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# Common and distinct circulating microRNAs in four neurovascular disorders

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ABSTRACT

*Background:* Familial cerebral cavernous malformations (FCCM), Sturge-Weber Syndrome (SWS), and hereditary hemorrhagic telangiectasia (HHT) are driven by genetic mutations causing varying vascular dysmorphism and risk of brain bleeding. Cerebral microbleeds (CMBs) are associated with the aging process with less characterized genetic drivers. This study hypothesizes that common and distinct circulating microRNAs (miRNAs) can reflect mechanisms of vascular dysmorphism and bleeding, which can serve as potential biomarkers in clinical contexts.
*Methods:* Differentially expressed (DE) plasma miRNAs ($p < 0.05$, FDR corrected, absolute fold change [|FC|]> 1.5]) were identified between patients with FCCM, SWS, HHT, and CMB, compared to age and sex matched healthy patients. Ingenuity Pathway Analysis as well as transcriptome integration analyses were performed to identify gene targets of the DE miRNAs and their associated pathways. Preselected miRNAs were validated using ddPCR.
*Results:* Eleven circulating DE miRNAs were identified in FCCM, 40 in SWS, 41 in HHT, and 26 in CMB ($p < 0.05$, FDR-corrected, [|FC|]>1.5]). Further analyses showed that 18 DE miRNAs were commonly dysregulated in any two of the studied neurovascular disorders. The PI3K-Akt and ROBO SLIT signaling pathways were identified across all four disorders. The plasma levels of four miRNAs were further validated ($p < 0.05$) using ddPCR.
*Conclusion:* The common dysregulated miRNAs across neurovascular disorders reflect shared mechanistic pathways underlying vascular dysmorphism and bleeding. These findings pave the way for further mechanistic exploration of these miRNAs, and their potential clinical application for disease monitoring and therapeutic intervention.

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## 1. Introduction

Familial cerebral cavernous malformations (FCCMs), Sturge-Weber syndrome (SWS), hereditary hemorrhagic telangiectasia (HHT), and cerebral microbleeds (CMBs) are cerebrovascular dysmorphisms with aberrant vasculature and varying associated risks of hemorrhage [1–6]. Common clinical symptoms also include focal neurological deficits and epilepsy [2,5,7,8]. Currently, the diagnosis of these cerebrovascular anomalies relies primarily on neuroimaging [9–14]. Differentiating FCCMs from CMBs is particularly challenging, as imaging is time-intensive and may be confused by biological mimics or even technical artifacts [15–17]. Circulating molecules reflecting these neurovascular diseases may enhance the diagnosis and assessment of their severity and progression, potentially complementing imaging signatures.

Despite their distinct features, FCCM, SWS, HHT, and CMBs share common pathological processes, including endothelial disruption and inflammation [18–21]. Additionally, increased permeability has been observed in HHT, FCCM, and SWS because of abnormal pericyte-endothelial cell interaction, dysregulated tight junctions, and thin vessels walls [22–24]. FCCM, SWS, and HHT are all genetic diseases with shared dysregulated signaling pathways in endothelial cells (ECs) [8,25–29]. A commonly dysregulated pathway in ECs identified between FCCM, SWS, and HHT is PIK3 signaling [29–32]. FCCM, SWS, and HHT also share common dysregulated biochemical processes, such as fibronectin and Notch signaling [30,33–40]. The Brain Vascular Malformation Consortium (BVMC) has been studying commonalities and distinct features of FCCM, SWS, and HHT for 15 years, including an ongoing study of common and distinct plasma protein biomarkers and cytokines (NINDS/NCATS U54NS065705, ClinicalTrials.gov ID NCT01764529, NCT04717427, NCT01158807).

On the other hand, CMBs, which are associated with the aging brain, result from vascular dysmorphism related to cerebral amyloid angiopathy (CAA) or hypertensive microangiopathies [41], and involve local bleeding with associated inflammatory response in the neuroglial milieu [42,43]. Thus, FCCM, SWS, HHT, and CMB can serve as a paradigm for identifying biomarkers of pathological vascular disruption and associated bleeding.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate post-transcriptional activity through targeting 3′ untranslated regions of mRNAs [44,45]. Circulating miRNAs have been shown to mediate intracellular signaling pathways and cell-to-cell communication [46]. A dysregulation of the miRNome has also been observed in brain injury and during the development of brain vasculature [44]. Furthermore, studies have suggested that circulating miRNAs may reflect tissue-specific clinical changes, serve as markers of disease progression, and can be used to monitor treatment effects [47]. Finally, recent studies have reported that the miRNA profiles can be altered during the clinical course of cerebrovascular diseases, offering the potential for early, non-invasive diagnostic tools [48].

This study aims to characterize miRNA expression profiles, miRNomes, across FCCM, SWS, HHT, and CMB. It is hypothesized that common differentially expressed (DE) miRNAs and gene targets will be identified across the four diseases. Integrated miRNA-mRNA analyses are hypothesized to reveal shared molecular pathways related to vascular dysmorphism and bleeding across different pathologies, and distinct associations reflecting unique disease features. This approach aims to generate novel mechanistic hypotheses, and to identify the potential role of miRNAs as diagnostic biomarkers and therapeutic targets, complementing other research examining circulating protein biomarkers in the same diseases.

## 2. Materials and methods

### 2.1. Study design and participants

Patients with HHT and SWS were enrolled in the BVMC projects at multiple recruitment sites (Fig. 1, Table 1). SWS patients (n = 10; 22.3 ± 9.6 years, range = [7–40 years old]) were enrolled between April 2022 to May 2023 and were included if they had MRI-documented unilateral or bilateral leptomeningeal angiomas with or without neurological symptoms ([9]). HHT patients (n = 10; 24.4 ± 7.8, range = [18–41 years old]) were enrolled between December 2009 to February 2022 if they had a confirmed genetic mutation and/or fulfilled at least three of four Curaçao criteria for definite clinical HHT diagnosis: (1) spontaneous recurrent nosebleeds, (2) telangiectasias on lips, oral cavity, fingers or nose, (3) confirmation of pulmonary, hepatic, or brain AVMs, and/or (4) a diagnosed first-degree relative with the same criteria [9]. Seven of those 10 HHT participants had a confirmed brain AVM.

FCCM patients (n = 10; 26.5 ± 6.3 years, range = [18–37 years old]) were enrolled at the University of Chicago (UChicago) between January 2018 and April 2022 as part of ongoing biomarker discovery and validation in this disease (ClinicalTrials.gov ID NCT01764529) (Fig. 1, Table 1). Patients were enrolled based on the presence of multifocal CCM lesions on magnetic resonance imaging (MRI) with a known genetic mutation in *CCM* genes and/or a family history of CCMs [49]. Patients with lesion resection or any prior brain irradiation were excluded [50,51]. CMB patients (n = 10; 65.9 ± 6.7 years, range = [56–77 years old]) were also enrolled at UChicago between March 2018 and April 2023 if (1) they were over 50 years old, (2) had brain MRI with susceptibility weighted imaging (SWI)/Venbold sequences in the year prior to enrollment, and/or (3) had 2 or more microbleeds on brain MRI adjudicated by a neuroradiologist (Fig. 1, Table 1).

An additional group of Healthy Young (HY) control participants (n = 10; 23.1 ± 2.8 years, range = [19–28 years old]) were enrolled at UChicago between June 2021 and July 2022 if they did not have any medical or neurologic condition requiring ongoing follow-up or medical treatment in the preceding year nor used any recreational, psychoactive, or neuroleptic drugs in the prior year [52]. Healthy Old (HO) control participants (n = 10; 63.9 ± 8.3 years, range = [54–81 years old]) were also enrolled at UChicago between September 2018 to June 2023 if they were over 50 years old, had brain MRI with SWI/Venbold sequences in the year prior to enrollment with no microbleeds on brain MRI (Fig. 1, Table 1). Exclusion criteria for both CMB patients and control participants included concurrent brain disease or structural brain pathology, medical illness requiring hospitalization or surgery within the prior year, history of stroke or epileptic seizure within the past year, pregnancy or within six months postpartum, or reluctance for a blood stick.

The HY control participants were age and sex matched to the FCCM, SWS, and HHT patients. The HO control patients were age and sex matched to the CMB patients.

### 2.2. MiRNome sequencing

RNA was extracted from each sample of serum using the quantities specified in the Qiagen RNeasy Serum/Plasma kit (Venlo, Netherlands) manufacturer's protocol (maximum 200 μl). Extracted RNA samples were quantified using the Qubit RNA HS assay (ThermoFisher, Waltham, MA, USA) and quality was confirmed with the Bioanalyzer RNA pico chip assay (Agilent Technologies, Santa Clara, CA, USA). One nanogram of RNA was used as input for library preparation with the NextFlex small RNA-seq kit v4 (Revvity, Waltham, MA, USA). The plasma miRNome from each disorder was extracted and mapped to the human miRbase with sRNAbench (https://arn.ugr.es/srnatoolbox/srnabench/). The total read count generated from the sRNAbench output was used as input for the DESeq2 package [53,54]. A pairwise differential expression analysis on raw counts for mature sense miRNAs was conducted using DESeq2 ($p < 0.05$, Benjamini and Hochberg false discovery rate

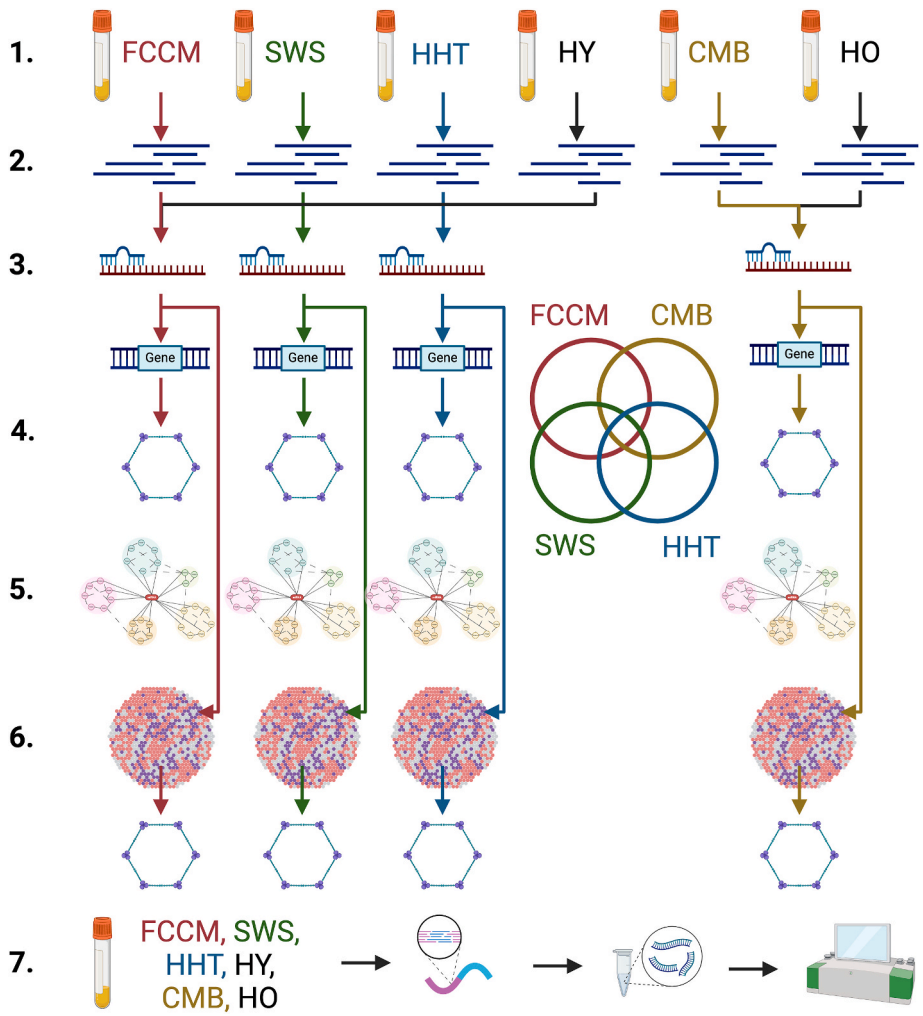


**Fig. 1.** Study design. (1) Patient plasma samples from Familial Cerebral Cavernous Malformation (FCCM, n = 10), Sturge-Weber Syndrome (SWS, n = 10), Hereditary Hemorrhagic Telangiectasia (HHT, n = 10), Cerebral Microbleed (CMB, n = 10), Healthy Young (HY, n = 10), and Healthy Old (HO, n = 10) were collected. (2) RNA was extracted from plasma samples and miRNA libraries were generated by small RNA-seq. (3) Each disease circulating miRNA levels were pooled and compared to respective controls to identify differentially expressed (DE) miRNA. (4) Ingenuity Pathway Analysis (IPA) was utilized as a bioinformatics tool to analyze miRNA target genes, pathways, and networks. (5) Top networks and hub genes were identified by IPA through clustering of genes interactions. (6) The lesional DE genes in each disease were identified for further analysis. (7) Selected miRNAs were validated through droplet digital PCR.

**Table 1**
Age and sex of study cohort.

| | FCCM (n = 10) | SWS (n = 10) | HHT (n = 10) | HY (n = 10) | p-value | CMB (n = 10) | HO (n = 10) | p-value |
|---|---|---|---|---|---|---|---|---|
| Age (mean ± SD) | 26.5 ± 6.3 | 22.3 ± 9.6 | 24.4 ± 7.8 | 23.1 ± 2.8 | *0.58* | 65.9 ± 6.7 | 63.9 ± 8.3 | *0.59* |
| Range (years) | 18–37 | 7–40 | 18–41 | 19–28 | | 56–77 | 54–81 | |
| Female (%) | 30 | 40 | 30 | 30 | *>0.9* | 60 | 50 | *0.65* |

Patients from Familial Cerebral Cavernous Malformation (FCCM, n = 10), Sturge-Weber Syndrome (SWS, n = 10), Hereditary Hemorrhagic Telangiectasia (HHT, n = 10), Cerebral Microbleed (CMB, n = 10), Healthy Young (HY, n = 10), and Healthy Old (HO, n = 10) groups were selected and matched based on age and sex.

[FDR]-corrected, |Fold Change [FC]|>1.5). HY was compared to FCCM, SWS, and HHT ($p < 0.05$, FDR-corrected; |FC|>1.5) while CMB was compared to HO ($p < 0.05$, FDR-corrected; |FC|>1.5). One sample from FCCM patients, 2 from HHT, 1 from CMB, and 1 from HY patient were discarded from differential expression analysis as they did not pass quality check thresholds or not did not have enough reads.

### 2.3. Functional analysis

The Ingenuity Pathway Analysis (IPA) platform (Qiagen) was used to determine downstream target genes of the DE miRNAs ($p < 0.05$, FDR-corrected) using TargetScan, TarBase, miRecords, and Ingenuity Knowledge Base [55]. Only highly predicted targets in humans with both (1) cumulative weighted context score (CWCS) < -0.4 based on the TargetScan algorithm and (2) experimentally validated gene targets from the IPA database were considered [56,57]. Pathways ($p < 0.05$, FDR-corrected) had associated (1) gene ratio (GR), defined as the number of genes targeted by miRNAs divided by the total number of genes, calculated and (2) interaction score (IS) computed as the number of unique miRNA-gene interactions associated with each pathway. Additional pathway analyses ($p < 0.01$, FDR corrected) were performed with targeted genes of only experimentally observed mRNA targets.

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) and UniProt Knowledgebase were also used to further

identify targeted genes [58–60].

### 2.4. Hub gene identification

The genes targeted by each of the DE miRNAs were used to create networks from IPA Knowledge Base. For each disease only the top networks with similar highest IPA score and number of focus genes were merged for further analysis. Hub genes were selected with the highest number of connections to other genes (minimum >11 connections). The networks were generated using QIAGEN IPA [55].

### 2.5. Unsupervised analyses of top pathways of DE miRNAs

Unsupervised analyses of the DE miRNAs for each disease were first performed. Several filtering criteria were defined to set stringent thresholds and increase the likelihood of identifying true functional miRNA-target-pathway interactions and dysregulation. These criteria were employed to minimize the effects of (1) false positive rates of miRNA-target binding (e.g., ranging from 20 % to 50 % in target prediction programs) [61,62], and (2) false positive predictions of miRNA bindings (e.g., resulting in functional suppressions) [56,63,64]. These selection criteria for unsupervised analyses included (1) GR (i.e., the number of genes targeted/total number of genes in the pathway (2) IS (i.e., the number of genes targeted in the pathway), (3) experimentally, validated gene targets, (4) the number of DE miRNAs targeting genes in the pathway, and (5) p-value ($p < 0.01$, FDR corrected). Filtering pathways with a higher IS yields a higher likelihood of at least one true functional miRNA-target-pathway interaction and functional suppression. Additionally, filtering with a higher GR keeps a set proportion of interactions required to be considered for larger pathways.

### 2.6. Transcriptome and supervised pathway analyses

The supervised analyses of the DE miRNAs for each disease were then performed using previously published transcriptomes. The IPA-targeted genes of the DE miRNAs ($p < 0.05$, FDR-corrected) were filtered using the transcriptome(s) of CCM lesional neurovascular units [$p < 0.05$, FDR corrected; log2(FC) > 1.5] [65,66], SWS microvascular EC line [$p < 0.05$, FDR corrected; log2(FC) > 1.5] [67], HHT tissue ($p < 0.1$, FDR corrected) [68], and CMB lesional tissue [$p < 0.05$, FDR corrected; log2(FC) > 1.5] (Srinath et al., in preparation).

Gene targets were filtered for supervised analyses to be only highly predicted targets, defined as having a human CWCS < −0.4, or experimentally validated gene targets from the IPA database [56,57]. The pathways of each transcriptome ($p < 0.01$, FDR corrected) had associated IS and GR computed for each pathway in each disease.

### 2.7. Droplet digital PCR validation

The plasma levels of five miRNAs including *miR-487b-3p*, *miR-4732-5p*, *let-7e-5p*, *miR-486-3p*, *miR-409-3p* were quantified through droplet digital PCR (ddPCR) to validate the differential miRNome analyses. These miRNAs were selected as (1) they were DE in at least two of the neurovascular diseases or also DE in an independent cohort, (2) had a log2(FC) > 20 or IS > 300 and log2(FC) > 6, and (3) they were at detectable concentrations in the samples tested. *Refer to Supplemental Methods for additional details about the ddPCR assays, cohort matching, blood collection and processing, RNA extraction, and miRNA expression profiling.*

### 2.8. Study approval and ethics

This study was approved by the UChicago Institutional Review Board (IRB) protocol IRB20-0518 and BVMC IRB protocols cIRB19-27677, cIRB19-27678, and cIRB19-27676 conducted in accordance with the Declaration of Helsinki and its later amendments, as well as the Belmont Report.

## 3. Results

### 3.1. DE miRNAs in FCCM

Differential analysis of the plasma miRNome of FCCM patients identified 11 DE miRNAs ($p < 0.05$, FDR corrected, |FC|>1.5) (Supplementary Table 1). Among these, nine DE miRNAs were only identified in the plasma of FCCM patients, but not in the other 3 neurovascular disorders (Table 2, Supplementary Table 1). Gene network analysis of the targets of these DE miRNAs revealed *MYC* and *SNAI* as hub genes unique to FCCM (connections>11) (Supplementary Fig. 1, Supplementary Table 2). Unsupervised analyses of these DE miRNAs showed that the top pathways ($p < 0.01$, FDR corrected) only identified in FCCM included “Extracellular matrix organization” and “Assembly of collagen fibrils and other multimeric structures” (Supplementary Fig. 2A, Supplementary Table 3). Supervised analyses with the CCM transcriptome showed that among the top pathways identified with the most gene targets were the “Synaptogenesis Signaling Pathway,” “Neurovascular Coupling Signaling Pathway,” and “Extracellular matrix organization” pathway ($p < 0.01$, FDR corrected) (Supplementary Fig. 3A, Supplementary Table 4). Notably, supervised analyses identified the “Extracellular matrix organization” pathway as a top pathway in the transcriptome of CCMs [65,66] which was also found in unsupervised analyses.

### 3.2. DE miRNAs in SWS

A total of 40 DE miRNAs ($p < 0.05$, FDR corrected, |FC|>1.5) were identified in the plasma of SWS patients (Supplementary Table 5). Of these, 26 DE miRNAs were uniquely identified in the plasma of SWS patients (Table 2, Supplementary Table 5). Gene network interaction analysis pinpointed *ARID1A* as a hub gene unique to SWS, exhibiting high connectivity (connections>11) within the top disease-specific networks (Supplementary Fig. 4, Supplementary Table 6). Unsupervised pathway analyses of these DE miRNAs found the “IL-7 Signaling Pathway” as a top pathway ($p < 0.01$, FDR corrected) only identified in SWS (Supplementary Fig. 2B, Supplementary Table 7). Further supervised analyses showed that “IL-6 Signaling” and “IL-10 Signaling” were identified as top pathways ($p < 0.01$, FDR corrected) in the transcriptome of SWS [67] (Supplementary Fig. 3B, Supplementary Table 8). Of interest, several interleukin pathways were identified in both unsupervised and supervised analyses that interact through the JAK-STAT pathway.

### 3.3. DE miRNAs in HHT

HHT patient plasma showed 41 DE miRNAs ($p < 0.05$, FDR corrected, |FC|>1.5) (Supplementary Table 9). Twenty-four DE miRNAs were found only in the plasma of HHT patients (Table 2, Supplementary Table 9). *WNT5A* was identified as a hub gene unique to HHT with extensive network connections (connections>11) (Supplementary Fig. 5, Supplementary Table 10). In addition, the unsupervised analyses showed that “Signaling by PDGF” and “FOXO-mediated transcription of cell cycle genes” were top pathways ($p < 0.01$, FDR corrected) only in HHT (Supplementary Fig. 2C, Supplementary Table 11). Moreover, supervised analyses on the transcriptome of HHT defined “TR/RXR Activation” as a top pathway ($p < 0.01$, FDR corrected) [68] (Supplementary Fig. 3C, Supplementary Table 12). No pathways were similar between unsupervised and supervised analyses.

### 3.4. DE miRNAs in CMB

In CMB, 26 miRNAs were differentially expressed ($p < 0.05$, FDR corrected, |FC|>1.5) (Supplementary Table 13). Among these, 23 DE

**Table 2**
Commonly dysregulated miRNAs among any two or more neurovascular disorders.

| *MiRNA* (Interaction Score)[a] | FCCM | | SWS | | HHT | | CMB | |
|---|---|---|---|---|---|---|---|---|
| | *$Log_2$ Fold Change* | *Adjusted p-value* | *$Log_2$ Fold Change* | *Adjusted p-value* | *$Log_2$ Fold change* | *Adjusted p-value* | *$Log_2$ Fold Change* | *Adjusted p-value* |
| *miR-487b-3p (37)* | −24.28 | <0.001 | | | −21.87 | <0.001 | | |
| *miR-431-5p (105)* | +23.38 | <0.001 | | | | | −22.61 | <0.001 |
| *let-7d-3p 9 (33)* | | | −5.09 | 0.022 | −7.17 | <0.001 | | |
| *let-7e-5p (536)* | | | −4.13 | 0.027 | −6.94 | <0.001 | | |
| *miR-122-5p (157)* | | | +3.37 | 0.012 | +2.57 | 0.036 | | |
| *miR-126-5p (66)* | | | −4.15 | 0.002 | −2.87 | 0.037 | | |
| *miR-1307-3p (51)* | | | −7.60 | <0.001 | −8.51 | <0.001 | | |
| *miR-181b-5p (211)* | | | −4.16 | 0.022 | −9.96 | <0.001 | | |
| *miR-18a-5p (161)* | | | −3.53 | 0.029 | −3.70 | 0.041 | | |
| *miR-28-5p (149)* | | | −3.81 | 0.030 | −9.23 | <0.001 | | |
| *miR-423-5p (695)* | | | +1.94 | 0.012 | +2.03 | 0.010 | | |
| *miR-425-3p (73)* | | | −21.87 | <0.001 | −21.34 | <0.001 | | |
| *miR-433-3p (86)* | | | −5.13 | 0.030 | −8.97 | <0.001 | | |
| *miR-4433b-5p (173)* | | | −5.36 | 0.013 | −7.40 | <0.001 | | |
| *miR-486-3p (978)* | | | −5.36 | 0.029 | −25.54 | <0.001 | | |
| *miR-486-5p (93)* | | | +2.09 | 0.015 | +2.00 | 0.040 | | |
| *miR-223-3p (158)* | | | | | +1.79 | 0.027 | +0.87 | 0.042 |
| *miR-409-3p (18)* | | | | | −22.04 | <0.001 | −8.78 | 0.002 |

[a] Interaction score for each miRNA represents the number of gene targets corresponding to that miRNA as determined by Ingenuity Pathway Analysis. Eighteen differentially expressed (DE) miRNA were identified in two of the neurovascular disorders ($p < 0.05$, FDR corrected). One DE miRNA was found between Familial Cerebral Cavernous Malformation (FCCM) and Hereditary Hemorrhagic Telangiectasia (HHT), one between FCCM and Cerebral Microbleed (CMB), 14 DE miRNAs between Sturge-Weber Syndrome and HHT, and two DE miRNAs between HHT and CMB. No DE miRNAs were found across three or four of the diseases studied.

miRNAs were specific to CMB (Table 2, Supplementary Table 13). Network analysis highlighted *EZH2* as a hub gene unique to CMB, demonstrating significant connectivity (connections>11) within the dysregulated gene set (Supplementary Fig. 6, Supplementary Table 14). Top pathways only associated with CMB ($p < 0.01$, FDR corrected) identified using unsupervised analyses included "Transcriptional Regulation by MECP2" (Supplementary Fig. 2D, Supplementary Table 15). Further supervised analyses with the transcriptome also identified "Transcriptional Regulation by MECP2" as a top pathway ($p < 0.01$, FDR corrected) in CMBs (Supplementary Fig. 3, Supplementary Table 16).

### 3.5. Commonly DE miRNAs in more than one neurovascular disorder

Eighteen DE miRNAs were common in any two of the neurovascular disorders studied (Table 2). One DE miRNA was common between FCCM and HHT, one between FCCM and CMB, two DE miRNAs between HHT and CMB, and 14 DE miRNAs between SWS and HHT (Fig. 2). There was no DE miRNAs found in common in at least three of the diseases studied.

### 3.6. MiR-487b-3p was downregulated in both FCCM and HHT

*MiR-487b-3p* ($p < 0.05$, FDR corrected) was downregulated in both FCCM (log2FC = −24.3) and HHT (log2FC = −21.9) (Table 2, Supplementary Table 1, Supplementary Table 9). IPA showed that *miR-487b-3p* targeted eight genes involved in 17 pathways ($p < 0.01$, FDR corrected) linked to apoptosis, signaling dysregulation, and inflammation (Fig. 3A). In addition, *MAP2K4* and *PRKCA* were identified as targets of *miR-487b-3p* (Fig. 3A–Supplementary Table 17, Supplementary Table 18). Pathways linked to these genes included apoptosis, tumor necrosis factor receptor 1 (TNFR1), ERBB, roundabout (ROBO) receptor, and inflammation-related signaling pathways (Fig. 3A–Supplementary Table 17, Supplementary Table 18). *MAP2K4*, a central component of the MAPK signaling pathway, is involved in regulating cell growth, differentiation, and apoptosis.

Additionally, *PRKCA* is involved in TNFR1, ERBB, ROBO receptor, and inflammation-related signaling pathways, responsible for both vascular and immune responses. TNFR1 signaling is critical for mediating inflammatory responses and apoptotic signaling. Furthermore, the ROBO receptor pathway is involved in neuronal guidance and angiogenesis.

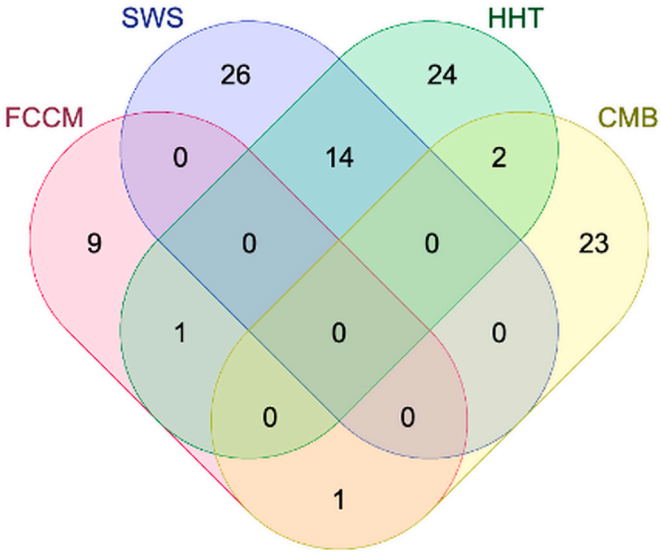


**Fig. 2.** Venn diagram of differentially expressed miRNAs identified between Familial Cerebral Cavernous Malformation (FCCM), Sturge-Weber Syndrome (SWS), Hereditary Hemorrhagic Telangiectasia (HHT) and Cerebral Microbleed (CMB). Eleven differentially expressed (DE) miRNAs were identified in the plasma of FCCM, 40 in SWS, 41 in HHT, and 26 in CMB. Of interest, one was common between FCCM and HHT, one between FCCM and CMB, two between HHT and CMB, and 14 between SWS and HHT. No DE miRNA was found to be shared across three or more disorders.

### 3.7. MiR-431-5p was upregulated in FCCM and downregulated in CMB

*MiR-431-5p* ($p < 0.05$, FDR corrected) was upregulated in FCCM (log2FC = 23.4) and downregulated in CMB (log2FC = −22.6) (Table 2, Supplementary Table 1, Supplementary Table 13). IPA revealed *miR-431-5p* targets 16 genes involved in 13 pathways ($p < 0.05$, FDR corrected) linked to immune signaling, stress response, and vascular development with targets to angiopoietin genes (*ANGPT2*, *ANGPT4*) and tumor necrosis factor family genes (*TNFSF8*) (Fig. 3B). The pathways included the TLR3 cascade, metabolic regulation pathways, and

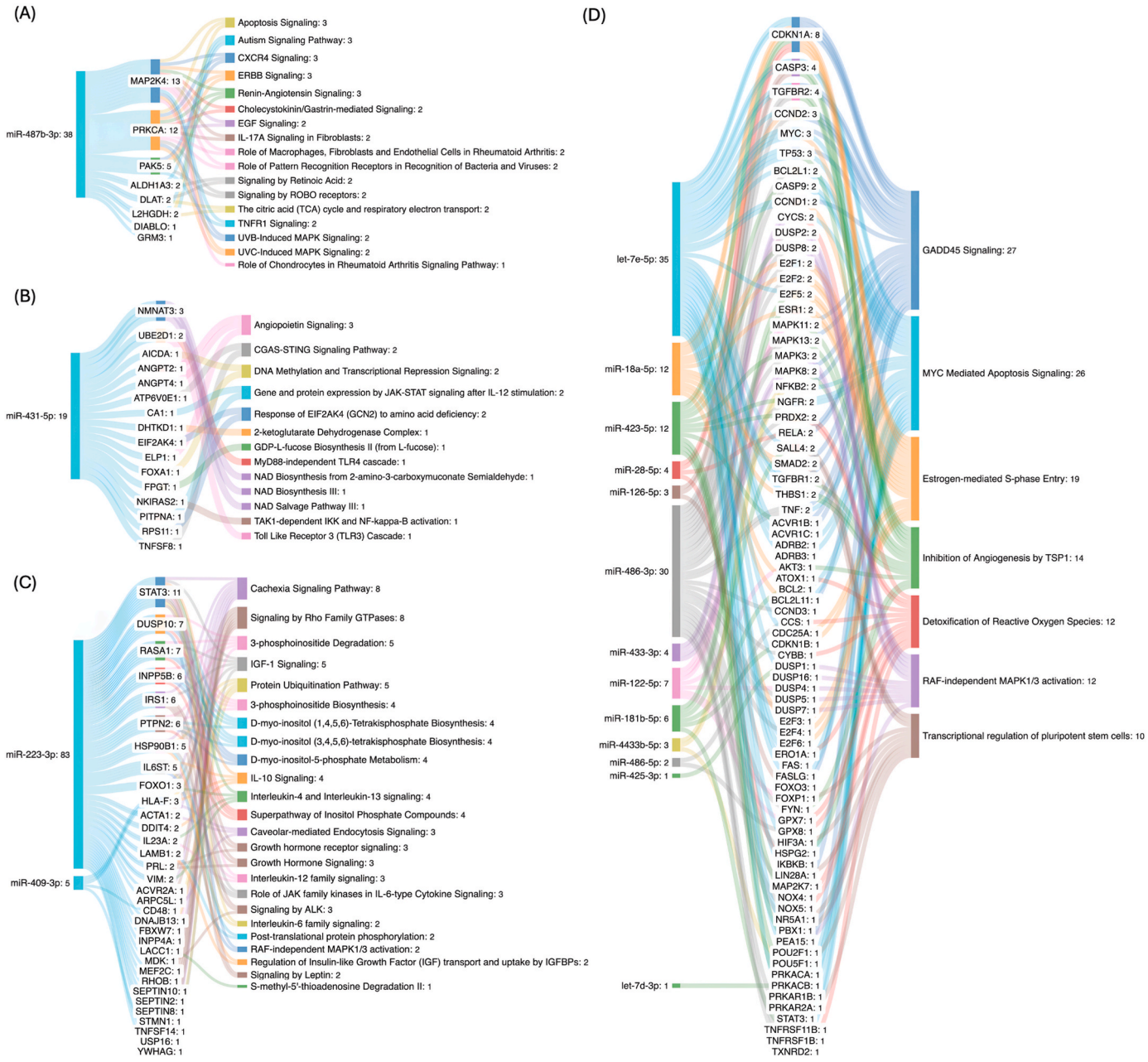


**Fig. 3.** Gene targets and pathways of commonly identified dysregulated miRNAs in studied neurovascular disorders. The gene targets and associated pathways of the miRNAs that were shared between disorders were analyzed through Ingenuity Pathway Analysis. (**A**) *MiR-487b-3p* was downregulated in Familial Cerebral Cavernous Malformation (FCCM) and Hereditary Hemorrhagic Telangiectasia (HHT) with links to apoptosis, signaling dysregulation, and inflammation ($p < 0.01$, FDR corrected). (**B**) *MiR-431-5p* was upregulated in FCCM while downregulated in Cerebral Microbleed (CMB) with links to immune response and metabolic dysregulation ($p < 0.05$, FDR corrected). (**C**) *MiR-223-3p* was upregulated, while *miR-409-3p* was downregulated, in both CMB and HHT with links to intracellular signaling cascades ($p < 0.01$, FDR corrected). (**D**) Three miRNAs were upregulated and 11 downregulated in Sturge-Weber Syndrome and HHT with links to cellular growth and stress response ($p < 0.01$, Interaction Score $\geq 10$, Gene Ratio $\geq 0.43$).

angiopoietin signaling (Fig. 3B–Supplementary Table 17, Supplementary Table 19). *ANGPT2* plays a key role in vascular remodeling and EC junction integrity, while *TNFSF8* is also involved in immune response and inflammation. Additionally, the TLR3 pathway mediates immune responses to cellular damage.

### 3.8. MiR-223-3p was upregulated and miR-409-3p was downregulated in both CMB and HHT

*MiR-223-3p* ($p < 0.05$, FDR corrected) was upregulated and *miR-409-3p* downregulated in both HHT (respectively log2FC = 1.8, log2FC = −22.0) and CMB (respectively log2FC = 0.9, log2FC = −8.8) (Table 2, Supplementary Table 9, Supplementary Table 13). IPA identified that *miR-223*-3p targeted 30 genes, which are involved in 24 pathways ($p < 0.01$, FDR corrected), and that *miR-409*-3p targeted three genes, which are involved in four pathways ($p < 0.01$, FDR corrected) (Fig. 3C). *MiR-223-3p* targeted *STAT3*, *RHOB*, and Septin genes (*SEPTIN2*, *SEPTIN8*, *SEPTIN10*). *STAT3*, a transcription factor, is involved in cell survival, apoptosis, and immune regulation. The Rho signaling pathway, mediated by *RHOB*, is known to control actin cytoskeleton dynamics and cell movement. *MiR-409-3p* targeted *TNFSF14*, *HLA-F*, and *CD48* (Fig. 3C–Supplementary Table 18, Supplementary Table 19). These

miRNAs were linked to intracellular signaling cascades such as Rho signaling, IGF-1 signaling, and interleukin signaling (Fig. 3C).

### 3.9. Three miRNAs were upregulated and eleven downregulated in SWS and HHT

Fourteen miRNAs ($p < 0.05$, FDR corrected) were common between SWS and HHT with 11 downregulated and three upregulated across both diseases (Table 2, Supplementary Table 5, Supplementary Table 9). These miRNAs targeted 78 genes and were involved in seven pathways [$p < 0.01$, FDR corrected, IS $\geq 10$, GR $\geq 0.43$] (Fig. 3D). Genes targeted by these miRNAs included *BCL2*, *MAPK3*, and *MYC* (Fig. 3D–Supplementary Table 18, Supplementary Table 20). The targeted genes were identified to be involved in pathways including overactive angiogenesis and defective blood vessel maturation result in fragile vessel networks.

### 3.10. Shared gene targets of differentially expressed miRNAs

Seventeen common gene targets ($p < 0.05$, FDR-corrected) were a target of at least two DE miRNAs in each neurovascular disorder (Fig. 4). These genes had reported roles in cellular processes, from survival and proliferation to signaling and structural regulation. *BCL2L1* emerges as a key regulator of apoptosis and cell survival. Cyclin D2 (*CCND2*) drives cell cycle progression by promoting the transition from G1 to S phase, while *CDKN1A* acts as a gatekeeper, inhibiting cyclin-dependent kinase complexes to arrest the cell cycle when needed. In addition, several genes also play an important role in signaling cascades. *PTEN* serves as a control on the PI3K-Akt cascade, a pathway central to cell growth and survival. Furthermore, *RAB15* facilitates vesicle trafficking, contributing to intracellular signaling dynamics. *TGFBR2* maintains cell growth and differentiation. At the interface of neurotransmission, *GRIN2B* encodes a subunit of the NMDA receptor, essential for synaptic signaling and plasticity.

Immune and inflammatory systems were also modulated by several genes targeted by these miRNAs. *POU2F2* regulates immune responses through its role as a transcription factor, while *NKIRAS2* modulates inflammatory pathways. There were also genes which control the structural and adhesion dynamics. *NF2*, a tumor suppressor, regulates cell adhesion and migration, preventing unwarranted cell proliferation. Furthermore, the zinc finger proteins *(ZBTB16, ZIK1, ZNF23, ZNF256, ZNF544, ZNF772, ZNF791)* control gene expression and transcriptional regulation. Finally, functional analysis of these 17 gene targets through DAVID identified 28 ($p < 0.05$, count>2) linked pathways that included the PI3K-Akt signaling and cellular senescence pathways.

### 3.11. Shared pathways across neurovascular diseases

Ten pathways ($p < 0.01$, FDR corrected, IS $\geq 21$, GR $\geq 0.21$) were commonly identified between CMB, FCCM, HHT, and SWS based on the DE miRNAs in each disorder (Fig. 5A). These pathways highlighted mechanisms that regulate stem cell function, neuronal guidance, ion transport, immune signaling, and extracellular matrix remodeling (Fig. 5A). The pathways for each disorder were further analyzed based on whether the circulating miRNAs found to be dysregulated targeted the lesional transcriptomes established for each condition. Eleven pathways ($p < 0.05$, FDR corrected) were found common between CMB, FCCM, HHT, and SWS. These pathways involve immune response, tissue fibrosis, and neural development with mechanisms of cell signaling, inflammation, myelination, and cellular stress response (Fig. 5B). The ROBO SLIT Signaling pathway emerged through both analyses and across all disorders.

### 3.12. Validation of miRNA expression through droplet digital PCR

A panel of DE miRNAs including *let-7e-5p*, *miR-486-3p*, *miR-487b-3p*, *miR-409-3p*, and *miR-4732-5p* were further quantified by ddPCR (Supplementary Table 21). The plasma levels of *let-7e-5p* levels were lower in SWS ($p = 0.002$) and HHT ($p = 0.0006$) compared to HY samples (Fig. 6A), consistent with miRNome sequencing results (*let-7e-5p*: SWS log2FC = −4.1; HHT log2FC = −6.9). Further analyses showed that the plasma levels of *let-7e-5p* were able to distinguish HY subjects from both SWS and HHT patients with 100 % of sensitivity and 90 % of specificity (area under the curve [AUC] = 92.78 % and 90.56 % respectively; Supplementary Fig. 7). Additionally, the plasma levels of *miR-486-3p* were lower for SWS patients ($p < 0.0001$) and HHT ($p = 0.0004$) compared to HY samples (Fig. 6B), validating the initial results (*miR-486-3p*: SWS log2FC = −5.4; HHT log2FC = −25.5). The plasma levels of *miR-486-3p* were also able to distinguish HY from both SWS and HHT patients with 100 % of sensitivity and 90 % of specificity (AUC = 97.78 % and 94.44 % respectively; Supplementary Fig. 7).

Of interest, *miR-487b-3p* levels were lower in HHT ($p = 0.001$) compared to HY, consistent with miRNome sequencing results (*miR-487b-3p*: HHT log2FC = −21.9, FCCM log2FC = −24.3), while FCCM showed a trend toward lower levels ($p = 0.06$) (Fig. 6C). The plasma levels of *miR-487b-3p* were able to distinguish HHT patients from HY subjects with 89 % sensitivity and 90 % specificity (AUC = 90.56 %; Supplementary Fig. 7). The same analyses showed that the plasma levels of *miR-487b-3p* were able to distinguish FCCM patients from HY subjects with 80 % and 60 % sensitivity/specificity (AUC = 74.5 %; Supplementary Fig. 7).

The plasma levels of *miR-409-3p* were lower in HHT patients compared to HY subjects ($p = 0.002$; Fig. 6D), also seen in initial results (*miR-409-3p*: HHT log2FC = −22.0). Further analyses showed that the plasma levels of *miR-409-3p* were able to differentiate HHT patients from HY subjects with 100 % and 80 % sensitivity/specificity (AUC = 90 %; Supplementary Fig. 7). Interestingly, CMB samples did not show a difference from HO ($p = 0.1620$) in *miR-409-3p* (Fig. 6D). Finally, the

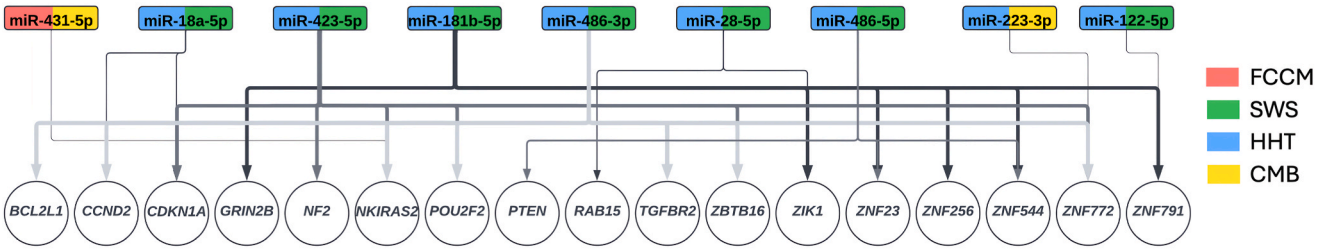


**Fig. 4.** Network of common gene targets among studied neurovascular disorders. The gene targets of the differentially expressed miRNAs in Familial Cerebral Cavernous Malformation (FCCM), Sturge-Weber Syndrome, Hereditary Hemorrhagic Telangiectasia (HHT), and Cerebral Microbleed (CMB) were identified. The genes in each disorder were compared and revealed 17 gene targets were targeted by at least two miRNAs in FCCM, SWS, HHT, and CMB. Of all the miRNAs that targeted these genes, nine of the miRNAs were shared among two diseases. The nine miRNAs that were shared between two disorders were then used to create a miRNA-mRNA network with the 17 gene targets.

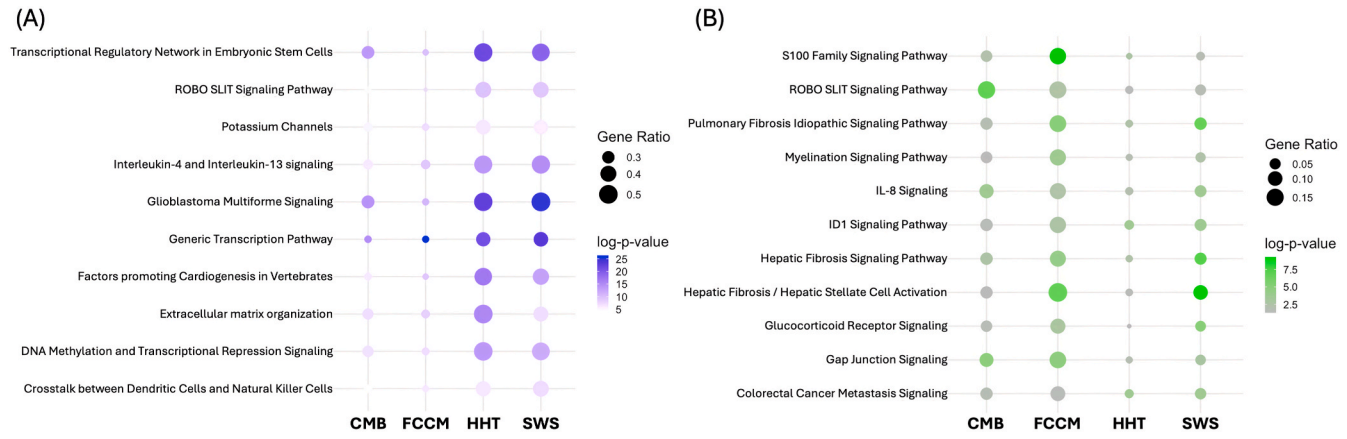


**Fig. 5.** Dot plots of shared pathways among studied neurovascular disorders. (**A**) Ingenuity Pathway Analysis was queried to identify pathways based on the differentially expressed (DE) miRNA of each disease and targeted genes. Ten shared pathways were identified between Cerebral Microbleed, Familial Cerebral Cavernous Malformation, Hereditary Hemorrhagic Telangiectasia, Sturge-Weber Syndrome ($p < 0.01$, FDR corrected, interaction score $\geq 21$, gene ratio $\geq 0.21$). The interaction score was defined as the number of genes targeted by miRNA in that pathway. Gene ratio was defined as the number of genes targeted by miRNA divided by the total number of genes in the pathway. (**B**) The targeted genes of the DE miRNAs in each disorder were filtered based on the identified transcriptome of each disorder. Ingenuity Pathway Analysis revealed eleven pathways shared between disorders ($p < 0.05$, FDR corrected).

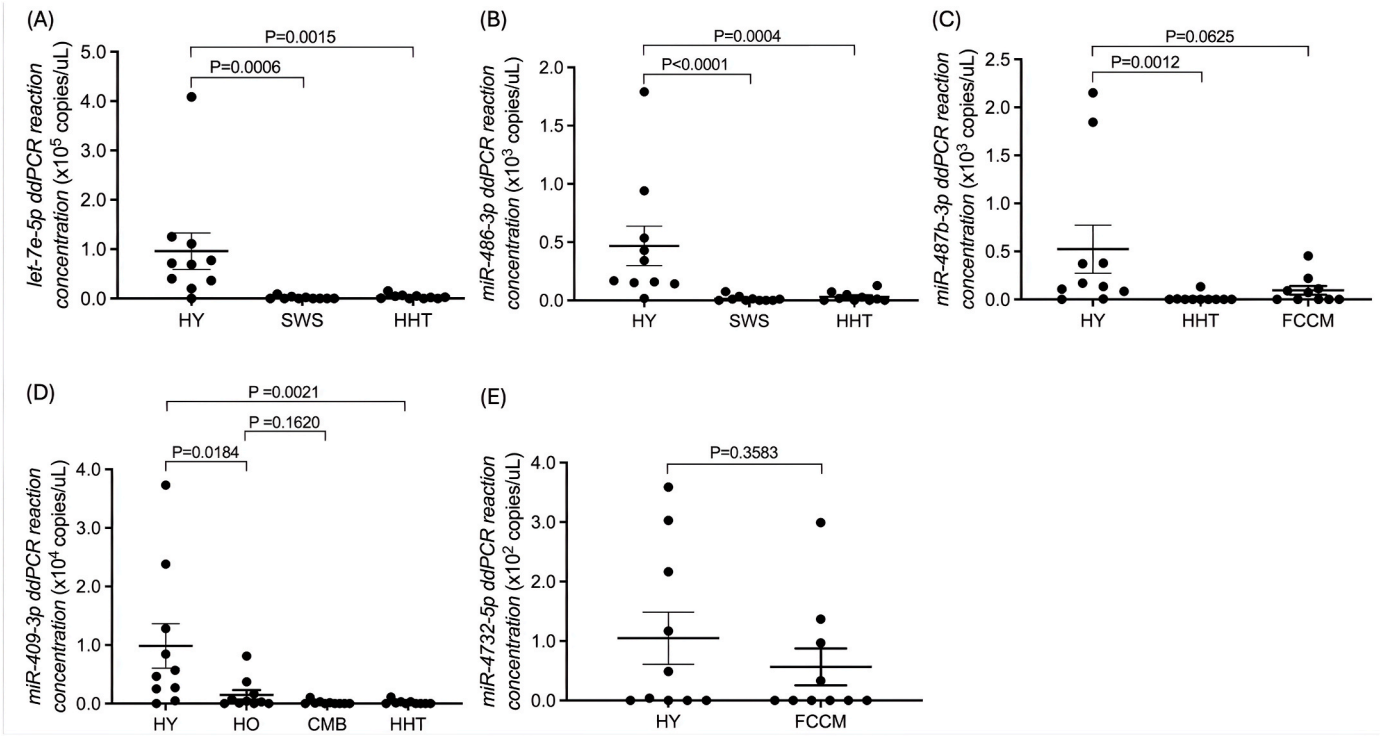


**Fig. 6.** Droplet Digital PCR miRNA validation. Sturge-Weber syndrome (SWS, n = 9), hereditary hemorrhagic telangiectasia (HHT, n = 9), familial cerebral cavernous malformation (FCCM, n = 10), cerebral microbleed (CMB, n = 10), healthy young (HY, n = 10), and healthy old (HO, n = 10) samples were assessed through droplet digital PCR to validate small RNA sequencing results. **(A)** *Let-7e-5p* levels were lower in SWS ($p = 0.002$) and HHT ($p = 0.0006$) compared to HY. (**B**) *MiR-486-3p* levels were lower in SWS ($p < 0.0001$) and HHT ($p = 0.0004$) compared to HY. **(C)** *MiR-487b-3p* levels were lower in HHT ($p = 0.001$) compared to HY but not in FCCM ($p = 0.06$) compared to HY. **(D)** *MiR-409-3p* was lower in HHT ($p = 0.002$) and HO ($p = 0.02$) when compared to HY. (**E**) *MiR-4732-5p* was not found to be statistically different in FCCM from HY by ddPCR ($p = 0.36$). The levels of miRNA are presented as copies/μL adjusted for any cDNA or sample dilution. Amplitudes were inspected to determine if any No Call reported were from all negative droplets or high positive droplet readings. Statistical Test: Mann-Whitney *U* test.

levels of *miR-4732-5p* levels were not different between FCCM and HY samples (Fig. 6E).

Supplemental Results provide additional information on gene targets, pathways linked to DE miRNAs in each neurovascular condition, and transcriptome pathways.

## 4. Discussion

### 4.1. Common and distinct mechanisms in different neurovascular diseases

Our study focused on four neurovascular diseases, which share some common mechanistic features despite distinct pathobiology.

Extravasation of immune infiltrating macrophages in perivascular spaces has been noted around FCCMs [69], SWS [70], HHT [71] as well as in CMBs [72]. FCCMs and CMBs also both show vessel dilation and extravasation of red blood cells [2,72], while hyalinization can be observed around vessels in SWS and CMB [22,73].

Both HHT and FCCM patients harbor brain lesions and systemic haploinsufficiency [9]. FCCM arises from an inherited germ line mutation in one of the genes *KRIT1*, *CCM2*, or *PDCD10* [2]. Mutations in endothelial receptors *ENG*, *ACRVL1/ALK1*, or *SMADA4* are associated with the three major subtypes of HHT [26]. Lesions in FCCM and HHT form from "second hit" somatic mutations in brain microvascular endothelium [25,74–78]. Finally, SWS is associated with a non-inherited somatic mutation in the *GNAQ* gene, primarily affecting ECs [8,27,28,79]. Of interest, a *PIK3CA* GOF mutation in mutant ECs has been shown to increase PI3K-mTOR signaling and stimulate lesional growth of CCMs [32]. HHT and SWS also show increased PI3K/AKT activation [29–31]. Several other pathways have also been suggested to be common between these disorders. RAS/ERK and MAPK pathways were found dysregulated in both SWS [31,80,81] and FCCM [25,82–84]. Transforming growth factor (TGF)-β signaling has been reported in both HHT [85] and FCCM [86]. Moreover, several prominent pathways involved in functions ranging from endothelial migration, angiogenesis, and proliferation across these neurovascular diseases have been suggested [87–94].

Fibronectin was shown to be more expressed in human cerebral cavernous malformation (CCM), including sporadic form and FCCM, lesional pericytes than in epilepsy tissue [34], while aberrant fibronectin remodeling has been observed in ECs following CCM1/2 loss [35]. Transcriptome analyses of SWS lesions showed that fibronectin mRNA was significantly overexpressed and under-expressed in parenchymal and meningeal vessels, respectively, when compared to epilepsy control [33]. A dysregulation of fibronectin processes has also been suggested during AVM genesis by acting as a potential biochemical mediator between both YAP/TAZ signaling and increased integrin expression in an HHT mouse model [39,40]. Moreover, dysregulated fibronectin remodeling and extracellular matrix structure were observed around CCMs in mice, with altered EC contractility and attenuated endothelium integrity. In SWS, altered fibronectin expression has been linked to abnormal proliferation of blood vessels and potential response to hypoxia [35,95].

CMBs may harbor similar pathologic signaling axes identified in FCCM, SWS, and HHT. Of interest, Mohan et al. (2023) suggested that Notch and TGF-β signaling pathways may be dysregulated in the EC transcriptome of aging mice and associated with greater risk of CMB genesis [96]. Interestingly, mutations in *NOTCH3* are known to drive cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, where CMBs are radiological hallmarks [97]. Moreover, alterations in neuronal networks and microglial-neuronal interactions have been associated with neurovascular degeneration and breakdown of the blood-brain barrier (BBB) in CMB genesis [98].

This study is the first to comprehensively analyze the role of circulating miRNAs across four neurovascular disorders: FCCM, SWS, HHT, and CMB. The findings highlight both common and specific miRNA signatures as well as their associated pathways, shedding light on shared and unique mechanisms underlying these conditions. Furthermore, this work points toward common potential biomarkers and therapeutic targets.

### 4.2. Commonly identified dysregulated miRNAs

Two miRNAs, *let-7e*-5p and *miR-486*-3p were DE in the plasma of patients with SWS and HHT (Table 2). Previous research has proposed *let-7e-5p* as a biomarker for ischemic stroke [99] and that a downregulation of this miRNA contributed to endothelial progenitor cell dysfunction [100]. Moreover, *let-7e-5p* has been shown to promote thrombus organization, revascularization, and migration in endothelial progenitor cells [100] as well as reinstate proangiogenic properties in METTL3 depleted ECs [101]. Taken together, this evidence suggests a role for the downregulation of *let-7e-5p* in SWS and HHT in the impairment of vascular repair and stability. Additionally, *let-7e-5p* was previously found higher in FCCM and associated with ADAMTS5, which has been implicated in lesion pathogenesis [102,103]. Lower levels of *miR-486-3p* may be involved in elevated neuroinflammation, as indicated by the reduction of neuroinflammation and microglial polarization via SIRT2 due to delivery of *miR-486-3p* after subarachnoid hemorrhage [104]. Masliah-Planchon et al. (2013) reported a role of *miR-486-3p* in altering the PTEN pathway [105]. Srinath et al. (2023) showed that *miR-486-3p* was downregulated in CCM patients with recent symptomatic hemorrhage (Srinath et al., 2023). These results may suggest that *miR-486-3p* has a role in vascular homeostasis.

The differential analyses also showed that *miR-487b-3p* was commonly dysregulated in the plasma of patients with FCCM and HHT (Table 2). Downregulation of *miR-487b-3p* suggests an adaptive role in angiopoietin signaling for lesion stabilization to counteract vascular damage by promoting endothelial repair [106,107]. *MiR-487b-3p* has also been shown to regulate TGF-β and inhibit TGF-β induced apoptosis [108]. Taken together, the role of TGF-β in cerebrovascular disease pathogenesis may involve *miR-487-3p* [109]. Interestingly, *miR-487b-3p* was shown to be upregulated in a different cohort of FCCM patients [103].

Previous studies have suggested *miR-409-3p* as a circulating biomarker for patients with cerebral small vessel disease associated vascular dementia [110]. Moreover, *miR-409-3p* has been shown to be involved in endothelial senescence (Hu et al., 2021), VSMC apoptosis and proliferation [111], and hemorrhagic transformation and BBB damage [112]. Additionally, several studies have demonstrated that *miR-409-3p* is involved in neuroinflammation through pathways regulating inflammatory cytokine production in reactive astrocytes [113] as well as the migration and activation of microglia [114,115].

The plasma of patients with FCCM and CMB showed that *miR-431-5p* was dysregulated (Table 2). This miRNA has been shown to inhibit angiogenesis and lymphangiogenesis through suppressing the secretion of VEGF-A and ANG2 by targeting ZEB1 [116]. In addition, *miR-223-3p,* DE in HHT and CMB, has been shown to regulate neuroinflammation, including microglial and astrocytic activation, via the NLRP3 inflammasome across hypoxic and neurodegenerative contexts [117–119]. *MiR-223-3p* has been shown to inhibit M1 polarization of macrophages [120], through suppression of Notch signaling, and microglia in cerebral ischemia [121]. In a separate study in ischemic stroke [122], showed that *miR-223-3p* inhibition promoted NOTCH2 expression, apoptosis, and attenuated angiogenesis [122]. Moreover, the Notch mediated effects on neuroinflammation and angiogenesis of *miR-223-3p* may offer another potential axis for Notch signaling dysregulation in CMB and HHT [30,97]. Finally, *miR-223-3p* was shown to activate the PI3K/AKT signaling pathway by regulating EGFR expression [123].

### 4.3. Common pathways across neurovascular disorders

A dysregulation of the PI3K-Akt pathway has been shown to promote vascular permeability and inflammation [124]. In FCCM, the disruption of ECs has been shown to be mediated by PI3K-mTOR signaling [29]. Similarly, in HHT, mutant ECs have been linked to dysregulated PI3K/Akt signaling, contributing to vascular abnormalities and increased risk of bleeding [30,125]. Studies have suggested a role of PI3k-Akt activity in SWS [126]. Activity of a downstream molecule, mTOR, is increased in SWS brain tissue [127] promoting proliferation and reduced apoptosis in ECs [8,27,80,128].

*MiR-223-3p*, *miR-487b-3p*, and *miR-431-5p* target genes such as *ROBO2* and *SLIT3*, which play pivotal roles in preserving endothelial barrier function and reducing vascular permeability [129]. Dysregulation of ROBO SLIT compromises endothelial barrier stability, potentially leading to pathological bleeding and lesion formation [129–131].

Therapies are under investigation to inhibit Rho kinase, a downstream regulator of the ROBO SLIT pathway [132] to reduce BBB permeability [133,134]. Recent advances in cancer research have further highlighted the role of SLIT2 in vascular and immune modulation [135]. Mechanistically, SLIT2 mediates these effects via ROBO1 and ROBO2, operating through PI3K-γ signaling, a pathway previously shown to drive tumor-associated macrophage polarization and tumor progression across various cancer models [135]. The potential crosstalk between ROBO, SLIT, and PI3K-γ signaling pathways suggests a complex regulatory network impacting both vascular integrity and immune cell behavior [135].

Additionally, a commonly identified pathway in HHT and CMB included thrombospondin-1 mediated angiogenesis inhibition, which may reflect the hallmark bleeding tendencies and fragile vessel networks [30,136–138]. GADD45 signaling emerged in CMB and FCCM and oncogene-induced senescence in CMB, SWS, and HHT, linking miRNA dysregulation to cellular stress responses and vascular aging [139]. *GADD45* is an important regulator in cellular senescence and as part of the NF-κB-Gadd45-MAPK pathway linked to a critical regulatory pathway in many vascular disorders [140,141].

### 4.4. Unique miRNAs expressed in FCCM

In ischemia-reperfusion injury in rats, *miR-26b-5p* has been shown to reduce the production of inflammatory cytokines and reactive oxygen species (ROS) by negatively regulating the expression of N-MYC and PTEN, a potent suppressor of PI3K signaling [142]. *MiR-26-5p* also has been shown *in vitro* to target *Smad1*, a downstream TGF-β signaling molecule [143]. This may suggest that the *miR-26b-5p* downregulation found in FCCM plasma is reflective of an uninhibited inflamed microenvironment with elevated oxidative stress in the brain. Similarly elucidating miRNA regulation of ROS production and related injury, *in vitro* ischemic-reperfusion injury models reveal that *miR-199a-5p* exacerbates neuronal injury via ROS production and apoptosis by targeting *Brg1* [144]. Additionally, it reduces cell viability by attenuating the CAV-1/MEK/ERK pathway [145]. On the other hand, in an ischemic stroke model, *miR-199a-5p* reduced neuronal apoptosis, inflammatory cytokine production, and infarct volume by targeting *Ddr1*, a potential signaling mediator of extracellular matrix remodeling in the BBB [146]. Furthermore, *miR-199a-5p* has been shown to reduce BBB disruption after ischemic stroke by enhancing Akt signaling via suppression of PIK3R2 expression leading to increased VEGF and Claudin-5 [147]. Hypoxia was shown to induce underexpression of *miR-199a-5p* in a human brain EC line [148], consistent with its downregulation in circulating blood in FCCM, which has been known to harbor hypoxic microenvironments. Sponging of *miR-191-5p*, another miRNA downregulated in FCCM patient plasma, led to the subsequent increased expression of CDK6, enhanced cell migration, and EC angiogenesis in an *in vitro* model of ischemia-reperfusion injury [149]. In *in vivo* cerebral infarction, *miR-191-5p* downregulation attenuated targeting of *Bdnf*, a regulator of angiogenesis, and was associated with improved neurological deficits and reduced infarct volume [150]. *MiR-191-5p* was also associated with alleviated microglial-induced injury by suppressing the microglial MAPK signaling pathway [151]. Taken together, the downregulation of *miR-191-5p* in FCCM plasma may be reflective of varying mechanisms of response to dysregulation involving altered angiogenesis and microglial activation. Furthermore, *miR-491-5p*, downregulated in FCCM, has previously been associated with spontaneous hemorrhagic transition in the serum of patients with ischemic stroke [152]. *MiR-491-5p* has also been linked to oxidative-induced cell injury in a traumatic brain injury (TBI) model through the inhibition of metallothionein-2 [153]. Interestingly, *miR-491-5p* downregulation promotes cerebral blood flow and neovascularization in a TBI model [154]. The direction of how miRNAs are dysregulated in other models of brain disease may differ from FCCM, likely reflecting miRNA specificity to models studied.

### 4.5. Unique miRNAs expressed in SWS

Knockdown of *miR-17-5p*, downregulated in the plasma of SWS patients, has been shown to decrease cerebral microvascular density and blood flow, ROS production by cerebral ECs, apoptosis, cell senescence, and infarct volume in aged mice with ischemic stroke [155]. Additionally, the knockdown of *miR-17-5p* was shown to partially increase PTEN expression and decrease PI3K/Akt phosphorylation *in vitro* [155]. Furthermore, inhibition of PTEN expression via *miR-17-5p* was validated in an *in vitro* radiosensitized glioma model and showed a decrease in HIF-1α expression, a marker of hypoxia [156]. *MiR-17-5p* plasma levels were lower in ischemic stroke patients and targeted *SOS1*, a gene linked to ROS and inflammation in hypoxic/reoxygenation injury [157]. A decrease in *miR-17-5p* was also shown to increase the expression of NFATC3, which downregulated tight junction protein expression in ECs, therefore increasing BBB permeability [158]. Further indicating miRNA regulation of the BBB, a downregulation in *miR-17-5p* increased MMP3 expression which disrupted tight junctions and increased permeability in brain microvascular ECs [159]. Hypoxia was shown to alter BBB permeability in an *in vitro* ICH model through *miR-27a-3p* targeting *ARHGAP2* and tight junction protein expression elevation via the ARHGAP2/Wnt/β-catenin signaling pathway [160]. Furthermore, *miR-27a-3p* was shown to target *GSK3β*, activate Wnt/β-catenin signaling, upregulate claudin-5 and occluding expression, and finally reduce barrier leakage in human brain ECs [161]. Taken together, *miR-27a-3p* downregulation in SWS patient plasma potentially indicates a regulatory effect on BBB disruption in SWS.

In SWS, *miR-37b-3p* was identified to be downregulated in patient plasma. *MiR-27b-3p* inhibition, in lipopolysaccharide-activated microglia, resulted in reduced expression of pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-1β, and microglial apoptosis [162]. *MiR-27b-3p* has been further shown to regulate neuroinflammation by targeting the *Hoxa5*/Gm5106 axis [163]. Additionally, *miR-21-5p* deteriorates BBB permeability by targeting tissue inhibitor of matrix metalloproteinases and enhancing NF-κB signaling as seen in a TBI model with human brain microvascular endothelial cells (HBMECs) [164]. Exosomal *miR-21-5p* also displayed pro-angiogenic activity in human umbilical vein endothelial cells (HUVECs) by enhancing VEGF and VEGFR2 expression [165], while preserving against neuronal apoptosis post-subarachnoid hemorrhage (SAH) by targeting the PTEN/Akt pathway [166]. *MiR-21-5p* has also been shown with network analysis and functional enrichment to have miRNA-mRNA and miRNA-circRNA interactions involved in apoptosis and wound healing [167].

### 4.6. Unique miRNAs expressed in HHT

*ENG* knockdown HUVECs displayed downregulated expression of *miR-454-3p* and attenuated angiogenic processes [168]. Thus, *miR-454-3p* may reflect aberrant angiogenic activity of ECs in HHT. Similarly downregulated in HHT plasma, *miR-140-5p* has been shown to be downregulated in SAH patient serum [169]. When delivered via mesenchymal stem cell derived extracellular vesicles in an SAH mouse model, *miR-140-5p* reduced post-SAH brain injury. This effect was mediated by epigenetically upregulating activation of the cAMP/PKA/CREB pathway through inhibition of *HDAC7* and reducing pro-inflammatory cytokine expression and M1 polarization of microglia [169]. In contrast, inhibition of *miR-140-5p* was associated with attenuation of mitochondrial uncoupling and hypertension by targeting *Bcl-xL* possibly indicating disease and tissue-specific dysregulation [170]. Taken together, reduced expression of *miR-140-5p* in HHT plasma may reflect reduced inhibition of damaging neuroinflammation.

*MiR-29b-3p*, *miR-495-3p*, and *let-7b-5p*, only the latter of which was upregulated in HHT patient plasma, have shown evidence in various *in vitro* contexts of regulatory effect on signaling components within the TGF-β signaling cascade [171–173]. More specifically, *miR-29b-3p* inhibition promoted collagen production through the VEGFA/TGF-β axis

[173], *miR-495-3p* regulated angiogenesis through TGF-β1 and SMAD signaling pathways [171], and *let-7b-5p* can bind and suppress *TGFBR1* [172]. *MiR-29b-3p* has also been shown to mediate the downregulation of VE-cadherin expression and upregulation of MMP9 expression, attenuating barrier integrity of HBMECs [174]. Moreover, *let-7b-5p* counteracts migration and proliferation of PDGF-induced pulmonary artery smooth muscle cells [175]. *MiR-29b-3p* notably targeted *Pdgf-b* and reduced atrial fibrosis and remodeling [176]. Additionally, *miR-30c-5p* was shown to inhibit proliferation and migration of VSMCs by targeting *SDC2* and attenuating activation MEK/ERK signaling [177].

### 4.7. Unique miRNAs expressed in CMB

*MiR-25-3p* from hypoxic glioma cells was shown to target *PHLPP2* in macrophages and activate PI3K/AKT-mTOR signaling to promote polarization of macrophages to an M2 phenotype [178]. Furthermore, a decrease in *miR-25-3p* was associated with elevated TLR4 and NLRP3 expression, thereby suppressing microglial activation and increasing cytokine release [179]. Additionally, *miR-25-3p* upregulation was negatively associated with its validated targets *Klf4* and *Pten*—genes involved in cell cycle regulation and inflammation—and positively associated with inflammatory cytokines [180]. Interestingly, the TLR4 and KLF4 signaling are also known contributors to CCM formation, by mediating the gut-brain axis and dysregulating ROCK signaling respectively [75]. Aside from inflammation, *miR-25-3p* also promotes downstream VEGFR2 phosphorylation and associated angiogenesis in response to senescence in ECs due to aging [181]. Additionally, *miR-25-3p,* upregulated in ICH, targets *Nox4*, and has a protective effect by inhibiting oxidative stress and reducing hematoma volume after ICH [182]. Thus, *miR-25-3p* plays a role in regulating neuroinflammation and response to oxidative stress. In an ischemic stroke model, M2 microglial EVs-derived *miR-23a-5p* resulted in downregulation of TNF, MMP3, and NF-κB p65 expression, while increasing ZO-1, occludin, and claudin-5 expression and associated BBB leakage [183]. Taken together, the downregulation of *miR-23a-5p* in CMB plasma could reflect BBB disruption. *MiR-29b-2-5p* was previously found to target *PSEN1* and downregulate Aβ plaque formation *in vitro* as well as in an AD mouse model [184]. Thus, CAA's association and low expression of *miR-29b-2-5p* in CMB patient plasma may be indicative of a pro-Aβ forming environment contributing to lesion formation.

### 4.8. Limitations

While this study provides significant insights into miRNA regulation in neurovascular disorders, the relatively small sample size and cross-sectional design may limit the generalizability of the findings. The small sample size (n = 10 subjects per disease) enrolled in this study may lead to an increased false positive rate of DE miRNAs. This may limit the generalizability of the findings Future studies with larger, independent cohorts are needed for validation the DE miRNAs identified in this generating hypothesis study. Additionally, sample processing at multiple sites may introduce variability to plasma and RNA quality, but all sites used a standardized blood processing protocol to minimize variability. The miRNAs were assessed at a single point in time. Their stability at multiple points in time in the same patients must be verified, or their variation in response to disease progression or confounding morbidities. While the cohorts were matched for age and sex there may be additional confounding factors that may influence miRNA expression independently of disease pathology, such as chronic illnesses or medications. Future studies should include comprehensive comorbidity profiling and stratified analyses to isolate disease-specific signals. The associations herein do not prove causation but motivate novel mechanistic hypotheses about the role of the respective miRNAs. It also remains unclear if these miRNAs can be used for therapeutic gene silencing in the respective diseases.

Admittedly, the choice of diseases was rather arbitrary, as other pathologies could have been examined with vascular dysmorphism or bleeding. We took advantage of parallel projects examining common and distinct circulating protein biomarkers and cytokines in these same pathologies, and our discoveries herein will allow correlation with the protein biomarkers. We examined FCCM and not sporadic cases, HHT and not sporadic AVMs, and we examined CMB without attempting to characterize their amyloid or hypertensive etiologies. These pilot discoveries will need to be pursued within individual diseases for specific mechanistic and clinical questions, as is being done with biomarkers of hemorrhage in CCM [185].

## 5. Implications and future directions

The identification of shared pathways, such as the PI3K-Akt and ROBO SLIT signaling pathways, highlights potential biomarkers of disease activity and therapeutic targets that may be applicable across multiple neurovascular disorders. Rapamycin, inhibiting PI3K signaling is already being considered as potential therapy in both HHT and CCM [186,187]. Targeting miRNAs which modulate these pathways, could also provide a therapeutic strategy for restoring endothelial function and preventing vascular dysregulation. Disorder-specific miRNAs and associated pathways point toward more tailored biomarker and therapeutic approaches for each condition when considering potential microRNA targets.

Germline mutations in FCCM and HHT may show systemic endothelial dysfunction and maladaptive responses to vascular injury. Unique miRNAs to FCCM regulate oxidative stress, inflammation, and BBB permeability, implicating them in recurrent hemorrhages and chronic lesion development. In contrast, HHT-specific miRNAs suggest dysregulation in angiogenesis and inflammatory suppression, reinforcing the distinct pathophysiology of HHT. SWS and CMBs are primarily focal vascular disorders. SWS appears to be driven by miRNAs implicated in angiogenesis, neuroinflammation, and BBB integrity. However, CMBs involved miRNAs that regulated endothelial dysfunction, oxidative stress, and inflammatory pathways. Their miRNA signatures reflect local disease activity rather than inherited endothelial pathology. Additionally, CMB miRNAs were linked to a pro-amyloid environment. These miRNA differences could serve as biomarkers to help distinguishing these disorders from each other. Moreover, while overlapping pathways exist across these neurovascular conditions, each condition presents a distinct miRNA signature providing insight into potential mechanisms that differentiate its pathophysiology.

A systematic review reporting the association between miRNAs and the physiopathogenesis as well as clinical course of defined neurovascular diseases has recently been published [188]. Mechanistic studies in preclinical models should be conducted to establish how the miRNAs identified herein may be associated with disease pathology and their clinical course. Future studies with larger cohorts are needed to validate these miRNA signatures. Analytic validations of miRNA levels and their reproducibility are important. Robustly controlled, independent cohorts would be needed to further validate individual candidate miRNA levels for either unimodal or multimodal model generation for disease stratification. Furthermore, biomarker validation studies should postulate specific contexts of use, and relevant patient selection, follow up, and outcome assessment.

## CRediT authorship contribution statement

Janne Koskimäki: Data Curation, Visualization, Writing – review & editing, Writing – original draft, Formal analysis. Aditya Jhaveri: Data Curation, Methodology, Validation, Visualization, Writing – review & editing, Writing – original draft, Investigation, Formal analysis. Abhinav Srinath: Writing – review & editing, Investigation. Akash Bindal: Writing – review & editing, Writing – original draft, Investigation. Diana Vera Cruz: Software, Data Curation, Writing – review & editing, Formal analysis, Visualization. Geetha Priyanka Yeradoddi: Software, Data

Curation, Writing – review & editing, Formal analysis, Visualization. Rhonda Lightle: Writing – review & editing, Investigation. Justine Lee: Writing – review & editing, Writing – original draft, Investigation. Agnieszka Stadnik: Writing – review & editing, Investigation. Javed Iqbal: Writing – review & editing, Roberto Alcazar-Felix: Writing – review & editing, Investigation. Stephanie Hage: Writing – review & editing, Investigation. Sharbel Romanos: Writing – review & editing. Robert Shenkar: Writing – review & editing. Jeffrey Loeb: Writing – review & editing. Marie E. Faughnan: Writing – review & editing. Shantel Weinsheimer: Writing – review & editing, Investigation. Helen Kim: Writing – review & editing. Romuald Girard: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. Issam A. Awad: Resources, Writing – review & editing, Project administration, Methodology, Conceptualization, Funding acquisition, Supervision.

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## Declaration of competing interest

I.A.A. is a consultant for Neurelis and Ovid Therapeutics. H.K. is a consultant for Neurelis, Ovid Therapeutics, and Recursion Pharmaceuticals. J.Lo. serves as the Chief Clinical Strategist for the Sturge Weber Foundation. All other authors declare no conflicts of interest.

## Abbreviations

| | |
|---|---|
| **Aβ** | amyloid-β |
| **BBB** | blood-brain barrier |
| **BVMC** | Brain Vascular Malformation Consortium |
| **CAA** | cerebral amyloid angiopathy |
| **CCM** | cerebral cavernous malformation |
| **CMB** | Cerebral Microbleed |
| **CWCS** | cumulative weighted context score |
| **CCND2** | Cyclin D2 |
| **DAVID** | Database for Annotation, Visualization, and Integrated Discovery |
| **ddPCR** | droplet digital PCR |
| **DE** | differentially expressed |
| **EC** | endothelial cell |
| **FC** | fold change |
| **FCCM** | Familial Cerebral Cavernous Malformation |
| **FDR** | false discovery rate |
| **GEO** | Gene Expression Omnibus |
| **GR** | Gene Ratio |
| **HBMEC** | human brain microvascular endothelial cells |
| **HHT** | Hereditary Hemorrhagic Telangiectasia |
| **HO** | Healthy Old |
| **HUVEC** | human umbilical vein endothelial cells |
| **HY** | Healthy Young |
| **IPA** | Ingenuity Pathway Analysis |
| **IRB** | Institutional Review Board |
| **IS** | Interaction Score |
| **miRNA** | microRNA |
| **MRI** | magnetic resonance imaging |
| **ROS** | reactive oxygen species |
| **ROBO** | roundabout |
| **SAH** | subarachnoid hemorrhage |
| **SWI** | susceptibility weighted imaging |
| **SWS** | Sturge-Weber Syndrome |
| **TBI** | traumatic brain injury |
| **TGF** | transforming growth factor |
| **TNF** | tumor necrosis factor |
| **TNFR1** | tumor necrosis factor receptor 1 |
| **UChicago** | University of Chicago |
| **VSMC** | vascular smooth muscle cell |

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.102189.

## Data availability

The small-RNA sequencing data is available in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) database and is accessible through GEO series accession numbers GSE288162 and GSE287906. The CCM transcriptomic sequencing data is available in the National Center for Biotechnology Information's GEO database and is accessible through GEO series accession number GSE130176. Any additional data will be provided from the corresponding authors upon reasonable request.

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