Cerebral cavernous malformations form an anticoagulant vascular domain in humans and mice

How to cite:

Lopez-Ramirez, Miguel Alejandro; Pham, Angela; Girard, Romuald; Wyseure, Tine; Hale, Preston; Yamashita, Atsuki; Koskimäki, Janne; Polster, Sean; Saadat, Laleh; Romero, Ignacio A.; Esmon, Charles T.; Lagarrigue, Frederic; Awad, Issam A.; Mosnier, Laurent O. and Ginsberg, Mark H. (2019). Cerebral cavernous malformations form an anticoagulant vascular domain in humans and mice. Blood, 133(3) pp. 193–204.

For guidance on citations see FAQs.

© 2018 American Society of Hematology

https://creativecommons.org/licenses/by-nc-nd/4.0/

Version: Accepted Manuscript

Link(s) to article on publisher’s website:
http://dx.doi.org/doi:10.1182/blood-2018-06-856062

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online’s data policy on reuse of materials please consult the policies page.
Cerebral Cavernous Malformations Form an Anticoagulant Vascular Domain

Miguel Alejandro Lopez-Ramirez¹, Angela Pham¹, Romuald Girard², Tine Wyseure³, Preston Hale¹, Atsuki Yamashita³, Janne Koskimäki², Sean Polster², Laleh Saadat², Ignacio A. Romero⁴, Charles T. Esmon⁵, Frederic Lagarrigue¹, Issam A. Awad², Laurent O. Mosnier³, and Mark H. Ginsberg¹.*

¹Department of Medicine, University of California San Diego, La Jolla, CA, USA.
²Neurovascular Surgery Program, The University of Chicago, Chicago, IL USA.
³Department of Molecular Medicine, Scripps Research Institute, La Jolla, CA, USA.
⁴Department of Life, Health, and Chemical Sciences, Biomedical Research Network, The Open University, Milton Keynes, UK.
⁵Coagulation Biology Laboratory, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA.
⁶These authors contributed equally.

*Correspondence should be addressed to M.H.G., email: mhginsberg@ucsd.edu

Word counts for text and abstract: 3456 and 180
Figure/Table count: 7
Reference count: 52
Key Points

Increased levels of protein C activation co-factors form an anticoagulant vascular domain in cerebral cavernous malformations.

Plasma levels of soluble TM may represent a biomarker for CCM risk of hemorrhage.
Abstract

Cerebral cavernous malformations (CCM) are common brain vascular dysplasias prone to acute and chronic hemorrhage with significant clinical sequelae. The pathogenesis of recurrent bleeding in CCM is incompletely understood. Here we show that central nervous system (CNS) hemorrhage in CCM is associated with locally elevated expression of the anticoagulant endothelial receptors thrombomodulin (TM) and endothelial protein C receptor (EPCR). TM levels are increased in human CCM lesions and in the plasma of patients with CCMs. In mice, endothelial-specific genetic inactivation of Krit1 (Krit1ECKO) or Pdcd10 (Pdcd10ECKO), which cause CCM formation, result in increased levels of vascular TM and EPCR, and in enhanced generation of activated protein C (APC) on endothelial cells. Increased TM expression is due to upregulation of transcription factors KLF2 and KLF4 consequent to the loss of KRIT1 or PDCD10. Increased TM expression contributes to CCM hemorrhage, because genetic inactivation of one or two copies of the Thbd gene decreases brain hemorrhage in Pdcd10ECKO mice. Moreover, administration of blocking antibodies against TM and EPCR significantly reduced CCM hemorrhage in Pdcd10ECKO mice. Thus, a local increase in the endothelial co-factors that generate anticoagulant APC can contribute to bleeding in CCMs and plasma soluble TM may represent a biomarker for hemorrhagic risk in CCMs.
Introduction

Cerebral Cavernous Malformations (CCM) are common central nervous system (CNS) vascular anomalies that occur either sporadically or heritably and affect ~1/200 humans.\(^1\) Mutations of three genes *KRIT1* (CCM1), CCM2, or *PDCD10* (CCM3) are associated with development of CCM, cerebral venous capillary dysplasias with clusters of endothelium filled with blood and prone to hemorrhage.\(^2,3\) The *KRIT1*\(^{+/−}\) genotype is the most common cause of the familial form of CCM, whereas the *PDCD10*\(^{+/−}\) genotype often results in a more severe clinical manifestation of the disease.\(^4,5\) Unlike sporadic CCM, where a single lesion is typically observed, familial CCM are characterized by multiple vascular lesions believed to be due to random inactivation of the normal allele.\(^1,4,6\) Moreover, a combination of chronic bleeding and episodes of acute hemorrhagic stroke, associated with CCM, cause focal neurological deficits, headaches, epileptic seizures, and occasionally death.\(^7\) In addition, patients with *PDCD10* mutations are more likely to present significant CCM hemorrhages earlier in life.\(^4\) Although the annual symptomatic hemorrhage rate varies largely among different studies from 0.25 to 22.9% per patient-year,\(^2,8\) it is thought that all CCM harbor occult bleeding.\(^7,8\) Natural history studies and MRI analysis have identified prior CCM bleeding within a year as a predictor of repeated hemorrhage and subsequent clinical sequelae.\(^7,9,10\) However, the detailed molecular mechanisms underlying the pathogenesis of CNS hemorrhage in CCM remains elusive.
Recently, we performed genome-wide transcriptome analysis of the acute effects of inactivation of \textit{Krit1} in murine brain endothelial cells (BMEC) and found increased levels of \textit{Thbd} mRNA, which encodes the natural anticoagulant receptor, thrombomodulin (TM).\footnote{TM levels are notably low in normal young brain endothelium, it plays a role in the thromboresistant properties of the brain.\cite{12,13}} TM binds thrombin and, while bound, thrombin fails to convert fibrinogen into insoluble fibrin, and instead catalyzes formation of activated protein C (APC). APC generation is enhanced by the presence of the endothelial cell protein receptor (EPCR),\cite{13,14} the mRNA of which is also increased with loss of \textit{Krit1} in BMECs.\footnote{TM levels are notably low in normal young brain endothelium, it plays a role in the thromboresistant properties of the brain.\cite{12,13}}

Because APC is a potent natural anticoagulant, high levels of TM have been associated with a bleeding disorder\cite{15,16} and used as a biomarker of endothelial cell dysfunction,\cite{17} we hypothesized that increased TM and EPCR could create a local bleeding diathesis in CCM.

Here we show that TM is upregulated in CCM lesions, and in the plasma of individuals with the sporadic or familial form of the disease. In two acute models of CCM, we report marked increases in brain endothelial TM and EPCR following genetic inactivation of \textit{Krit1} or \textit{Pdcd10}. Increased expression of TM but not EPCR was ascribable to elevation of the transcription factors KLF2 and KLF4 in CCM. Increased TM expression contributes to CCM hemorrhage, because genetic inactivation of one or two copies of the \textit{Thbd} gene decrease brain bleeding in \textit{Pdcd10\textsuperscript{ECKO}} mice. Moreover, administration of blocking antibodies against TM and EPCR reduced CNS bleeding in \textit{Pdcd10\textsuperscript{ECKO}} mice. Thus we propose that
upregulation of TM and EPCR in CCM endothelium forms an anticoagulant vascular domain that contributes to the bleeding-induced morbidity in CCM.

**Material and methods**

**Patient recruitment.** From May 2015 to November 2017, 77 patients (mean age=34.26 ± 18.53 years, range = [5.18 - 76.02]) with confirmed diagnosis of CCM (38 sporadic, 21 CCM1 and 18 CCM3) and 10 healthy subjects (mean age=33.26 ± 7.49 years, range = [23.0 - 43.75]) were enrolled for this study. The recruitment of patients was performed in conjunction with their routine clinical evaluations or follow-up. All participants underwent informed consent to participate in this research in accordance to the Declaration of Helsinki, and approved by The University of Chicago Institutional Review Board (IRB). The ethical principles guiding the IRB are consistent with The Belmont Report, and comply with the rules and regulations of The Federal Policy for the Protection of Human Subjects (56 FR 28003).

As per currently accepted disease categorization, CCM cases were classified as sporadic if they harbored a solitary lesion on the most sensitive susceptibility weighted imaging (SWI) MRI sequences, or a cluster of lesions associated with a developmental venous anomaly (DVA). They were classified as familial if they harbored multifocal CCM lesions, a family history of CCM in a first-degree blood relative or a mutation genotyped at a CCM gene locus. Patients with CCM lesion resection or any prior brain irradiation were excluded.
Genetically-modified Mice. The endothelial-specific conditional Krit1 or Pdcd10 null mice were generated by breeding transgenic mice expressing endothelial specific Pdgfb promoter driven tamoxifen-regulated Cre recombinase, iCreERT2, in combination with loxP-flanked Krit1 exon 5 (Krit1^{fl/fl}) (Pdgfb-iCreERT2; Krit1^{fl/fl}) mice or in combination with loxP-flanked Pdcd10 exon 4 and 5 (Pdcd10^{fl/fl}, a generous gift from Wang Min, Yale University) (Pdgfb-iCreERT2; Pdcd10^{fl/fl}). All experiments were performed using aged matched Krit1^{fl/fl} or Pdcd10^{fl/fl} littermates on the same C57BL/6 background. Thbd^{fl/fl} mice (a generous gift from Hartmut Weiler, Medical College of Wisconsin) were crossed with Pdgfb-iCreERT2; Pdcd10^{fl/fl} mice to generate Pdgfb-iCreERT2; Pdcd10^{fl/fl}; Thbd^{fl/wt} and Pdgfb-iCreERT2; Pdcd10^{fl/fl}; Thbd^{fl/fl} mice. Mice were administered 50 µg of 4-hydroxy-tamoxifen (Sigma-Aldrich, H7904) by intragastric injection on Postnatal day 1 inducing Cre activity and endothelial Krit1 or Pdcd10 gene inactivation in the littermates bearing the iCreERT2 (Krit1^{ECKO} or Pdcd10^{ECKO}). These mice and control Krit1^{fl/fl} or Pdcd10^{fl/fl} mice were sacrificed on the indicated postnatal days. All animal experiments were carried out in compliance with animal procedure protocols approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Isolation and purification of primary brain microvascular endothelial cells (BMEC). Adult 2 to 4 month old mice were sacrificed and their brains were removed and dropped into ice-cold buffer A (10 mM HEPES, 1x penicillin-
streptomycin, 0.5% bovine serum albumin (BSA) in DMEM) and processed as previously described.\textsuperscript{11}

**Genetic Inactivation of Krit1 or Pdcd10 in BMEC.** BMEC at passages 1-3 were maintained at 37 °C in 95% air and 5% CO\textsubscript{2} and were grown to 85 % confluence and treated for 48 h with 5 \(\mu\)M 4-hydroxy-tamoxifen (Sigma-Aldrich, H7904) after which the medium was replaced with medium lacking 4-hydroxy-tamoxifen\textsuperscript{11} and cells were harvested at the time indicated.

**Bleeding quantification.** \(Pdcd10^{ECKO}\) and littermate controls \(Pdcd10^{fl/fl}\) were treated with inhibitory antibodies (4.6 mg/kg) against mouse TM (MTM-1701\textsuperscript{20}) and (2.6 mg/kg) mouse EPCR (rcr-16\textsuperscript{21}) at postnatal (P) day 7 and euthanized at day P10. \(Pdcd10^{ECKO};Thbd^{ECKO/wt}\), \(Pdcd10^{ECKO};Thbd^{ECKO}\), \(Pdcd10^{ECKO}\) and littermate controls \(Pdcd10^{fl/fl}\) were euthanized at day P10. Brains were harvested and transferred into 500 \(\mu\)l of PBS 1x (SampleA) and incubated for 3 min with gentle movement. Retinas were transferred into 1 ml PFA 4% for 20 min and incubated at RT. After 3 washes with PBS 1x, retinas were dissected to remove cornea, lens and expose extravascular blood from the retina using 100 \(\mu\)l of PBS 1x (SampleB). Extravascular bleedings from brains (SampleA) and retinas (SampleB) were then processed. For brains we use 200 \(\mu\)l of SampleA while for retinas we used 50 \(\mu\)l of SampleB plus 150 \(\mu\)l of PBS. Acetic acid was added to a final concentration of
1% (v/v) in order to lyse the extravasated red blood cells and hemoglobin was subsequently measured using a microplate reader (VersaMax, Molecular Devices Corporation) set to 405 nm.

**Statistical analysis.** Data are expressed as means +/- standard error of the mean (S.E.M). For all experiments, the number of independent experiments (N) is indicated. The sample sizes were estimated with two-sample t test (two tailed). Two-tailed unpaired Student’s t-test was used to determine statistical significance. For multiple comparisons, one-way ANOVA follow by Tukey’s post hoc test was used. (*P<0.05, **P<0.01, ***P<0.001).
Results

Increased TM expression in CCM lesions

We recently performed genome-wide transcriptome analysis of the effects of acute inactivation of Krit1 in murine brain endothelial cells (BMEC) and observed increased Thbd mRNA that encodes a major anticoagulant endothelial receptor, TM. TM expression is modest in normal brain microvasculature relative to systemic blood vessels, suggesting that the brain is a hemostatically “privileged” environment to avert hemorrhage. To examine whether TM expression is increased in human CCM, we stained lesions obtained at surgery. TM staining was increased in the endothelium of human CCM lesions in comparison to lesion-free brain tissue from a CCM patient or non-neurological disease control (Figure 1A). Furthermore, increased expression of TM in CCM endothelial cells was confirmed by a ~ 2.9 fold increase in THBD mRNA in CCM lesion endothelial cells isolated by laser capture microdissection (Figure 1B).

We next examined venous plasma levels of soluble TM in familial and sporadic forms of CCM. Significantly increased soluble TM was detected in patients with familial CCM due to mutations in KRIT1 (CCM1) or PDCD10 (CCM3) (mean=3.57 ng/ml ± 0.64) compared to healthy subjects (mean=2.78 ng/ml ± 0.11). There was also a significantly increased soluble TM in sporadic CCM patients (mean=3.06 ng/ml ± 0.03) (Figure 1C). Thus CCM lesions arising from loss of function of either KRIT1 (CCM1) or PDCD10 (CCM3) led to an increase in TM protein and mRNA levels in CCM lesions and an increase of TM in the plasma.
Loss of brain endothelial KРИT1 or PDCD10 increases the expression of TM

To confirm that reduced expression of KRIT1 causes increased TM expression in human brain endothelium, we analyzed the effect of silencing of KRIT1 on TM expression in a human brain endothelial cell line. Infection of hCMEC/D3 cells with a virus encoding shRNA for KRIT1 (Figure 2A) significantly increased expression of TM mRNA and protein (Figure 2B,C). Immunofluorescence also revealed elevated levels of membrane bound TM protein in KRIT1-depleted human brain endothelial cells (Figure 2D). Flow cytometry analysis showed that surface TM protein increased ~1.7 fold in KRIT1-depleted cells (Figure 2E,F). An increase in human brain endothelial TM following loss of KRIT1 was accompanied by an increased capacity of brain endothelial cells to generate APC (Figure 2G). The increased APC generation was specific because blocking antibodies against TM or EPCR reduced APC generation (Figure 2G).

We used BMECs isolated from mice bearing floxed alleles of Krit1 (Krit1fl/fl) or Pdcd10 (Pdcd10fl/fl) and an endothelial-specific tamoxifen-regulated Cre recombinase (Pdgfb-iCreERT2), to investigate the effect of acute genetic inactivation of CCM genes on brain endothelial TM expression. Consistent with results observed in human brain endothelial cells, TM protein levels were significantly increased in 4-hydroxy-tamoxifen treated Pdgfb-iCreERT2; Krit1fl/fl (Krit1ECKO) BMECs when compared to control Krit1fl/fl BMECs (Figure 3A). Increased APC generation was also observed following genetic inactivation of Krit1 in BMECs (Data not shown). In addition, acute genetic inactivation of Pdcd10 in 4-
hydroxy-tamoxifen treated \( Pdgfb-iCreERT2;Pdcd10^{fl/fl} \) (\( Pdcd10^{ECKO} \)) BMECs caused a ~2.3 fold increase in TM protein levels as assessed by Western blotting (Figure 3B). Consistent with increased protein abundance, RT-qPCR analysis confirmed a dramatic increase of \( Thbd \) mRNA levels in both \( Krit1^{ECKO} \) (~8 fold) and \( Pdcd10^{ECKO} \) (~8.8 fold) BMECs when compared to control \( Krit1^{fl/fl} \) or \( Pdcd10^{fl/fl} \) BMECs, respectively (Figure 3C).

**KLF2 and KLF4 regulate expression of endothelial TM in CCM**

Transcription factors KLF2 and KLF4 are regulators of endothelial anticoagulant gene expression.\(^{24-27}\) We confirmed that the changes in brain endothelial TM, at the protein and mRNA levels, were associated with a large increase in transcription factors KLF2 and KLF4 in both \( Krit1^{ECKO} \) and \( Pdcd10^{ECKO} \) BMECs (Figure 3D,E). Therefore, to test whether a rise in KLF2 and KLF4 are inducers of TM during CCM, we used lentivirus-mediated transduction to ectopically express KLF2 and KLF4 in human endothelial cells (Figure 4A) at levels that we previously showed to suppress a target gene, \( Thbs1 \).\(^{11}\) Overexpression of KLF2 and KLF4 led to significant increase in TM mRNA levels when compared to control GFP-infected cells (Figure 4B). We also observed that ectopic expression of KLF2 or KLF4 resulted in ~4.2 fold or ~5.8 fold increases in TM protein, respectively as determined by Western blot analysis (Figure 4C). Flow cytometry analysis showed that membrane bound endothelial TM was ~4 fold-increased in KLF4 overexpressing cells when compared to control infected cells (Figure 4D,E). Moreover, we observed that reducing the expression of KLF2 and KLF4 (Figure
4F,G) prevented increased TM expression in KRIT1-depleted human brain endothelial cells (Figure 4H). Together these observations suggest that the rise in KLF2 and KLF4 lead to increased brain endothelial TM in CCMs.

**Brain endothelial TM increase is accompanied by hemorrhage in Pdcd10<sup>ECKO</sup> mice**

We used Pdcd10<sup>ECKO</sup> mice to investigate the distribution and expression of TM protein in experimental murine CCMs. At P9, Pdcd10<sup>ECKO</sup> hindbrains exhibited visible hemorrhagic CCM lesions (Figure 5A) and bleeding was confirmed in hematoxylin and eosin-stained sections (Figure 5B), and immunofluorescence staining of sections for erythrocytes using anti-TER119 (Figure 5C). Double staining of P9 Pdcd10<sup>ECKO</sup> cerebella for TM and Isolectin B4 showed increased TM staining of the luminal surface of CCMs (Figure 5D). In contrast, P9 Pdcd10<sup>fl/fl</sup> cerebellum showed minimal expression of TM (Figure 5D). To confirm this increase in TM expression, we isolated Pdcd10<sup>ECKO</sup> hindbrains and quantified TM protein and mRNA levels. Consistent with results observed in vitro and in situ, Pdcd10<sup>ECKO</sup> hindbrains showed ~10 fold and ~3.3 fold increase in TM protein and mRNA levels, respectively, as assessed by Western blot or RT-qPCR analysis, respectively (Figure 5E,F). There was no increase in PECAM1 abundance as assessed by Western blot, confirming that the increased TM was not due to increased density of blood vessels (Data not shown). Moreover, we observed increase in TM levels in heart but not in lung or intestine tissue from Pdcd10<sup>ECKO</sup> mice (Figure 5F). We also noted that expression of both KLF2 and KLF4 was
markedly increased in \textit{Pdcd10}^{ECKO} hindbrain tissue (Figure 5G) corroborating previous findings.\textsuperscript{28} The \textit{Pdcd10}^{ECKO} mice developed retinal vascular lesions in the leading front of the vascular plexus with extensive hemorrhages (Figure 5H,I) associated with a dramatic increase in TM staining and the increased bleeding was confirmed by increased hemoglobin (Figure 5I,J). Loss of function mutation in \textit{KRIT1} is the most common cause of the familial form of CCM. Staining of P11 \textit{Krit1}^{ECKO} hindbrains for TM revealed an increase in TM staining of the luminal Isolectin B4-positive CCM endothelial cells (Supplemental Figure 1); however, TM staining was clearly less intense that that observed in \textit{Pdcd10}^{ECKO} CCM. In contrast to \textit{Pdcd10}^{ECKO} mice, \textit{Krit1}^{ECKO} mice exhibited much fewer hemorrhagic CCM lesions (Supplemental Figure 1). Thus, CCM in \textit{Pdcd10}^{ECKO} and \textit{Krit1}^{ECKO} mice, like humans, express increased TM in CCM, neurovascular lesions that lead to brain hemorrhage.

\textbf{EPCR expression in the brain is upregulated in CCM but is not regulated by KLF2 or KLF4}

EPCR, an endothelial transmembrane protein that presents protein C to the thrombin-TM complex\textsuperscript{14} is expressed in the brain vasculature and plays a crucial role in the vascular anticoagulant pathway\textsuperscript{29,30} by facilitating protein C activation.\textsuperscript{31} We next investigated whether EPCR levels were also altered in CCMs. \textit{PROCR (EPCR)} mRNA and protein expression was increased in human CCM in comparison to control brain tissue (Figure 6A,B). Quantitative RT-PCR and Western blot analysis confirmed the upregulation of EPCR mRNA and protein in
Krit1ECKO and Pdcd10ECKO BMECs (Figure 6C,D,E). Consistent with results observed in vitro, Pdcd10ECKO hindbrains showed ~2 fold increase in Procr (Epcr) mRNA abundance (Figure 6F). Increased Procr mRNA was specific to lesion areas because we did not observe an increase in other vascular beds of Pdcd10ECKO mice analyzed (Figure 6F). In contrast to TM, ectopic expression of KLF2 or KLF4 did not change EPCR protein or mRNA levels in endothelial cells as assessed by Western blot or RT-qPCR analysis, respectively (Figure 6G,H).

**Blocking TM and EPCR attenuates vascular hemorrhage in Pdcd10ECKO mice**

To assess the role of endogenous endothelial TM in CCM hemorrhages, we examined the impact of genetic inactivation of endothelial Thbd in Pdcd10ECKO mice. Pdcd10ECKO;ThbdECKO/wt mice exhibited a significant reduction in brain bleeding (~ 1.6 fold decrease) as did Pdcd10ECKO;ThbdECKO mice (~1.8 fold decrease) when compared to Pdcd10ECKO littermates (Figure 7A). Moreover the upregulation of TM and EPCR contributed to brain hemorrhage in Pdcd10ECKO mice, because blocking antibodies against mouse TM and mouse EPCR reduced bleeding (Figure 7B) as signaled by a ~2 fold reduction in extractable hemoglobin in Pdcd10ECKO mice (Figure 7B). These data suggest that CCMs caused by mutations of KRIT1 or PDCD10 lead to the formation of an anticoagulant vascular domain due to increased expression of TM and EPCR driven in part by transcription factors KLF2 and KLF4 thereby contributing to increased hemorrhage (Figure 7C).

**Discussion**
CNS bleeding is a primary source of morbidity and mortality in CCM patients. We report that CCM endothelial cells manifest elevated expression of TM and EPCR mRNA and protein. This cell-autonomous increased expression of TM and EPCR is a direct result of a decrease of the products of KRIT1 or PCDC10, two genes whose loss of function leads to CCM formation. A rise in expression of KLF2 and KLF4, two transcription factors already implicated in CCM pathogenesis, can account for increased TM expression; however, these transcription factors did not account for the upregulation of EPCR expression. The increased abundance of TM and EPCR on the endothelial cell surface results in increased capacity to support generation of APC, a potent endogenous anticoagulant that inactivates the coagulation cofactors, Factor Va and Factor VIIa. Increased TM expression levels contributed to CCM hemorrhage because genetic inactivation of one or two copies of the Thbd gene in endothelial cells decrease brain bleeding in Pdcd10\textsuperscript{ECKO} mice. Moreover, administration of blocking antibodies against TM and EPCR significantly reduced CCM hemorrhage in Pdcd10\textsuperscript{ECKO} mice. These studies identify an increase in expression of two endothelial co-factors for generation of anticoagulant APC in CCM and indicate that the formation of an anticoagulant endothelial domain in CCM lesions can contribute to bleeding. Thus, manipulation of the balance between anticoagulant and cytoprotective effects of APC could serve to reduce the incidence and morbidity of CCM bleeding.

The elevation of endothelial surface TM and EPCR can contribute to bleeding in CCM. Cerebral capillary dysplasias with hemosiderin deposition detected by
MRI\textsuperscript{10,32} or histology\textsuperscript{3,10,33} are hallmarks of CCM lesions. Electron microscopic studies have documented loss of tight junctions in some CCM\textsuperscript{34} and KRIT1 enables Rap1 stabilization of endothelial cell-cell junctions.\textsuperscript{35,36} Indeed, we have recently found that loss of tight junctions is one of the earliest manifestation of inactivation of Krit1 or Pcdc10 in BMEC.\textsuperscript{11} Disruption of brain interendothelial junctions in CCMs\textsuperscript{11,37,38} could trigger the recurring micro-hemorrhages that cause hemosiderin deposition. Importantly, bleeding is much less evident in other cerebrovascular diseases that exhibit loss of blood-brain barrier (BBB) integrity due to disruption in brain endothelial tight and adherens junctions.\textsuperscript{39} Normal brain vasculature maintains restricted levels of TM and EPCR compared to systemic blood vessels\textsuperscript{22,40} suggesting that the brain is a pro-hemostatic environment. Our finding that loss of CCM1 or CCM3 causes a marked increase of EPCR and TM on the surface of CCM endothelial cells suggests that the CCM form an anticoagulant vascular domain within the brain that causes a local bleeding diathesis.

Familial and sporadic forms of CCMs exhibited significant increases in soluble TM in plasma, suggesting that this may be a biomarker for patients at elevated risk of bleeding. Elevated levels of TM in plasma have been documented and are associated with endothelial dysfunction\textsuperscript{17,41} and bleeding disorders.\textsuperscript{15-17} As discussed above, the increased TM can reflect an increased formation of APC in lesions which, in turn, leads to anticoagulant activity\textsuperscript{13} that facilitates bleeding. As the number of potentially new therapeutic interventions in CCMs grows,\textsuperscript{11,42-45} the
identification of markers to identify patients with elevated risk of hemorrhage becomes imperative. Indeed, recent studies\textsuperscript{46,47} showed that levels of inflammatory and angiogenic molecules in peripheral blood plasma could predict CCM clinical behavior, and this work now identifies soluble TM as a new putative biomarker candidate in this disease.

Sangwung et al\textsuperscript{48} show that endothelial cell loss of KLF2 and KLF4 results in substantial loss of TM and we report here that overexpression of these transcription factors can drive TM expression, as would be predicted from previous studies.\textsuperscript{24-26} There is now compelling evidence that elevation of KLF2 and KLF4 make important contributions to the pathogenesis of CCM.\textsuperscript{11,28,42,49} Endothelial-specific deletion of KLF2 and KLF4 in adults leads to lethality\textsuperscript{48} indicating that inhibition of these transcription factors is unlikely to be a suitable therapeutic strategy. Here we add the upregulation of TM to the growing list\textsuperscript{11} of KLF2 and KLF4 targets that can contribute to the pathogenesis of CCM disease and might serve as therapeutic targets. In contrast, we could not ascribe the rise in EPCR expression to KLF2 and KLF4. In particular, depleting KRIT1 increased EPCR and TM expression in cultured endothelial cells whereas over expression of KLF2 and KLF4 only increased TM. Thus, the cell autonomous increase in EPCR does not appear to be a consequence of increased expression of KLF2 and KLF4 or of TM. The links between loss of CCM genes and increased EPCR expression could be a fruitful area for future work.
The work reported here suggests new potential avenues for the management of CNS hemorrhage in CCM. Insights gleaned from bleeding in Hemophilia increased our awareness that excessive or unbalanced APC generation can actively contribute to bleeding and that the anticoagulant activity of APC is a valid target for drug development.\textsuperscript{50,51} However, APC is a multifunctional protease and independently of its anticoagulant activity, APC induces cytoprotective effects on endothelial cells and neurons that are dependent on protease activated receptor (PAR) 1, PAR3, and EPCR. These cell signaling activities of APC are central to the primary mechanism of action for APC’s neuroprotective effects in a variety of acute and chronic neuropathologies, including ischemic stroke, traumatic brain injury, chronic cerebral ischemia, amyotrophic lateral sclerosis, and multiple sclerosis.\textsuperscript{52} Strategies targeting the protein C system aimed at mitigating or preventing bleeding in CCM, therefore, need to be specific for APC anticoagulant activity while leaving APC’s neuroprotective activities unaltered. This was also illustrated by our experiments where inhibition of all APC activities (anticoagulant and cytoprotective) using the anti-mouse (A)PC antibody SPC-54\textsuperscript{51} caused premature death and cerebral thrombosis in \textit{Pdcd10\textsuperscript{ECKO}} mice (data not shown). Several anticoagulant-specific strategies targeting the protein C pathway have been proposed for control of bleeding in hemophilia patients with inhibitors, including anti-APC and anti-protein S antibodies specifically targeting APC’s anticoagulant activity, APC-resistant Factor Va, and others.\textsuperscript{50,51} Whether these strategies can be safely adapted to CCM will have to be determined since the safety window in hemophilia and CCM is likely quite different and targeting of the
acute CNS bleeds versus the risk of re-bleeding in the 1 year window of increased vulnerability that follows such bleeds will be an important consideration for this.\textsuperscript{7,8}

Another consideration is that the high level of APC in CCM lesions could diminish morbidity by means of its cytoprotective effects that include protection of vascular and blood-brain-barrier integrity.\textsuperscript{14,29} Indeed, signaling-selective 3K3A-APC with minimal anticoagulant activity reduced tissue-type plasminogen activator-induced bleeding in a murine ischemic stroke model.\textsuperscript{53} indicating that consequences of the enhanced APC generation in CCM might be more complex. New studies are needed to appreciate these complexities and to evaluate what strategies targeting the different components of the protein C system will be most advantageous in CCM. The work reported here motivates the development of such novel strategies for the management of CCM hemorrhage and suggests new biomarkers that could serve to identify those patients with elevated risk of bleeding as potential subjects for such new therapeutic strategies.

**Acknowledgments**

We thank Angioma Alliance for providing us with human CCM specimens. The authors thank Selina Chang, Riya Verma, Tiffany S. Shamas and Shady Soliman for technical assistance. We are grateful to Jennifer Santini for microscopy technical assistance and the UCSD School of Medicine Microscopy Core. The anti-EPCR antibodies (rcr-252, rcr-2 and rcr-16) were generously made available by Dr. Kenji Fukudome (Saga Medical School, Saga, Japan). Thbd\textsuperscript{fl/fl} mice were the generous gift of Dr. Hartmut Weiler (Blood Research Institute, Milwaukee, WI).
This work was supported by NIH grants NS092521 (to M.H. Ginsberg and I.A. Awad), K01HL133530 (to M.A. Lopez-Ramirez), HL104165, HL130678, and HL142975 (to L.O.M.), HL078784 (to M.H. Ginsberg), and the University of California, San Diego School of Medicine Microscopy Core (P30 NS047101).

**Authorship Contributions**

M.A.L.R designed and performed the experiments, performed analysis and interpretation of the data, generation of the figures and wrote the paper; A.P., P.H. performed and analyzed histological and gene expression experiments in culture and conditional knockout mice; R.G., J.K., S.P., I.A. performed, analyzed and interpreted the RNA transcriptome of human CCM lesions and human thrombomodulin plasma levels; T.W., A.Y., L.O.M. performed, analyze and interpreted protein C activation and bleeding studies; R.G., A.P., P.H., T.W., A.Y., I.A. and L.O.M helped in writing the manuscript; F.L., I.A.R., L.O.M provided reagents and help in interpretation of data, CTE provided critical reagents, and M.H.G. conceived the project, performed overall study design, analysis and interpretation of the data, and wrote the paper.

**Conflict of Interest Disclosures**

UCSD and TSRI hold intellectual property rights related to APC-resistant Factor Va and some uses of APC mutants on which LOM is listed as inventor. LOM is a founder and a member of the Board of Directors of Hematherix LLC. The other authors declare no competing financial interests.
References


**Figure Legends**

**Figure 1. Increased TM in CCM.** (A) Immunofluorescence staining of TM (red) and collagen IV (green) of human CCM, lesion-free brain tissue matched to the CCM patient (2 samples) and brain tissue from a healthy subject as control. Asterisks show vascular lumen of CCM lesions. Bar, 100 µm (n=4). (B) Expression levels of *THBD* as assessed by RNA-seq of endothelial cells isolated from human CCM lesions (SEM, n=5). (C) Plasma levels of soluble TM in patients with sporadic CCM (SEM, n=38), CCM1 (SEM, n=21), CCM3 (SEM, n=18) and normal controls (SEM, n=10). **, P<0.01, ***, P<0.001; determined by an unpaired two samples t-test.

**Figure 2. Loss of KRIT1 increases TM in human brain endothelial cells.** hCMEC/D3 cells were transduced with shKRIT1 or shControl using lentivirus. (A) KRIT1 ShRNA induced a ~30% decrease in KRIT1 mRNA in hCMEC/D3 cells as determined by RT-qPCR. (B) KRIT1-depleted hCMEC/D3 cells expressed ~2 fold
as much TM mRNA as ShControl cells (SEM, n=4). (C) Silencing of KRIT1, using shKRIT1, in hCMEC/D3 cells resulted in ~ 2.9 fold increase in TM protein levels (SEM, n=3). (D) Representative confocal images of TM (red) staining in hCMEC/D3 cells transduced with shKRIT1 or shControl. Nuclei were counterstained with DAPI (blue). Bar, 200 \( \mu \text{m} \) (n=2). (E) Relative abundance of TM in hCMEC/D3 cells transduced with shKRIT1 or shControl as assessed by flow cytometry. (F) Mean fluorescence intensity (MFI) of membrane bound TM in shKRIT1 when compared to shControl transduced hCMEC/D3 cells in B (SEM, n=3). (G) Assessment of protein C activation on hCMEC/D3 cells transduced with shKRIT1 or shControl. Thrombin (IIa) and protein C (PC) were added to shKRIT1 or ShControl brain endothelial cells in the presence or absence of blocking antibodies anti-Thrombomodulin (aTM) or anti-EPCR (rcr252). As controls the non-blocking anti-EPCR (rcr2) antibody was used (SEM, n=3).

**Figure 3. Inactivation of Krit1 or Pdcd10 increases the expression of TM in murine brain endothelial cells.** (A) Quantification of TM protein from three independent biological replicates in Krit1\(^{ECKO}\) (KO) compared with Krit1\(^{fl/fl}\) (Flox) BMECs (SEM, n=3). (B) Quantification of TM protein from three independent biological replicates in Pdcd10\(^{ECKO}\) (KO) compared with Pdcd10\(^{fl/fl}\) (Flox) BMECs (SEM, n=3). (C) RT-qPCR analysis of Thbd mRNA in Krit1\(^{ECKO}\) or Pdcd10\(^{ECKO}\) BMECs compared with Krit1\(^{fl/fl}\) or Pdcd10\(^{fl/fl}\) BMECs (control), respectively (SEM, n=3). (D-E) Analysis of Klf2 and Klf4 mRNA by RT-qPCR in Krit1\(^{ECKO}\) BMECs in D or in Pdcd10\(^{ECKO}\) BMECs in E compared with Krit1\(^{fl/fl}\) or Pdcd10\(^{fl/fl}\) BMECs.
(control), respectively (SEM, n=3). *, P < 0.05; **, P < 0.01; ***, P < 0.001; determined by Student’s t test.

**Figure 4. KLF2 and KLF4 regulate expression of endothelial TM.** (A) HUVECs were transduced with lentivirus encoding shKRIT1, KLF2, or KLF4, and the increase in KLF2 or KLF4 mRNA relative to cells transduced with lentivirus encoding shControl (for shKRIT1) or GFP (for KLF2 or KLF4) was measured by RT-qPCR (SEM, n=5, data published36). (B) Analysis of THBD mRNA levels by RT-qPCR in HUVECs transduced with lentivirus encoding KLF2 or KLF4 compared with lentivirus encoding GFP as control (SEM, n=3 or 4). (C) Analysis of TM protein levels in HUVECs transduced with lentivirus encoding KLF2 or KLF4 as assessed by Western blot analysis; lentivirus encoding GFP was used as control (SEM, n=3). (D) Relative abundance of TM in GFP+ HUVECs transduced with lentivirus encoding KLF4-GFP or GFP control as assessed by flow cytometry. (E) Mean fluorescent intensity (MFI) of TM in KLF4-GFP+ cells when compared to control GFP+ transduced HUVEC cells in D (SEM, n=3). (F) Analysis of KLF2 mRNA levels by RT-qPCR in hCMEC/D3 cells transduced with lentivirus encoding ShKRIT1 or Scrambled control followed by transfection with KLF2 and KLF4 specific SiRNAs (SiK2/K4) or SiRNA control (SiCtrl) (SEM, n=4). (G) Analysis of KLF4 mRNA levels by RT-qPCR in hCMEC/D3 cells transduced with lentivirus encoding ShKRIT1 or lentivirus encoding Scrambled control followed by transfection with KLF2 and KLF4 specific SiRNAs (SiK2/K4) or SiRNA control (SiCtrl) (SEM, n=4). (H) Silencing of KLF2 and KLF4 (SiK2/K4) using specific
siRNAs prevented increased TM expression in KRIT1-depleted hCMEC/D3 cells. Cells were transduced with lentivirus encoding shKRIT1 compared to cells transduced with lentivirus encoding shControl (SEM, n=4). *, P < 0.05; **, P < 0.01; ***, P < 0.001; determined by Student’s t test.

**Figure 5. Loss of **Pdcd10** increased expression of brain endothelial TM. (A)** Pdcd10ECKO hindbrains show prominent hemorrhagic lesions Bar, 2 mm. (B) Hematoxylin and eosin staining of cerebellar sections from Pdcd10ECKO mice and Pdcd10fl/fl littermate controls. Arrows indicate bleeding into the brain parenchyma. Arrowhead indicates normal cerebral vasculature. Higher-magnification images are shown on the right panel. Bar, 100 μm (n=4 mice in each group). (C) Immunofluorescence staining of blood cells using TER119 (red) and label of the microvasculature using Isolectin B4 (white) of cerebellar sections from Pdcd10ECKO or Pdcd10fl/fl mice at P9; DAPI staining (blue) was used to reveal nuclei, Bar, 100 μm. Higher-magnification images are shown on the right panel, Bar, 50 μm. (n=3 mice in each group). (D) Immunofluorescence staining of TM (green) or Isolectin B4 (white) of cerebellar sections from Pdcd10ECKO and Pdcd10fl/fl mice. Asterisks, vascular lumen of CCM lesions. Bar, 50 μm. (n=4 mice in each group). (E) Quantification of TM in cerebellar tissue in Pdcd10ECKO and control Pdcd10fl/fl littermates as assessed by Western blot analysis (SEM, n=4 or 7 mice in each group). (F) Analysis of Thbd mRNA levels by RT-qPCR in cerebellar, heart, intestine and lung tissue in Pdcd10ECKO compared with control Pdcd10fl/fl littermates (SEM, n=6 or 8 mice in each group). (G) Analysis Klf2 and Klf4 mRNA
levels by RT-qPCR in cerebellar tissue in \( Pdcd10^{ECKO} \) compared with control \( Pdcd10^{fl/fl} \) littermates (SEM, \( n=7 \) mice in each group). (H) Whole-mount P9 retina show hemorrhage in \( Pdcd10^{ECKO} \) mice but not in retinas of littermate controls \( Pdcd10^{fl/fl} \) mice. (I) Maximum-intensity projection of whole-mount P9 retinal vasculature at the angiogenic growth front stained for TM (green), TER119, blood cells, (red) and isolectin B4 (turquoise) \( (n=4) \). (J) Measurement of hemoglobin content of extravasated erythrocytes from retinal vasculature at P10 in \( Pdcd10^{ECKO} \) and control \( Pdcd10^{fl/fl} \) littermates. **, \( P < 0.01 \); ***, \( P < 0.001 \); determined by Student’s \( t \) test and one-way ANOVA follow by Tukey’s post hoc test.

**Figure 6. Increased EPCR in CCMs.** (A) Immunohistochemistry of EPCR (brown precipitate) of human CCM and brain tissue from a healthy subject as control. Asterisks show vascular lumen of CCM lesions. Bar, 100 \( \mu \)m \( (n=3) \). (B) Analysis of \( PROCR \) (EPCR) mRNA levels by RT-qPCR in human CCM lesions or control brain tissue used in \( A \) (SEM, \( n=4 \) CCM tissue, \( n=1 \) lesion-free brain tissue from CCM patient, \( n=2 \) from healthy subject brain tissue and \( n=2 \) from post-mortem specimens as control). (C) RT-qPCR analysis of \( Procr \) (\( Epcr \)) mRNA in \( Krit1^{ECKO} \) or \( Pdcd10^{ECKO} \) BMECs compared with \( Krit1^{fl/fl} \) or \( Pdcd10^{fl/fl} \) BMECs (control), respectively (SEM, \( n=3 \)). (D) Quantification of EPCR protein in 9 days \( Krit1^{ECKO} \) (KO) compared with \( Krit1^{fl/fl} \) (Flox) BMECs (SEM, \( n=3 \)). (E) Quantification of EPCR protein in 20 days \( Pdcd10^{ECKO} \) (KO) compared with \( Pdcd10^{fl/fl} \) (Flox) BMECs (SEM, \( n=3 \)). (F) Analysis of \( Epcr \) mRNA levels by RT-qPCR in cerebellar, heart, intestine and lung tissue in \( Pdcd10^{ECKO} \) and control \( Pdcd10^{fl/fl} \) littermates (SEM, \( n=4 \) or 7
mice in each group). (G) Analysis of EPCR protein levels in HUVECs transduced with lentivirus encoding KLF2 or KLF4 as assessed by Western blot analysis; lentivirus encoding GFP was used as control (SEM, n=3). (G) Analysis of EPCR mRNA levels by RT-qPCR in HUVECs transduced with lentivirus encoding KLF2 or KLF4 compared with lentivirus encoding GFP as control (SEM, n=3 or 4). *, P < 0.05; determined by Student’s t test and one-way ANOVA follow by Tukey’s post hoc test.

**Figure 7. TM and EPCR create anticoagulant vascular domain in CCMs.** Measurement of hemoglobin content of extravasated erythrocytes from hindbrains at P10. (A) Decreased cerebellar hemorrhages are observed in Pdcd10 \textsuperscript{ECKO};Thbd \textsuperscript{ECKO}/\textsuperscript{wt} or Pdcd10 \textsuperscript{ECKO};Thbd \textsuperscript{ECKO} when compared to Pdcd10 \textsuperscript{ECKO} littermates. Pdcd10 \textsuperscript{fl/fl} is a non-CCM lesion control (SEM, n=5 or 8). (B) Blocking antibodies against TM (4.6 μg/g) and EPCR (2.6 μg/g) or IgG (7.2 μg/g) control were injected retroorbitally into Pdcd10 \textsuperscript{ECKO} or control Pdcd10 \textsuperscript{fl/fl} littermate controls at P7 (SEM, n=3 or 13). (C) Schematic diagram of the formation of an anticoagulant domain in CCM lesion. Increased expression of KLF2 and KLF4 transcription factors lead to upregulation of brain endothelial TM. In addition, EPCR expression is upregulated in CCMs independently of KLF2 and KLF4. Increased levels of TM and EPCR create anticoagulant domains that predispose the CCM lesions to hemorrhages.
Figure 2

A) KRIT1 mRNA levels relative to ShControl

B) TM RNA levels relative to ShControl

C) Western blot

D) Immunofluorescence

E) Flow cytometry

F) Mean Fluorescence Intensity (MFI) of TM

G) APC generated (nM)