Decreased Levels of MicroRNAs-28-5p, -361-3p and Increased Insulin-Like Growth Factor 1 mRNA Levels in Mononuclear Cells from Hereditary Hemorrhagic Telangiectasia Patients

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Decreased Levels of MicroRNAs-28-5p, -361-3p and Increased Insulin-Like Growth Factor 1 mRNA Levels in Mononuclear Cells from Hereditary Hemorrhagic Telangiectasia Patients

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Abstract

Hereditary hemorrhagic telangiectasia (HHT) is a rare vascular disorder inherited in an autosomal dominant manner. Patients with HHT can develop vascular dysplasias called telangiectasias and arteriovenous malformations (AVMs). Our objective was to profile and characterize microRNAs (miRs), short-noncoding RNAs that regulate gene expression post-transcriptionally, in HHT patient derived peripheral blood mononuclear cells (PBMCs). PBMCs, comprised mostly of lymphocytes and monocytes, have been reported to be dysfunctional in HHT. A total of 40 clinically confirmed HHT patients and 22 controls were enrolled in this study. PBMCs were isolated from 16 ml of peripheral blood and purified for total RNA. MiR expression profiling was conducted with a human miR array analysis. Select dysregulated miRs and miR targets were validated with RT-qPCR. Of the 377 miRs screened, 41 dysregulated miRs were identified. MiR-28-5p and miR-361-3p, known to target insulin-like growth factor 1 (IGF1), a potent angiogenic growth factor, were found to be significantly downregulated in patients. Consequently, IGF1 mRNA levels were found to be significantly elevated. Our research successfully identified miR dysregulation and elevated IGF1 mRNA levels in HHT PBMCs. This novel discovery represents a potential pathogenic mechanism that could be targeted to alleviate clinical manifestations of HHT.

Key words: hereditary hemorrhagic telangiectasia (HHT), microRNA (miR) dysregulation, peripheral blood mononuclear cells (PBMCs)
**Introduction**

Hereditary hemorrhagic telangiectasia (HHT) is a rare, autosomal dominant, vascular disorder that affects 1 in 5000 to 10,000 people worldwide (Kjeldsen et al. 1999; Donaldson et al. 2014). HHT is characterized by angiogenic dysregulation leading to the formation of telangiectasias and arteriovenous malformations (AVMs). Telangiectasias, occurring in skin and mucocutaneous tissue (Shovlin et al. 2000; Faughnan et al. 2011) and organ arteriovenous malformations (AVMs) are direct connections between arteries and veins, without intervening capillary beds. Approximately 90% of HHT patients develop nasal telangiectasias that can cause chronic epistaxis, and at least 20% develop chronic bleeding from gastrointestinal telangiectasias (Shovlin et al. 2000; Faughnan et al. 2011). AVMs may develop in the pulmonary (PAVM), hepatic (LVM) and cerebral vasculature (CAVM), and put the patient at risk of life-threatening hemorrhage, as well as complications due to shunting, such as stroke, brain abscess and high-output cardiac failure (Shovlin et al. 2000; Faughnan et al. 2011; Guttmacher et al. 1995).

HHT arises from heterozygous mutations in at least 3 known genes, endoglin (ENG, chromosomal locus 9q34), activin receptor-like kinase 1 (ACVRL1, chromosomal locus 12q1), and mothers against decapentaplegic homolog 4 (SMAD4, chromosomal locus 18q21) (McAllister et al. 1994; Johnson et al. 1996; Gallione et al. 2004). These genes encode proteins that are involved in the transforming growth factor beta (TGFβ)/bone morphogenetic protein (BMP) signalling pathway. ENG encodes for a TGFβ co-receptor, while ACVRL1 encodes for a TGFβ Type I receptor (Pardali et al. 2010; Goumans et al. 2009). ENG and ACVRL1 mutations define HHT Type 1 (HHT1) and 2 (HHT2), respectively, and generally display distinct clinical manifestations, although overlap of the clinical spectrum is not uncommon. SMAD4 encodes for an intracellular TGFβ signalling protein and mutations in SMAD4 typically result in Juvenile Polyposis syndrome in addition to HHT (JP-HHT) (Johnson et al. 1996).

Haploinsufficiency of the respective gene products dysregulate TGFβ/BMP signalling, adversely affecting endothelial cell proliferation, migration, cell recruitment and differentiation during angiogenesis (Bourdeau et al. 2000; Abdalla & Letarte, 2005; Lebrin et al. 2004). Additionally, homozygous knockdown of ENG or ACVRL1 in adult mice results in an HHT phenotype, confirming their role in HHT pathogenesis (Park et al. 2009; Choi et al. 2014; Garrido-Martin et al. 2014). Various cells in the inflammatory/repair response, including lymphocytes,
monocytes/macrophages, neutrophils and leukocytes, rely on TGFβ signalling, endoglin and activin receptor-like kinase 1 for normal function (Shull et al. 1992; Kulkarni et al. 1993; Larsson et al. 2001; Ishida et al. 2004; Doetschman et al. 2012; Dingenouts et al. 2015). It has been hypothesized that AVM formation involves an impaired response to injury that may include the dysfunction of peripheral blood mononuclear cells (PBMCs) (Dingenouts et al. 2015).

PBMCs are composed mostly of lymphocytes and monocytes, and have been implicated in HHT pathogenesis (Verhoeckx et al. 2015; Post et al. 2010). In vivo HHT1 mouse models have demonstrated that aberrant TGFβ signalling impairs the homing capacity of PBMCs to sites of inflammation (Post et al. 2010). This reduced migratory capacity has been postulated to contribute to the development of angiogenic dysplasia, prolonged inflammation and increased infection rates observed in HHT patients (Guilhem et al. 2013; Peter et al. 2014). Lymphopenia or reduced levels of CD4/8 T and natural killer cells have also been reported in HHT patients (Guilhem et al. 2013). However, the exact role PBMCs play in HHT pathogenesis is still unclear.

MicroRNAs (miRs) are small (19 to 25 nucleotides long) endogenous non-coding RNA species that regulate gene expression post-transcriptionally through RNA interference. (Lagos-Quintana et al. 2001; Lee et al. 1993; Wightman et al. 1993) These highly conserved species act as guides that deliver enzymatic complexes to silence mRNAs. MiRs have been shown to silence mRNAs by two mechanisms that are dependent on miR-mRNA complementarity. Perfect complementarity leads to mRNA cleavage, while imperfect complementarity results in ribosome destabilization. Approximately 2000 miRs have been confidently identified in humans, and have been shown to be involved in a variety of human diseases, cellular functions, and pathways, including the TGFβ signalling pathway and angiogenesis (Wightman et al. 1993; Landgraf et al. 2007; Ardekani & Naeini, 2010; Butz et al. 2012; Hammond et al. 2015).

The expression and function of miRs associated with HHT remain largely unexplored. Our aim was to profile and characterize miRs in PBMCs from HHT patients through comparative miR array profiling and RT-qPCR validation. We hypothesize that the miR profile of HHT PBMCs will be dysregulated compared to controls, and hope our findings will contribute to the understanding of the pathogenetic mechanisms involved in the development of HHT.


**Materials and Methods**

**Patient Recruitment**

A total of 16 mL of peripheral blood was obtained from patients clinically diagnosed with HHT (according to Curaçao’s diagnostic criteria for HHT (Shovlin et al. 2000)) and age and gender matched controls from the Toronto HHT Centre at St. Michael’s Hospital, Toronto, Canada. A total of 40 HHT patients between the ages 18 and 65 were recruited. HHT patients who exhibited significant anemia (hemoglobin < 100g/L) or pregnancy were excluded from the study. Informed written consent was obtained from all participants involved in the study. All protocols that involved human samples were approved by the Research Ethics Board of St. Michael’s Hospital, University of Toronto in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

**Human Peripheral Blood Mononuclear Cell Layer (Buffy Coat) Isolation**

Peripheral blood was collected into two BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes and processed immediately. Blood samples were centrifuged at 4°C for 30 mins at 1650 g. Half of the uppermost layer of plasma was aspirated and the buffy coat collected. A total of 10 mL of cold (4°C) PBS was added to the buffy coat and centrifuged for 5 mins at 300 g to wash. The PBMC pellet was further processed for total RNA isolation.

**Total RNA Isolation of Peripheral Blood Mononuclear Cells**

A Qiagen® miRNeasy Mini Kit was used to isolate total RNA from PBMCs according to the manufacturer’s protocol. The PBMC pellet was resuspended in 700 µL of QIAzol lysis reagent and homogenized. The homogenate was incubated at room temperature (21-25°C) for 5 mins. 140 µL of chloroform was then added to the homogenate and vigorously shaken for 15 s. This mixture was incubated at room temperature (21-25°C) for 3 mins and then centrifuged at 4°C for 15 mins at 12000 g. The upper aqueous phase was transferred to a 1.5 mL Eppendorf tube and 1.5 volumes of 100% ethanol was added and thoroughly mixed. A maximum of 700 µL of this mixture was transferred to an RNeasy® Mini column and centrifuged at 8000 g for 15 s at room temperature (21-25°C). The eluate was discarded. This was repeated with the remainder of the sample. The column was washed with buffers RWT and RPE in series via centrifugation. Finally, 40 µL of RNase free water was added to the column and centrifuged at 8000 g for 1 min.
to elute RNA. RNA quality and concentration was assessed with a NanoDrop™ 2000 spectrophotometer. RNA samples were then stored at -80°C until analysis. All equipment used was DNase, RNase and pyrogen free.

**MicroRNA Array Analysis of PBMC Total RNA**

An Applied Biosystems TaqMan™ Human MicroRNA Array covering 377 miRs (Card A v2.0) was used to profile miRs in PBMCs. Prior to miR profiling, the samples were reverse transcribed into cDNA with a TaqMan™ MicroRNA Reverse Transcription (RT) Kit and Megaplex™ RT Primers. The RT reaction had a total volume of 7.5 µL that included 3 µL of total RNA (600 ng), 0.8 µL of Megaplex™ RT primers (10x), 0.2 µL of dNTPs with dTTP (100mM), 1.5 µL of MultiScribe™ reverse transcriptase (50 U/µL), 0.8 µL of 10x RT buffer, 0.9 µL of MgCl₂ (25 mM), 0.1 µL of RNase inhibitor (20 U/µL) and 0.2 µL of nuclease-free water. The thermocycling procedure was carried out as follows: 40 cycles of 16°C for 2 min, 42°C for 1 min and 50°C for 1 s, followed by 85°C for 5 min and lastly a 4°C hold step. The PCR reaction had a total volume of 900 µL that included 450 µL of 2x TaqMan™ universal PCR master mix (no AmpErase™ UNG), 444 µL of nuclease-free water and 6 µL of Megaplex™ RT product. Subsequently, 100 µL of the PCR reaction was dispensed into each port of the Array Card A v2.0. Following the manufacturer’s instructions the card was sealed and centrifuged. The PCR reaction was carried out with a 384 well TaqMan™ Low Density Array block on a ViiA 7 Real-Time PCR System (Applied Biosystems). PCR and thermocycling parameters were automatically inputted by the included SDS v2.2 setup file (SDS.txt). Results were analyzed using the RQ Study software (Applied Biosystems™) and normalized to U6 RNA. MiRs of interest were selected based upon a fold change of 1.5 and greater and/or consistency amongst samples (Liu et al. 2012; Sokolov et al. 2012).

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis (RT-qPCR) for MiRs**

RT-qPCR was performed to validate select dysregulated miRs identified by the miR array analysis. Total RNA samples were reverse transcribed into cDNA with a TaqMan™ MicroRNA RT Kit (Applied Biosystems). Reaction components included: 0.15 µL of dNTPs (100 mM), 1 µL of MultiScribe™ Reverse Transcriptase (50 U/µL), 1.5 µL of RT Buffer (10x), 0.19 µL of
RNase inhibitor (20 U/μL), 4.16 μL of nuclease-free water, 3 μL of RT primer (5x) and 5 μL of the RNA sample (total RNA 20 ng). RT reaction was carried out as follows: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and 4°C hold. MiR qPCR was done in a total volume of 10 μL with a Real-Time PCR Assay Kit (Applied Biosystems). The reaction contained: 1 μL of RT product, 0.5 μL of PCR primer (20x), 5 μL of 2x TaqMan™ universal PCR master mix (no AmpErase™ UNG) and 3.5 μL of nuclease-free water. The PCR reaction was carried out on a ViiA 7 Real-Time PCR System (Applied Biosystems) as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification of individual miR expression was carried out with the $2^{-\Delta\Delta CT}$ method and normalized against endogenous U6 RNA.

**MicroRNA Target Identification**

The characterization of miR function was accomplished by the use of published literature derived from online journal databases, namely, Google Scholar and PubMed. MiR targets were identified and cross-referenced using published literature and online miR databases, including miRTarBase (Chou et al. 2018), mirDIP (Tokar et al. 2018), mirPath v.3 (Vlachos et al. 2015) and TarBase v.8 (Karagkouni et al. 2018).

**RT-qPCR for mRNA**

A total of 500 ng of purified RNA was used for RT with the Omniscript RT Kit (Qiagen). The total reaction volume was 20 μL and contained the following components: 2 μL of 10x RT buffer, 1 μL (10 U) of RNase inhibitor, 200 ng of random primer (Qiagen), 2 μL of 5mM dNTPs, 1 μL (4 U) of reverse transcriptase and nuclease-free water was added to adjust the volume. The RT thermocycling protocol was as follows: 37°C for 1 hr followed by a 5 min, 85°C incubation period to inactivate the enzyme. For ENG and ACVRL1 mRNA expression a SYBR-Green Gene Assay Kit (Thermo Fisher Scientific) was used. The sequences of the primers were as follows: ENG, 5’-AGCTGACTCTCCAGGCATCC-3’ (forward), 5’-GCAGCTCTGTGTTGACC-3’ (reverse); ACVRL1, 5’-GTGAGAGTGTGGCCGTC-3’ (forward), 5’-CATGTCTGAGGCGATGAAGC-3’ (reverse); and β-actin, 5’-AGCCTCGCCTTTGCGGTC-3’ (forward), 5’-CTGGTGCGCTGGGCG-3’ (reverse). A total reaction volume of 10 μL was used for qPCR that contained the following components: 5 μL of 2x PowerUp™ SYBR™ green master mix, 1 μL of RT product, 200 nM of each primer and
nuclease-free water was added to adjust the reaction volume. A ViiA7 qPCR System (Thermo Fisher Scientific) was used to perform qPCR. The thermo-cycling protocol used was 95 °C for 10 min and 40 cycles of 95°C for 15 Sec and 60°C for 1 min. A dissociation curve was generated for each reaction to determine the specificity of amplification. Normalization against β-actin was carried out to determine the relative levels of target genes with the $2^{-\Delta\Delta C_t}$ method.

A TaqMan™ reporter probe was used to measure expression levels of IGF1 mRNA in HHT patient and control PBMCs. Primer sequence for the target was (5’-3’): IGF1 forward GCTCACCTTCACCAGCTCTGCCA; and IGF1 reverse TCCGACTGCTGGAGCCATACC. The total volume of the qPCR reaction was 10 µL and contained the following components: 2 µL RT product, 0.5 µL TaqMan™ primer, 5 µL of 2x TaqMan™ universal PCR master mix (no AmpErase™ UNG) and 2.5 µL of nuclease-free water. The qPCR thermo-cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 1 min. Normalization against β-actin was carried out to determine the relative levels of target genes with the $2^{-\Delta\Delta C_t}$ method.

**Enzyme-Linked Immunosorbent Assay (ELISA) for IGF1 Plasma Protein**

A human IGF1 ELISA kit (Abcam, ab100545) was used to measure plasma IGF1 protein levels in HHT patients and controls. All reagents, standards and samples were prepared according to the manufacturer’s instructions. A total of 100 µL of plasma sample and standard were added to the appropriate wells and incubated with gentle shaking for 2.5 hours at room temperature. After incubation the solution was discarded and the wells were thoroughly washed 4 times with 300 µL of 1X Wash Solution. After the final wash, residual liquid was removed and 100 µL of 1X Biotinylated IGF1 Detection Antibody was added to each well and incubated for 1 hour at room temperature with mild shaking. After incubation the solution was discarded and the wash step repeated. 100 µL of 1X HRP-Streptavidin was added to each well and incubated with gentle shaking for 45 min at room temperature. The solution was discarded, the wash step repeated and 100 µL of TMB One-Step Substrate Reagent was added to each well and incubated with gentle shaking for 30 min in the dark. 50 µL of Stop solution was then added to each well. The optical density at 450 nm was read using an eMax ELISA Microplate Reader. Data was analyzed by calculating the mean value of duplicated readings for each standard and sample.
Statistical Analysis

A two-tailed Student’s $t$-test was used to determine significant differences between the mean of HHT patient and control groups. A $p$-value ($P$) of $<0.05$ was determined to be statistically significant.

Results

Participants Enrolled

A total of 40 clinically confirmed HHT patients were enrolled in this study. The mean age for the HHT patient group was 45.2 ± 11.1 (27 females and 13 males) and the mean age of the control group (n=22) was 46.2 ± 12.9 (15 females and 7 males, Table 1). HHT patients with PAVMs were diagnosed by thoracic CT or pulmonary angiography. Patients with CAVMs were diagnosed by MRI and those with LVMs by contrast-enhanced computed tomography, MRI or Doppler ultrasonography. Patient mean age, gender, AVM type and genotype of the HHT patient group are shown in Table 1.

Decreased ENG and ACVRL1 mRNA Levels in HHT Patient PBMCs

ENG and ACVRL1 mRNA levels in HHT patient and control PBMCs were relatively quantified by RT-qPCR, normalized using β-actin. ENG and ACVRL1 mRNA levels were found to be significantly lower in HHT patient PBMCs compared to controls, $P < 0.01$ and $<0.05$, respectively (Figure 1). Relative levels of ENG mRNA in the HHT and control group were, 0.007±.0047 and 0.02±.013, respectively, and ACVRL1 mRNA levels in the HHT patient and control group were, 0.0004±.0004 and 0.0015±.001, respectively. PBMCs displayed higher levels of ENG expression (Combined mean Ct of HHT and control groups: 22.4) compared to ACVRL1 (Combined mean Ct of HHT and controls groups: 26.7) in both HHT patients and controls. In addition, ENG and ACVRL1 mRNA levels were consistently decreased in both HHT1 (n=8) and HHT2 (n=8) patients.

Dysregulated MiR Array Profile in HHT Patient PBMCs

A total of 377 human miRs were analyzed in the array analysis. The analysis returned 41 dysregulated miRs defined as having a fold-change of 1.5 and greater, and 14 miRs were selected based on consistency amongst samples. The dysregulated miRs included miR-1-3p, -
20b-5p, -28-5p, -29c-3p, -30b-5p, -133a-3p, -143-3p, -214-3p, -361-3p, -374a-5p, -518d-3p, -618, -654-5p, and -708-5p (Figure 2). Of these miRs, 9 were upregulated (miR-1-3p, -20b-5p, -30b-5p, -133a-5p, -361-3p, -374a-5p, -618, -654-5p and -708-5p) and 5 were down-regulated (miR-28-5p, -29c-3p, -143-3p, -214-3p and 518d-3p). MiRs-28-5p, -30b-5p, 361-3p and 374a-5p were selected for RT-qPCR validation because they were the most consistently dysregulated amongst samples. Relative levels of all 14 dysregulated miRs are shown in Supplementary Figure 1.

**Decreased Levels of MiR-361-3p and MiR-28-5p in HHT Patient PBMCs**

Level of miRs -28-5p, -30b-5p, -361-3p and -374a-5p were validated by RT-qPCR, using specific primers and U6 RNA as normalization, in HHT patients and controls. As shown in Figure 3A and 3C, RT-qPCR validation returned significant changes of miR-28-5p and -361-3p levels between HHT patients and controls. Interestingly, miR-361-3p was determined to be upregulated by the array analysis, but downregulated as determined by RT-qPCR. It is well known that variability exists between arrays and RT-qPCR, but RT-qPCR remains the gold standard for the relative quantification of miRs and is necessary to validate miR array data (Git et al. 2010; Chugh & Dittmer, 2012). MiR-30b-5p and -374a-5p levels were not found to be significantly different between HHT patients and controls (Figure 3B and D, respectively).

Relative levels of miR-30b-5p were 0.146 ± 0.021 and 0.164 ± 0.019 for HHT patient and control groups, respectively. MiR-374a-5p demonstrated relative levels of 0.067 ± 0.026 and 0.079 ± 0.008 for HHT patient and control groups, respectively. However, miR-28-5p and 361-3p were found to be significantly decreased in HHT patient PBMCs compared to controls, \( P < 0.05 \), (Figure 3A and C, respectively). Relative levels of miR-28-5p were 0.009 ± 0.002 and 0.013 ± 0.001 for HHT patient and control groups, respectively. MiR-361-3p returned relative levels of 0.002 ± 0.009 and 0.004 ± 0.001 for HHT patient and control groups, respectively. MiRs-28-5p and -361-3p were shown to both target IGF1 based on published literature and various miR databases (miRTarBase (Chou et al. 2018), mirDIP (Tokar et al. 2018), mirPath v.3 (Vlachos et al. 2015) and TarBase v.8 (Karagkouni et al. 2018)).

**Increased IGF1 mRNA Levels in HHT Patient PBMCs**

IGF1 mRNA levels were quantified by RT-qPCR and normalized to \( \beta \)-actin. Levels of IGF1 mRNA were significantly increased in HHT patients compared to controls \( (P < 0.01) \), shown in
Figure 4. Relative levels of IGF1 mRNA in HHT patients and controls were $2.1E-05 \pm 1.7E-05$ and $6.09E-06 \pm 4.2E-06$, respectively. PBMCs from HHT patients consistently displayed higher expression of IGF1 (Mean Ct: 29) compared to control PBMCs (Mean Ct: 31). Additionally, IGF1 plasma protein levels were not significantly different between HHT patient and control groups. Plasma IGF1 protein levels in HHT patients and controls were $95 \text{ng/mL} \pm 21$ and $105 \text{ng/mL} \pm 16$, respectively (Figure 5).

Discussion

HHT is a rare genetic disorder inherited in an autosomal dominant fashion whereby patients develop hemorrhagic vascular dysplasias called telangiectasias and AVMs. PBMCs are a heterogeneous cellular population, comprised of lymphocytes and monocytes, and provide an in vitro model for studying processes such as immunity, inflammation, vascular repair and angiogenesis (Dingenouts et al. 2015). PBMC dysfunction has been identified in HHT in the form of lymphopenia and reduced monocyte migration (Guilhem et al. 2013; Post et al. 2010); however, the exact role of PBMCs in HHT pathogenesis is still unclear. In addition, how miRs might be involved in HHT is not fully understood. Our goal was to profile and characterize miRs in HHT patient derived PBMCs.

Sanz-Rodriguez et al have identified reduced ENG upregulation in activated monocytes from HHT1 and HHT2 patients (Sanz-Rodriguez et al. 2004). In the present study, we determined that freshly isolated PBMCs from both HHT1 and HHT2 patients displayed significantly reduced ENG and ACVRL1 mRNA levels. Of interest, decreased ENG and ACVRL1 expression was exhibited in both HHT1 and HHT2 patients. This suggests a reciprocal relationship between the expression of ENG and ACVRL1.

Research on miR dysregulation in HHT is quite limited. We have previously identified elevated miR-210 levels in the circulation of HHT patients with PAVMs (Zhang et al. 2013), and have suggested that miR-210 may represent a valuable biomarker for the identification of PAVMs in patients with HHT. In the same year, a study by Tabruyn et al reported that miR-205, an inhibitor of TGFβ signaling, was downregulated in the circulation of HHT1 and HHT2 patients (Tabruyn et al. 2013). In this study, we identified miR dysregulation in PBMCs derived from HHT patients that included a significant downregulation of miRs-28-5p and -361-3p shown by RT-qPCR. MiRs-143-3p, -518d-3p, -618 and -654-5p demonstrated the largest fold changes, but
were not found to be enriched in HHT patient and control PBMCs. Additionally, these miRs are not well characterized and/or not known to play a functional role in PBMCs. MiR-361-3p has been shown to function in potently proangiogenic Tie2-expressing monocytes through the regulation of IGF1 (Wang et al. 2016). MiR-28-5p has also been shown to target IGF1, with the overexpression of miR-28-5p resulting in a significant reduction of IGF1 transcripts in hepatocellular carcinoma cells (Shi and Teng et al. 2015). Here we show that IGF1 mRNA levels are significantly increased in PBMCs from HHT patients compared to controls. Therefore, it is possible that the increase of IGF1 transcripts are a result of the decreased levels of miRs-28-5p and -361-3p. PBMCs were studied as a whole because of the importance of monocyte-lymphocyte interaction in the angiogenic process (Ramello et al. 2014; Naldini et al. 2015; Naldini et al. 2005). The study of individual cells from this population could limit the importance of this interaction.

PBMCs, specifically the monocyte fraction, have been shown to express and secrete IGF1 in a paracrine manner to modulate and coordinate various processes that include inflammation, vascular repair and angiogenesis (Delafontaine et al. 2004; Dimova & Djonov, 2017). Additionally, PBMCs have been reported to express the IGF1 receptor and are susceptible to IGF1 stimulation (Kooijman et al. 1992). Recently, the phosphatidylinositol 3-kinase (PI3K/AKT) pathway has been implicated in HHT pathogenesis, where its inhibition in an HHT2 mouse model attenuated AVM formation (Ola et al. 2016). It has been shown that IGF1 plays a predominant role in the regulation of angiogenesis through the stimulation of the PI3K/AKT pathway (Delafontaine et al. 2004; Bach, 2014; Friedrich et al. 2018). Our finding of similar IGF1 plasma protein levels between HHT patients and controls suggests that IGF1 may be overexpressed locally at sites of inflammation or wounding, thereby stimulating abnormal angiogenesis and potentially contributing to AVM formation without necessarily increasing systemically measured IGF1. This postulation is in line with the three event hypothesis that describes the requirement of a local angiogenic trigger, either inflammation, infection or wounding, for AVM formation to occur (Tual-Chalot et al. 2015). PBMCs and IGF1 could be involved in these processes and play an important role in HHT pathogenesis.
In conclusion, we identified miR dysregulation in PBMCs derived from HHT patients. The finding of decreased miR-28-5p and miR-361-3p, and elevated IGF1 mRNA levels, may represent an important pathogenic mechanism that warrants further study.

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Conflict of Interest

The authors declare no conflicts of interest.
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Table 1. Summary of HHT Patient and Control Demographics

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<th>HHT Patients (n=40)</th>
<th>Controls (n=22)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>45.2 ± 11.1</td>
<td>46.2 ± 12.9</td>
</tr>
<tr>
<td>Number (%) of Females</td>
<td>27 (67.5)</td>
<td>15 (68.2)</td>
</tr>
<tr>
<td>Female Age (years) (SD)</td>
<td>43.2 ± 10.4</td>
<td>46.5 ± 11.7</td>
</tr>
<tr>
<td>Number (%) of Males</td>
<td>13 (32.5)</td>
<td>7 (31.8)</td>
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<td>Male Age (years) (SD)</td>
<td>49.6 ± 11.7</td>
<td>45.5 ± 16.1</td>
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Number of Mutations:

- **ENG**: 19 (47.5)
- **ACVRL1**: 14 (35)
- **SMAD4**: None
- **Unknown**: 2 (5)
- **Undetermined†**: 5 (12.5)

Number of patients with AVMs:

- **PAVM**: 23 (57.5)
- **PAVM and CAVM**: 2 (5)
- **PAVM and LVM**: 4 (10)
- **CAVM**: 4 (10)
- **No AVM**: 7 (17.5)

Data presented as mean ± standard deviation (SD) or n (%).

* Patients screened negative for known HHT mutations, including **ENG**, **ACVRL1** and **SMAD4**

† Patients that have not yet undergone genetic testing

Abbreviations: HHT, hereditary hemorrhagic telangiectasia; **ENG**, endoglin; **ACVRL1**, activin receptor-like kinase 1; **SMAD4**, mothers against decapentaplegic homolog 4; **PAVM**, pulmonary arteriovenous malformation; **CAVM**, cerebral arteriovenous malformation; **LVM**, liver arteriovenous malformation.
**Figure legends**

**Figure 1. RT-qPCR of ENG and ACVRL1 mRNA Levels in HHT Patient and Control PBMCs.** A) RT-qPCR returned significantly lower ENG mRNA levels in HHT patients compared to controls ($P < 0.01$). B) ACVRL1 mRNA levels were significantly lower in HHT patients compared to controls ($P < 0.05$).

Abbreviations: HHT, Hereditary Hemorrhagic Telangiectasia patients; CON, Controls.

**Figure 2. Fold Change of Dysregulated MiRs in HHT Patient PBMCs Relative to Controls.** Of the 377 miRs screened 41 miRs were dysregulated, defined as a fold change of 1.5 and greater, and 14 miRs were selected based on consistency of dysregulation in HHT patients and controls. Of the 14 miRs, 9 were upregulated and 5 were downregulated (shown above). MiR-654-5p returned the largest positive fold change of 5, while miR-361-3p demonstrated the lowest positive fold change of 1.5. MiR-143-3p demonstrated the largest negative fold change of 3. MiR-28-5p returned a negative fold change of 1.8, miR-30b-5p returned a positive fold change of 1.5 and miR-374a-5p demonstrated a positive fold change of 1.6.

Abbreviations: HHT, hereditary hemorrhagic telangiectasia; PBMCs, peripheral blood mononuclear cells.

**Figure 3. RT-qPCR of MiR-28-5p, -30b-5p, -361-3p and -374a-5p Levels in HHT Patient and Control PBMCs.** A) RT-qPCR returned significantly lower levels of miR-28-5p in HHT patients compared to controls ($P < 0.05$). B) MiR-30b-5p levels were not significantly different in HHT patients compared to controls. C) RT-qPCR returned significantly lower levels of miR-361-3p in HHT patients compared to controls ($P < 0.05$). D) MiR-374a-5p levels were not significantly different in HHT patients compared to controls.

Abbreviations: HHT, Hereditary Hemorrhagic Telangiectasia patients; CON, Controls.

**Figure 4. RT-qPCR of IGF1 mRNA Levels in HHT Patient and Control PBMCs.** RT-qPCR returned significantly higher IGF1 mRNA levels in HHT patient PBMCs compared to controls ($P < 0.01$).

Abbreviations: HHT, Hereditary Hemorrhagic Telangiectasia patients; CON, Controls.
Figure 5. ELISA of IGF1 Protein Levels in HHT Patient and Control Plasma. Plasma protein levels of IGF1 were not significantly different between HHT patient and control groups. The mean amount of IGF1 protein in the HHT patient group and control group was 95 ng/mL ± 21 and 105 ng/mL ± 15, respectively.

Abbreviations: HHT, Hereditary Hemorrhagic Telangiectasia patients; CON, Controls.

Supplementary Figure 1. Relative Expression of Dysregulated MiRs Identified by the Array Analysis.

Displayed is the relative expression of the 14 dysregulated miRs identified by the array analysis. Of the 14 miRs 9 were upregulated and 5 were downregulated in HHT patient PBMCs.

Abbreviations: HHT, Hereditary Hemorrhagic Telangiectasia; CON, Controls.
A) ENG

B) ACVRL1

182x77mm (300 x 300 DPI)
Dysregulated MiRs in HHT Patient PBMCs

Relative Fold Change

Increase in HHT patient PBMCs
Decrease in HHT patient PBMCs
n=8

175x90mm (300 x 300 DPI)
A) MiR-28-5p

B) MiR-30b-5p

C) MiR-361-3p

D) MiR-374a-5p

182x159mm (300 x 300 DPI)
IGF1 Protein Levels in Plasma

Average IGF1 Plasma Concentration (ng/mL)

CON (16)  HHT (24)

0  50  100  150

86x72mm (300 x 300 DPI)