1.7%)
unknown	0 (0%)	1 (0.6%)	1 (0.3%)	1 (0.9%)	1 (1%)	2 (1.2%)
no epistaxis	4 (3.2%)	11 (6.5%)	15 (5.1%)	5 (6.9%)	11 (11%)	16 (9.3%)
total	124	170	**294**	72	100	**172**

Table 2b - First occurence of epistaxis in age categories, stratified for gender, in Dutch and Antillean patients with HHT1

	HHT1 Dutch			HHT1 Antillian		
age category, N (%)	**males**	**females**	**total**	**males**	**females**	**total**
0-10 yrs	49 (64.5%)	79 (69.9%)	128 (68.3)	32 (66.7%)	31 (54.4%)	63 (60%)
11-20 yrs	16 (21.1%)	13 (11.5%)	29 (15.3%)	11 (22.9%)	14 (24.6%)	25 (23.8%)
21-30 yrs	2 (2.6%)	9 (7.9%)	11 (5.8%)	1 (2.1%)	3 (5.3%)	4 (3.8%)
31-40 yrs	3 (3.9%)	4 (3.6%)	7 (3.7%)	1 (2.1%)	3 (5.3%)	4 (3.8%)
41-50 yrs	5 (6.6%)	0 (0%)	5 (2.6%)	0 (0%)	0 (0%)	0 (0%)
51-60 yrs	0 (0%)	1 (0.9%)	1 (0.5%)	0 (0%)	0 (0%)	0 (0%)
61-70 yrs	0 (0%)	1 (0.9%)	1 (0.5%)	0 (0%)	0 (0%)	0 (0%)
unknown	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.7%)	1 (0.9%)
no epistaxis	1 (1.3%)	6 (5.3%)	7 (3.7%)	3 (6.3%)	5 (8.8%)	8 (7.6%)
total	76	113	**189**	48	57	**105**

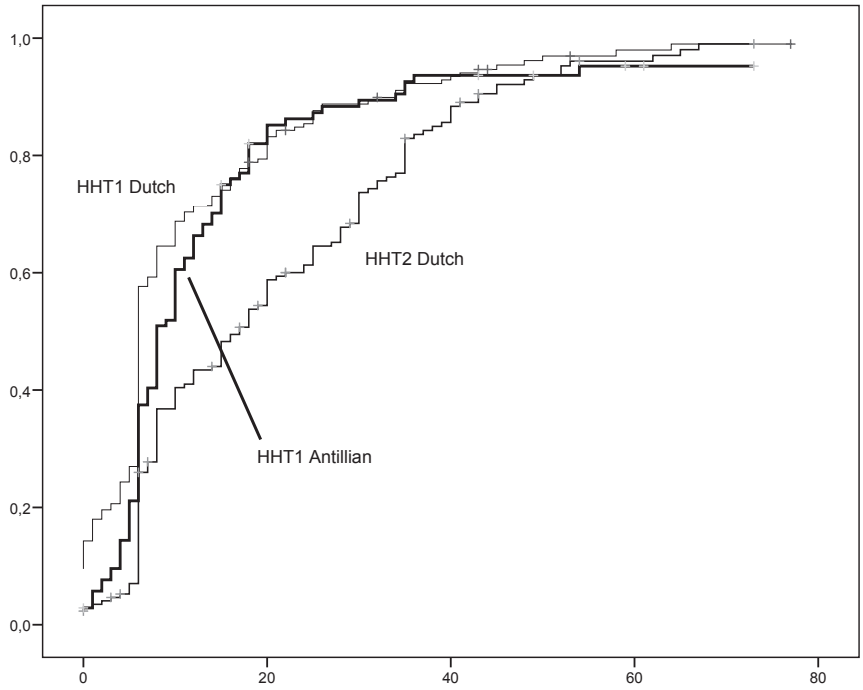


Figure 1 - Kaplan-Meier curve showing probability of being affected with epistaxis for Dutch HHT1 and HHT2 subjects and Antillian HHT1 patients. Age categories are on the X-axis, on the Y-axis the proportion of persons with epistaxis. Persons without epistaxis were not included in this analysis

Severity of epistaxis

The severity of epistaxis was strongly correlated with the age of the patients (figures 2 and 3). Patients without epistaxis were younger than patients with epistaxis grade 1, patients with epistaxis grade 1 were younger than patients with epistaxis grade 2, and so on ($p < 0.001$). This effect was statistically significant for both men and women, and Dutch patients with HHT1 and HHT2. Data for Antillean patients did not reach significance due to the lower number of cases; (data not shown).

Effect of treatment

The information about nasal treatment was limited. Since the patients did not usually know the specific treatment modality, *e.g.* YAG laser or argonplasma coagulation, the various treatment modalities were grouped together. 37 HHT1 patients (18 men and 19 women) and 37 HHT2 patients (24 men and 13 women) were treated, often several times and with several different modalities. Lasting benefit occurred in only 16% of the treated patients. The percentage of successful treatment would be lower when not the number of patients, but the number of treatments was taken into account.

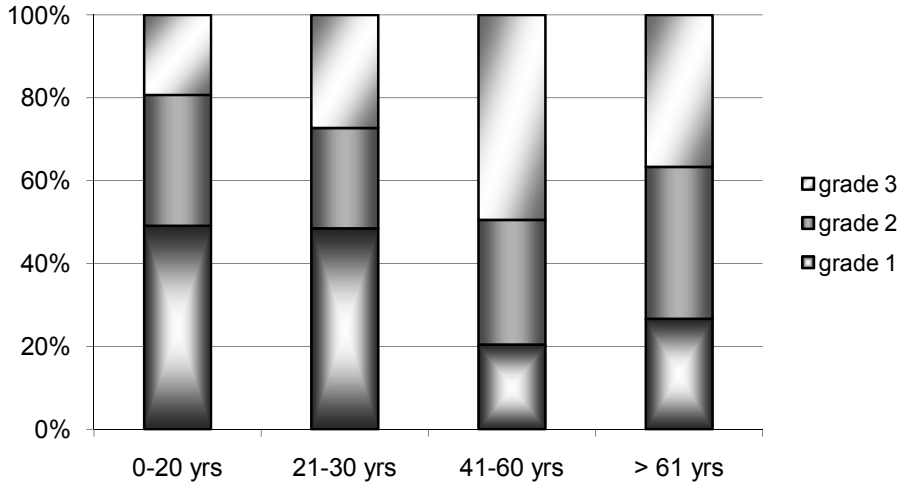


Figure 2 - Severity of epistaxis in patients with HHT1 by age category

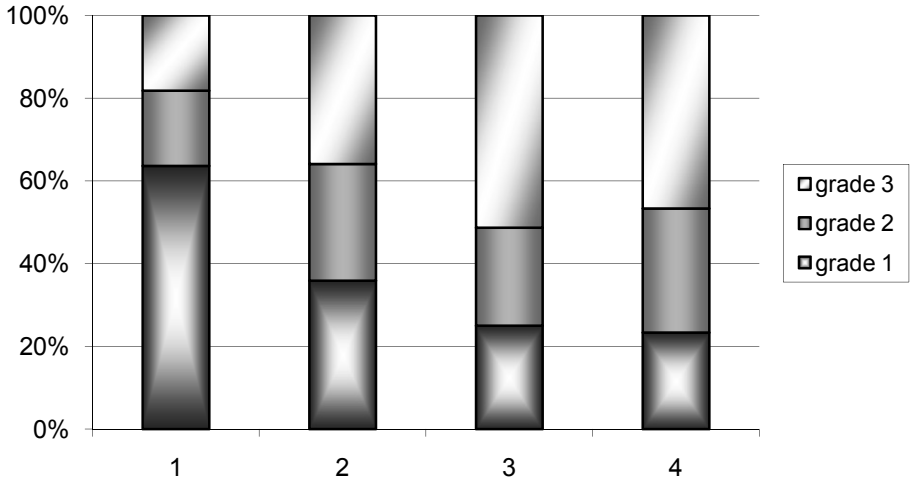


Figure 3 - Severity of epistaxis in patients with HHT2 by age category

Discussion

In this study a retrospective analysis was performed to establish phenotypic variability regarding the severity and age of onset of epistaxis, comparing HHT1 and HHT2. Furthermore, within HHT1 and HHT2, the influence of gender and ethnicity was analyzed.

The overall frequency of spontaneous recurrent epistaxis did not differ significantly between patients with HHT1 (95%) compared to HHT2 (91%). These percentages are in concordance with the presence of nasal telangiectases in (approximately) the same population; nasal telangiectases were present in 95% of patients with HHT1 and 92% with

HHT2 [13] and similar to the frequencies published in earlier studies [15,17,18]. Furthermore, in HHT1 nosebleeds are present at an earlier age in both males and females. Within HHT1, no difference was found between Dutch patients or patients from the Dutch Antilles.

Earlier studies also describe an earlier onset of epistaxis in HHT1 than HHT2. Bayrak-Toydemir *et al*.[18] found in both HHT1 and HHT2 that all patients (100%) above 40 years, had epistaxis, Lesca *et al*.[17], described persons without epistaxis only in HHT2 (9% above 60 years). Sabba *et al*.[15] found epistaxis in 91% of HHT1 patients and 95% of the HHT2 patients.

In our cohort, absence of epistaxis occurred more often in women (22) than in men (9), in both HHT1 (3.2% versus 6.5%) and in HHT2 (6.9% versus 11%). It is possible that several patients without epistaxis might still develop nosebleeds, because of their age at the time of the questionnaire.

It has been suggested that supplemental estrogens might have beneficial influence on epistaxis [22]. But only 4 of 22 women without epistaxis were known to use estrogens (data not shown).

The clustering of patients into age categories created a relationship between age of onset and epistaxis. Although this is a cross sectional analysis, it can be a very useful tool for clinicians because it is a reflection of the age-related penetrance. Stratification of the data by age category also has the benefit of circumventing bias created by referral at a younger age. Correction for referral bias by excluding probands from the analysis was not performed in this study. We have shown in earlier publications, that the data on nasal telangiectases (the cause of nosebleeds) did not change significantly when excluding the probands [13].

HHT1 patients were significantly younger than patients with HHT2. This is not surprising, since patients with HHT1 have more often a PAVM or CAVM [12]. Therefore more family members with severe complications or symptoms will be known in these families, promoting screening of the other family members and resulting in a younger age at presentation. To avoid interference of our results by this age difference, age categories were established. These show that the onset of epistaxis is significantly earlier in HHT1 than in HHT2, as well in men as in women. Accordingly, nasal telangiectases occurred also earlier in HHT1 in a comparable but not totally similar population [13]. At age 20 years, 84% of the patients with HHT1 reported epistaxis, compared to only 57% of the patients with HHT2. No difference was detected comparing the onset of epistaxis in Dutch HHT1 patients with Antillean HHT1 patients.

The clinical diagnosis of HHT is based on the Curaçao criteria [5]. One of the 4 criteria is spontaneous recurrent epistaxis. Three criteria are required for a definite clinical diagnosis. Usually, a family history of HHT is present, but PAVMs and CAVMs are rare, especially in HHT2 [12]. Furthermore, patients under the age of 20 years with HHT2 have dermal and oral telangiectases only in a minority of the patients, 38.5% and 30.8% [13]. Epistaxis is present in 57% of the patients and nasal telangiectases in 67% of the patients under the age 20 years. These figures indicate that the clinical diagnosis can be missed easily in patients with HHT2

at or under age 20 years. Especially in these age categories and especially in HHT2, a clinical diagnosis cannot be excluded when manifestations of HHT are absent and warrants DNA analysis, for certainty.

Severity of epistaxis

The severity of epistaxis is difficult to establish. The amount of blood loss and the duration of the bleeding may vary in time and is hard to quantify. Therefore only the frequency of epistaxis in the preceding year has been taken into account in this study. The mean age of patients increased significantly with increasing frequency of epistaxis, suggesting increasing number of nosebleeds with increasing age. The significant aggravation with age was present in both males and females in Dutch patients with HHT1 and HHT2. The influence of treatment on the frequency of epistaxis has not been evaluated because only 13% of the patients with HHT1 and 21% of the patients with HHT2 were known to have been treated. Moreover details of the treatment were not available and only 12 of 74 patients reported lasting improvement of the treatment. These poor results are in accordance with the literature, which indicated that failure of short term palliation is the rule and that the natural history is usually not altered. [23]. It should be mentioned that septodermatoplasty according to Saunders with the Ross modification seems promising in case of transfusion dependent epistaxis [24-26]. In the present study only 14 patients had some form of dermatoplasty.
In conclusion, in HHT1 and HHT2 the majority of patients develop epistaxis. In HHT1 epistaxis occurs earlier in life compared to HHT2 in both males and females. Comparison within HHT1 and HHT2 revealed no significant differences, suggesting the onset or severity of epistaxis is not influenced by gender. In HHT1 onset of epistaxis was similar for both Antillean and Dutch HHT1 patients (males and females). This means that different ancestral backgrounds (different genetic backgrounds?) apparently do not influence this manifestation of the disease, which is confirmed by similar frequencies of epistaxis in different populations. The number of nosebleeds increases with increasing age in Dutch patients with HHT1 and HHT2.

References

1. Guttmacher AE, Marchuk DA, White RI Jr. Hereditary hemorrhagic telangiectasia. N Engl J Med 1995; 333: 918-924.
2. Haitjema T, Balder W, Disch FJ, Westermann CJ. Epistaxis in hereditary haemorrhagic telangiectasia. Rhinology 1996; 34: 176-178.
3. Plauchu H, de Chadarevian JP, Bideau A, Robert JM. 1989. Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. Am J Med Genet 32:291-297.
4. 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 Hemorrhagic Telangiectasia. J Med Genet. 2009 Jun 29. [Epub ahead of print]
5. 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
6. Lesca G, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Riviere 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.
7. Letteboer TG, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum Genet 2005; 116: 8-16.
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). The Lancet 2004; 363: 852-859.
9. Cole SG, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet. 2005; 42:577-582.
10. 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 Part A 2006; 140: 2155-2162.
11. Gallione CJ, Scheessele EA, Reinhardt D, Duits AJ, Berg JN, Westermann CJ, 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.
12. Letteboer TG, Mager HJ, Snijder RJ, Koeleman BP, Lindhout D, Ploos van Amstel HK, Westermann KJ . Genotype - phenotype relationship in Hereditary Hemorrhagic Telangiectasia. J Med Genet 2006; 43: 371-377.
13. Letteboer TG, Mager HJ, Snijder RJ, Lindhout D, Ploos van Amstel HK, Zanen P, Westermann KJ. Genotype-phenotype relationship for localization and age distribution of telangiectases in hereditary hemorrhagic telangiectasia. Am J Med Genet A. 2008 1; 146: 2733-2739.
14. 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: 585-590.
15. Sabbà C, Pasculli G, Lenato GM, Suppressa P, Lastella P, Memeo M, Dicuonzo F, Guant G. Hereditary hemorrhagic telangiectasia: clinical features in ENG and ALK1 mutation carriers. J Thromb Haemost. 2007 5:1149-1157
16. 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
17. 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; French-Italian-Rendu-Osler Network. Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. Genet Med. 2007; 9: 14-22.
18. Bayrak-Toydemir P, McDonald J, Markewitz B, Lewin S, Miller F, Chou LS, Gedge F, Tang W, Coon H, Mao R. Genotype-phenotype correlation in hereditary hemorrhagic telangiectasia: mutations and manifestations. Am J Med Genet 2006; 140: 463-470.

19. Bossler AD, Richards J, George C, Godmilow L, Ganguly A. Novel mutations in ENG and ACVRL1 identified in a series of 200 individuals undergoing clinical genetic testing for hereditary hemorrhagic telangiectasia (HHT): correlation of genotype with phenotype. Hum Mutat. 2006; 27: 667-675
20. Haitjema T, Disch F, Overtoom TT, Westermann CJ, Lammers JW. Screening family members of patients with hereditary hemorrhagic telangiectasia. Am J Med 1995; 99: 519-524.
21. 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; 116: 324-328.
22. Estrogen treatment of hereditary hemorrhagic telangiectasia. A double-blind controlled clinical trial. Vase P.,Acta Med Scand. 1981; 209 :393-396.
23. Shah RK, Dhingra JK, Shapshay SM. Hereditary hemorrhagic telangiectasia: a review of 76 cases Laryngoscope. 2002; 112 : 767-773
24. Saunders W. Hereditary hemorrhagic telengiectasia: effective treatment of epistaxis by septal dermoplasty Acta Otolaryngol. 1964 58; 497-502.
25. Geisthoff UW, Fiorella ML, Fiorella R. Treatment of recurrent epistaxis in HHT. Curr Pharm Des. 2006; 12:1237-42.
26. Fiorella ML, Ross D, Henderson KJ, White RI Jr. Outcome of septal dermoplasty in patients with hereditary hemorrhagic telangiectasia. Laryngoscope. 2005;115: 301-305

Chapter 3.4

Assessment of intestinal vascular malformations in patients with hereditary hemorrhagic telangiectasia and anemia

van Tuyl SA, Letteboer TG, Rogge-Wolf C, Kuipers EJ,
Snijder RJ, Westermann CJ, Stolk MF

Eur J Gastroenterol Hepatol. 2007; 19: 153-158

Abstract

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder with mucocutaneous telangiectasia and visceral arteriovenous malformations. Mutations of endoglin and Activin A receptor like kinase-1 have different phenotypes, HHT1 and HHT2, respectively. The gastrointestinal tract is frequently affected, but limited information is available on the relationship with genotype.

The aim of this study is to determine whether different genotypes have different phenotypes with respect to intestinal telangiectasia. HHT patients, referred for anemia underwent videocapsule endoscopy. Chart review was performed for information on genotype and HHT manifestations.

Twenty-five patients were analyzed (men/women 13/9, mean age 49±15 years.), 14 HHT1, eight HHT2 and three without known mutation. Epistaxis occurred in 96% of patients. Gastroduodenoscopy revealed telangiectasia in 7/12 (58%) HHT1 and 3/8 (38%) HHT2 patients. Videocapsule endoscopy found telangiectasia in all HHT1 and 5/8 (63%) HHT2 patients. In 9/14 HHT1 patients, telangiectasia were large. Telangiectasia in the colon was restricted to 6/11 (55%) HHT1 patients. Hepatic arteriovenous malformations were present in 1/7 HHT1 and 5/6 HHT2 patients.

Thus, large telangiectasia in small intestine and colon appear to occur predominantly in HHT1. Hepatic arteriovenous malformations are mainly found in HHT2. In HHT patients with unexplained anemia, videocapsule endoscopy should be considered to determine the size and extent of telangiectasia and exclude other abnormalities.

Introduction

HHT or Rendu-Osler-Weber's disease is a hereditary autosomal dominant disorder with variable expression. Telangiectases are present at characteristic sites like the lips, oral cavity, nose and fingers in about 90% of the patients. Epistaxis frequently is the first manifestation of the disease. The gastrointestinal tract is frequently involved, but the exact prevalence of the intestinal lesions is not known [1-3]. Gastrointestinal hemorrhage is seen in 10-45% of patients [4-6]. Other manifestations of HHT are due to visceral arteriovenous malformations, which can occur in lungs, liver and brain.

So far, mutations of endoglin (*ENG*; OMIM 131195) on chromosome 9, of Activin A receptor like kinase-1 (*ALK-1*; OMIM 601284) on chromosome 12 and MADH4 on chromosome 18 have been demonstrated to be the genetic basis of HHT [7-9]. These different genotypes have different phenotypes. The *ENG* mutation predisposes to the HHT1 phenotype associated with pulmonary arteriovenous malformations (PAVM) and cerebral arteriovenous malformations (CAVM) [10]. Mutations in the *ACVRL1* gene, the HHT2 phenotype, are associated with a later onset, a milder phenotype and with hepatic arteriovenous malformations (HAVM) [10,11]. MADH4 mutation is associated with the combination of HHT and juvenile polyposis [8].

Little information is available, however, on the prevalence of gastrointestinal vascular malformations in patients with HHT. Telangiectases are located preferentially in the proximal stomach [12-14]. Patients above 50 years are more likely to have gastric or duodenal telangiectases than patients younger than 50 years [14]. Data concerning the involvement of the small intestine in HHT are sparse. Until recently, complete examination of the small intestine could only be achieved by intraoperative enteroscopy. With the advent of videocapsule endoscopy (VCE) the entire small intestine can be visualized in a non-invasive way [15].

We therefore investigated the relationship between HHT phenotype, with an emphasis on the occurrence of small intestinal telangiectasia as assessed by VCE, and the genotype. These data may have implications for the understanding and clinical management of intestinal blood loss in HHT patients.

Methods

Patients

Twenty-five patients with known HHT, based on the Curaçao criteria were selected because their medical history revealed anemia insufficiently explained by epistaxis [16]. All patients except numbers 3, 9 and 20 are a subgroup of the HHT population previously described by Letteboer *et al*.[7,11]. A chart review of HHT manifestations was performed for all the patients. Subgroup analysis was performed for genotype, age and sex. The presence of visceral arteriovenous malformations was established by chest radiograph, abdominal ultrasound, computed tomography-scanning, magnetic resonance imaging or angiography. In patients

who underwent gastroduodenoscopy, the telangiectases were classified depending on the presence in esophagus, stomach or duodenum. For description of telangiectasia in the duodenum, data from gastroduodenoscopy and VCE were used. If a colonoscopy was performed, the presence or absence of telangiectasia was noted. All patients underwent VCE.

Videocapsule endoscopy

VCE was performed using the Given Imaging M2A wireless capsule (Given Imaging, Yoqneam, Israel), as described previously [17]. In brief, a swallowable capsule is propelled by intestinal peristalsis taking two pictures per second of the intestine. Images are transmitted to antennas on the abdominal wall for registration, and can be downloaded to a work station.

The VCE images were reviewed by one of four gastroenterologists with extensive experience of using this technique, without knowledge of the results of HHT mutation analysis. Vascular malformations were classified for location (stomach, proximal duodenum, small intestine and colon) and for number [none, few (≤ 5) or multiple (> 5)]. As the size of the telangiectasia cannot be measured adequately at VCE, they were assessed as large or small. This qualification was reassessed by a second reviewer. In case of large and small telangiectasia in the same patient, they were classified as large.

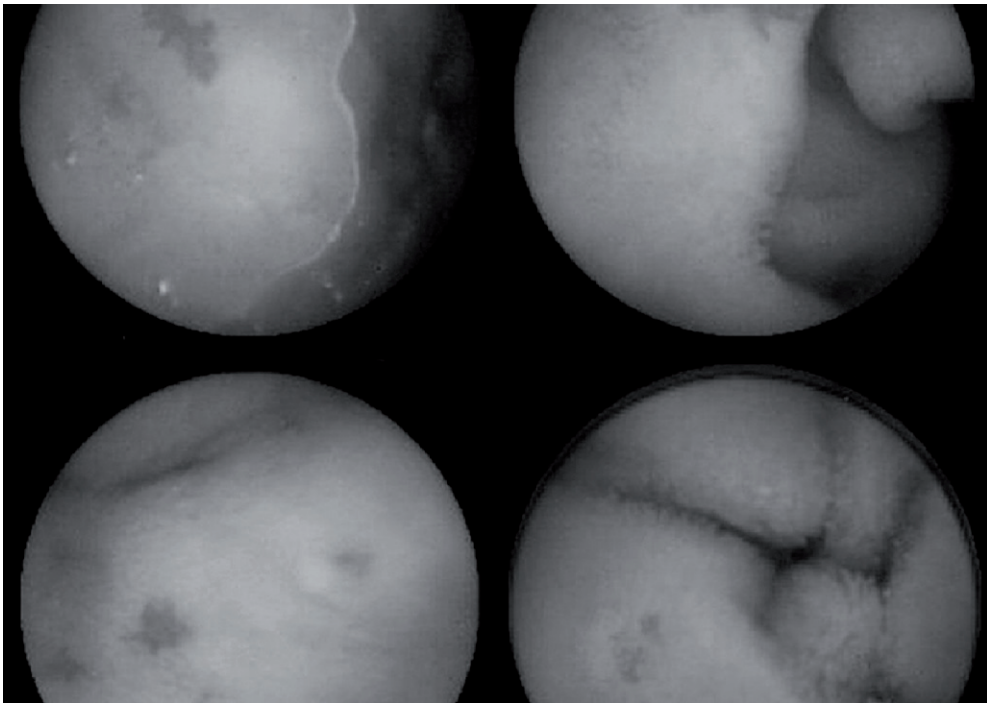


Figure 1 - Small intestinal telangiectasia detected by videocapsule endoscopy in four different hereditary hemorrhagic telangiectasia

Statistics

Parametric results were compared by two-sided Student's *t*-test. Group proportions were compared with $\chi 2$ test or Fisher's exact test where appropriate. A $p < 0.05$ was considered significant.

Table 1 - Phenotypic differences between HHT1 and HHT2 patients

	HHT1	HHT2	P
n	14	8	
age (years)	56 ±14.9	47± 13.9	ns
sex (men/women)	7/7	6/2	ns
hemoglobin (mmol/l)	5.8± 0.9	5.6± 1.6	ns
gastric AVM			
total	7/12 (58%)	3/5 (60%)	ns
large	3/12 (25%)	1/5 (20%)	ns
small intestine AVM			
total	14/14 (100%)	5/8 (63%)	0.005
large	9/14 (64%)	1/8 (13%)	0.02
colonic AVM			
total	6/11 (55%)	0/7(0%)	0.03
large	2/11 (18%)	0/7 (0%)	ns
HAVM	1/7 (14%)	5/6 (83%)	0.02
epistaxis	13/14 (93%)	7/8 (88%)	ns
PAVM	9/13 (69%)	0/8 (0%)	0.003
CAVM	2/9 (22%)	0/8 (0%)	ns

AVM, arteriovenous malformations; CAVM, cerebral arteriovenous malformations; HAVM, hepatic arteriovenous malformations; HHT1, hereditary hemorrhagic teleangiectasia 1; HHT2, hereditary hemorrhagic teleangiectasia 2; PAVM, pulmonary arteriovenous malformations

Results

Twenty-five patients were analyzed (men/women 14/11, mean age 49±17 years, range 18-72 years). Fourteen patients carried the *ENG* mutation (HHT1) and eight patients the *ACVRL1* mutation (HHT2). In three patients, the family mutation was not known (HHT?). No significant difference was found between HHT1 and HHT2 patients concerning age, sex and hemoglobin level (Table 1).

Arteriovenous malformations of the digestive tract

Telangiectasia were found in the entire digestive tract in 23/25 patients (92%), at gastroduodenoscopy in 14/21 patients (67%), at VCE in 19/25 (76%), and at colonoscopy in 6/19 patients (32%). All telangiectases in the duodenum that were detected by conventional endoscopy were confirmed by VCE.

Upper digestive tract

Twenty-one patients underwent a gastroduodenoscopy. This revealed upper gastrointestinal telangiectasia in 10 (48%) patients. One patient (5%) had a small telangiectasy in the distal esophagus. Ten patients (48%) had gastric telangiectases, four of them had large telangiectasia. Six patients (29%) had duodenal telangiectases, three of them had both gastric and duodenal telangiectasia (Tables 2 and 3). No significant differences were found in the prevalence of lesions between HHT1 and HHT2.

Small intestine

All patients underwent VCE without complications. The average gastric transit time was 25±35 min and small intestinal transit time was 264±91 min. The cecum was reached in 96%. Small bowel telangiectases were found in 19/25 (76%) patients (Fig. 1). In all HHT1 patients, telangiectases were present in the small intestine. In 9/14 (64%) patients, multiple large telangiectases were seen, whereas the other patients had multiple small telangiectases. Telangiectases were present in 5/8 (63%) of HHT2 patients. Multiple large telangiectases were found in one patient and few small telangiectases were found in four patients. The occurrence of telangiectasia and large telangiectasia was therefore more frequent in HHT1, than in HHT2 patients ($p = 0.005$ and 0.02, respectively). In four patients, active bleeding was seen during VCE, three of them were HHT1 patients. In two HHT? patients, multiple small telangiectasia were found and in one HHT? patients VCE was without abnormalities.

Six HHT1 patients were younger than 50 years, three of them had large telangiectasia and the other three had small telangiectasia. Eight HHT1 patients were older than 50 years and 7/8 (88%) of them had multiple large telangiectases and one (13%) had multiple small telangiectases (p = NS). Three HHT2 patients were younger than 50 years and had no abnormalities at VCE, whereas all five patients older than 50 years had telangiectasia ($p = 0.02$). No major abnormalities except telangiectasia were detected by VCE. In one patient, the presence of a polyp in the proximal small intestine was suggested, which could not be confirmed by push enteroscopy. In two other patients, superficial erosions in the stomach were found.

Colon

Colonoscopy was performed in 19 patients. Telangiectases were found in six (32%) patients. Two of them had a solitary large telangiectasy, one patient had a small solitary lesion and three had multiple small telangiectases. All six patients with telangiectasia belonged to the 11 examined HHT1 patients (55%). None of the HHT2 patients had telangiectasia in the colon (p = 0.03).

Liver

HAVM was present in six patients. In HHT1 patients 1/7 (14%) had a HAVM, and in HHT2 patients 5/6 (83%) had a HAVM (p = 0.02). The presence of a HAVM was not known in seven HHT1 patients and in two HHT2 patients. The two HHT? patients who were examined had no HAVM.

HHT manifestations outside the digestive tract

Spontaneous recurrent epistaxis was present in 24/25 patients (96%) and no difference between HHT1 and HHT2 patients was found. PAVM were present in 11 patients (tables 2 and 3). In case of HHT1 patients 9/13 (69%) had a PAVM, whereas in HHT2 patients no PAVM was found (p = 0.003). Two patients with HHT? had a PAVM. In one HHT1 patient and in one HHT? patient, the presence of a PAVM was not examined.

Eighteen patients were analyzed for the presence of a CAVM. CAVM was present in two HHT1 patients and in one patient without a known mutation (p = NS).

Discussion

HHT is a relatively rare disorder, but many patients affected by this disease suffer from recurrent occult, or overt gastrointestinal bleeding, often leading to anemia. As a result, these patients may be presented to a gastroenterologist for diagnosis and treatment. Depending on the site of telangiectasia, treatment with argon-plasma coagulation can be considered and in case of diffuse abnormalities, treatment with estrogens may be tried [4,18-21]. Longacre *et al*.[4] also described the possibility of treatment with danazol, a weak androgen, or aminocaproic acid, which inhibits fibrinolysis. There is however, limited information on the distribution of telangiectasia in the small intestine. The majority of studies examined patients by gastroduodenoscopy and colonoscopy, but little information is available on the small intestinal manifestations of HHT as this part of the digestive tract was difficult to examine using endoscopic techniques. The proximal small intestine can be investigated by push enteroscopy, but for the endoscopic assessment of the entire small intestine, until recently an intraoperative enteroscopy was mandatory.

Table 2 - HHT1 manifestations

N	Sex	Age (years)	Hb (mmol/l)	Epistaxis	PAVM	HAVM	CAVM	Stomach	Ileum	Colon
2	M	26	6.4	+	+	np	–	none	multiple, small	none
3	F	52	7.1	–	np	np	np	np	multiple, large	np
4	M	58	5.6	+	+	np	–	none	multiple, large	none
6	M	57	5.3	+	+	–	–	multiple, small	multiple, large	multiple,small
10	F	23	7.4	+	+	–	–	np	multiple, large	np
13	F	42	5.2	+	+	np	–	none	multiple, large	few, small
14	F	18	5.4	+	–	np	+	none	few, small	solitary, small
15	M	46	5.8	+	+	–	–	multiple, large	multiple, large	none
17	F	52	7.4	+	+	–	–	few, large	multiple, large	none
18	M	60	4.7	+	–	np	–	none	multiple, large	solitary, large
19	F	51	6.9	+	+	–	np	few, small	multiple, small	solitary, large
21	M	72	4.9	+	–	np	np	few, small	multiple, large	none
22	M	34	5.2	+	–	–	+	few, large	multiple, small	few, small
23	F	60	5.6	+	+	+	–	multiple, small	few, small	np

CAVM, cerebral arteriovenous malformations; F, female; HAVM, hepatic arteriovenous malformations; Hb, hemoglobin; HHT1, hereditary hemorrhagic teleangiectasia 1; M, male; np, not performed; PAVM, pulmonary arteriovenous malformations

Table 3 - HHT2 manifestations

N	Sex	Age (years)	Hb (mmol/l)	Epistaxis	PAVM	HAVM	CAVM	Stomach	Ileum	Colon
1	M	72	4.7	+	–	+	np	multiple, large	multiple, small	none
7	M	61	3.8	+	–	–	–	none	multiple, small	none
8	F	43	3.3	+	–	+	–	few, small	none	none
9	M	71	7.5	+	–	+	np	none	solitary, small	np
11	M	33	6.5	+	–	+	–	none	none	none
12	M	56	6.7	+	–	np	np	few, small	multiple, large	none
16	M	50	7.2	+	–	np	np	none	none	none
24	F	67	5.2	–	–	+	–	none	few, small	none

CAVM, cerebral arteriovenous malformations; F, female; HAVM, hepatic arteriovenous malformations; Hb, hemoglobin, HHT2, hereditary hemorrhagic teleangiectasia 2; M, male; np, not performed; PAVM, pulmonary arteriovenous malformations.

This is an invasive procedure. Enteroclysis is not reliable for the detection of superficial mucosal abnormalities like telangiectasia. VCE is a new and non-invasive technique for endoscopic evaluation of the small intestine.

The presence of telangiectasia in the small intestine of HHT patients as assessed by VCE has been reported previously in a single study [22]. In this study, gastroduodenoscopy and VCE were performed in 20 HHT patients without knowledge of the underlying mutation. The prevalence of telangiectasia was reported to be 56%, with a preferential occurrence of telangiectasia in older patients.

In the present study, telangiectasia of the digestive tract were found in 92% of patients, which is much higher as compared with the study of Ingrosso *et al*.[22]. The difference will probably be explained by selection as our study includes only patients with anemia. Ingrosso *et al*.[22] describe patients with hemoglobin level varying between 5.3 and 9.7 mmol/l and report a lower hemoglobin level in patients with telangiectasia. Although our study also includes colonic telangiectasia, this does not account for the difference because all patients with colonic telangiectasia also had gastric or small intestinal telangiectasia. An interesting observation was the absence of telangiectases at colonoscopy in HHT2 patients whereas telangiectases in the colon were found in 43% of the HHT1 patients. In the small intestine, telangiectases were found at VCE in all HHT1 patients, with large telangiectases in 9/14 (64%). In only one HHT2 patient was a large telangiectasy found, four HHT2 patients had a few small telangiectases. Four patients (2, 4, 7 and 24) who were examined in our study had no abnormalities at gastroduodenoscopy and colonoscopy, but multiple large telangiectases at VCE. The absence of abnormalities at conventional endoscopy in HHT patients therefore does not exclude the presence of large telangiectasia in the small intestine.

On the basis of previous reports, the major confounding factor in the gastrointestinal manifestations of HHT is age, because telangiectasia is more frequent in patients older than 50 years [14]. The only other study using VCE for the detection of small intestinal telangiectasia also reported that patients without telangiectasia had a mean age of 45 years compared with a mean age of 63 years if telangiectasia were present [22]. These findings are compatible with a study by Plauchu *et al*.[23] describing 324 HHT patients with gastrointestinal bleeding in 15%. Half of the patients were older than 58 years at the initial manifestation of gastrointestinal bleeding as compared with the manifestation of epistaxis in half of the patients before the age of 20 years.

So far, no consistent difference in the gastrointestinal manifestations of HHT1 and HHT2 could be established [10,11]. In a study comparing 39 HHT1 patients and 16 HHT2 patients [24], more severe gastrointestinal bleeding was reported in HHT1 patients. Berg *et al*.[10] describe data from 49 HHT1 patients and 34 HHT2 patients, and found no significant difference for the prevalence of gastrointestinal hemorrhage between both groups. A separate analysis of the prevalence of gastrointestinal bleeding in patients older than 60 years found predominance for HHT2 patients (37 vs. 11%), although this did not reach statistical significance [10].

Our study found a higher prevalence of large telangiectasia in HHT1 patients older than 50 years, although this did not reach significance. In HHT2 patients, the presence of telangiectasia was restricted to patients older than 50 years. Despite the significantly higher prevalence of telangiectasia in the small intestine and colon in HHT1, the level of anemia was comparable in both genotypes. The question remains to be resolved whether telangiectases increase in size and number during life. VCE might be a valuable tool to address this issue in a research setting.

It is difficult to determine whether there are true phenotypical differences in the patients of this study between HHT1 and HHT2 with respect to gastrointestinal localization, because the patients were selected largely on account of anemia, which was insufficiently explained by epistaxis. To confirm the presence of a true phenotypical difference, a systematic investigation in large groups of HHT patients without anemia should be performed using VCE.

Recently, double balloon enteroscopy has been introduced, in which an enteroscope is introduced through the entire small bowel by sequential insufflation and desufflation of balloons on the tip of the endoscope and an overtube [25,26]. This technique allows investigation of the entire small intestine without the need for a laparotomy. It offers the possibility to take biopsies or perform argon-plasma coagulation of telangiectasia. This technique might therefore be of relevance for HHT patients with unexplained blood loss from telangiectases identified by VCE, because they can be treated by argon-plasma coagulation [19,27,28]. Although solid data are sparse, there seems to be a good correlation between images obtained by VCE and conventional endoscopy. A study by Hartmann *et al*.[29] compared the findings of VCE and intraoperative enteroscopy in 47 patients and VCE had a sensitivity of 95% and a specificity of 75%, considering intraoperative enteroscopy as the reference. One study prospectively compared the findings of VCE and double balloon enteroscopy in 35 patients [30]. In this study, VCE demonstrated abnormalities of the small intestine in 28 patients that were confirmed in 20 patients (71%). Further comparative studies of VCE and double balloon enteroscopy are needed to establish the correlation between both the techniques.

In this study, several patients with severe anemia (patients 8, 11 and 24) showed no or few small intestinal telangiectasia. Although a negative gastrointestinal endoscopy (gastroduodenoscopy, VCE and colonoscopy) in these patients does not completely exclude an intestinal source of bleeding, it strongly points to epistaxis as the main cause of anemia. As VCE is now accepted as a diagnostic tool in the analysis of obscure gastrointestinal bleeding, it should be considered in case of unexplained anemia in HHT patients [31,32]. In this way, alternative diagnoses can be excluded and in case of large telangiectasia in the small intestine, endoscopic treatment can be considered in case of anemia requiring chronic iron supplementation or frequent transfusions.

Conclusion

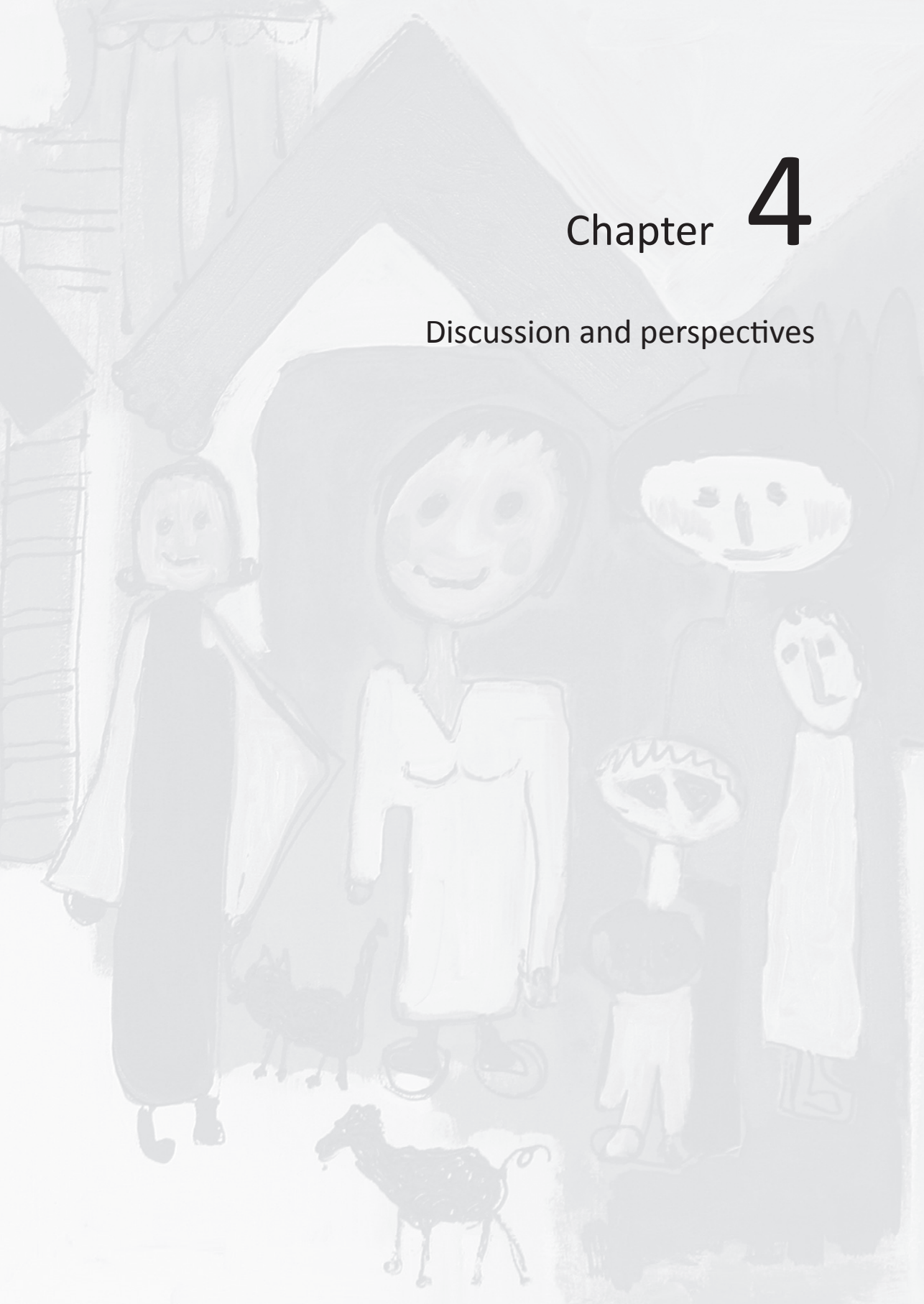
HHT frequently affects the digestive tract, resulting in anemia because of bleeding telangiectasia. Large telangiectasia in the small intestine and the colon mainly occur in HHT1 patients, whereas HAVM is predominantly present in HHT2 patients. This has become more relevant with the emerging possibilities of new endoscopic techniques for the treatment of small intestinal telangiectasia. In HHT patients with unexplained anemia, VCE should be considered to determine the size and extent of telangiectasia and exclude other abnormalities.

References

1. Guttmacher AE, Marchuk DA, White RI Jr. Hereditary hemorrhagic telangiectasia. N Engl J Med 1995; 333: 918-924.
2. Kjeldsen AD, Kjeldsen J. Gastrointestinal bleeding in patients with hereditary hemorrhagic telangiectasia. Am J Gastroenterol 2000; 95: 415-418.
3. Sharma VK, Howden CW. Gastrointestinal and hepatic manifestations of hereditary hemorrhagic telangiectasia. Dig Dis 1998; 16: 169-174.
4. Longacre AV, Gross CP, Gallitelli M, Henderson KJ, White RI Jr, Proctor DD. Diagnosis and management of gastrointestinal bleeding in patients with hereditary hemorrhagic telangiectasia. Am J Gastroenterol 2003; 98: 59-65.
5. Reilly PJ, Nostrant TT. Clinical manifestations of hereditary hemorrhagic telangiectasia. Am J Gastroenterol 1984; 79: 363-367.
6. Smith CR, Bartholomen LG, Caine JC. Hereditary hemorrhagic teleangiectasia and gastrointestinal hemorrhage. Gastroenterology 1963; 44: 1-6.
7. Letteboer TG, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum Genet 2005; 116: 8-16.
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; 363: 852-859.
9. Westermann CJ, Rosina AF, De VV, 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 2003; 116: 324-328.
10. 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: 585-590.
11. Letteboer TG, Mager JJ, Snijder RJ, Lindhout D, Ploos van Amstel JK, Westermann CJ. Genotype-phenotype relation in hereditary hemorrhagic teleangiectasia. J Med Genet 2006; 43: 371-377.
12. Vase P, Grove O. Gastrointestinal lesions in hereditary hemorrhagic telangiectasia. Gastroenterology 1986; 91: 1079-1083.
13. Marwick T, Kerlin P. Angiodysplasia of the upper gastrointestinal tract. Clinical spectrum in 41 cases. J Clin Gastroenterol 1986; 8: 404-407.
14. Proctor DD, Price J, Henderson KJ, White RI. Does the number and/or distribution of arteriovenous malformations change with age in patients with hereditary hemorrhagic teleangiectasia. IVth HHT Scientific Conference; Tenerife, Canary Islands 2001. p.69.
15. Iddan G, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. Nature 2000; 405: 417.
16. 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; 6: 66-67.
17. van Tuyl SA, Stolk MF, Timmer R. Clinical application of video capsule endoscopy. Scand J Gastroenterol Suppl 2003; 239: 24-28.
18. Sargeant IR, Loizou LA, Rampton D, Tulloch M, Bown SG. Laser ablation of upper gastrointestinal vascular ectasias: long term results. Gut 1993; 34: 470-475.
19. Morris AJ, Mokhashi M, Straiton M, Murray L, Mackenzie JF. Push enteroscopy and heater probe therapy for small bowel bleeding. Gastrointest Endosc 1996; 44:394-397.
20. van Cutsem E, Rutgeerts P, Vantrappen G. Treatment of bleeding gastrointestinal vascular malformations with oestrogen-progesterone. Lancet 1990; 335: 953-955.
21. Hisada T, Kuwabara H, Tsunoda T, Kaneko K, Kubota S, Miwa Y, et al. Hereditary hemorrhagic telangiectasia showing severe anemia which was successfully treated with estrogen. Intern Med 1995; 34:589-592.
22. Ingrosso M, Sabba C, Pisani A, Principi M, Gallitelli M, Cirulli A, Francavilla A. Evidence of small-bowel involvement in hereditary hemorrhagic telangiectasia: a capsule-endoscopic study. Endoscopy 2004; 36: 1074-1079.
23. Plauchu H, de Chadarevian JP, Bideau A, Robert JM. Age-related clinical profile of hereditary hemorrhagic

telangiectasia in an epidemiologically recruited population. Am J Med Genet 1989; 32: 291-297.

24. Kjeldsen AD, Moller 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.
25. May A, Nachbar L, Wardak A, Yamamoto H, Ell C. Double-balloon enteroscopy: preliminary experience in patients with obscure gastrointestinal bleeding or chronic abdominal pain. Endoscopy 2003; 35: 985-991.
26. Yamamoto H, Sugano K. A new method of enteroscopy: the double-balloon method. Can J Gastroenterol 2003; 17: 273-274.
27. Cello JP, Grendell JH. Endoscopic laser treatment for gastrointestinal vascular ectasias. Ann Intern Med 1986; 104: 352-354.
28. Askin MP, Lewis BS. Push enteroscopic cauterization: long-term follow-up of 83 patients with bleeding small intestinal angiodysplasia. Gastrointest Endosc 1996; 43: 580-583.
29. Hartmann D, Schmidt H, Bolz G, Schilling D, Kinzel F, Eickhoff A, et al. A prospective two-center study comparing wireless capsule endoscopy with intraoperative enteroscopy in patients with obscure GI bleeding. Gastrointest Endosc 2005; 61: 826-832.
30. Hadithi M, Heine GD, Jacobs MA, van Bodegraven AA, Mulder CJ. A prospective study comparing video capsule endoscopy with double-balloon enteroscopy in patients with obscure gastrointestinal bleeding. Am J Gastroenterol 2006; 101: 52-57.
31. Jensen DM. Current diagnosis and treatment of severe obscure GI hemorrhage. Gastrointest Endosc 2003; 58: 256-266.
32. Leighton JA, Goldstein J, Hirota W, Jacobson BC, Johanson JF, Mallery JS, Waring JP, Baron TH, Faigel DO. Obscure gastrointestinal bleeding. Gastrointest Endosc 2003; 58: 650-655.



Chapter 4

Discussion and perspectives

Focus of this thesis

The primary goal of this thesis was to study the genetic causes of HHT and the relationship between genotype and phenotype. HHT, like many other human monogenic disorders, shows considerable clinical variability as well as genetic heterogeneity. Understanding the genetic causes and how they relate to clinical expression and course of disease is of significant importance for accurate and reliable genetic counseling of patients and families with HHT and for the development of strategies for management and prevention of the disease or its complications.

Therefore, we started to characterize the molecular and genetic heterogeneity of HHT families (chapter 2). Once mutations in the majority of families were found, the identification of carriers and non-carriers in families together with well described phenotypes, allowed for an extensive analysis of the genotype-phenotype relationship (chapter 3). This resulted in knowledge of the frequency of HHT1 and HHT2 and of the different manifestations in both subtypes. Furthermore, within HHT1 and HHT2, stratification of the symptoms (epistaxis, telangiectases and AVMs) for gender provided gender specific prevalences. This resulted in a further improvement of genetic counseling in HHT and in the possibility to more precisely address intra- and interfamilial variability.

Molecular and genetic heterogeneity

Mutation analysis was performed in several hundred probands of families suspected of HHT. The main focus was on the *ENG* and *ACVRL1* genes. Sequence analysis was performed on the exons and their flanking intronic sequences that contain at least the splice site consensus sequences. These regions were amplified using PCR. When no mutation was found, subsequent MLPA analysis was performed to identify deletions or duplications, which increased the mutation detection rate only slightly (chapter 2.1 and 2.2).

In HHT probands, a broad spectrum of mutations of both genes was found. Among pathogenic mutations we observed missense mutations, nonsense mutations, splice site mutations and small and large deletions and insertions/duplications.

The majority of families show their own private mutation (for the distribution of the mutations described in chapter 2.1, see figure 1). A few apparently independent probands share the same mutation. Several of these mutations have been shown to be of the same ancestral origin, either by genealogical analysis or by haplotyping.

The deleterious effects on gene expression of nonsense, frame shift and splice site mutations is easy to understand, the pathogenicity of missense mutations is less unambiguous. In *ACVRL1*, the majority of missense mutations are found in exon 7 and exon 8, encoding the kinase domain. It is a region in which different amino acid substitutions for the same codon are reported and in which missense mutations are not tolerated (deleterious) [1-6]. As expected, these missense mutations occur at positions that are highly conserved

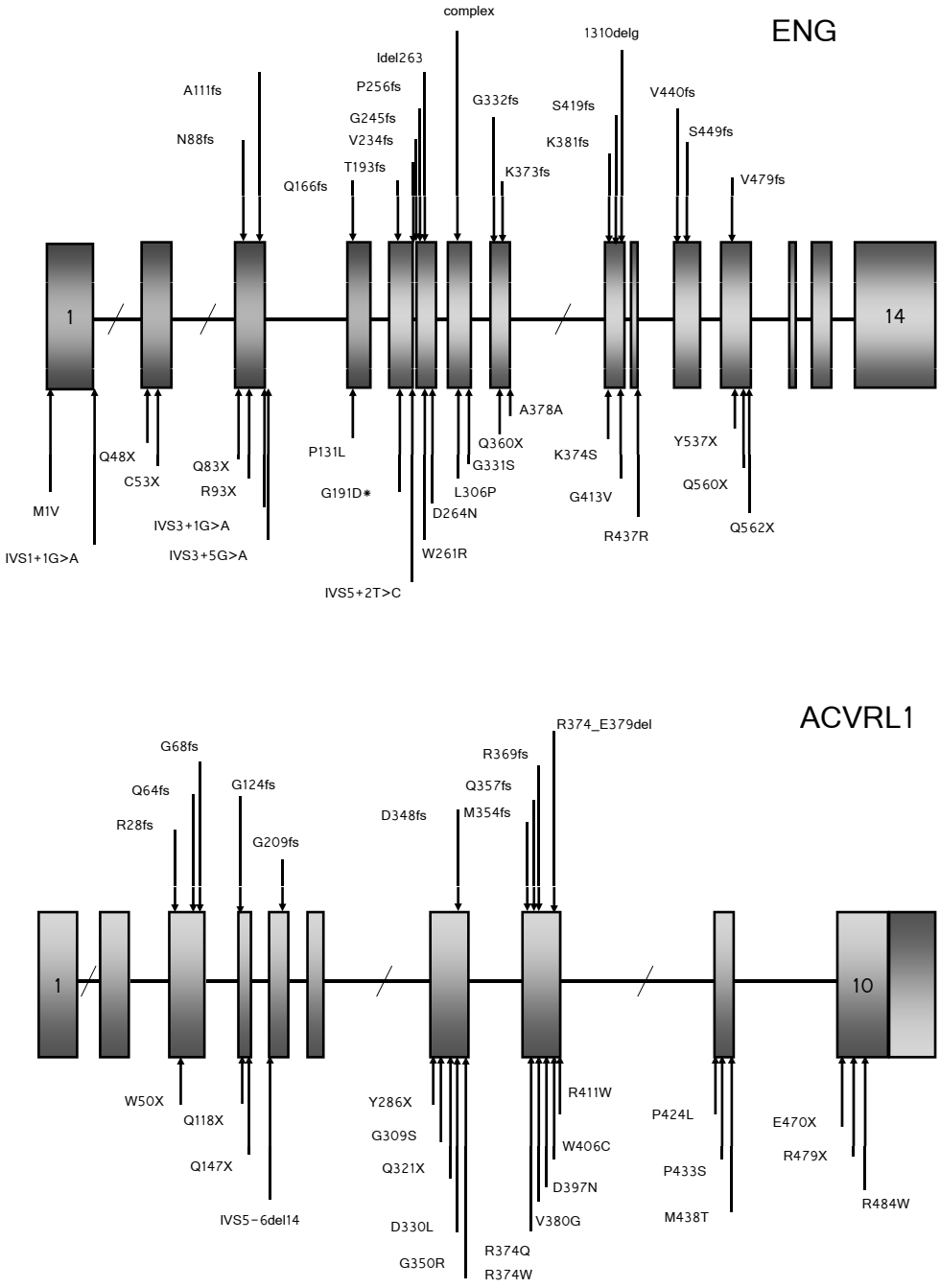


Figure 1 - Mutation spectrum of the mutations detected *ENG* (HHT1) and *ACVRL1* (HHT2) in the probands described in chapter 2.1

between species and among other type 1 receptors. These mutations are predicted to have structural effects, creating misfolding and a nonfunctional protein [5,7].

In *ENG*, the majority of the mutations are nonsense mutations and frame shifts mutations that result in a premature stop of translation. The missense mutations, in contrast to those in the *ACVRL1* gene, are in the *ENG* gene less restricted to certain exonic regions.

The third gene analyzed was *SMAD4*. Mutations in *SMAD4*, causing JP-HHT, are found in a small proportion of probands, less than 3% (chapter 2.3). In JP-HHT, *SMAD4* mutations are mainly restricted to the last four exons of the gene (MH2 domain). In contrast, mutations in *SMAD4* causing only juvenile polyposis are found throughout the gene. Although suggestive for a specific domain involved in JP-HHT, identical mutations have been found in probands with juvenile polyposis and in probands with the JP-HHT phenotype (chapter 2.3)[8]. Moreover, Gallione *et al*.[8] showed that although the *SMAD4* mutations in JP-HHT patients do show a tendency to cluster in the MH2 domain, mutations in other parts of the gene can also be associated with the combined syndrome [9]. Because of the age related penetrance of juvenile polyposis and the fact that not all mutation carriers develop symptomatic polyps, absence of polyposis symptoms does not obviate screening for *SMAD4* mutations. Therefore, screening for SMAD4 mutations is warranted when in an HHT positive proband, mutations in *ENG* and *ACVRL1* are excluded.

Are there more or less “severe” mutations?

The mutation spectra in *ENG* and *ACVRL1* confirm loss of function as the predominant causal mechanism in HHT1 and HHT2. Some hypothesized that the type of mutation can play a role in the severity of the disease. Two publications hinted towards a more severe effect of truncating mutations compared to missense mutations [10,11]. Bayrak-Toydemir *et al*.[10] described two or less organs affected in 69% of patients with an *ENG* missense mutation, compared to 38% with a truncating mutation. Lesca *et al*.[11] found more patients with epistaxis (HHT1 and HHT2), telangiectases (HHT2) and PAVMs (HHT1) comparing truncating mutations to missense mutations. In contrast, Sabba *et al*.[12] described in HHT2 more patients with PAVMs and HAVMs, associated with a specific splice site mutation compared to missense mutation and truncating mutations. They found no difference between truncating mutations and missense mutations [12].

In our study we focused on the gene involved and not on the type of mutation involved, because the effect of the mutation is in most cases hard to predict. Functional analysis of the mutations will be helpful for establishing the relationship between mutation and phenotype.

Mutation detection rates, ENG versus ACVRL1

Mutation detection rates in HHT probands differ between different countries. This is most likely caused by the way HHT patients and families were ascertained. Obviously, the more

patients with a clinically confirmed diagnosis (instead of possible diagnosis or even unlikely diagnosis), the higher the mutation detection rate. The mutation analyses presented in this thesis were performed in a cohort of patients that were referred to a single HHT expertise centre (Nieuwegein), assuring a uniformly established clinical diagnosis. This approach resulted in a detection rate of > 90%, the highest reported. Others have found percentages ranging from 63% to 88%, when looking at clinically confirmed probands only [2,3,13-18]. Our results show and confirm that in patients with a clinical diagnosis (that fulfill the diagnostic criteria), the vast majority of patients have mutations in *ENG* or *ACVRL1*.

In probands with a possible or unlikely clinical diagnosis (< 3 of the Curaçao criteria) the mutation detection rate was still 32% (chapter 2.2), showing the clinical criteria are useful, but when used too strictly, patients will be missed and consequently escape clinical surveillance.

When comparing the involvement of *ENG* versus *ACVRL1* in HHT, there are clear differences between countries. In The Netherlands, there is a predominance of *ENG* mutations in HHT probands (the ratio *ENG* versus *ACVRL1* is 1.32). An *ENG* predominance is also observed in Canada and Denmark (HHT1/HHT2 of 1.6 and 2.0 respectively). In France, Spain and Italy however, *ACVRL1* mutations are more prevalent (HHT1/HHT2 of 0.51, 0.54 and 0.41 respectively) [1,2,19].The cause of these geographical differences is not obvious. After correction for founder effects, Bayrak-Todemir *et al.*[10] found virtually no difference between HHT1 and HHT2 (53% and 47%). In contrast, when assuming that identical mutations are mostly from a common ancestor, one would expect similar relative contributions of the two respective genes in our HHT population. This is indeed the case in the Netherlands (40 *ENG* mutations versus 31 *ACVRL1* mutations). Lesca *et al.*[2] found a higher prevalence of *ACVRL1* mutations, also after correction for founder effects. If indeed geographical factors exist, this would add to the complexity of the phenotypic variability (environmental factors, genetic background) within HHT and its subtypes.

Other candidate genes for HHT

In some HHT families, gene analysis does not reveal a pathogenic mutation in *ENG*, *ACVRL1* or *SMAD4*. These families are not atypical HHT families, since HHT manifestations are present in probands and their relatives, and probands fulfill the Curaçao criteria. The proportion of probands with particular visceral manifestations, are within the range of those found for genetically confirmed HHT1 and HHT2 (chapter 3.1).

Of course, it cannot be excluded that in families in which no pathogenic mutation was detected (by our tests) in *ENG*, *ACVRL1* or *SMAD4*, they could still have a mutation in these genes, albeit in regions not investigated (promotor region, intronic regions or untranslated regions). Genes other than *ENG*, *ACVRL1* or *SMAD4* might be the cause of HHT in these families. To date, other candidate regions for HHT map to chromosome 5q31 and chromosome 7p14 [20.21]. For both loci linkage has been found in isolated families with

"typical" HHT. No genes have been found in these loci nor have there been reports on other families that could be linked to these chromosomal regions. The families without a confirmed mutation from our cohort, however, are too small to perform informative linkage analysis. An alternative approach considered for these families is analyzing the "exome" i.e. all exons of all genes by next generation sequencing.

Juvenile polyposis is not only associated with *SMAD4*, but also with mutations in *BMPR1A*. In contrast JP-HHT patients have only been shown to carry mutations in *SMAD4*. There have been no reports of juvenile polyposis patients with mutations in *BMPR1A* with HHT-like symptoms.

Mutations in *BMPR2* are associated with an autosomal dominant form of pulmonary arterial hypertension with a penetrance of around 20%. Pulmonary hypertension (PH) can also occur in patients with HAVM. It is important to recognize that pulmonary hypertension in HHT can be distinguished into two different types; (1) associated with liver AVMs, secondary to high cardiac output (PH) and (2) associated with pulmonary arterial hypertension (PAH) caused by arteriopathy. In HHT, PH is not frequent and PAH is rare, although not systematically evaluated. Since the first publication on PAH in HHT, a variety of mutations in *ACVRL1* have been found to predispose for the development of PAH in HHT2 [22-25]. *ACVRL1* and *BMPR2* operate on TGFβ receptors and share intracellular pathways through SMAD1/5/8. This on top of *SMAD4* and *BMPR1A* as causes of JP further supports the crosslinks between BMP signaling and HHT and can be of help considering potential candidate genes. To date, *SMAD1*, *SMAD5*, *SMAD7*, *BMPRII* and *BMPR1A*, all genes part of the TGFβ/BMP signaling pathway have already been excluded in a panel of HHT families without a causative mutation in *ENG*, *ACVRL1* or *SMAD4* [2].

Phenotypic variability

This thesis adds to the knowledge of the variability of the clinical manifestations of HHT1 and HHT2, namely epistaxis, telangiectases and visceral manifestations). At the start of our analyses, accurate data on the prevalence of the symptoms for HHT1 and HHT2 were hardly available. This was because of the lack of large numbers of patients and families that were genotypically as well as clinically well characterized. Thus, the establishment of the genotype in HHT families in our study resulted in the opportunity to discriminate between HHT1 and HHT2 and between carriers and non carriers. This combined with an accurate phenotypic description gave insight in the phenotype-genotype relationship (chapter 3).

Epistaxis, telangiectases, AVM

In HHT epistaxis is usually the presenting symptom and may commence before the age of 20 years. We found that at this age 80% of the patients with HHT1 reported epistaxis, compared to only 54% of the patients with HHT2. Nosebleeds appear earlier in life in HHT1

but at age 40, the majority of patients have nosebleeds, with no difference between HHT1 and HHT2 (chapter 3.3). We found that telangiectases of the nasal mucosa have a higher frequency and start to appear earlier in life than those of the oral mucosa or dermal sites in both HHT1 and HHT2. Telangiectases in the oral and nasal mucosa are detected earlier in life in HHT1 patients. In the age category 0-20 year more than 90% of HHT1 patients had nasal telangiectases, whereas in HHT 2 only 67% had those. The earlier onset of nasal telangiectases in HHT1 is in line with the earlier onset of epistaxis. Dermal lesions are more frequent and appear earlier in life in HHT2. In both HHT1 and HHT2 the number of sites affected increase with increasing age (cross sectional study, chapter 3.2).

In chapter 3.1 it is established that PAVMs are more frequent in HHT1 than in HHT2 (48.7% - 5.3%). CAVM was detected in 14.6% of HHT1 whereas they were present in 1.3% of HHT2 patients, not significantly different after correction for referral bias, but clearly a striking difference. HAVMs were found significantly more often in HHT2 patients (40.6% - 7.6%). Telangiectases in the GI tract were investigated in a small cohort of HHT patients, referred with unexplained anemia. In these patients, telangiectases in the GI tract were found in 92% of the patients. In the small intestine, telangiectases were more frequently present and generally larger in HHT1 patients compared to HHT2 patients. Colonic telangiectases were only detected in HHT1 patients (chapter 3.4).

Potential pit-falls in genotype-phenotype analyses

In this thesis we provide an extensive genotype-phenotype analysis. Establishing a genotype-phenotype relation is highly dependent on several factors. First and most important is the way the patients were ascertained. For example, if the majority of patients had been referred by a pulmonologist, a preponderance of PAVMs is likely to occur. This would result in a selected population with a skewed genotype-phenotype analysis; the most obvious patients, with the most severe manifestations are than included and less severe are left out. An attempt to correct for this referral bias is mandatory.

Secondly, it is important to use the proper diagnostic tools to detect the manifestations; tools that are the gold standard. Using less sensitive testing methods will leave manifestations undetected, missing less severe symptoms and influencing the established phenotype.

Lastly, one should always be aware of the fact that some symptoms can be a phenocopy, not associated with the disease. For example, in children, nosebleeds are common, not always associated with HHT, but with for example trauma or nose picking or coagulopathy. In the elderly, telangiectases can be present without an association with HHT. Sun exposure can also induce telangiectases sometimes mistaken for telangiectases in HHT. It is therefore important that different manifestations of the disease are screened for by an HHT centre with expertise.

In our genotype-phenotype analysis we tried to correct for the above pit-falls. Still, referral bias and bias in phenotypic description cannot be excluded and remains likely

to exist. This study is performed retrospectively. In the last years, a new technique has been introduced to detect PAVMs in HHT patients; the transthoracic echocardiography. It appears to be more sensitive and is able to detect smaller PAVMs than CT scanning [26,27]. Transthoracic echocardiography is a technique in which a right to left shunt is detected on echocardiography after injecting saline with tiny air bubbles to creating contrast. When the contrast is present in the left atrium within four cycles it is considered to result from an intracardiac shunt (for example patent foramen ovale, PFO), but when they reach the atrium after four cycles or more it is considered to be a pulmonary right-left shunt (RLS). The genotype-phenotype analysis in this thesis has been performed before the introduction of the contrast echocardiography and not all patients had CT performed. This is likely to contribute to an underestimation of PAVMs in the HHT patients. Indeed, in a recent publication on the Dutch HHT cohort, the proportion of patients with a PAVM was found to be higher in both HHT1 and HHT2 [28]. Using TTCE 85% of the patients with HHT1 had pulmonary RLS, this was 35% in HHT2. With high resolution CT scanning (the gold standard) PAVMs were detected in 58% of the HHT1 patients and 18% of the HHT2 patients. TTCE does not only detect RLS that are treatable PAVMs, but also small, clinically irrelevant PAVMs. Furthermore, pulmonary shunts can appear to be present in 6% of persons without HHT family mutation and in the normal population as well [27,28]. TTCE could prove be too sensitive for clinical use in HHT, but is very useful as screening method (simple and patient friendly) preceding high resolution CT.

HAVM screening remains controversial in HHT. HAVMs are in the majority of cases without symptoms. Patients with an asymptomatic HAVM will not be treated; yet they have the knowledge of having an HAVM. Therefore HAVMs are not screened for in all patients; only patients that have symptoms (liver bruit, heart failure, liver failure, abnormal liver enzymes, and abdominal pain) are screened for HAVMs. The same holds true for CAVM. Although the most recent recommendation, according to international guidelines [29] is to screen routinely adult patients and children with possible or definite HHT for CAVM (at the time of diagnosis), this was not the case in the cohort we analyzed in this study. CAVM screening was optional in the majority of the patients and some patients declined.

Comparing the phenotype with other publications

Large genotype-phenotype relation studies (chapter 3) have now been performed in several countries [3,10-12, 30]. For *SMAD4*, the genotype-phenotype analyses are lacking, because *SMAD4* mutations are rare, often *de novo* and consequently, the number of affected family members is very limited. The HHT1-HHT2 comparisons of the different groups are depicted in table 1.

In these reports more PAVMs are found in HHT1 compared to HHT2. CAVM is in general more frequent in HHT1 compared to HHT2. The exception is the report of Kjeldsen *et al.* [30], but in their study CAVM was not screened for, only mentioned when present (symptomatic) which resulted in a single patient in HHT1 and a single patient in HHT2. All authors report more HAVM in HHT2, concurring with our results.

Table 1 - Results of genotype-phenotype analyses of HHT patients in different countries

	HHT1	HHT2	HHT1	HHT2	HHT1	HHT2
mean age	44 yr	52 yr	35 yr	46 yr	43.1 yr	49.0 yr
M:F	0.67	0.67	0.91	0.92	1.65	1.14
number of patients	93	250	61	50	45	77
origin	**France / Italy**		**USA, Utah**		**Italy**	
epistaxis	96.8%	89.2%	100% @40	100% @ 40	91% (41/45)	95% (73/77)
TA	97.8%	93.2%	100% @40	100% @ 40	93% (42/45)	91% (68/75)
PAVM	54% (27/50)	12.8%(19/149)	59% (36/61)	29% (13/45)	75.5% (34/45)	44.1% (34/77)
CAVM	9.1% (2/22)	4% (2/50)	16.4% (10/61)	2% (1/50)	20.9% (9/43)	0/55 (0%)
HAVM	43.5% (20/46)	57.6% 87/151	1.7% (1/59)	27.7% (13/47)	60% (27/45)	83.1% (64/77)
GI			18% (7/39)	21% (8/39)	60% (18/30)	51.1% (24/47)
remarks	only mutation carriers included		havm screening after auscultation (bruit) only mutation carriers included		only mutation carriers included	
reference	*Lesca et al.; 2007*		*Bayrak-Toydemir et al.; 2006*		*Sabba et al., 2007*	

The genotype-phenotype analysis of the Italian group [12] showed remarkable high frequencies for the visceral manifestations, for which the cause is not obvious. In their study only patients with a confirmed genetic diagnosis were used, whereas in most other studies also patients clinically confirmed according to the Curaçao criteria were included. One would expect reduced prevalences when only genetically confirmed probands and relatives are included, because this group contains a larger proportion less severely affected patients (with only two or even one of the clinical criteria). More sensitive screening methods were used by the Italians, which could explain their findings. However, especially their frequencies of visceral manifestations found in HHT2 are strikingly high, albeit confirmed by Bossler *et al.* [3]. Bossler *et al.* [3] however, only included probands and not (maybe less severely affected) family members introducing an overestimation. The fact that the number of HHT2 patients in these analyses is limited might also have contributed to this high percentage of PAVMs in HHT2. It is also prominent that the Italian cohort is the only patient population in which more men participated than women, again suggesting some kind of referral bias.

Differences between men and women

In our study we stratified the data for gender and found trends for a higher frequency for the visceral manifestations in women. Significantly more women had a PAVM in HHT1 and a HAVM in HHT2. Also, we observed a higher frequency of dermal telangiectases in women, significantly in women with HHT1, particularly on the hands. Lesca *et al.*[11] could not confirm

HHT1	HHT2	HHT3	HHT1	HHT2	
?	?	?	52.6 yrs	57.2 yrs	**mean age**
0.48	0.47	0.70	0.86	3	**M:F**
77	50	73	39	16	**number of patients**
USA, Pennsylvania			**Denmark**		**origin patients**
78% (60/77)	92% (46/50)	67% (49/73)	74.4%		**epistaxis**
65% (50/77)	80% (40/50)	68.5% (50/73)			**TA**
67.5% (52/77)	48% (24/50)	67% (49/73)	56.2% (18/32)	22.2% (2/9)	**PAVM**
9.1% (7/77)	0	12.3% (9/73)	2.5% (1/39)	6.2% (1/16)	**CAVM**
2.6% (2/77)	14% (7/50)	2.7% 92/73)	ND	ND	**HAVM**
9.1% (7/77)	10% (5/50)	15.1% (11/73)	56.4% (22/39)	18.8% (3/16)	**GI**
only probands included			CAVM not screened for; 7 HHT1 and 7 HHT2 did not screen for PAVM		**remarks**
Bossler et al.; 2006			*Kjeldsen et al.; 2005*		**reference**

the influence of sex on PAVMs, but they also found a strong and significant preponderance of HAVMs in women. There are no other publications describing higher frequencies in women, but most of these studies have only limited numbers of patients.

It remains difficult to compare studies from different regions and different groups. As mentioned before, ascertainment and specifics of the investigated cohorts play a very important role (mean age, only clinically confirmed patients, only genetically confirmed patients, screening techniques). True regional differences in frequencies of the symptoms cannot be ruled out either; environmental factors or modifier genes (with different frequencies in different populations) could also play a role and explain different prevalences.

HHT and migraine

A number of studies suggest a relationship between right to left shunt and migraine especially the relationship between patent foramen ovale (PFO) and migraine [31,32]. Subsequent retrospective analysis of migraine in HHT revealed a significantly higher frequency of migraine in patients with a PAVM (21%) compared to patients without PAVM (13%) [33], the prevalence in a normal population being 10-12%. Furthermore, it was shown that in a cohort of patients the frequency of migraine decreased from 45% before embolization to 34.5% after embolization [34]. Especially the reduction of migraine with aura was striking, 33% before to 19% after embolization. Recently, in a prospective study the frequency of

migraine in HHT with PAVM was shown to be 29%, whereas this was 15% without a PAVM. The frequency of migraine with aura was 24% and 6%. It was established that the presence of migraine with aura was an independent predictor of PAVM, and PAVM an independent predictor of migraine with aura [35].

The pathogenesis of migraine is complex and not well known, this also accounts for the relation between intrapulmonary shunts and migraine. The association between intrapulmonary shunting and migraine with aura appears consistent, the association between intracardiac shunt and migraine remains surrounded by controversy mainly because of conflicting data on the effect of closure of the PFO [36,37].

Genetic counseling

The genotype-phenotype analysis in this thesis provides insight in the prevalence of symptoms in HHT1 and HHT2, applicable for genetic counseling of patients at least in The Netherlands, with the exception of the prevalences of PAVMs. As above mentioned, using more sensitive screening methods, more PAVMs are detected, although possibly not all clinically significant PAVMs.

In chapter 3.1, we proposed to counsel a prevalence of PAVM of 45-50% in HHT1 and of 3-5% in HHT2. Using chest CT these percentages were higher; 58% in HHT1 and 18% in HHT2 [28]. The latter prevalences are probably more accurate when counselling HHT families.

The different percentages for men and women should be used with care, since differences between men and women (although significant) have not yet been confirmed by others. It is recommended to emphasize that for a number of specific manifestations women tend to be more affected than men. The frequencies of the different manifestations per age category provide also the opportunity to estimate the chance for a family member to be affected without any symptoms (for example without epistaxis and telangiectases) at a certain age. This is especially helpful when DNA analysis is not available or possible (chapter 3).

DNA analysis is the suitable tool in clinical practice for identifying carriers. Determination of genetic status can prevent unnecessary screening and determine which patients should be monitored regularly.

Follow up

In case of a clinically or genetically confirmed diagnosis, it is advised to refer the patient to an HHT centre for screening of manifestations and follow-up examinations. Screening for PAVM will be performed using TTCE initially. When a pulmonary RLS is suspected a high resolution CT scan should be performed to image the PAVM and decide whether or not treatment is warranted. In a recent study a grading system was used grading TTCE severity into three categories. It was shown that only grade 3 (most severe) shunts were associated

with a PAVM detectable on HRCT (PPV 0.83) and considered for treatment [38]. When these results are reproduced, this means that PAVMs can be detected and graded using the TTCE and patients with low grade shunts do not have to be submitted to chest CT scan and radiation exposure.

In patients without a PAVM, screening should be repeated after puberty, before and after pregnancy and otherwise every five year [29]. When an AVM or RLS is documented or not excluded, antibacterial prophylaxis should be provided for at risk procedures and screenings intervals will be determined by the pulmonologist. Screening for CAVMs was not performed regularly until now. Screening for CAVMs is common practice in the USA, but not in Europe. The new international guidelines recommend that patients with HHT should be screened for CAVM at the time of diagnosis using MRI with and without contrast. Whether or not this recommendation will be followed in all countries remains to be seen. Screening for HAVM will be performed when clinical data are suggestive for HAVM, using Doppler ultrasonic echography or CT scanning. Screening for GI telangiectases will be performed when occult blood is present in the stool or anemia exists, anemia not explained by epistaxis.

Children

Publications on the manifestations of HHT in children are limited, especially on the prevalence of AVMs in children. The rate of complications of PAVMs and CAVMs was considered low in children [39,40]. Whether this is due to a lower prevalence of AVMs in children was not clear. Recently, results of a prospective study in which children were screened for CAVMs and PAVMs was published. In HHT1 60.7% (17/28) of the children had a PAVM and 14.2% (4/28) of the children a CAVM. In HHT2 this was 12.5% for PAVM (4/32) and 6.2% for CAVM (2/32) [41]. Some of the children with an AVM were symptomatic and treatment was warranted.

In The Netherlands screening of children consists of obtaining medical history and a general physical examination, including nasal inspection by a specialist, chest-X-ray and oximetry, as is suggested in the international guidelines [29]. When normal, screening will be repeated after puberty and at the age of 18 years.

Genetic modifiers

We and others have shown phenotypic differences between HHT1 and HHT2. Apart from these differences between HHT1 and HHT2, there is also a considerable variability in penetrance and expression between families with the same mutation and within families. The mechanisms leading to the intra- and interfamilial variability in HHT, for the visceral manifestations, the site-specific telangiectases (mucosa versus skin) or epistaxis are not obvious. There could be involvement of genetic modifiers.

Mice heterozygous for *Eng* or *Acvrl1* demonstrate the clinical phenotype of HHT, the severity being dependent on the genetic background of the mice [42,43]. The overlap in prenatal

vascular abnormalities of *Acvrl1, Tgfbr1, Tgfbr2, Eng* and *Tgfb1* homozygous knockout mice is considerable. For the latter, genetic modifier genes have been shown to influence this pre-natal lethal phenotype [44-47]. It is therefore possible that there are also genetic factors within the TGFβ pathway that modulate the phenotypic variation in HHT. Different modifiers may be differentially expressed in patients, resulting in AVMs or telangiectases in different organs with different frequencies. Their interaction with the *ENG* or *ACVRL1* might differ, dependent on the differential roles that *ENG* and *ACVRL1* play in the TGFβ/BMP signaling pathway. In contrast, there may be specific modifiers for HHT1 and specific modifiers for HHT2, which again could be tissue specific. Most likely the differences between HHT1 and HHT2 and the differences within families are caused by a combination of multiple modifier genes interacting with several (maybe tissue specific) environmental factors in a very complex manner. This will be subject of future investigations.

Environmental factors

That environmental factors play a role is particularly suggested by the localization of dermal telangiectases; the sun exposed dermal sites (face and hands) are most often affected. In a non-HHT population telangiectases are associated with sun exposure, smoking and increasing age [48]. Although these telangiectases are different from the telangiectases found in HHT, the association found in that population might also hold true for the HHT population. Indeed the telangiectases in HHT do occur predominantly on dermal sites that are sun exposed or are prone to other external influences. Furthermore, age is a prominent factor in HHT, as the number of sites affected increase with increasing age. The finding of a high frequency of telangiectases in the nasal and oral mucosa (compared to sun exposed dermal) might be a reflection of the density and superficial localization of arteries in mucosal tissue in general. It is very likely that the higher frequency is explained by a combination of genetic and environmental factors. Environmental factors such as local stress, infection, minor trauma, exposure to toxic agents and temperature changes. Apart from telangiectases one could speculate that environmental factors are contributing to all manifestations in HHT.

Future prospects

Apart from the identification of additional HHT genes, the search for modifier genes is a good next step in HHT research. We are currently conducting a SNP analysis in HHT patients comparing mutation carriers with a PAVM and mutation carriers without a PAVM for common variations within their genome. We focus on genomic regions syntenic to murine modifier loci known to influence TGFβ1-dependent vascular development. We also will screen SNPs within candidate genes encoding TGFβ and BMP signaling components, including *ENG* and *ACVRL1*. We hope via this analysis to be able to demonstrate that other loci can be important in the pathogenesis for PAVMs and maybe other AVMs. Understanding

the molecular mechanisms of genetic interaction will hopefully give insight into the etiology of AVM in HHT patients.

In conclusion

The results published in this thesis have improved the knowledge of HHT. In families suspected of HHT, DNA analysis reveals a mutation in the vast majority of the probands in *ENG* or *ACVRL1*. Once a genetic diagnosis is set, a general risk profile for the different clinical manifestations can be given to probands and family members according to the type of HHT. Screening for clinical manifestations is advised for affected family members.

The risk profile of the clinical manifestations is population based. An explanation for the intrafamilial variability has not been found, but is focus of future research. Identifying modifier genes or environmental factors influencing the expression of the HHT genes will benefit carriers greatly. It gives the opportunity to a better understanding of the reasons behind the development of symptoms and offers therefore a more personal risk profile. This will improve the management of the disease, make it more individual based and will personalize and improve the genetic counseling.

References

1. 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.
2. 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; French Rendu-Osler Network. Distribution of ENG and ACVRL1 (ALK1) mutations in French HHT patients. Hum Mutat. 2006; 27: 598.
3. Bossler AD, Richards J, George C, Godmilow L, Ganguly A. Novel mutations in ENG and ACVRL1 identified in a series of 200 individuals undergoing clinical genetic testing for hereditary hemorrhagic telangiectasia (HHT): correlation of genotype with phenotype. Hum Mutat. 2006; 27: 667-675.
4. 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; French Rendu-Osler Network. Molecular screening of ALK1/ACVRL1 and ENG genes in hereditary hemorrhagic telangiectasia in France. Hum Mutat. 2004; 23: 289-299.
5. 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.
6. Abdalla SA, Letarte M. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. J Med Genet. 2006; 43: 97-110
7. 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, Epub ahead of print
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). The Lancet 2004; 363: 852-859.
9. 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, Kini U, Leedom T, Ludman M, Lux A, Maisenbacher M, Mazzucco S, Olivieri C, Ploos van Amstel JK, Prigoda-Lee N, Pyeritz RE, Reardon W, Vandezande K, Waldman JD, White RI Jr, Williams CA, Marchuk DA. Overlapping spectra of SMAD4 mutations in juvenile polyposis (JP) and JP-HHT syndrome. Am J Med Genet A. 2010; 152: 333-339.
10. Bayrak-Toydemir P, McDonald J, Markewitz B, Lewin S, Miller F, Chou LS, Gedge F, Tang W, Coon H, Mao R. Genotype-phenotype correlation in hereditary hemorrhagic telangiectasia: mutations and manifestations. Am J Med Genet A. 2006; 140: 463-470.
11. 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; French-Italian-Rendu-Osler Network. Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. Genet Med. 2007; 9: 14-22.
12. Sabbà C, Pasculli G, Lenato GM, Suppressa P, Lastella P, Memeo M, Dicuonzo F, Guant G. Hereditary hemorrhagic telangiectasia: clinical features in ENG and ALK1 mutation carriers. J Thromb Haemost. 2007; 5: 1149-1157.
13. Prigoda NL, Savas S, Abdalla SA, Piovesan B, Rushlow D, Vandezande K, Zhang E, Ozcelik H, Gallie BL, Letarte M. Hereditary haemorrhagic telangiectasia: mutation detection, test sensitivity and novel mutations. J Med Genet. 2006; 43: 722-728.
14. Brusgaard K, Kjeldsen AD, Poulsen L, Moss H, Vase P, Rasmussen K, Kruse TA, Hørder M. Mutations in endoglin and in activin receptor-like kinase 1 among Danish patients with hereditary haemorrhagic telangiectasia. Clin Genet. 2004; 66: 556-561.
15. Fontalba A, Fernandez-L A, García-Alegria E, Albiñana V, Garrido-Martin EM, Blanco FJ, Zarrabeitia R, Perez-Molino A, Bernabeu-Herrero ME, Ojeda ML, Fernandez-Luna JL, Bernabeu C, Botella LM. Mutation study of Spanish patients with hereditary hemorrhagic telangiectasia. BMC Med Genet. 2008; 1; 9: 75.
16. Schulte C, Geisthoff U, Lux A, Kupka S, Zenner HP, Blin N, Pfister M. High frequency of ENG and ALK1/ACVRL1 mutations in German HHT patients. Hum Mutat. 2005; 25: 595.
17. Lenato GM, Guanti G. Hereditary Haemorrhagic Telangiectasia (HHT): genetic and molecular aspects. Curr Pharm Des. 2006; 12: 1173-1193.
18. Richards-Yutz J, Grant K, Chao EC, Walther SE, Ganguly A. Update on molecular diagnosis of hereditary hemorrhagic telangiectasia. Hum Genet. 2010; 128: 61-77.

19. 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: 295
20. Cole SG, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet 2005; 42: 577-582.
21. 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; 140: 2155-2162.
22. 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; 345: 325-334
23. Abdalla SA, Gallione CJ, Barst RJ, Horn EM, Knowles JA, Marchuk DA, Letarte M, Morse JH. Primary pulmonary hypertension in families with hereditary haemorrhagic telangiectasia. Eur Respir J. 2004; 23: 373-377.
24. 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; 40: 865-871.
25. Faughnan ME, Granton JT, Young LH. The pulmonary vascular complications of hereditary haemorrhagic telangiectasia. Eur Respir J. 2009; 33: 1186-1194.
26. van Gent MW, Post MC, Luermans JG, Snijder RJ, Westermann CJ, Plokker HW, Overtoom TT, Mager JJ. Screening for pulmonary arteriovenous malformations using transthoracic contrast echocardiography: a prospective study. Eur Respir J. 2009; 33: 85-91.
27. Gazzaniga P, Buscarini E, Leandro G et al. Contrast echocardiography for pulmonary arteriovenous malformations screening: does any bubble matter? Eur J Echocardiogr 2009; 10: 513-518.
28. van Gent MW, Post MC, Snijder RJ, Westermann CJ, Plokker HW, Mager JJ. Real prevalence of pulmonary right-to-left shunt according to genotype in patients with hereditary hemorrhagic telangiectasia: a transthoracic contrast chocardiography study. Chest. 2010; Feb 12. [Epub ahead of print]
29. 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 Hemorrhagic Telangiectasia. J Med Genet. 2009 Jun 29. [Epub ahead of print]
30. 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.
31. Post MC, Budts W. The relationship between migraine and right-to-left shunt: Fact or fiction? Chest. 2006 ;130: 896-901.
32. Schwerzmann M, Nedeltchev K, Lagger F, Mattle HP, Windecker S, Meier B, Seiler C. Prevalence and size of directly detected patent foramen ovale in migraine with aura. Neurology. 2005; 65: 1415-1418
33. Post MC, Letteboer TG, Mager JJ, Plokker TH, Kelder JC, Westermann CJ. A pulmonary right-to-left shunt in patients with hereditary hemorrhagic telangiectasia is associated with an increased prevalence of migraine. Chest 2005; 128: 2485-2489.
34. Post MC, Thijs V, Schonewille WJ, Budts W, Snijder RJ, Plokker HW, Westermann CJ. Embolization of pulmonary arteriovenous malformations and decrease in prevalence of migraine. Neurology. 2006; 66: 202-205.
35. Post MC, van Gent MW, Plokker HW, Westermann CJ, Kelder JC, Mager JJ, Overtoom TT, Schonewille WJ, Thijs V, Snijder RJ. Pulmonary arteriovenous malformations associated with migraine with aura. Eur Respir J. 2009; 34: : 882-887.
36. Dowson A, Mullen MJ, Peatfield R, Muir K, Khan AA, Wells C, Lipscombe SL, Rees T, De Giovanni JV, Morrison WL, Hildick-Smith D, Elrington G, Hillis WS, Malik IS, Rickards A. Migraine Intervention With STARFlex Technology (MIST) trial: a prospective, multicenter, double-blind, sham-controlled trial to evaluate the effectiveness of patent foramen ovale closure with STARFlex septal repair implant to resolve refractory migraine headache. Circulation. 2008; 18 :117: 1397-1404.
37. Butera G, Biondi-Zoccai GG, Carminati M, Caputi L, Usai S, Bussone G, Meola G, Delogu AB, Sheiban I, Sangiorgi G. Systematic review and meta-analysis of currently available clinical evidence on migraine and patent foramen ovale percutaneous closure: much ado about nothing? Catheter Cardiovasc Interv. 2010; 75: 494-504

38. van Gent MW, Post MC, Snijder RJ, Swaans MJ, Plokker HW, Westermann CJ, Overtoom TT, Mager JJ. Grading of pulmonary right-to-left shunt with transthoracic contrast echocardiography: does it predict the indication for embolotherapy? Chest. 2009; 135: 1288-1292.

39. Maher CO, Piepgras DG, Brown RD Jr, Friedman JA, Pollock BE Cerebrovascular manifestations in 321 cases of hereditary hemorrhagic telangiectasia. Stroke 2001; 32: 877–882.

40. Shovlin CL, Jackson JE, Bamford KB, Jenkins IH, Benjamin AR, Ramadan H, Kulinskaya E. Primary determinants of ischaemic stroke/brain abscess risks are independent of severity of pulmonary arteriovenous malformations in hereditary haemorrhagic telangiectasia. Thorax 2008; 63: 259–266.

41. Al-Saleh S, Mei-Zahav M, Faughnan ME, MacLusky IB, Carpenter S, Letarte M, Ratjen F. Screening for pulmonary and cerebral arteriovenous malformations in children with hereditary haemorrhagic telangiectasia. Eur Respir J. 2009; 34: 875-881.

42. Bourdeau A, Faughnan ME, Letarte M. Endoglin-deficient mice, a unique model to study hereditary hemorrhagic telangiectasia. Trends Cardiovasc Med 2000; 10: 279-285.

43. Torsney E, Charlton R, Diamond AG, Burn J, Soames JV, Arthur HM. Mouse model for hereditary hemorrhagic telangiectasia has a generalized vascular abnormality. Circulation 2003; 107: 1653-1657.

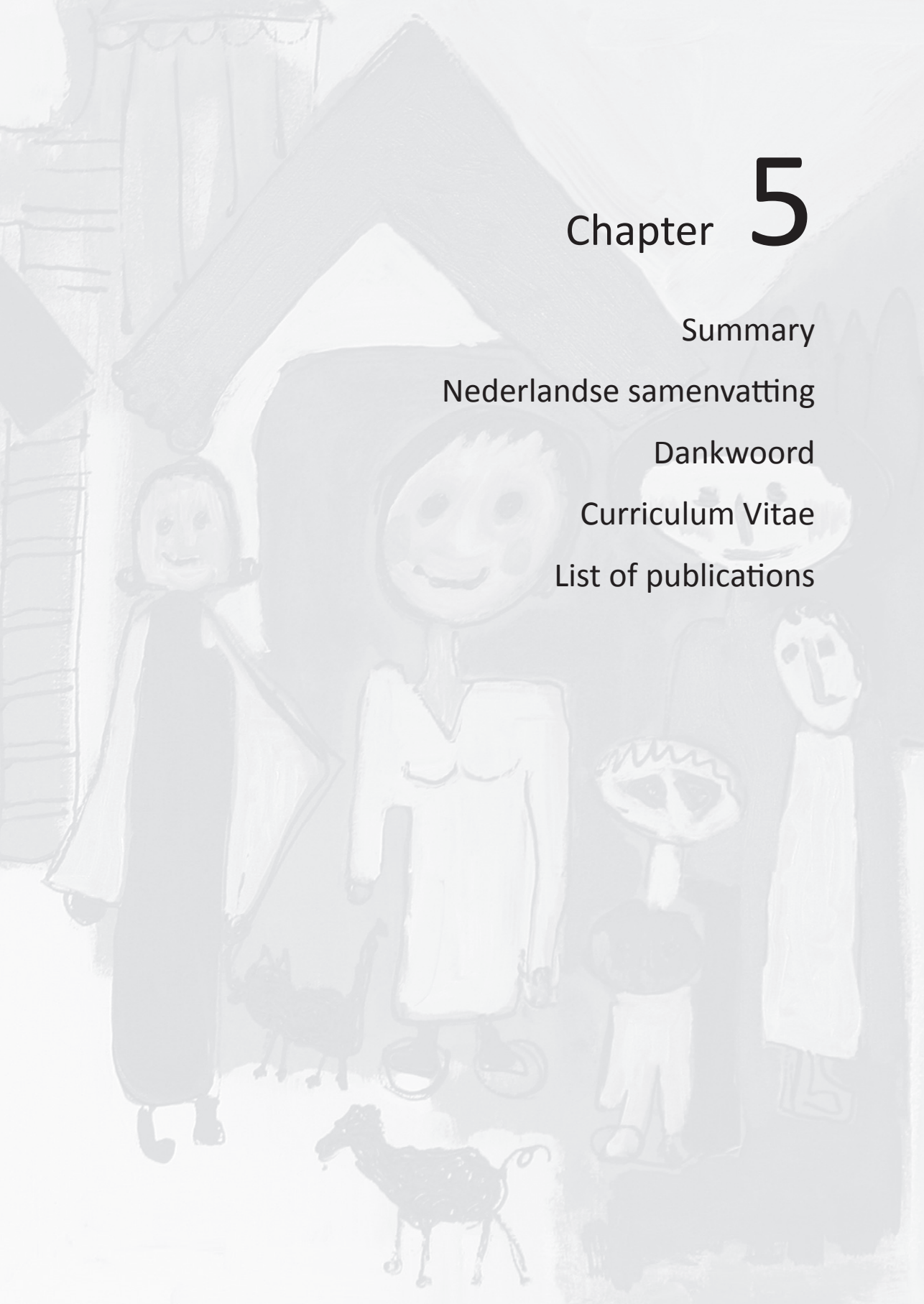
44. Bonyadi M, Rusholme SA, Cousins FM, Su HC, Biron CA, Farrall M, Akhurst RJ. Mapping of a major genetic modifier of embryonic lethality in TGF beta 1 knockout mice. Nat Genet. 1997; 15: 207-211

45. Tang Y, McKinnon ML, Leong LM, Rusholme SA, Wang S, Akhurst RJ. Genetic modifiers interact with maternal determinants in vascular development of Tgfb1(-/-) mice. Hum Mol Genet 2003; 12: 1579-1589.

46. Tang Y, McKinnon ML, Leong LM, Rusholme SA, Wang S, Akhurst RJ. Genetic modifiers interact with maternal determinants in vascular development of Tgfb1(-/-) mice. Hum Mol Genet. 2003; 12: 1579-1589.

47. Tang Y, Lee KS, Yang H, Logan DW, Wang S, McKinnon ML, Holt LJ, Condie A, Luu MT, Akhurst RJ. Epistatic interactions between modifier genes confer strain-specific redundancy for Tgfb1 in developmental angiogenesis. Genomics. 2005; 85: 60-70.

48. Kennedy C, Bastiaens MT, Bajdik CD, Willemze R, Westendorp RG, Bouwes Bavinck JN; Leiden Skin Cancer Study. Effect of smoking and sun on the aging skin. J Invest Dermatol 2003; 120: 548-554.



Chapter 5

Summary

Summary

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber (ROW) syndrome is an autosomal dominant disease characterized by vascular malformations in multiple organ systems. HHT has an age-related penetrance and variable clinical expression. The clinical symptoms are caused by direct arteriovenous connections without an intervening capillary bed. This can result in a range of manifestations, from smaller mucocutaneous telangiectases to large visceral arteriovenous malformations (AVM). The clinical manifestations of HHT include recurrent epistaxis, multiple telangiectases, at characteristic sites (lips, oral cavity, nose, fingers, gastrointestinal telangiectases) and AVMs, mostly in the lung (PAVM), the liver (HAVM) or the brain (CAVM).

The clinical diagnosis HHT is established using the Curaçao criteria. At least three of the following four criteria are required for a certain clinical diagnosis: spontaneous and recurrent epistaxis, telangiectases at characteristic sites, visceral manifestations (gastrointestinal telangiectases, PAVM, CAVM, HAVM) and a first degree relative with HHT. In the presence of two criteria, the diagnosis is considered possible.

Mutations in the endoglin (*ENG)* and activin A receptor type-like kinase 1 (*ACVRL1)* genes have been shown to be associated with HHT; mutations in *ENG* cause HHT1 and mutations in *ACVRL1* cause HHT2. A combined syndrome of juvenile polyposis and HHT is caused by mutations in the *SMAD4* gene.

The results of DNA analysis of the *ENG* and *ACVRL1* genes is described in **chapter 2.1**. DNA analysis is performed in 104 probands referred by a tertiary referral centre (Nieuwegein). Almost all patients were from the Netherlands. Mutation analysis is performed using sequencing of protein encoding exons and their flanking intronic sequences and using polymerase chain reaction for amplification of the DNA. Sequence variants are detected in 97 probands (92%), 55 probands carry an *ENG* mutation and 42 probands have an *ACVRL1* mutation. In 7 families no mutation is detected. In *ENG* 40 different mutations are found, in *ACVRL1* 31 different mutations. The majority of the mutations are de novo (not reported previously). All kinds of mutations are found: missense mutations, splice site mutations, nonsense mutations and small deletions and insertions.

Because the technique used in chapter 2.1 does not contain the search for large deletions or duplications, a subsequent analysis was performed. In **chapter 2.2** the results of Multiplex Ligation-dependent Probe Amplification (MLPA) are presented. In a (now larger) cohort of probands (123), without a pathogenic mutation after DNA analysis as described in chapter 2.1, MLPA analysis revealed 4 different mutations in 5 probands. In the *ENG* gene one deletion and two duplications are found and in the *ACVRL1* gene one large deletion is detected. Two of these MLPA mutations were found in a cohort of patients referred the HHT expertise centre (with a certain clinical diagnosis), the other three mutations were found

in a cohort of probands of whom clinical data were unavailable. All 5 probands appeared to have a certain and confirmed clinically diagnosis. These data, combined with data in the literature confirms that, although the search for large deletions or duplications is necessary, they will be found only in a small proportion of the HHT patients (1.5-5%). MLPA analysis is nowadays standard in DNA diagnostics.

To find out how often HHT symptoms are associated with *SMAD4* mutations, DNA analysis of the *SMAD4* gene is performed, in patients without a history of juvenile polyposis but with HHT symptoms (**chapter 2.3).** DNA analysis is performed in 30 patients without a mutation in *ENG* or *ACVRL1*. *SMAD4* mutations are found in 3 of these 30 patients. In one patient, the medical history revealed that colonic polyps had been discovered using endoscopy. In another patient, colon cancer was diagnosed and upon examination the patient appeared to have multiple hamartomatous polyps. None of the patients reported a family history. Calculating the proportion of *SMAD4* mutations in the whole HHT population, we show that an estimated 2-3% of the HHT patients harbour a *SMAD4* mutation as the cause of the symptoms. Genetic testing of *SMAD4* is therefore advised when no mutations are found in *ENG* or *ACVRL1*. Upon finding a *SMAD4* mutation, screening for polyps is warranted.

In chapter 2, the molecular and genetic characterization of the HHT families was performed. Once a mutation in the majority of the families is found, the identification of carriers and non-carriers is possible. This combined with well described phenotypes, allows for an extensive analysis of the genotype-phenotype correlation (**chapter 3**).

The results of genotype-phenotype analyses for AVMs are reported in **chapter 3.1.** For these analyses 584 patients with a clinically or genetically confirmed HHT older than 16 years are included. A pulmonary arteriovenous malformation (PAVM) is significantly more frequent in HHT1 compared to HHT2 (48.7% and 5%) and a hepatic arteriovenous malformation (HAVM) is found more often in HHT2 (40.6%. versus 7.6%). A cerebral arteriovenous malformation (CAVM) is detected in 14.6% of the patients with HHT1 compared to 1.3% of the HHT2 patients. Gender analyses reveal that women are more prone to have visceral manifestations. In HHT1 and HHT2 more women than men have an HAVM and in HHT1 more women than men were found to have a PAVM. With these data, genetic counselling of HHT patients or family members can be given accurate.

Telangiectases are the hallmark for the clinical diagnosis HHT. Despite that, there was only limited data on telangiectases as a symptom of HHT. Therefore, we analysed the localization and age distribution of telangiectases for HHT1 and HHT2, described in **chapter 3.2**. We show that in HHT1 and HHT2 telangiectases in the nasal mucosa appear earlier in life and are present at a higher prevalence than telangiectases in the oral mucosa or at dermal

sites. Oral and nasal mucosal telangiectases are present earlier in life in subjects with HHT1, whereas dermal telangiectases are more frequent and appear earlier in life in HHT2. In both HHT1 and HHT2, the number of sites involved increases with increasing age. Gender differences are observed for dermal telangiectases, differences between men and women are only found for dermal telangiectases, which are more frequent in women, significantly in HHT1 and particularly on the hands.

Telangiectases are very important to establish the clinical diagnosis HHT and bleeding from nasal telangiectases is often the presenting symptom. In **chapter 3.3** a study is undertaken to evaluate the age of onset and the severity of epistaxis in HHT patients, comparing both subtypes (HHT1 and HHT2). It is a retrospective study, questionnaire based, performed in a cohort of 466 HHT patients. The data are stratified for age, gender and ancestral background, to evaluate their influence on the severity of the phenotype. In patients with HHT1, epistaxis occurs significantly earlier in life than in patients with HHT2, in both men and women and in both patients from the Netherlands and from the Dutch Antilles. The number of nosebleeds increases with increasing age, in HHT1 and HHT2, without gender differences. In our study, gender or ancestral background has no apparent influence on the occurrence or severity of epistaxis.

Many HHT patients suffer from gastrointestinal bleeding. Gastroduodenoscopy and colonoscopy is a routine procedure giving information on duodenum and colon. However knowledge is lacking about the small intestine in HHT, because until recently a non-invasive technique was not available. In **chapter 3.4** the relationship between gastrointestinal telangiectases and the HHT genotype is investigated, with an emphasis on the small intestine using video capsule endoscopy (VCE). In a cohort of 25 patients with confirmed HHT and with anemia that could not be explained by epistaxis, the gastrointestinal tract is visualized to detect telangiectases. Telangiectases are present in almost all HHT patients (92%) with the highest frequency of telangiectases in the small intestine. These were more frequent in HHT1 and in general larger in HHT1. Telangiectases in the colon are detected less frequently compared to the small intestine. Colonic telangiectases are only found in the HHT1 population and are always accompanied by telangiectases elsewhere in the gastrointestinal tract. Patients with telangiectases isolated to the small intestine only and no telangiectases detectable by gastroduodenoscopy or colonoscopy are also present. This proves that the absence of telangiectases using conventional endoscopy in HHT patients does not exclude the presence of telangiectases in the small intestine. In patients with unexplained anemia, video capsule endoscopy should therefore be considered in order to cover the entire gastrointestinal tract.

In **chapter 4** the results of the previous chapters are discussed and evaluated. The results published in this thesis have improved our knowledge of HHT. In families suspected of HHT, a general risk profile for the different clinical manifestations can be given to probands and family members according to the type of HHT. However, the risk profile of the clinical manifestations remains population based. An explanation for the question, why the one family member is affected with all symptoms whereas the other has only epistaxis, remains open and will be focus of future research. Identifying modifier genes or environmental factors influencing the expression of the HHT genes will possibly generate a more personalized risk profile.

Nederlandse samenvatting

Samenvatting

Hereditaire hemorrhagische teleangiëctasiën (HHT) ook de ziekte van Rendu-Osler-Weber (ROW) genoemd is een autosomaal dominant overervende aandoening, met als belangrijkste verschijnsel vaatafwijkingen op meerdere locaties in het lichaam. HHT heeft een leeftijdsafhankelijke penetrantie en een variabele expressie. De verschijnselen worden veroorzaakt door directe verbindingen tussen arteriën en venen, zonder capillairen (haarvaten). Dit kan resulteren in teleangiëctasiën in de mucosa of op huid, maar ook tot grotere arterioveneuze malformaties (AVMs). De klinische verschijnselen van HHT zijn regelmatige bloedneuzen, meerdere teleangiëctasiën (lippen, mondholte, vingers, maag/darmen) en arterioveneuze malformaties in met name de longen (PAVM), de lever (HAVM) of de hersenen (CAVM).

Klinisch wordt de diagnose HHT gesteld op basis van Curaçao criteria. Tenminste drie van de volgende vier criteria moeten aanwezig zijn voor de klinische diagnose HHT: regelmatig spontane bloedneuzen, teleangiëctasiën op karakteristieke plaatsen, gastrointestinale teleangiëctasiën of AVM (PAVM, CAVM, HAVM) en een eerste graad verwante met HHT.

Mutaties in het *ENG* gen en het *ACVRL1* gen zijn geassocieerd met HHT; mutaties in het *ENG* gen veroorzaken HHT1 en mutaties in het *ACVRL1* gen HHT2. De combinatie van de aandoening juveniele polyposis èn HHT, wordt veroorzaakt door mutaties in het *SMAD4* gen.

De resultaten van DNA onderzoek van het *ENG* gen en het *ACVRL1* gen bij 104 probandi, verwezen door het HHT centrum in Nieuwegein worden beschreven in **hoofdstuk 2.1**. Bijna alle patiënten komen uit Nederland. Mutatie analyse is verricht met behulp van sequentie analyse van alle exonen en de flankerende intron sequenties, die geamplificeerd zijn met een polymerase ketting reactie. Er zijn varianten gevonden in 97 probandi (92%); 55 probandi hadden een *ENG* mutaties, 42 probandi een *ACVRL1* mutatie. In 7 families is geen mutatie gevonden. In *ENG* zijn 40 verschillende mutaties gevonden, in *ACVRL1* 31. Alle type mutaties zijn gevonden: missens mutaties, “splice-site” mutaties, nonsens mutaties, en kleine deleties en inserties. Naar grotere deleties en duplicaties is in deze studie niet gezocht.

In families waarin geen mutatie is gevonden met de techniek beschreven in hoofdstuk 2.1, is vervolgens gezocht naar grote deleties en duplicaties. De resultaten daarvan worden gepresenteerd in **hoofdstuk 2.2.** Gebruik makend van Multiplex Ligation-dependent Probe Amplification (MLPA), is in een, inmiddels uitgebreidere, cohort probandi (123 probandi) zonder mutatie, gezocht naar deleties/duplicaties in *ENG* en *ACVRL1.* Er werden 4 verschillende mutaties in 5 probandi gedetecteerd. In het *ENG* gen zijn 1 deletie en 2 duplicaties gevonden, in het *ACVRL1* gen is een grote deletie ontdekt. Twee van de MLPA mutaties werden gevonden in een cohort met een zekere klinische diagnose, verwezen door het HHT centrum. De overige drie mutaties werden gevonden in een cohort van

probandi, waarvan kennis van de klinische verschijnselen ontbrak. Alle 5 probandi met een grote deletie/duplicatie hebben een zekere klinische diagnose op basis van de Curaçao criteria. Deze gegevens gecombineerd met gegevens uit de medische literatuur, tonen dat onderzoek naar grote deleties en duplicaties geïndiceerd is, maar dat ze slechts in een kleine groep patiënten de oorzaak zijn van HHT (1.5%-5%). In het diagnostisch DNA onderzoek, uitgevoerd in ons centrum, wordt MLPA techniek inmiddels standaard uitgevoerd.

Om te onderzoeken hoe frequent *SMAD4* mutaties de oorzaak zijn van HHT, is in **hoofdstuk 2.3** DNA onderzoek verricht naar *SMAD4* mutaties in een groep HHT patiënten zonder aanwijzingen voor de ziekte juveniele polyposis. DNA analyse is verricht bij 30 patiënten, zonder een mutatie in *ENG* of *ACVRL1*. *SMAD4* mutaties zijn gevonden in 3 van deze 30 patiënten. Uit de medische gegevens van één patiënt bleek dat met behulp van endoscopie poliepen in de dikke darm waren geconstateerd. Bij een andere patiënt werd dikke darmkanker geconstateerd en met behulp van endoscopisch onderzoek bleken er ook meerdere hamartomateuze poliepen in de dikke darm aanwezig. Geen van de patiënten rapporteerde een verdachte familieanamnese. Als de gehele HHT populatie in ogenschouw wordt genomen, dan kan berekend worden dat ongeveer 2-3% van alle HHT patiënten, een *SMAD4* mutatie heeft als oorzaak van de HHT. Genetisch onderzoek van *SMAD4* wordt geadviseerd als er geen mutatie wordt gevonden in *ENG* of *ACVRL1*. Als een *SMAD4* mutatie wordt gevonden, dan is screenend onderzoek naar poliepen geïndiceerd.

In hoofdstuk 2 is de genetische en moleculaire heterogeniteit van HHT families in kaart gebracht. Nu in de meerderheid van de families een mutatie is aangetoond, is het beter mogelijk aangedane familieleden te onderscheiden van niet aangedane familieleden. Dit gecombineerd met een goed omschreven fenotype, maakt een nauwkeurige genotype-fenotype analyse mogelijk (**hoofdstuk 3**).

De resultaten van genotype-fenotype analyse voor de AVMs worden gerapporteerd in **hoofdstuk 3.1**. Voor deze analyse werden 584 patiënten met een klinisch dan wel genetisch bevestigde diagnose HHT (ouder dan 16 jaar) geïncludeerd. Een pulmonale arterioveneuze malformatie (PAVM) blijkt significant vaker aanwezig bij HHT1 patiënten dan bij HHT2 (48.5% en 5%) en een arterioveneuze malformatie van de lever (HAVM) komt frequenter voor bij HHT2 patiënten (7.6% en 40%). Een cerebrale arterioveneuze malformatie (CAVM) is aanwezig bij 14.5% van de HHT1 patiënten, vergeleken met 1.3% van de HHT2 patiënten. De analyse toonde verder dat vrouwen vaker verschijnselen hebben van HHT dan mannen. In HHT1 en HHT2 bleken meer vrouwen een HAVM te hebben dan mannen en in HHT1 bleken meer vrouwen een PAVM te hebben. Met behulp van deze data kan genetische counseling nauwkeurig worden gegeven aan HHT patiënten en hun familieleden.

Ondanks het belang van teleangiëctasiën voor het stellen van de klinische diagnose HHT, was er nog weinig bekend over de relatie tussen leeftijd van de patiënt en teleangiëctasiën. In **hoofdstuk 3.2** wordt de lokalisatie van teleangiëctasiën per leeftijdscategorie beschreven, voor zowel HHT1 als HHT2. Hierbij wordt aangetoond dat in zowel HHT1 als HHT2 teleangiëctasiën in de slijmvliezen van de neus eerder en vaker aanwezig zijn dan teleangiëctasiën in het mondslijmvlies of op de huid. Teleangiëctasiën van de mond- en neusslijmvliezen worden op jongere leeftijd gevonden in patiënten met HHT1, terwijl teleangiëctasiën van de huid vaker en eerder voorkomen bij HHT2 patiënten. Bij zowel HHT1 als HHT2, neemt het aantal plaatsen met teleangiëctasiën toe met het stijgen van de leeftijd. Verschillen tussen mannen en vrouwen werden alleen gevonden voor teleangiëctasiën van de huid; deze komen vaker voor bij vrouwen, vooral op de handen en voornamelijk in HHT1.

Teleangiëctasiën zijn een belangrijk kenmerk van HHT, bloedingen uit teleangiëctasiën in het neusslijmvlies zijn vaak het eerste symptoom in HHT. In **hoofdstuk 3.3** wordt een studie gepresenteerd, waarin de leeftijd waarop neusbloedingen in HHT1 en HHT2 ontstaan wordt beschreven. Daarnaast wordt de ernst van de neusbloedingen in kaart gebracht. Het betreft een retrospectieve studie in een cohort van 466 HHT patiënten, verricht met behulp van vragenlijsten. De gegevens uit de vragenlijsten zijn geanalyseerd, rekening houdend met leeftijd, geslacht of land van herkomst. Neusbloedingen komen op jongere leeftijd voor bij HHT1 dan bij HHT2. Dit geldt voor zowel mannen als vrouwen en voor zowel Nederlandse patiënten als patiënten met een Antilliaanse afkomst. Het aantal neusbloedingen neemt toe met het toenemen van de leeftijd, bij beide subtypen (HHT1 en HHT2) en onafhankelijk van het geslacht. In deze studie is er geen invloed gevonden van het geslacht of het land van herkomst op ernst en de leeftijd waarop neusbloedingen voor het eerst optreden.

Vele HHT patiënten krijgen bloedingen in het maag-darmkanaal. Onderzoek van de maag en de dikke darm wordt regelmatig verricht, onderzoek van de dunne darm is lastiger, omdat een niet invasieve techniek tot voor kort niet beschikbaar was. In **hoofdstuk 3.4** worden de teleangiëctasiën van het maag-darmkanaal onderzocht van patiënten met HHT1 of HHT2. Dit met het accent op de dunne darm en gebruik makend van videocapsule endoscopie. In een cohort van 25 personen met een bevestigde diagnose HHT en met bloedarmoede (niet veroorzaakt door neusbloedingen) is onderzoek verricht. Teleangiëctasiën zijn aangetoond in bijna alle patiënten (92%), de meeste teleangiëctasiën zijn gevonden in de dunne darm, vaker in HHT1 en over het algemeen groter in HHT1. Teleangiëctasiën in de dikke darm zijn alleen gevonden in HHT1 patiënten en altijd in combinatie met teleangiëctasiën elders in het maag-darmkanaal. Er zijn ook patiënten met teleangiëctasiën in alleen dunne darm, zonder teleangiëctasiën in de maag of dikke darm. Dit toont aan dat gebruik maken van conventionele onderzoekmethodes (scopieën) niet altijd toereikend is. Daarom wordt geadviseerd videocapsule endoscopie te overwegen bij HHT patiënten met een onverklaarde anemie.

In **hoofdstuk 4** worden de resultaten uit de voorgaande hoofdstukken bediscussieerd en geëvalueerd. De resultaten uit dit proefschrift hebben de kennis over HHT vergroot. In families verdacht van HHT is het mogelijk het subtype HHT te bepalen en daarmee een inschatting te geven op de kans op de verschillende verschijnselen. Deze inschattingen zijn wel op basis van populatie onderzoek. Een antwoord op de vraag waarom het ene familielid alle verschijnselen krijgt en het andere familielid alleen bloedneuzen, is er nog niet en onderwerp van toekomstig onderzoek. Het identificeren van genen of omgevingsfactoren die daarop invloed hebben, zal het mogelijk maken een meer persoonsgebonden risico inschatting te maken.

Dankwoord

Dankwoord

Vroeger was een tocht met een klipper een grote en langdurige onderneming, met een uitgebreide bemanning. De bijdrage van ieder lid van de bemanning was essentieel, voor een goede vaart en een juiste koers. Zonder een goede samenwerking was het schip slecht bestuurbaar en opkruisen al helemaal onmogelijk. Klippers bereikten door hun slanke bouw, het grote zeiloppervlak en een ingewerkte bemanning hoge snelheden, toch bleef geduld een schone zaak. Bij luwte was er weinig vooruitgang en de bemanning was over het algemeen lang van huis.

Dit proefschrift is eveneens een lange tocht geweest, voor elkaar gebracht door een uitgebreide bemanning. De tocht was een succes, de klipper ligt nu afgemeerd, de lading is goed overgekomen. Ik ben iedereen die dat heeft mogelijk gemaakt veel dank verschuldigd, zonder bemanning was dit niet gelukt. Speciaal wil ik, de kapitein, de stuurman en alle matrozen bedanken, in willekeurige volgorde.

Dr. C.J.J. Westermann, beste Kees, jij hebt een cruciale rol gespeeld in deze tocht. Je niet aflatende enthousiasme voor het HHT onderzoek is voor mij een grote stimulans geweest en heeft zeer motiverend gewerkt. Bij het onderzoek naar de vele aspecten van HHT staan bij jou de patiënten en de patiëntenzorg centraal, dat is waar het om gaat. Je bent in het HHT onderzoek de spin in het web en zorgt daarbij ook nog eens goed voor je medeonderzoekers; ook voor de broodnodige ontspanning. Ik zal je inspanning, begeleiding en de ontspanning nooit vergeten en hoop daar nog lang van te mogen genieten. Ook de andere leden van het HHT centrum wil ik bedanken, speciaal Hans-Jurgen Mager, Repke Snijder en Elly Vermorken.

Prof. dr. D. Lindhout, beste Dick, ik weet niet goed waar te beginnen met je te bedanken. Je bent in drukke en ook moeilijke tijden altijd bereikbaar geweest en dat waardeer ik enorm. Ondanks andere onderzoeklijnen die je aandacht vroegen en vragen, heb je altijd aandacht gehad voor de verschillende aspecten van het HHT onderzoek en ben je altijd weer met nieuwe ideeën gekomen. Als resultaten worden besproken heb je vaak net een andere blik op de zaak, net vanuit een andere richting, en daardoor verfrissend. Ik heb daar enorm veel van geleerd. Mange tak !!

Dr. J.H.K. Ploos van Amstel, beste Hans Kristian, mijn interesse in HHT is eigenlijk door jou gewekt. Mijn eerste contact met HHT was gedurende mijn laboratorium stage, waarin we startten met het DNA onderzoek van het ENG gen en het ACVRL1 gen, samen met Richard Zewald. Daarna heb je mij begeleid bij het daarop volgende onderzoek en het schrijven van de verschillende artikelen. Zo zijn we samen in het HHT onderzoek verzeild geraakt. Vooral voor je ontspannen begeleiding en niet aflatende geduld ben ik je zeer dankbaar. Aan onze trippen naar de tweejaarlijkse HHT meetings bewaar ik zeer goede herinneringen.

Dr. R.A. Akhurst, PhD. Dear Rosemary, thank you for the opportunity to work with you on HHT research, I am very grateful for our collaboration. I will never forget the start, my time in your laboratory, because of the work and the progress, but also because of the way

I was included in the lab. The lab, you and your family made my stay unforgettable, it felt like home. It is a pity San Francisco is so far away… I hope we can continue (and expand) our collaboration and I will be able to enjoy your patience, inspiration and humour for a long time to come. I look forward to more shared meetings, they are very motivating…

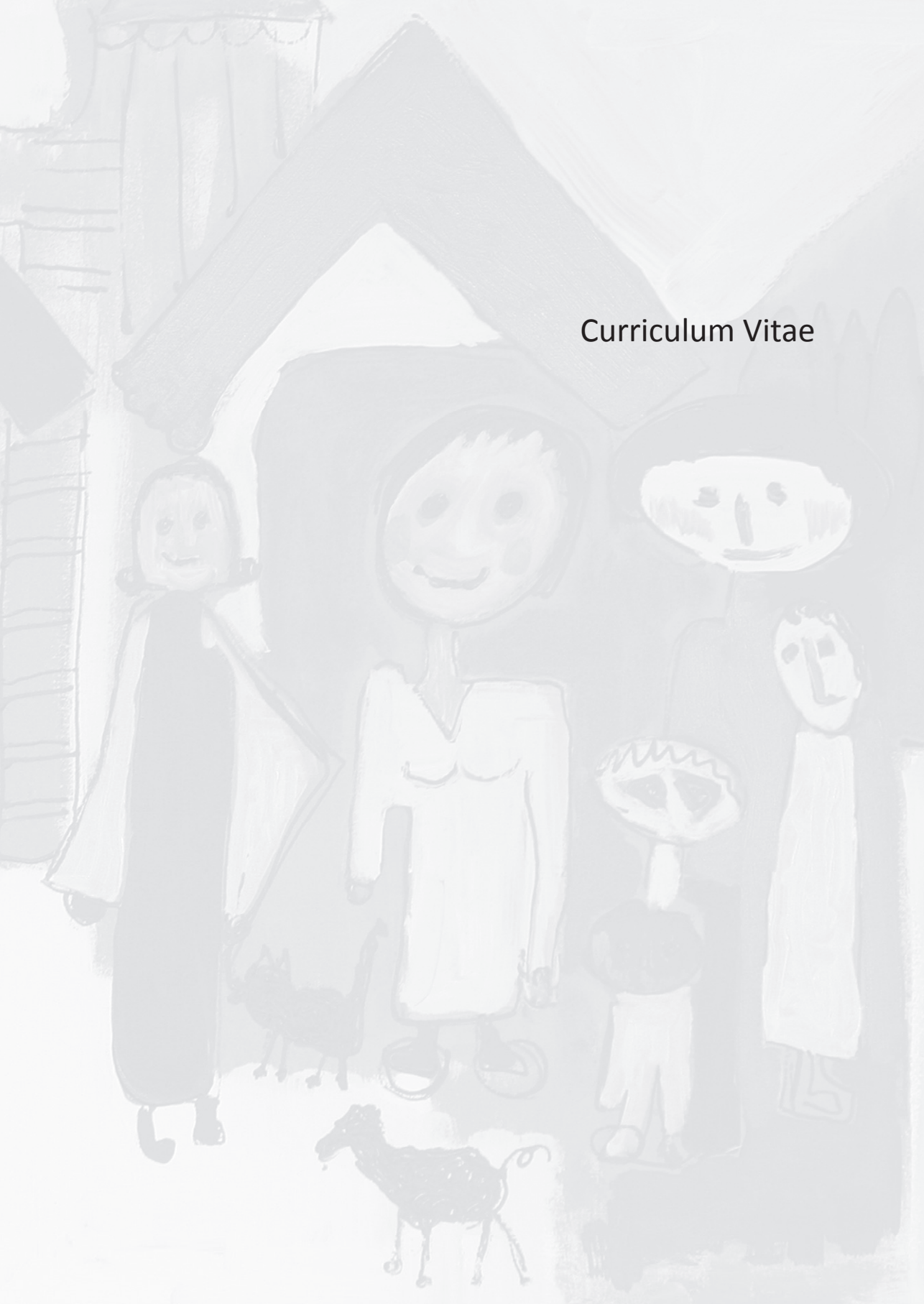
De afdeling klinische genetica en mijn lieve collega's, wil ik bedanken voor het geduld dat ze opgebracht hebben. Deze trip heeft lang geduurd en veel energie gevergd, dat ging wel eens ten koste van andere (lopende) zaken en van mijn humeur. Ik wil iedereen hartelijk danken voor het opgebrachte geduld. Het DNA laboratorium dank ik voor het verrichte DNA onderzoek en Eric Hennekam voor het genealogisch onderzoek van de HHT families; beiden onmisbare onderdelen van het onderzoek. Verder wil ik ook speciaal Vivian en Wilmy bedanken voor hun hulp met de laatste loodjes…

Lieve pa, ma en Bas, jullie onvoorwaardelijke vertrouwen in deze missie is een baken voor mij geweest. Ik ben daarvoor heel dankbaar en besef dat het veel geduld van jullie heeft gevraagd. Didam en Maartensdijk waren oases van rust, waar ik met volle teugen heb genoten en mijn hoofd heb kunnen leeg maken. Zonder jullie was deze missie niet mogelijk geweest. Dorus, thanks for the walks….

Mijn vrienden en familie ben ik ook dank verschuldigd. Ik ben me bewust van het feit dat ik de laatste tijd niet veel tijd voor jullie heb gehad. Ik troost me bij de gedachte dat iedereen beseft dat het niet altijd om kwantiteit gaat, maar vooral om kwaliteit. Toch zal ook de kwantiteit meer aandacht krijgen, beloofd !!

Lieve Gerrit-Jan en Yes, vanaf het begin van het onderzoek wist ik dat, bij een promotie, ik jullie zou vragen om paranimfen te zijn. Mijn gevoel zei dat dat zo moest en zo hoorde… Jullie zijn geweldig paranimfen en nog betere vrienden, ik ben er trots op jullie naast me te hebben staan.

Dit schip heeft de zeilen gestreken, daar ben ik trots op. Ze wordt klaar gemaakt voor de volgende trip. Daaronder valt natuurlijk ook verder onderzoek ten bate van de HHT patiënten, die ik ook heel hartelijk dank.
Maar nu eerst: vakanties plannen !



Curriculum Vitae

Curriculum Vitae

Tom Letteboer werd geboren op 9 februari 1967 te Zwolle. In 1985 werd het diploma Voorbereidend Wetenschappelijk Onderwijs aan het Liemers College te Zevenaar behaald. Na een jaar de studie Medische Biologie te hebben gevolgd aan de Universiteit Utrecht, startte hij in 1986 met de studie geneeskunde aan dezelfde universiteit. In 1992 werd het doctoraal examen met goed gevolg afgelegd. Tijdens de studie geneeskunde waren er verschillende extracurriculaire activiteiten, waaronder lid van het bestuur der MSFU " Sams" in de functie commissaris reductiebureau en verkoop. Na het behalen van het doctoraal examen geneeskunde volgde een onderzoeksproject van 6 maanden aan de afdeling Cancer Biology van de Cleveland Clinic Foundation in Cleveland, Ohio (begeleiders Dr. P. Paul en Dr. M.J. Coppes), waarbij de genetische defecten van familiaire polyposis coli werden gekarakteriseerd. De coschappen werden gestart in 1992, met als speciale coschappen radiodiagnostiek in het Academische Ziekenhuis Utrecht, Intensive Care Pediatrie in het Wilhelmina Kinderziekenhuis te Utrecht en sociale geneeskunde op de afdeling revalidatie in Klinikum Passauer Wolf in Bad Griesbach, Duitsland. Op 26 mei 1995 werd het artsexamen gehaald.

Aansluitend aan het artsexamen, was hij anderhalf jaar aangesteld als research fellow aan de afdeling Cancer Biology van de Cleveland Clinic Foundation (begeleider Dr. C. Colmenares). Dit werd gevolgd door een arts-assistentschap op de afdeling kindergeneeskunde in het Sophia Ziekenhuis in Zwolle.

De ervaringen met het moleculair biologisch onderzoek gecombineerd met het arts-assistentschap kindergeneeskunde prikkelden de interesse voor de klinische genetica. Na twee jaar arts-assistent op de afdeling klinische genetica aan de Universiteit Utrecht, startte in 2001 de opleiding tot klinische geneticus (opleider Prof. dr. F.A. Beemer). Tijdens de opleiding tot klinische geneticus begon het onderzoek naar hereditaire hemorrhagische teleangiëctasiën, leidend tot een vruchtbare samenwerking met het HHT centrum in het St. Antonius ziekenhuis te Nieuwegein. In 2004 werd de eerste "Robert I. White Jr., M.D. Young HHT Clinician of the Year Award" ontvangen uitgereikt door HHT Foundation International. Sinds januari 2005 is hij werkzaam als klinisch geneticus aan de afdeling Klinische Genetica van de Divisie Biomedische Genetica van het Universitair Medisch Centrum Utrecht.

List of publications

List of publications

Hester van Wieringen, **Tom G.W. Letteboer**, Rob Rodrigues Pereira, Sanne de Ruiter, Walter A.F. Balemans, Dick Lindhout. *Diagnostiek van foetale alcoholspectrumstoornissen.* Ned Tijdschr Geneeskd. 2010; 154: A331.

Westermann CJ, Kitonyi GW, **Letteboer TG**. *Is hereditary hemorrhagic telangiectasia rare in the black race? The first sub-Saharan mutation.* Haemophilia. 2010 Jun 28, Epub ahead of print

Letteboer TG, Mager HJ, Snijder RJ, Lindhout D, Ploos van Amstel HK, Zanen P, Westermann KJ. *Genotype-phenotype relationship for localization and age distribution of telangiectases in hereditary hemorrhagic telangiectasia.* Am J Med Genet A: 2008; 146A: 2733-2739.

van Tuyl SA, **Letteboer TG**, Rogge-Wolf C, Kuipers EJ, Snijder RJ, Westermann CJ, Stolk MF. *Assessment of intestinal vascular malformations in patients with hereditary hemorrhagic teleangiectasia and anemia.* Eur J Gastroenterol Hepatol: 2007; 19:153-158.

Gallione CJ, Richards JA, **Letteboer TG**, Rushlow D, Prigoda NL, Leedom TP, Ganguly A, Castells A, Ploos van Amstel JK, Westermann CJ, Pyeritz RE, Marchuk DA. *SMAD4 mutations found in unselected HHT patients.* J Med Genet: 2006; 43: 793-797.

Bliek J, Terhal P, van den Bogaard MJ, Maas S, Hamel B, Salieb-Beugelaar G, Simon M, **Letteboer T**, van der Smagt J, Kroes H, Mannens M. *Hypomethylation of the H19 gene causes not only Silver-Russell syndrome (SRS) but also isolated asymmetry or an SRS-like phenotype.* Am J Hum Genet: 2006; 78: 604-614.

Rump P, **Letteboer TG**, Gille JJ, Torringa MJ, Baerts W, van Gestel JP, Verheij JB, van Essen AJ. *Severe complications in a child with achondroplasia and two FGFR3 mutations on the same allele.* Am J Med Genet A: 2006; 140: 284-290.

Post MC, **Letteboer TG**, Mager JJ, Plokker TH, Kelder JC, Westermann CJ. *A pulmonary right-to-left shunt in patients with hereditary hemorrhagic telangiectasia is associated with an increased prevalence of migraine.* Chest: 2005; 128: 2485-2489.

Letteboer TG, Mager JJ, Snijder RJ, Koeleman BP, Lindhout D, Ploos van Amstel JK, Westermann CJ. *Genotype-phenotype relationship in hereditary hemorrhagic telangiectasia.* J Med Genet: 2006; 43: 371-377.

Letteboer TG, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. *Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients.* Hum Genet: 2005; 116: 8-16.

Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Jüppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, **Letteboer T**, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML. *LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development.* Osteoporosis-Pseudoglioma Syndrome Collaborative Group. Cell: 16; 107: 513-523.

Paul P, **Letteboer T**, Gelbert L, Groden J, White R, Coppes MJ. *Identical APC exon 15 mutations result in a variable phenotype in familial adenomatous polyposis.* Hum Mol Genet. 1993; 2: 925-931.