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EXECUTIVE SUMMARY

Executive summary of the 12th HHT international scientific conference

Jillian W. Andrejecsk[1] · Anna E. Hosman[2] · Luisa M. Botella[3] · Claire L. Shovlin[4] · Helen M. Arthur[5] · Sophie Dupuis-Girod[6] · Elisabetta Buscarini[7] · Christopher C. W. Hughes[1] · Franck Lebrin[8,9] · Christine L. Mummery[10] · Marco C. Post[11] · Johannes J. Mager[2]

Abstract Hereditary hemorrhagic telangiectasia is an autosomal dominant trait affecting approximately 1 in 5000 people. A pathogenic DNA sequence variant in the *ENG*, *ACVRL1 or SMAD4* genes, can be found in the majority of patients. The 12th International Scientific HHT Conference was held on June 8–11, 2017 in Dubrovnik, Croatia to present and discuss the latest scientific achievements, and was attended by over 200 scientific and clinical researchers. In total 174 abstracts were accepted of which 58 were selected for oral presentations. This article covers the basic science and clinical talks, and discussions from three theme-based workshops. We focus on significant emergent themes and unanswered questions. Understanding these topics and answering these questions will help to define the future of HHT research and therapeutics, and ultimately bring us closer to a cure.

Keywords HHT · Hereditary hemorrhagic telangiectasia · Endoglin · Activin receptor-like kinase 1 (ALK1) · Arteriovenous malformation · Epistaxis

Summary of basic science talks

Jillian W. Andrejecsk

HHT models

The genetic cause of most HHT cases has been known for over 20 years; most cases are due to pathogenic DNA sequence variants in *ENG* (endoglin/ENG/CD105),

Co-first authors: Jillian W. Andrejecsk and Anna E. Hosman.

✉ Franck Lebrin
F.Lebrin@lumc.nl

1 Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, CA, USA

2 Department of Pulmonology, St. Antonius Hospital, Koekoekslaan 1, 3435 CM Nieuwegein, The Netherlands

3 Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Ramiro de Maeztu 9, 28040 Madrid, Spain

4 Vascular Science, National Heart and Lung Institute, Imperial College London, London, UK

5 Institute of Genetic Medicine, Centre for Life, Newcastle University, Newcastle NE1 3BZ, UK

6 Hospices Civils de Lyon, Genetic Unit and HHT Reference Center, Bron, School of Medical and University Lyon 1, Lyon, France

7 Gastroenterology and Endoscopy Department, Reference Center for Hereditary Hemorrhagic Telangiectasia, Maggiore Hospital, ASST Crema, Crema, Italy

8 Einthoven Laboratory for Experimental Vascular Medicine, Department of Internal Medicine (Nephrology), Leiden University Medical Center, Leiden, The Netherlands

9 CNRS UMR 7587, INSERM U979, Institut Langevin, ESPCI, Paris, France

10 Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands

11 Department of Cardiology, St. Antonius Hospital, Koekoekslaan 1, 3435 CM Nieuwegein, The Netherlands

ACVRL1 (encoding activin receptor-like kinase 1, ALK1) and *SMAD4*. Yet, it is still not completely understood how these mutations lead to the characteristic vascular malformations—telangiectases and arteriovenous malformations (AVMs). To advance understanding, new models are needed and several important advances were presented in this year's meeting.

Mouse models

In protein signaling, SMAD4 is downstream of two TGF-type receptor complexes—the ALK1/BMPR2 complex which is affected in HHT, and the ALK5/TGFBR2 complex. Two groups developed SMAD4 mouse models, reporting HHT phenotypes similar to the ALK1 and ENG models, suggesting signaling is initiated predominantly through ALK1. Ola et al. presented an EC-specific SMAD4 deletion that resulted in an increase in EC proliferation and a decrease in mural coverage. AVMs formed in this model and were dependent on canonical SMAD4 signaling. Meadows, Crist et al. (see Abstracts OR3 and OR54) described a similar phenotype with less defined hierarchy in vessels and used the model to identify downstream targets VEGFR2 and TEK.

Bailly et al. presented a BMP9 knockout (KO) mouse model that displayed liver, kidney and lung problems. Focusing on liver defects, white spots and hemorrhage were observed in the KO mouse, as well as enlarged vessels and sinusoids and fibrosis. There were also gender differences, with females experiencing more liver defects. Oh et al. developed a new mouse model combining suppression and overexpression. Overexpression of ALK1 in both *Alk1* KO mice and *Eng* KO mice prevented AVM formation, suggesting ALK1 overexpression could be an effective therapeutic strategy for both HHT1 and HHT2.

Macrophage recruitment, rather than angiogenic stimulation, may play a crucial role in the formation of AVMs in HHT2, as presented in the 2015 meeting [1]. This was expanded upon this year, in relation to the role of ENG on macrophage function in HHT1. ENG is expressed in macrophages, suggesting that HHT1 patients with mutations in ENG may have an impaired innate immune response. Botella et al. developed a myeloid lineage specific ENG KO mouse and investigated the response to infection. A decrease in production of immune cytokines was observed, and ENG KO mice developed spontaneous infection. Additionally, there was an impaired response to peritoneal infection, with a decrease in the phagocytic activity of peritoneal macrophages and a decrease in the migration of immune cells to the site of inflammation [2].

Several other novel mouse models were discussed, including transmammary delivery of antibodies targeting BMP9 and BMP10 developed by Marambaud et al. [3]. Adenoviral vectors expressing CRISPR/CAS9 and targeting ALK1, were reported by Kim et al. (see Abstract OR53), which induced a 14% deletion of *Alk1* in WT mouse brains. In this model, when Ad-CRISPR-ALK1 was delivered and angiogenesis was stimulated with VEGF, brain AVMs developed. Arthur et al. (see Abstract OR4) described a model developed to dissect the role of ENG in paracrine mediated effects on angiogenesis. Conditioned media was collected from cardiosphere derived cells, and in vitro and in vivo experiments showed the paracrine signals that induced angiogenesis were ENG dependent [4].

Models utilizing human cells

Andrejecsk et al. (see Abstract OR52) developed an in vitro telangiectasia model incorporating perfused vessels. This microfluidic-based model utilized human ECs and mural cells, mimicking an artery-capillary-vein network, and recapitulated the vessel overgrowth and increased branching observed in telangiectases in patients. The platform provides a clean model that can be used to test hypotheses involving lesion initiation and progression, and the effects of flow.

Orlova et al. derived induced pluripotent stem cells (iPSCs) from a rare mosaic patient. Fibroblasts were negative for mutations, and erythroblasts were positive for an ENG mutation. Both cell types were isolated, iPSCs were made and then differentiated into ECs. A downregulation was observed in the iPSC-derived ECs from erythroblasts, but not from fibroblasts. No differences were observed between the two cell types in expression of CD31, proliferation, barrier function or 2D sprouting assays. This work provides a renewable, scalable source of patient-derived ECs—an important step for humanized models.

Blood flow in the development of AVMs

Blood flow velocity has a profound effect on vessel remodeling during development and disease. Vessels expand or contract to adapt to blood flow, and abnormal responses to flow are thought to play a role in AVM formation. Eichmann et al. reported an inducible *Alk1* KO mouse, and found that with loss of *Alk1*, AVMs developed in the retina only close to the optic nerve, where flow is highest. In 2D studies, it was found that flow potentiated BMP9 signaling and that flow promoted ENG-ALK1 complex formation [5].

Under flow, in 2D culture, WT ECs migrate against the direction of flow. ECs lacking *Eng*, however reverse their direction and migrate in the same direction as flow. Jakobsson et al. used a mosaic loss of *Eng* to demonstrate a similar result in vivo. Mutant cells showed reduced migration against the direction of blood flow, and accumulated on the venous side of the capillary beds. These results imply that the origin of AVM formation may be venous [6].

Siekmann et al. have created the first ENG KO zebrafish model. AVMs form during fin regeneration, and, as in Jakobsson's mouse model, KO ECs in the zebrafish preferentially localized in the venous regions. Additionally, arteries and veins were greatly enlarged in this model, but cell number did not increase. A change in cell shape was responsible for the increased vessel diameters in the ENG KO fish, and cells were less aligned with flow—further indicating a disruption in the normal response of ECs to flow when deficient in ENG. These larger arteries and veins promoted very high flow, effectively preventing perfusion of capillaries; when flow was altered by capturing red blood cells with optical tweezers, capillaries were reperfused. This mechanism may be an important contributor to the progression and enlargement of AVMs—when capillaries stop being perfused, they may regress, and the higher flow in larger vessels may stimulate remodeling to increase diameter [7].

Flow is known to be important in AVM formation in diseases other than HHT. Notch4 overexpression or underexpression in ECs can cause AVMs, and these can be reversed by normalizing Notch, as presented by Wang et al. (see Abstract OR40). Flow and resistance to flow is important in AVM formation under Notch dysregulation. Nitric oxide synthase, which modulates vascular tone, is a common molecular mediator of AVM formation, whether by Notch dysregulation or in HHT [8].

HHT genetics

While > 85% of HHT cases are caused by mutations in either *ENG or ALK1* and ~ 2% by mutations in *SMAD4*, many cases have no known genetic cause. Further, there is phenotypic variability in HHT, even in families with the same mutation—an HHT mutation is necessary, but that alone is not sufficient to cause an AVM in a specific organ. With this in mind, many investigators have utilized the most recent advances in gene sequencing to search for novel mutations—not only in *ENG*, *ACVRL1* and *SMAD4*, but also in non-coding regions, promoters, regulatory regions and possible modifier genes. Further, the ENG structure has been elucidated with new advances in structural biology by Jovine and colleagues—allowing specific mapping of mutations (see below).

Brilliant et al. (see Abstract OR48) found 2 additional new variants using exome chips, mapped to the newly described structure of ENG (see below) and hypothesized that certain variants would be associated with a higher risk of related clinical anomalies. The first variant was found in the orphan domain of ENG, and is potentially associated with polycystic kidney disease. The second was found in the linker between the orphan and ZP domains, and potentially associated with anomalies in peripheral vascular and other cardiovascular sites.

Using next generation sequencing of 91 patients, Giraud et al. (see Abstract OR49) identified novel pathogenic variants in *ALK1, ENG* and *SMAD4*; and additionally found potentially pathogenic variants in the promoter region of *ENG* and in *RASA1*. Giraud warned, though, that bioinformatics results may be difficult to interpret because the significance of a large number of these variants is unknown.

It is still unclear if a loss of heterozygosity in the HHT genes—*ENG, ALK1, SMAD4* and *BMP9*—is needed to form a lesion. Is a complete loss of protein needed, or is a drop below a certain threshold sufficient? Such a mechanism could explain the great variability of phenotypes found in HHT patients even between family members with the same mutation. One contributing factor may be modifier genes. These could act outside of the direct signaling pathway to reduce protein levels and initiate lesions. It is difficult to study these modifiers because they often have very small effects and, like *ENG* and *ACVRL1*, the effects may also be context-dependent. Akhurst et al. identified one modifier gene in a Dutch cohort of 200 patients—protein tyrosine phosphatase non-receptor type 14 (PTPN14). PTPN14 has a strong association with ALK1 and with gene products of, the endocytotic, Hippo-Yap-Taz, PI3 K and Notch pathways. Knocking out PTPN14 results in increased angiogenesis, as demonstrated in human umbilical artery endothelial cells (HUAEC) in a 3D angiogenesis assay. The specific mechanism is still not known, but it is thought that PTPN14 may stabilize ALK1 and ENG [9, 10].

ENG structure

Exciting new insights into the 3D structure of ENG and BMP9 were presented at this year's meeting by Jovine and colleagues. This group used fusion to the MBP protein to successfully crystalize the ENG-BMP9 complex, when it was previously not possible. The crystal structure of ENG revealed two ZP and two orphan domains, with binding domains for BMP9 in one of the orphan domains. The binding site is located within a hydrophobic pocket, and mutations that alter this hydrophobicity are most likely to prevent ligand binding—even if it is only resulting from the exchange of a single amino acid.

The structure also provided insight into a question about dissociation constants. The $K_{D(off)}$ of the orphan domain alone is 276 nM, while $K_{D(off)}$ of the whole molecule is 10 nM—even though the ZP region has no binding sites for BMP9. The ZP regions do provide a site for the crosslinking of a dimer structure, however, and the multiple binding sites more efficiently capture the ligand—if BMP9 is released from one binding site, the second is in close proximity—i.e. an increase in avidity. Additionally, this model of the ENG dimer fits perfectly with a model of ALK1, and the structures revealed that ActRIIB is unable to bind when both

ALK1 and ENG are present—for the type 2 receptor to bind, ENG must leave the complex [11].

HHT therapeutics

Potential drug targets

The advances in animal models also provided insights into potential drug targets. Meadows et al. (see Abstract OR3) utilized their SMAD4 KO mouse to identify the Ang2/TEK (or Tie2) pathway. Out of 184 genes that overlapped in their NGS and ChIP sequencing analyses, TEK was one of the most affected genes; this was confirmed by showing a 50% decrease in levels of TEK in the retinal capillaries of the SMAD4 KO mouse. Siekmann et al. discussed CXCR4 (and its ligand CXCL12) in the context of the zebrafish model. CXCR4 is expressed in tip cells during angiogenesis, and required for proper arterial patterning during fin regeneration [12]. Lebrin et al. (see Abstract OR39) provided evidence for a direct link between ALK1 and VEGF signaling in ECs to promote HHT2. Decreased expression of VEGFR1 was observed in these mice, leading to a decrease in the ability of tip cells to sense the VEGF gradient and a subsequent delay in migration with misaligned filipodia. Inhibition of VEGF signaling blocks pathological angiogenesis and vascular malformations in the model.

Rossi et al. (see Abstract OR58) observed that ENG KO mice bleed for longer than controls. It was observed that ENG can bind platelets and that there is a decrease in the ability to capture platelets when ENG is knocked out. It is possible that ENG is an important mediator of platelet capture, potentially through its RGD motif, providing a novel mechanism for targeting in the management of epistaxis and gastrointestinal bleeding.

Repurposing existing drugs

Marambaud et al. discussed the potential of a BMPR2 activator, Tacrolimus (FK506) to also treat HHT2. Tacrilomus has been used to treat familial pulmonary arterial hypertension (FPAH), caused by a mutation in BMPR2, and was included in a drug screen of 700 BMP9-ALK1 signaling activators. It was found to be effective at increasing levels of pSMAD1/5/8 and ID1 in HUVEC and C2C12 cells in a dose-dependent manner. Further, it inhibits Dll4 expression, promotes ALK1 cell surface disappearance, and inhibits VEGF-mediated activation of AKT. Efficacy was demonstrated in one patient EC line and in a mouse model with transmammary delivery of anti-BMP9/10—tacrilomus decreased the incidence of AVMs [13]. Other drug candidates are being screened for repurposing, including read-through drugs specifically targeting nonsense mutations as presented by Aldred et al. (see Abstract OR27). Faughnan et al. (see Abstract OR18) showed promising results in treatment of HHT related bleeding with Pazopanib 50 mg/day. This is an orally administered tyrosine kinase inhibitor blocking VEGF and is of interest as an anti-angiogenic treatment. Five/7 patients showed more than 50% decrease in epistaxis duration and 2/7 showed more than 50% decrease in epistaxis severity, resulting in improvement of hemoglobin levels.

Gene therapy

There are over 2000 clinical trials ongoing that involve gene therapy for different conditions. The largest number of current trials are targeting cancer, followed by monogenic diseases. HHT falls under the latter category—it is caused by a defect in one allele of a single gene, and is therefore a good candidate for gene therapy. Kasthuri, Asokan et al. are developing such a therapy based on adeno-associated viruses (AAVs). AAVs infect many animals, including humans, but are harmless and non-pathogenic. AAVs package and deliver recombinant DNA, and different strains of the virus target different tissues. AAV1 targets the vasculature, and so is ideal for treating HHT. There exists a major hurdle in using AAVs, however—20–60% of the population have pre-existing AAV neutralizing antibodies and are not eligible for treatment—the percentage depends on the AAV type. The Asokan group has developed through an iterative process CAM130, a mutated form of AAV1 that is not recognized by neutralizing antibodies. ENG and ALK1 AAVs have been developed, and tissue culture and mouse studies are about to begin [14].

An anti-ENG antibody and HHT symptoms

Many cancer therapeutics target the abnormal vasculature of tumors, and several companies are targeting the ALK1/ENG pathway as an adjunct to anti-VEGF therapies. Tracon Pharmaceuticals has developed an antibody targeting ENG—TRC105; Acceleron has developed a ligand trap targeting BMP9/10 called Dalantercept; and Pfizer has PF03446962, which targets ALK1. While these have demonstrated some effectiveness for certain indications in cancer, interestingly, they all trigger HHT-like side-effects.

Charles Theuer, president and CEO of Tracon Pharmaceuticals, attended the conference and gave two talks focused on their ENG antibody (TRC105), which overlaps with the BMP9-binding site in ENG. Tracon developed this therapeutic as an angiogenesis inhibitor to use in combination with, or as an alternative to, VEGF therapies in treating angiosarcoma, age-related macular degeneration and fibrosis, believing that ENG may be an escape pathway when anti-VEGF therapies are used alone. This idea is based on the observation that when VEGF is targeted, ENG

expression increases; and when ENG is targeted, there is an increase in the VEGF pathway. TRC105 consistently induces nosebleeds and telangiectasia, even with the lowest doses. Interestingly, when combined with anti-VEGF treatments, such as Bevacizumab, there is no amelioration of HHT-like side effects even though Bevacizumab has been shown to be effective in treating HHT patients. It is possible that the drug is too potent for anti-VEGF therapies to overcome these side effects. TRC105 has been used to successfully treat patients with angiosarcoma; some patients have been receiving it for several years—and all patients develop some form of HHT-like symptoms. Patients only develop small vessel lesions, however, and do not develop AVMs or other large vessel problems normally seen in HHT patients. Is this due to the fact that the only patients treated are those with mature vasculature? These patients could potentially provide insight into how lesions develop, and the role, if any, of VEGF in this process.

Summary of clinical talks

Anna E. Hosman

The aim of exchanging this scientific information is to stimulate the advances in the field so that patients suffering from HHT can be screened and treated optimally based on scientific evidence. Since the last HHT conference in 2015 large collaboration projects have been set up. Shovlin et al. presented the new VASCERN-HHT working group (see Abstract OR31) which has been established within the European Reference Network for Rare Multisystemic Vascular Diseases to bring together the views of HHT professionals and HHT patients/representatives in Europe, to establish priorities for action. Shovlin et al. also presented a new international collaborative venture (see Abstract OR35) with the UK 100,000 Genomes Project. This is a powerful NHS phenotyping database in combination with whole genome sequencing for the advancement of HHT genomics of which the first results are expected in 2017.

Management of pulmonary arteriovenous malformations

Pulmonary arteriovenous malformations (PAVMs) affect a high proportion of HHT patients and can lead to severe complications. However, timely screening and treatment can prevent serious complications [15]. Hosman et al. (see Abstract OR34) had previously shown a reduced life expectancy in unscreened and untreated HHT type 1 patients [16]. However, they have now shown that pre-emptive screening for PAVMs using transthoracic contrast echocardiogram (TTCE) and, when indicated, a chest CT scan and timely treatment of PAVMs increases and even normalizes the life-expectancy of HHT patients. Repeated screening is standard in many HHT centers but data on at what interval these should be performed are still required. Iyer et al. (see Abstract OR20) showed a de-novo PAVM formation rate of 0.7% per patient year when TTCE or CT were negative for PAVMs on screening, but no clinically significant PAVMs were identified in patients in the first 5 years of an initial negative screen. It was commented that negative CT scan without TTCE might not show small untreated PAVMs and might be incorrectly classified as negative, after which those undetected PAVMs might grow. The data suggest however that a 5-year interval in a TTCE negative patient is likely to be adequate.

Ishaque et al. (see Abstract OR16) presented the results of and success rate of the Microvascular Plug (MVP) in embolization of PAVMs. The device allows more distal embolization and a high rate of immediate angiographic occlusion was found. However, questions on recanalization rate in children, high costs and risk of migration arose and the audience was divided on the benefit of this device over other existing methods [17]. DePietro et al. (see Abstract OR19) investigated the role of graded TTCE as a post-treatment surveillance in patients with low grade pulmonary right to left shunt after embolization of PAVMs. Data show post-treatment TTCE might be effective in determining which patient requires CT follow-up after PAVM embolization. Although this technique will not likely replace the CT scan in all patients, it might save a select group with a low-grade shunt from unnecessary radiation exposure. Shovlin et al. (P21) demonstrated 13 of 24 patients with PAVMs showed silent cerebral ischemic changes on MRI. This raises the question whether patients with persistent PAVMs should receive pharmacological stroke prevention.

Management of hepatic vascular malformations

This topic was discussed to great extent during one of the workshops (see "Workshop 3: management of hepatic vascular malformations" section). Boillot et al. (see Abstract OR8) showed long term outcomes of 15 HHT patients who underwent liver transplantation as a result of complications of liver involvement of HHT. After transplantation clinics and quality of life normalized, however it was suggested there was radiological recurrence of HHT in 8/15 with a delay of 12 years (6–15), which were asymptomatic in all but one [18]. Lenato et al. (see Abstract OR37) analyzed hepatic involvement and atrial fibrillation in geriatric patients. 68.4% of patients had chronic anemia 14.4% had signs of chronic atrial fibrillation. Elevated levels of gamma GT seemed to be a predictor for atrial fibrillation.

Management of cerebral vascular malformations

Cerebral vascular malformations (CVMs) occur more frequently in patients with an *ENG* mutation than patients with an *ACVRL1* mutation; 13.4% compared to 2.4% respectively [19, 20]. These can lead to severe hemorrhagic complications. Controversy remains concerning different areas of management of CVMs including timing of screening and methodology of treatment, especially in the case of silent CVMs. Hetts et al. (see Abstract OR11) made a comparison of MRI and digital subtraction angiography for the detection of CVMs in HHT patients. They conclude an MRI with gadolinium is the primary screening tool for CVMs. Kalani et al. (see Abstract OR10) reviewed a patient cohort ($n = 39$) with either single or multiple CVMs and outcomes of different treatment options. However, the true incidence of silent CVMs remains unclear. The aim is to further examine larger patient cohorts, increase the use of molecular genetic diagnostics for characterization of these cohorts to improve understanding of natural history of CVMs and improve patients prognostication, safety and treatment outcomes. Tayebi Meybodi et al. (see Abstract OR55) showed good long term outcomes of brain AVM obliteration using radiosurgery. After 5 years 60% was obliterated and it is a good option for patients with multiple lesions. It has minimal complications, but latency time and lower rate of cure in comparison to surgery and embolisation. Although there are more data available, no clear new recommendations were made and methodology of screening and treatment of CVMs remain a case by case decision.

Screening children

The methodology of screening children for HHT vary greatly between different HHT centers and was often discussed during this conference. The challenge is to find a good balance between invasive diagnostics to protect the few children at risk for devastating complications and not wanting to over diagnose the vast majority who live their childhood without problems. Hosman et al. (see Abstract OR13) presented a cohort of 436 pediatric patients, of whom 175 have a definite HHT diagnosis, with a follow-up period of 18 years and showed no PAVM related complications after screening with O2 saturations and physical examinations. They suggested it is safe to postpone TTCE and chest-CT until adult age [21]. Vickers et al. (see Abstract OR25) discussed the benefit of screening for brain AVMs in children affected by HHT. In their cohort of 49 patients no individual was identified who would have had a benefit of screening for brain AVMs as a child. However, it was commented that this is a small study population and larger studies have to be done before solid conclusions may be drawn. Several presentations and posters were dedicated to discussing the validity of the Curaçao criteria in children. Pahl et al. (see Abstract OR24) investigated the sensitivity and specificity of an altered version of the Curaçao criteria (specifying a required frequency of nosebleeds and number of telangiectases). Specificity was high in all age groups (96–100%) but sensitivity was low especially in children.

Epistaxis

Epistaxis is the symptom of HHT patients generally suffer the most from, often affecting their quality of life [22]. Severity can change with age, season, or various internal and external influences. Although various treatment options are available, by far most of them are still relatively short term solutions. Weber et al. (see Abstract OR12) showed 62% patients in a HHT cohort were vitamin D deficient and that deficient vitamin D levels are associated with higher epistaxis severity scores and longer bleeding duration compared to patients with normal vitamin D levels. This phenomenon has been described in earlier literature and might offer a simple treatment option to lessen epistaxis severity in a selected group of patients [23]. However, it should be considered whether this is not a causality loop in the case of HHT patients; patients with the most severe cases of epistaxis do not leave the house offering an explanation for the low vitamin D levels. Contis et al. (see Abstract OR14) showed promising results on epistaxis after treatment with 40 mg propranolol 2/day [24]. Concerns were raised regarding the potential to reduce cardiac compensation to hemorrhage. A phase 2 randomized trial (propranolol vs placebo) is planned.

Bevacizumab

Numerous posters and presentations were dedicated to treatment options involving bevacizumab. Varying applications and successes of bevacizumab treatment were discussed. Allred et al. (see Abstract OR5) showed treatment of high cardiac output with bevacizumab (5 mg/kg for at least 6 doses) could lead to a 13% reduction of cardiac index measured by right-heart catheterization and a decrease in cardiac output in 8 patients undergoing invasive hemodynamic assessment, although this was not significant. Dupuis-Girod et al. (see Abstract OR9) also showed positive long-temp results of Bevacizumab treatment for severe hepatic involvement in the Metafore study; 2 patients showed no response but are alive without liver transplantation, 5 showed a complete response, 3 were retreated with bevacizumab, 17 showed a partial response of whom 14 are alive, 1 awaiting liver transplantation and 2 have undergone liver transplantation. They conclude that a second or third treatment cycle or maintenances therapy with Bevacizumab are possible [25]. Iyer et al. (see Abstract OR6) presented multi-year clinical experience

with 34 patients, of whom 16 red blood cell transfusion-dependent, treated with intravenous bevacizumab for chronic nose and gastrointestinal bleeds. The majority of patients required additional doses in the maintenance faze, with a mean of 2.5 doses a year. The treatment interval used in this study was an induction scheme of 6 treatments every two weeks followed by a maintenance scheme of treatment every 4 weeks until week 26. The effect after stopping the treatment should be evaluated. This treatment was proven to be very effective with cessation of red blood cell transfusions and maintenance of adequate hemoglobin and iron levels in 80% of patients. Meir-Zahav et al. (see Abstract OR17) presented a small case series of 4 patients, with pulmonary arterial hypertension or pulmonary hypertension (PH) secondary to hepatic vascular malformations and high cardiac output, treated with Bevacizumab 5 mg/kg every 2 weeks for 6 courses. Retrospective analysis showed a significant decrease in pulmonary pressures and cardiac output in the PH patients, but not in the PAH patient. Dupuis-Girod et al. (see Abstract OR15) showed that the ALEGORI study, a randomized control phase II/III trial using Bevacizumab in a nasal spray, confirms outcomes of the NOSE study: Bevacizumab nasal spray does not work to reduce monthly epistaxis duration [26]. This may be due to the lack of effective transdermal delivery of the large Bevacizumab molecule in this form of application.

Other presentations of interest

Finnamore et al. (see Abstract OR7) reminded us of the importance of using dietary iron, as dietary intake is often below even the general population recommendations [27]. Furthermore, patients may choose to avoid specific food items that aggravate their epistaxis like food that is high in salicates [28].

Serra et al. (see Abstract OR22) described patient characteristics of HHT patients suffering from overt gastrointestinal involvement, making up a large proportion of the their HHT population. Predominant characteristics were postmenopausal women and proximal localization.

Vorselaars et al. (see Abstract OR26) reminded us that *SMAD4* pathogenic variants are associated with aortic dilation [29]. Significant aortopathy, both dilation of the aortic root and ascending aorta, was found in patients with a *SMAD4* mutation compared to HHT patients with other mutations and controls. The discussion rose whether HHT due to a *SMAD4* mutation might have more in common with other connective tissue diseases, like Loeys-Dietz syndrome, than previously thought.

McDonald et al. (see Abstract OR29) reported a patient with a mosaicism in whom the disease-causing mutation was not found in peripheral blood or saliva, but was present in hair bulbs. This suggests that mosaicism should be considered in patients/families with classic HHT but "negative" genetic results in the oldest clinically affected family member.

Boillot et al. (see Abstract OR8) showed long term outcomes of 15 HHT patients who underwent liver transplantation as a result of complications of liver involvement of HHT. After transplantation clinics and quality of life normalized, however it was suggested there was radiological recurrence of HHT in 8/15 with a delay of 12 years (6–15), which were asymptomatic in all but one [5].

Lenato et al. (see Abstract OR37) analyzed hepatic involvement and atrial fibrillation in geriatric patients. 68.4% of patients had chronic anemia 14.4% had signs of chronic atrial fibrillation. Elevated levels of gamma GT seemed to be a predictor for atrial fibrillation.

Workshop 1: immunity, injury, and inflammation in HHT and HHT vessels

Claire L. Shovlin and Luisa M. Botella

These relatively new topics for the HHT field were discussed in an interactive, joint clinical and scientific workshop. The known HHT pathogenic gene variants (in *ENG*, *ALK1* and *SMAD4*) affect proteins expressed on endothelial cells, but it is often overlooked that all three proteins are also co-expressed by other cell types, including the hemangioblasts [30] that give rise to endothelial, myeloid and lymphoid lineages, and in macrophages following the process of monocyte-macrophage differentiation [31].

The first workshop section considered *immunity*. In an illustrative exercise, HHT pathogenic variants in *ENG* and *ALK1* were initially postulated to modify immune responses in different ways based on the higher prevalence of brain abscesses and other unusual infections in HHT1/*ENG* patients. The importance of recognizing potential confounders was then emphasized: (i) Brain abscesses and other deep-seated infections are predominantly found in HHT patients with PAVMs [32, 33], and PAVMs are more common and severe in HHT1 patients; (ii) Bacteremia (infected blood) is normal after dental and other procedures [34], with PAVM-associated abscesses attributed to impaired pulmonary capillary removal of infected blood-borne particles [32, 33]; (iii) In the general population, bacterial infections are more severe in the setting of high iron levels [35], which, counterintuitively, are often found in HHT patients who use iron treatments and have transient iron overload states [33]. Nevertheless, the infections observed are unusual, and the discussion concluded with laboratory data demonstrating lesser magnitude responses by lipopolysaccharide (LPS)-stimulated macrophages from a myeloid-specific ENG knockout model (Engfl/fl LysMCre mice): The ENG-deficient macrophages demonstrated lower expression of proinflammatory

cytokines IL-1, IL-12, IL-6, CCL-20, and thrombospondin 1 compared to those with normal ENG expression [2]. The workshop presentations increased the proportion of participants who considered that HHT immunity was weaker than normal from 19 to 37%, although the most common response from patients was that in their day-to-day experience, their immune systems seemed stronger.

The second section reviewed laboratory data that *injury* increases endothelial expression of ENG and ALK1 [36, 37], and that vascular repair is abnormal if ENG or ALK1 are deficient [8]. HHT-independent injuries (such as external trauma [37]; mechanical stretching of vessels during respiration and peristalsis; gastrointestinal tract acidity; infection), and HHT-specific injuries were discussed. The latter include locally modified flow through HHT vessels, generally increased flow through all vessels in response to the high cardiac outputs [38], and the emerging evidence that therapeutic iron treatments required by most HHT patients may directly injure the endothelium [39]. In unbiased, replicate surveys, approximately 1 in 20 HHT patients using the treatments reported that iron tablets or infusions precipitate nosebleeds [40, 41]. The presentations increased the proportion of participants considering HHT patients respond less well to injury from 44 to 66%, but the most common response from patients was that their responses were no different to normal.

In the final workshop section, *inflammation* was discussed in more detail. Impaired resolution of inflammation in pan-ENG heterozygous mice, in a chronic colitis model [42], is now supplemented by similar findings in mice with myeloid-specific ENG deficiency [2]. ENG-deficient macrophages from Engfl/fl LysMCre knockout mice displayed reduced cytokine expression in response to peritoneal LPS (as noted above), and reduced phagocytic activity [2]. While the ENG-deficient mice were more likely to develop spontaneous infections by opportunistic bacteria, they also demonstrated better survival in the LPS/septic shock model, attributed to less exuberant inflammatory responses [2].

The take-home messages from the workshop were the importance of future immunophenotyping of HHT patients, and incorporation of the discussed processes into HHT pathogenic models: 85% of attendees thought that immunity and inflammation would influence the development of abnormal blood vessels in HHT.

Workshop 2: ALK1 and ENG signalling in HHT

Helen Arthur and Franck Lebrin

The goal of this workshop was to consider how signaling was altered following loss of ALK1 or ENG activity and how this contributed to HHT. A series of questions were discussed in turn.

Does TGFβ signalling have a role in HHT?

Mouse genetic studies have shown that loss of TGFβ1 ligand or TGFβ receptors (*Tgfbr1* or *Tgfbr2*) lead to yolk sac angiogenesis defects and an early embryonic lethal phenotype that are very similar to embryos which are null for one of the main HHT genes (*Alk1* and *Eng*). However, this is a common phenotype seen in a range of knockout mice and does not necessarily mean TGFβ1 signaling is directly involved in HHT. Also, analysis of the mouse neonatal retina has revealed that arteriovenous malformations form following postnatal loss of endothelial *Eng* (Eng-iKOe) or *Alk1* (Acvrl1-iKOe) [43, 44], and this phenotype is very similar to retinas where both BMP9 and BMP10 activity is blocked by antibody treatment of mouse pups [3]. In contrast, endothelial specific depletion of Tgfbr2 in the neonatal retina does not lead to AVMs, but results in failure of intraneural migration of the vasculature [45]. As AVMs are a very specific phenotype this is strong evidence that BMP9 and BMP10 are in the same vascular developmental pathway as ENG and ALK1. It is interesting that overexpression of ALK1 can rescue the AVM defects of Eng-iKOe retinas (in work presented at this meeting by Paul Oh) consistent with evidence that ENG helps to promote ALK1 signaling by facilitating ligand binding, and this role can potentially be bypassed if excess ALK1 protein is present on the endothelial cell surface. In vitro, extracellular domains of ENG and ALK1 bind BMP9 and BMP10 with high affinity, with recent structural studies of the ENG-BMP9 protein complex showing not only the sites of ENG-BMP9 interaction, but also predicted sites of ALK1 binding [11]. In contrast, ENG can only bind TGFβ1 when it is in complex with TGFBR2 protein. Taken together with the HHT-like phenotype of patients with *BMP9* mutations, it was agreed that the evidence points to the predominance of defects in the BMP9/10-ENG-ALK1 signaling axis in HHT.

However, these considerations do not mean TGFβ1 signaling has no role in HHT. ENG and ALK1 could be playing an important role in regulating the balance of BMP and TGFβ1 signaling in endothelial cells, by promoting activation of the BMP-SMAD1/5/8 pathway and countering the TGFβ1-SMAD2/3 pathway. Without the correct balance, endothelial cells are not able to undergo appropriate vascular growth and remodeling in response to angiogenic stimuli. We have known for some time that TGFBR1 (also known as ALK5) is down-regulated in *Eng* +/- mice, perhaps to compensate for reduced ALK1 signaling and help maintain the correct balance [46]. Therefore, TGFBR1 levels may influence progression of the clinical disease. In support of this idea, genetic variants of the *Adam17* gene affect rescue of the lethal embryonic phenotype of the *Tgfb1* null mouse. Variants at the *ADAM17* locus were subsequently shown to be associated with the presence of pulmonary AVM

in HHT1 patients, potentially by altering the shedding of TGFBR1 protein [47]. This finding supports the premise that the balance of TGFβ signaling is important in HHT.

One intriguing factor in this regard is SMAD4, as this protein plays a central role in both SMAD1/5/8 and SMAD2/3 pathways. It is required for nuclear translocation of phosphorylated SMAD proteins of both pathways enabling their transcriptional role downstream. Loss of function mutations in *SMAD4* are associated with an HHT-juvenile polyposis syndrome phenotype, exemplifying both the vessel-protective role of ENG/ALK1 signaling through SMAD1/5/8 and the tumor suppressive function of TGFβ signaling through SMAD2/3. However, (as reported independently by two groups at this meeting—Ola et al. and Meadows et al.) loss of *Smad4* in endothelial cells in the postnatal mouse retina leads to a very similar AVM phenotype to the HHT models discussed above. This suggests that BMP9/10-ALK1 activated SMAD1/5/8 signaling predominates in endothelial cells over TGFβ1-ALK5 activated SMAD2/3 signaling. However, this predominance may be limited to the early stages in retinal development, and it will be important to discover whether *Smad4* endothelial specific knockout mice develop AVM phenotypes in adult models of HHT such as the dermal wound healing model and VEGF stimulated cerebral model. To bring this argument full circle, HHT patients with *SMAD4* mutations were reported in this meeting with dilatation of the aortic root, a typical phenotype of Marfan and Loeys–Dietz patients. Loeys–Dietz Syndrome (LDS) is caused by mutations in genes of the TGFβ signaling pathway (*TGFBR1*, *TGFBR2*, *SMAD3*, *TGFB2*, *TGFB3*) which suggests that consideration of SMAD4 activity is important for both HHT and LDS clinical phenotypes. As was pointed out at the workshop, the more clinicians examine their patients the more they will see—it is the same for geneticists using pre-clinical disease models. Careful observation is paramount!

Is BMP9 anti-angiogenic or pro-angiogenic?

ENG and ALK1 are required for angiogenesis. In fact, ALK1-ECD-Fc ligand trap fusion proteins (eg Dalantercept) and anti-ENG antibodies (egTRC105) are in clinical trials to block activity of these receptors in anti-angiogenic cancer therapy. Yet, on the other hand, BMP9 appears to be a vascular quiescence factor that blocks VEGF stimulated angiogenic responses, with loss of BMP9and BMP10 activity leading to hypervascularity [48]. These opposing roles are paradoxical and challenging to reconcile. If BMP9/10, ENG and ALK1 are all in the same signaling pathway, why should signaling be both pro- and anti-angiogenic? It is also counter-intuitive to have relatively high circulating levels of vascular quiescent factors BMP9 and BMP10 at birth (with BMP9 continuing to rise postnatally), just at the same time as the postnatal vasculature is actively growing by angiogenesis. Discussion revolved around the possibility that we are considering angiogenesis in too simplistic a way. Angiogenesis is not "black and white". There is the classical sprouting and branching of new cells driven by VEGF that occurs via leading tip cells (providing directionality) and proliferating stalk cells (providing growth and expansion of the vascular plexus). On the other hand there also has to be a neovessel maturation step and this is where a quiescence factor such as BMP9 could be important. Although evidence from animal models suggests HHT is triggered by angiogenesis, defects in the maturation step could lead to vascular malformations typical of HHT. Considering angiogenesis in cancer, tumor neo-vessels have immature features and are leaky. If BMP9 promotes vascular stability then blocking BMP9 signaling with ALK1-ECD or anti-ENG may initiate vascular leakage allowing more efficient diffusion of drugs to tumor cells. Anti-ENG therapy also leads to release of soluble ENG so there will be indirect as well as direct consequences on the BMP9-ENG-ALK1 signaling axis. Overall, more careful consideration is needed of the role of this pathway in angiogenesis. For example, in females, angiogenesis is integral to the menstrual cycle and to the growth of the uterus in pregnancy, but there is no particular association of uterine AVMs in HHT patients. Furthermore, we still do not understand why different vascular beds (eg lung, liver, brain) show different susceptibilities for AVM occurrence in HHT. There is clearly much more work to do to understand the link between angiogenesis and HHT.

Is HHT an abnormal response to angiogenic signals?

Although angiogenesis is important, it could be altered vessel tone that plays a central role in AVM formation. A key regulator of vasodilation is endothelial nitric oxide synthase (eNOS), catalyzing release of nitric oxide (NO) for diffusion to neighboring SMCs to promote relaxation of resistance arteries. On the other hand, heterozygosity for *Eng* or *Alk1* mutations leads to eNOS uncoupling and the generation of increased superoxide (O2−) levels that could potentially inhibit vasoconstriction. There is still work to do to fully understand the role of eNOS in HHT. Interestingly, reversible cerebral vascular shunts are induced in vivo following activation of Notch signaling, and involve vasodilation of small vessels [8] via an eNOS-dependent mechanism. However, any role for Notch signaling in AVM formation in HHT remains to be elucidated. Recent work using zebrafish embryos showed that increased endothelial cell size was involved in shunt formation [7]. However in this case, vasoregulatory processes could not be involved as there are no pericytes or vascular smooth muscle cells associated with the affected vessels in these early embryos. In addition, recent work using mosaic analysis of Eng-iKOe postnatal

retinas revealed reduced migration of endothelial cells from the venous to the arterial side of the vascular network and the first visible changes prior to AVM formation occur at the site of the pre-capillary arteriole [6]. Further development of the AVM involves endothelial cell proliferation. Perhaps, the pre-capillary arteriole is where we should be focusing our investigations on AVM initiation. Vasorelaxation at this site would be expected to lead to increased blood flow and it has already been shown in zebrafish that blood flow exacerbates the development of AVMs [49]. In this way, the first step in forming an AVM may be vasorelaxation/or failure to effectively constrict a pre-capillary arteriole. This would increase blood flow, which may further enlarge the AVM by promoting increased endothelial cell proliferation. It will be important to determine all the different contributions of cell autonomous and external factors that contribute to an AVM.

It is also interesting to consider that the pre-capillary arteriole is also the primary site that is affected in lungs in pulmonary artery hypertension (PAH) and that some cases of PAH are due to *ALK1* mutations, though the majority of familial forms are due to mutations in *BMPR2*. Although the problem in PAH is one of vaso-occlusion rather than vessel enlargement, both HHT and PAH involve deregulated vascular cell proliferation. As the overlaps between HHT and PAH increase, and we better understand their etiology, there may be lessons to learn from current therapies for PAH patients that can be applied to HHT patients.

Although reported in the literature that HHT patients have higher circulating VEGF levels than the control population, this observation has to be viewed with caution. When using serum measurements, it has to be remembered that VEGF is found in platelets and may be released during the clotting process. VEGF protein in platelet free plasma provides a far more accurate measure of circulating VEGF. Workshop participants reported that neither adult nor pediatric HHT patients routinely have high circulating VEGF levels, but that increased VEGF is only seen when there are pulmonary AVMs or high levels of bleeding and is therefore likely to be a secondary response to hypoxia. On the other hand, several recent studies in mouse models of HHT have observed increased VEGF signaling in endothelial cells. The molecular mechanisms are varied: altered PTEN phosphorylation in the absence of *Alk1* [50], and altered VEGFR2 receptor recycling in the absence of *Eng* [6]. Further investigations are required to determine cell autonomous mechanisms of altered VEGF signaling in HHT.

Workshop 3: management of hepatic vascular malformations

Elisabetta Buscarini and Sophie Dupuis-Girod

The prevalence of hepatic malformations (HVMs) is particularly high in patients with a pathogenic *ACVRL1* mutation; whereas only 8% have symptomatic HVMs in cross sectional studies. Longitudinal series have shown an incidence of 3.6% per year of complications due to liver VMs in HHT. Only patients with symptomatic HVMs should be treated. The first line treatment consists of (I) treatment of high output heart failure should, similar to what is done for heart failure outside HHT, with special emphasis on escalating diuretics dosages; (II) treatment of portal hypertension is equal to the treatment of biliary cirrhosis, and (III) antibiotics in case of cholangitis. The results of these first line intensive treatments are to be judged within 6–12 months. If complicated liver VMs are refractory to first line treatment, liver transplantation is to be evaluated, whereas trans-arterial embolization is generally not an option due to its palliative effect and inherent risk of liver necrosis. Main indications for liver transplantation in the cases so far reported in literature have been high output heart failure, complicated portal hypertension and biliary ischemia. Literature data show that liver transplantation for complicated liver VMs has good outcome resulting in hemodynamic and clinical normalization and improved quality of life. Available data suggest that liver transplantation should be timely proposed before pulmonary resistances become fixed, and taking into account that complicated liver VMs in HHT represent a MELD-exception for transplantation. Boillot addressed surgical particularities in liver transplantations for HHT patients. Amongst other, the recipient hepatic artery should be removed and the donor artery should anastomosed to the origin of the recipient hepatic artery on the coeliac trunk, in order to prevent hepatic artery aneurysms, rupture and/or thrombosis. The use of Bevacizumab before liver transplantation does not change the surgical procedure but the interval between Bevacizumab and transplantation should be preferably 2–3 months. Although opinions were divided, on the basis of literature data, it was concluded that liver transplantation might be indicated in patients under 65 years old; in patients younger than 65 years not fit for transplantation, Bevacizumab could be tried and used as a "bridge-to-OLT" if patients respond; In patients older than 65 Bevacizumab can be proposed, with following maintenance dosages if patient respond [51]. However, when the audience was asked whether they would choose for bevacizumab or liver transplantation the majority of the audience would start with bevacizumab treatment.

Looking forward

This year's meeting highlighted the many advances that have been made in developing new models, detecting new causative mutations, clarifying signaling pathways, identifying

targets for therapeutics and treating patients. Each of these new insights has brought us closer to understanding important, unanswered questions. How do telangiectases and AVMs form? Does initiation occur during vascular development, or do injury and inflammation play an important role? Is it a requirement to completely lose the second, wildtype allele to form a lesion, or is a decrease in protein levels below a certain threshold enough? What are the specific roles of ALK1 and ENG, and how do they differ? What are the best treatment strategies to manage AVMs and epistaxis? Answering these questions will help to define the future of HHT research and therapeutics, and ultimately bring us closer to a cure.

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