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## Cerebral Cavernous Malformation: From Mechanism to Therapy

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### Abstract

Cerebral cavernous malformations (CCMs) are acquired vascular anomalies that constitute a common cause of central nervous system hemorrhage and stroke. The past two decades have seen a remarkable increase in our understanding of the pathogenesis of this vascular disease. This new knowledge spans genetic causes of sporadic and familial forms of the disease, molecular signaling changes in vascular endothelial cells that underlie the disease, unexpectedly strong environmental effects on disease pathogenesis, and drivers of disease endpoints such as hemorrhage. These novel insights are the integrated product of human clinical studies, human genetic studies, studies in mouse and zebrafish genetic models and basic molecular and cellular studies. This review addresses the genetic and molecular underpinnings of CCM disease, the mechanisms that lead to lesion hemorrhage, and emerging biomarkers and therapies for clinical treatment of CCM disease. It may also serve as an example for how focused basic and clinical investigation and emerging technologies can rapidly unravel a complex disease mechanism.

### Keywords

Cerebral cavernous malformation; cavernoma; cavernous angioma; vascular malformation

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Disclosures

None.

## Introduction

Cerebral cavernous malformations (CCMs) are a type of vascular lesion that form specifically in the central nervous system. CCMs are not a major underlying cause of cerebrovascular disease, but their investigation has been unusually fruitful and provided unexpected molecular and genetic insight into vascular development and disease. As discussed in greater detail below, multi-disciplinary studies of CCM disease performed by a group that includes clinicians, human geneticists, mouse and zebrafish biologists, cell biologists, and molecular biologists have culminated in an exceptionally rapid and deep understanding of disease pathogenesis.

This review summarizes the major developments in the CCM field over the past few decades, with the goal of highlighting recent advancements in the disease based on analysis of human genetics and murine models. Recent findings, such as the identification of PIK3CA mutations in CCM lesions, offer crucial insights into how genetics explain the highly variable penetrance in the phenotype and natural history of the disease. In addition to genetic factors, we discuss the importance of environmental contributors such as the microbiome and gut barrier in altering upstream inputs into the CCM signaling pathway. We emphasize the myriad ways in which CCM disease pathology is part of a complex network of pathways, operating at the intersection of hemodynamics, inflammation, and angiogenesis, and conclude with the current state of clinical trials and promising new avenues of therapeutics. The rapid rate of discovery in this field, and the close and productive interactions between the many different types of investigators working in it, have both brought the disease close to translational breakthroughs and provided a template for a modern approach to unraveling disease pathogenesis.

### CCM Human Genetics: Mechanisms of Familial and Sporadic Disease

**Heterozygous Mutation of CCM Genes: *KRIT1*, *CCM2*, or *PDCD10* Causes Familial CCM**—CCM disease can be broadly classified as either sporadic or familial. While sporadic CCMs typically present as solitary lesions in otherwise healthy individuals, familial CCMs typically present with a multiplicity of lesions and follow an autosomal dominant inheritance pattern<sup>1–3</sup>. Familial CCM has been shown to be caused by heterozygous loss of function (LOF) mutations in either *KRIT1*<sup>4, 5</sup>, *CCM2*<sup>6, 7</sup>, or *PDCD10*<sup>8</sup>. Mutations in these genes primarily consist of nonsense, frameshift, and canonical splice site mutations. Pathogenic missense mutations have been reported in all three genes; however, they make up the minority of reported mutations. Notably, there are 4 known founder mutations in the CCM genes that account for substantial fraction of familial CCM cases. The most common of these is NM\_194456.1(*KRIT1*):c.1363C>T(p.Gln455Ter) present in most Hispanic American cases of familial CCM<sup>5, 9</sup>. Another less frequent *KRIT1* founder mutation: NM\_194456.1(*KRIT1*):c.987C>A(p.Cys329Ter), has been found in several kindreds with Sardinian lineage<sup>10</sup>. A deletion spanning 77.6kb of *CCM2* resulting in deletion of exons 2 – 10 has been found in several Caucasian kindreds, it remains unclear whether all cases with this mutation are related or whether it has occurred independently in other families<sup>11</sup>. A splice mutation affecting *CCM2*:

NM\_031443.3(CCM2):c.30+5\_30+6delinsTT, has been found in seemingly unrelated Ashkenazi Jewish probands, possibly due to an historically ancient mutation<sup>12</sup>.

### Modifiers of Disease Severity

**Genotype-Phenotype Correlation.:** The clinical manifestations of familial CCM are highly heterogeneous. In many genetic diseases, the primary determinant of progression and severity is the causal mutation. However, this does not seem to be the case for CCM. A prime example of this is the common Hispanic founder mutation. Disease progression and severity in individuals with the Hispanic founder mutation is highly variable, despite sharing an identical pathogenic mutation<sup>13–16</sup>. The lack of correlation between clinical manifestations and mutation identity is consistent with the finding that the majority of germline mutations found in *KRIT1*, *CCM2*, and *PDCD10*—primarily frameshift, nonsense, and splice site mutations—result in loss of function. This suggests that the identity of mutations *within the same gene* does not have a significant impact on disease severity.

Though different mutations within a gene do not seem to impact disease severity, the identity of the mutated gene has been associated with several clinical characteristics. Many groups have noted an association between individuals with *KRIT1* mutations and cutaneous vascular lesions<sup>16–22</sup>. Individuals with a mutation in *CCM2* are more likely to be asymptomatic and have lower number of lesions compared to individuals with *KRIT1* or *PDCD10* mutations<sup>23</sup>. Familial CCM has been shown to be significantly more aggressive in individuals with a mutation in *PDCD10*<sup>24–26</sup>. The severity of CCM associated with *PDCD10* mutations is attributable to the role of *PDCD10* in the gut epithelium not shared with *KRIT1* or *CCM2* as discussed further below<sup>27</sup>.

**Genetic Modifiers.:** To identify genetic variants associated with disease severity, one group performed a large genetic association study of individuals with the *KRIT1* founder mutation that identified several genetic polymorphisms within inflammatory and immune response genes that are associated with total lesion count, number of large lesions, and intracerebral hemorrhage<sup>28</sup>. This analysis revealed associations between clinical disease presentation and variants in several genes including: *TGFBR2*, *CD14*, *IL-6R*, *MSR1*, *IGH*, and *TLR4*. Some of these genes have been shown to have critical roles in CCM pathogenesis, highlighting the importance of further evaluating the roles of these genes, and demonstrating the power of association studies in a genetically homogenous cohort. Identification of *TLR4* variants associated with disease severity are of particular interest owing to the direct role of *TLR4* in propagating CCM signaling (discussed below)<sup>27</sup>. These data suggest that polymorphisms in genes other than *KRIT1*, *CCM2*, and *PDCD10* may be important modifiers of CCM pathogenesis.

**Radiation-Induced CCMs.:** While the majority of sporadic CCMs occur in otherwise healthy individuals, numerous reports have shown that ionizing radiation is a potent inducer of CCM formation<sup>29–38</sup>. While pathologically similar to non-radiation induced sporadic CCMs, they have several distinct characteristics that hint at the underlying mechanisms driving CCM pathogenesis. One such characteristic is that individuals with radiation-induced CCMs often present with multiple lesions, in stark contrast to non-radiation induced

sporadic CCMs which almost always occur as a solitary lesion. Furthermore, it was found that occurrence of multiple radiation-induced CCMs is significantly associated with younger age at time of radiation treatment<sup>29</sup> and that the presence of multiple radiation induced CCMs may be related to higher doses of ionizing radiation<sup>37</sup>. Ionizing radiation has long been recognized as a potent source of DNA damage leading to genomic instability reviewed elsewhere<sup>39</sup>. The observation that radiation treatment may induce CCM formation—and that the multiplicity of lesions is related to radiation dose and age at radiation—support a key role for somatic mutations in CCM pathogenesis.

### **Role of Somatic Mutations in CCM Pathogenesis**

**Somatic Mutation of *KRIT1*, *CCM2*, or *PDCD10* drives CCM formation.** Although germline LOF mutations in *KRIT1*, *CCM2*, or *PDCD10* cause familial CCM as a clinical entity, they do not explain why CCMs present as focal lesions rather than a systemic vascular defect as might be expected if CCMs were the result of haploinsufficiency. This observation led to the hypothesis that a secondary, local event is necessary to initiate lesion formation; specifically, a somatic mutation in a CCM gene resulting in biallelic LOF. Somatic mutations resulting in biallelic LOF of *KRIT1*, *CCM2*, or *PDCD10* have been reported in both familial<sup>40–42</sup> and sporadic<sup>43</sup> CCMs. Furthermore, laser capture microdissection and immunohistochemical staining of the CCM proteins in the lesion endothelium has established that these mutations occur in endothelial cells<sup>41, 45, 46</sup>. Together these data show that while loss of a CCM gene is dominant at the level of an individual, it is recessive on a cellular level, requiring loss of the normal allele via somatic mutation prior to lesion formation (Figure 1).

**Activating *PIK3CA* Somatic Mutations in Familial and Sporadic CCMs.** The two-hit model of CCM pathogenesis elegantly explains why CCMs occur as focal lesions and why individuals with familial CCM have numerous lesions whereas sporadic cases almost always have a single lesion. The two-hit model has also proven true in mouse models of CCM (discussed below) where biallelic loss of *Krit1*, *Ccm2*, or *Pcd10* is required for lesion formation<sup>47, 48</sup>. One limitation of these models is that lesions only form during a brief window during early development. This observation is contradictory to what we find in human CCM disease where lesions may form throughout adulthood<sup>49, 50</sup>. This discrepancy may be explained by the recent discovery that many human CCMs harbor gain of function (GOF) mutations in the oncogene *PIK3CA* in addition to the previously noted LOF somatic mutations in the CCM genes. Somatic GOF mutations in *PIK3CA* are present in both sporadic<sup>43, 51, 52</sup> and familial CCMs of all three genotypes<sup>43, 51, 52</sup>. *PIK3CA* mutations co-occur with somatic CCM gene mutations indicating that in some sporadic CCMs no less than three independent somatic mutations (two LOF somatic mutations in a CCM gene, and a GOF somatic mutation in *PIK3CA*) occurred during lesion development (Figure 1). Single-nucleus DNA sequencing has shown that *PIK3CA* and CCM gene somatic mutations are present in the same clonal population of cells suggesting that the effects of these mutations synergize in a cell autonomous manner. Consistent with the finding of activating *PIK3CA* somatic mutations, several previous studies have noted a link between CCM pathogenesis and PI3K signaling<sup>44, 53</sup>. Indeed, mouse models of CCM that previously

only developed lesions during early development, when subjected to *PIK3CA* activation, develop lesions in adulthood.

Although activation of *PIK3CA* seems to be a common feature of resected human CCM lesions, it is unlikely that somatic activation of *PIK3CA* is *required* for lesion formation. Mouse models of CCM without *PIK3CA* perturbation suggest that congenital lesions may form in the absence of a *PIK3CA* mutation, perhaps due to a high basal level of PI3K signaling during development. Likewise, CCMs in humans may form in the absence of *PIK3CA* mutations during periods of elevated angiogenesis as may occur during growth or as a result of environmental factors. Furthermore, one important caveat of human CCM sequencing studies is that genetic analysis of lesions is limited to those which have been surgically resected, as asymptomatic lesions are rarely removed. This is a potent bias, selecting for aggressive lesions which are most likely to have activating mutations in *PIK3CA*. It is highly likely that a minority of all CCMs, but a majority of *symptomatic* CCMs, have activating mutations in *PIK3CA*.

**Activating MAP3K3 Somatic Mutations in Sporadic CCMs.:** As discussed above, previous work has established that familial and sporadic CCMs follow two-hit model, where somatic mutation(s) of a CCM gene initiates lesion formation. Recent work has found that many sporadic CCMs harbor a specific gain of function mutation in *MAP3K3* (p.I441M), the gene that encodes MEKK3<sup>51, 52</sup>. Furthermore, *MAP3K3* mutations have been found to co-occur with *PIK3CA* mutations, however initial data suggest that CCM and *MAP3K3* mutations are mutually exclusive<sup>52</sup>. This finding suggests that gain of function in *MAP3K3* and loss of function in CCM genes have similar functional consequences such that either are sufficient to initiate CCM formation (Figure. 1)—distinct from mutations in *PIK3CA* which exacerbate lesion growth but not required for lesion formation. The equivalent effects of CCM and *MAP3K3* mutations is supported by the direct role of the CCM complex in inhibiting MEKK3 activity (Figure. 2) such that increased MEKK3 activity may be achieved by either loss of the CCM complex or by gain of function in MEKK3.

**Lesion Formation and Growth.:** The identification of somatic mutations in familial and sporadic CCMs raises several important questions about CCM pathogenesis. Does heterozygous loss of *KRIT1*, *CCM2*, or *PDCD10* increase the rate of somatic mutations? One possible explanation for the high lesion burden is that heterozygous loss of a CCM gene, as is the case in familial CCM, causes cellular stress and/or genomic instability leading to an increase in the rate of somatic mutations. If true, one might expect a significant association between familial CCM and cancer—similar to hereditary cancer syndromes caused by mutations in DNA repair genes (e.g., Li-Fraumeni syndrome caused by mutations in *p53*, and Lynch syndrome caused by mutations in 4 mismatch repair genes). To date, no association between familial CCM and cancer has been noted.

Do somatically-mutated endothelial cells clonally expand similar to cancers? The initial studies identifying somatic mutations in familial and sporadic CCMs noted that the allele frequency of the somatic mutations was generally quite low, typically <10%<sup>40–42, 51</sup>. This observation may be in part explained by the presence of non-lesional cells in the sample diluting the mutant allele, however it may also suggest that CCM lesions are mosaic—

consisting of both somatic mutant and wild type cells. Direct evidence of mosaicism in human lesions is limited to staining of the CCM proteins showing incomplete staining of the lesional endothelium<sup>45, 46</sup>. However, recent studies of mouse models<sup>54, 55</sup> suggest that initially CCMs grow by clonal expansion but as they mature, they begin to incorporate wild type endothelial cells into the lesion.

### CCM signaling pathways: molecular and genetic insights

#### Regulation of MEKK3-KLF2/4 signaling is a primary role of the CCM complex

—Identification of the mutant genes responsible for CCM disease using positional cloning approaches in familial CCM disease patients revealed three non-homologous proteins with protein binding motifs, but no enzymatic or transcriptional activity. How three distinct proteins caused one clinical syndrome became clear when biochemical studies demonstrated that KRIT1, CCM2 and PDCD10 form a molecular complex<sup>56–58</sup>. However, gene expression studies did not reveal a vascular-specific pattern of these genes, and where and how this complex might be required to prevent vascular malformation remained unknown.

In vivo studies using genetic model organisms have revealed that the heterotrimeric “CCM complex” is required for cardiovascular development. Loss of *ccm1* or *2* function in zebrafish results in a dilated heart phenotype associated with failure to create a lumenized aortic outflow tract<sup>59–61</sup>. Constitutive global loss in mice also results in failure to create a lumenized branchial arch artery and early embryonic lethality<sup>59, 62</sup>. These phenotypes were reproduced following endothelial-specific gene deletion<sup>63–66</sup>, suggesting that CCM function is required specifically in endothelial cells, but a molecular mechanism of action and a facile animal model with which to investigate disease pathogenesis remained elusive. This barrier was broken with the seminal demonstration that deletion of CCM genes in neonatal mice is sufficient to confer robust CCM formation<sup>63</sup>. Using this neonatal model, numerous groups have subsequently demonstrated that endothelial loss of CCM complex function is sufficient to confer lesion formation, confirming that the endothelial cell is the site of action for the CCM complex.

How does an adaptor protein complex regulate endothelial cell function? A critical clue to the molecular basis of CCM function arose prior to the identification of the disease-causing genes from studies of the MAP3K3 kinase, MEKK3. A yeast two-hybrid screen performed using MEKK3 as bait identified strong interaction with an uncharacterized protein, OSM, later identified as CCM2. This interaction was confirmed biochemically and characterized structurally<sup>67, 68</sup>, establishing that the CCM complex binds MEKK3 through the CCM2 protein that also binds KRIT1 and PDCD10. Genetic loss of function studies in the mouse demonstrated a critical role for MEKK3 in endothelial cells<sup>69</sup> and corroborated human genetic studies that identified sporadic MEKK3 mutations in verrucous venous malformations<sup>70</sup>. Genetic studies in the developing heart defined the functional significance of this interaction, demonstrating that CCM function is required to negatively regulate MEKK3 function in vivo<sup>66</sup>. Importantly, this essential functional role was confirmed in the neonatal mouse brain, where loss of MEKK3 or either of its established downstream transcriptional effectors, KLF2 or KLF4, was found to be sufficient to prevent CCM formation following endothelial cell loss of CCM function<sup>71–73</sup>. These

studies have established the MEKK3-KLF2/4 signaling pathway as a central signaling axis in CCM disease pathogenesis.

**Modulators of CCM signaling: HEG1, CCM2L, STK24/25 and CDC42**—In addition to the core players of the CCM complex and MEKK3-KLF2/4 signaling pathway, genetic and biochemical studies have identified a number of other proteins that appear to modulate this pathway, but for which precise roles in CCM disease remain less understood.

**HEG1:** Forward genetic studies in fish demonstrated that loss of the transmembrane protein HEG1 conferred a big heart phenotype identical to that observed with loss of the CCM complex<sup>74</sup>. In mice, loss of HEG1 does not confer a developmental cardiovascular phenotype, but *Heg1<sup>-/-</sup>;Ccm2<sup>+/-</sup>* animals exhibit branchial arch artery defects identical to *Ccm2<sup>-/-</sup>* animals, consistent with action in a common pathway<sup>59</sup>. However, postnatal loss of HEG1 in endothelial cells does not confer or sensitize to CCM formation, and human genetic studies have not identified *HEG1* mutations in familial CCM disease<sup>75</sup>. Biochemical studies reveal that HEG1 directly binds KRIT1<sup>59, 76</sup>, and both proteins regulate endothelial junctions in cultured cells<sup>77, 78</sup>. The role of HEG1 remains incompletely understood, but present data suggest that HEG1 functions in association with the CCM complex in a manner that may be independent of MEKK3 but important for regulation of endothelial cell junctions. Biochemical and cellular studies suggest that an important mechanism for HEG1 regulation of endothelial junctions is the binding and localization of RASIP1<sup>78</sup>.

**CCM2L:** This is an orthologue of CCM2 with a highly homologous PTB domain by which it may also bind KRIT1<sup>79–81</sup>. Unlike CCM2, CCM2L is expressed specifically in endothelial cells in a dynamic pattern during angiogenic and cardiogenic processes<sup>79</sup>. Biochemical studies reveal that CCM2L, like CCM2, binds MEKK3<sup>79, 81</sup>, but whether CCM2L acts in a redundant manner with CCM2 to negatively regulate MEKK3 or as a dynamically expressed CCM2 antagonist remains controversial. Genetic interaction studies in zebrafish embryos support a redundant role with CCM2 in heart development<sup>80–82</sup>. In contrast, genetic studies in developing mice revealed no phenotype with loss of CCM2L and instead found that loss of CCM2L rescued lethal cardiovascular defects observed in *Heg<sup>-/-</sup>;Ccm2<sup>+/-</sup>* embryos. These findings suggested opposing roles for CCM2 and CCM2L, a conclusion also supported by biochemical studies demonstrating that CCM2L does not bind PDCD10, an essential component of the CCM complex<sup>79</sup>. Thus, CCM2L likely plays a modulatory role for CCM signaling in endothelial cells during cardiovascular growth. Whether that role is redundant with or opposing to that of CCM2 remains controversial, but there is presently no evidence that CCM2L participates in postnatal CCM lesion formation.

**STK24 and STK25:** The GCKIII serine/threonine kinases STK24 (aka MST3) and STK25 associate with the PDCD10/CCM3 protein through its focal adhesion targeting (FAT) domain in numerous contexts, including the CCM complex<sup>56, 83–85</sup>. Genetic studies in zebrafish reveal that loss of both STK24 and STK25 results in the big heart phenotype characteristic of CCM function loss<sup>84</sup>. Thus, it is likely that STK24/25 function is required for CCM complex function, but this hypothesis has not yet been tested in mice and

the identity of the substrate(s) of these serine-threonine kinases in this context remains unknown.

**CDC42:** CDC42 was identified as an unexpected modulator of CCM function and lesion formation following postnatal endothelial-specific deletion studies in mice. Loss of endothelial CDC42 was first noted to confer capillary-venous malformations in the angiogenic neonatal retina<sup>86</sup> and formation of lesions in the neonatal hindbrain that resembled CCMs both physically and spatially, and were associated with elevated endothelial levels of KLF4<sup>87</sup>. Like CCMs, vascular lesions conferred by endothelial CDC42 loss can be reversed by loss of KLF4, and biochemical studies revealed interaction between CDC42 and the CCM complex mediated by PDCD10<sup>87</sup>. These studies suggest that CDC42 may participate in CCM disease pathogenesis, perhaps in the still undefined mechanism by which the CCM complex negatively regulates MEKK3 activity in endothelial cells.

**Upstream MEKK3-KLF2/4 signaling in CCM disease**—Identifying key upstream signaling inputs that drive CCM disease is a priority because the nature of these inputs is expected to shed light on both disease pathogenesis and potential CCM therapies. In vitro and in vivo studies in mice have defined two major inputs to the MEKK3-KLF2/4 signaling pathway in endothelial cells: hemodynamic shear forces and inflammatory signals. Both have been implicated in CCM disease pathogenesis, as discussed below.

**The gut microbiome and brain endothelial TLR4 signaling define a CCM disease**

**gut-brain axis.:** Studies of MEKK3 signaling in cultured fibroblasts have demonstrated that it is required for innate immune inflammatory signaling in response to lipopolysaccharide (LPS) and interleukin 1b (IL1b)<sup>88</sup>. A first clue linking CCM disease to inflammatory signals in brain endothelial cells came from human genetic studies that associated polymorphisms in the genes encoding the LPS receptor TLR4 and its co-receptor CD14 with more severe familial CCM disease<sup>28</sup>. This connection remained mysterious until studies in mice serendipitously demonstrated that CCM lesion burden varied from severe to barely detectable depending upon the mouse facility the animals were housed in. Mice in a resistant colony only generated CCM lesions when they developed gram negative bacterial abscesses, suggesting that LPS derived from gram negative bacteria (GNB) drives lesion formation. This hypothesis was supported by the ability to prevent CCM lesion formation in susceptible animals with endothelial-specific loss of the LPS receptor TLR4<sup>89</sup>. Susceptibility to CCM lesion formation in mice tracked with the GNB content of the gut microbiome, and treatment of susceptible animals with antibiotics or housing in germ-free conditions prevented CCM formation<sup>89</sup>, indicating that LPS from GNB that reside in the gut microbiome is an essential driver of CCM formation in the brain (Figure 3). Importantly, these studies in mice are supported by analysis of the gut microbiome in humans with and without symptomatic CCM disease. These human studies confirm the importance of LPS derived from GNB and define a permissive microbiome, characterized by more abundant Bacteroides species, in human CCM disease<sup>90</sup>. These unexpected findings have established a particularly straightforward gut-brain disease axis in which GNB in the gut microbiome provide a ligand, LPS, required to activate TLR4 receptors and downstream MEKK3-KLF2/4 signaling in brain endothelial cells. They further identify inflammatory signaling as the primary driver of CCM formation



in vivo and suggest that future diagnostic and therapeutic strategies based on manipulation of the gut microbiome could be used to treat CCM disease.

**PDCD10 plays dual roles at both ends of the gut-brain CCM disease axis.:** Further evidence supporting the importance of a gut-brain axis in CCM disease has come from recent studies that address the role of PDCD10/CCM3. Studies of familial CCM disease have shown that germline loss of either KRIT1 or CCM2 results in a similar disease course, with symptomatic disease most frequently arising in middle age. In contrast, familial CCM disease due to germline loss of one PDCD10 allele has been found to result in earlier and more severe disease, often presenting as stroke in childhood<sup>25</sup>. Since the three CCM proteins contribute equally to the molecular CCM complex, the basis for this difference in natural history has not been clear. The answer to this puzzle turns out to be a dual role for PDCD10 in CCM disease. Genetic deletion studies in mice demonstrate that in the brain endothelium PDCD10 is required in much the same way as KRIT1 or CCM2, i.e., as a requisite component of the CCM complex<sup>27</sup>. In the gut epithelium, however, PDCD10 is required for the secretion of mucus by goblet cells, a critical part of the gut barrier that separates the billions of bacteria from the gut wall and vasculature. This function is not shared with KRIT1 and CCM2, and is likely mediated by PDCD10 participation in the STRIPAK complex that does not contain those proteins<sup>27</sup>. Germline loss of one PDCD10 allele reduces the gut barrier, thereby enabling translocation of GNB-derived LPS to the blood and accelerating the growth of CCM lesions in the brain that form following loss of PDCD10 in brain endothelial cells. These findings highlight the importance of the gut-brain axis for CCM disease, and that of the gut barrier in preventing CCM formation over an individual's lifetime.

**The role of hemodynamic activation of MEKK3-KLF2/4 signaling in CCM disease.:** The best characterized input to endothelial MEKK3-KLF2/4 signaling is hemodynamic shear force; thus it logical to consider that hemodynamic forces play a significant role in CCM disease. Studies in the developing zebrafish and mouse embryo have demonstrated that MEKK3-KLF2/4 signaling is regulated by fluid shear forces during cardiovascular development<sup>91–93</sup>. The CCM complex has been shown to regulate the MEKK3-KLF2/4 pathway in this context, and its manipulation alters cardiovascular developmental processes linked to fluid forces in the zebrafish embryo<sup>66, 94–98</sup>. These studies suggest that the CCM pathway may regulate MEKK3-KLF2/4 signaling stimulated by hemodynamic forces, but whether and to what extent pathway analysis in the developing embryo can be directly translated to CCM lesion formation in the postnatal brain remains unclear. CCM lesions in the brain first arise in post-capillary venules, a site of extremely low fluid shear. However, the beta blocker propranolol, an agent that reduces cardiac output and hemodynamic shear forces, ameliorates both zebrafish developmental phenotypes and postnatal CCM lesions conferred by CCM loss of function<sup>42</sup>. Future studies that focus on hemodynamic shear forces specifically in the venules and veins of the postnatal brain will better define the role of this upstream input in CCM pathogenesis.

**Downstream MEKK3-KLF2/4 signaling effectors in CCM disease—**A final signaling question remains the nature of the downstream MEKK3-KLF2/4 effectors that

drive CCM lesion formation. A very large number of signaling pathways have been found to be altered in CCM lesions, and thereby implicated in CCM pathogenesis, but functional data that test causal mechanism are more scarce.

**RHO/ROCK and endothelial junctions.:** Studies in cultured endothelial cells and zebrafish embryos have revealed that loss of CCM function results in activation of the RHO/ROCK signaling pathway with elevated levels of phospho-myosin light chain (pMLC) and altered vascular integrity<sup>84, 99–101</sup>. These findings have stimulated therapeutic efforts aimed at normalizing this pathway in CCM-deficient cells, e.g., with the use of the ROCK inhibitor Fasudil or antagonizing microRNA inhibition of the endothelial cell junction protein VE-cadherin, both of which can reduce lesion burden in mice<sup>97, 102</sup>. Mouse genetic studies demonstrate that reducing expression of either MEKK3 or KLF2 or KLF4 blocks the increase in pMLC conferred by CCM loss<sup>71</sup>. Confusingly, endothelial loss of MEKK3 also results in increased endothelial pMLC expression<sup>68</sup>. However, preclinical studies in mice suggest that ROCK inhibition can reduce CCM lesion burden<sup>102</sup>. Thus, changes in RHO/ROCK signaling appear to be downstream of increased MEKK3-KLF2/4 signaling and participate in CCM formation, but the molecular pathway between them remains undefined.

**End-MT and cell non-autonomous signals.:** The rise in expression of KLF4 and other mesenchymal cell markers has led to a model in which CCM-deficient endothelial cells undergo mesenchymal transition, thereby becoming pathogenic cells creating CCM lesions<sup>72</sup>. Recent lineage tracing studies in mice have further demonstrated that as CCM lesions grow they incorporate large numbers of unlabeled, presumably wild-type, endothelial cells<sup>54, 55</sup>. These findings have generated a model in which CCM-deficient cells drive early lesion formation (perhaps through the generation of endothelial progenitor-like cells<sup>55</sup>) with subsequent cell non-autonomous effects on neighboring wild-type endothelial cells that fuel later lesion growth<sup>54</sup>. One such cell non-autonomous mechanism may be secretion of ADAMTS5, a versican-degrading metalloprotease that has been shown to be augmented in CCM-deficient endothelial cells in vivo and in vitro, with effects on CCM growth that are genetically linked to altered versican proteolysis<sup>103</sup>. Another may be reduced expression of thrombospondin 1 (TSP1), a secreted anti-angiogenic protein that suppresses CCM formation<sup>104</sup>. Future testing of the role of wild-type endothelial cells in CCM lesion formation in human lesions will be particularly valuable in understanding this disease.

**PI3K-mTOR signaling is a critical downstream mechanism of CCM formation.:** Very recent mouse and human genetic studies have demonstrated an unexpected downstream mechanism by which CCM loss of function and MEKK3-KLF2/4 gain of function confer lesion formation: increased PI3K-mTOR signaling<sup>51</sup>. As noted above, mouse models have identified a strong association between sites of active angiogenesis and CCM formation. Following global endothelial cell loss of CCM function, neonatal mice form lesions specifically in the hindbrain and retina, two sites of active angiogenesis after birth<sup>63</sup>. If CCM gene deletion is induced at a later timepoint when those sites are less angiogenic, CCM formation is lost<sup>105</sup>. Conversely, addition of the angiogenic growth factor VEGF-A<sup>106</sup> or loss of the anti-angiogenic factor TSP1<sup>104</sup> augment CCM formation. These observations suggest that loss of CCM function alone is not sufficient for lesion formation, and that

other signals associated with active angiogenesis are also required. Since VEGF is a strong activator of PI3K signaling, CCMs arise in the venous system, and gain of function mutations in PIK3CA are known to drive venous and lymphatic malformations<sup>107–112</sup> we examined the interaction between CCM loss of function and PIK3CA gain of function in vivo. In the neonatal mouse, CCM loss of function and PIK3CA gain of function drove the formation of similar venous lesions, and strong synergy was observed when both were present. In a new adult mouse model in which Cre-encoding adeno-associated virus is injected directly into the mature brain, neither alone was sufficient to create a lesion but the combination resulted in classic cavernoma formation.

As described above, bulk DNA sequencing revealed PIK3CA gain of function mutations identical to those observed in human cancer in a majority of surgically resected human CCM lesions. Single nucleus DNA sequencing further revealed that CCM mutations and PIK3CA mutations arise in the same cell, supporting an intracellular molecular interaction. Consistent with these mouse and human genetic findings, both CCM loss of function and KLF4 gain of function increase endothelial PI3K-mTOR signaling, and Rapamycin, an approved mTORC1 inhibitor, potently blocks CCM formation in both neonatal and adult mice. These new studies are consistent with prior reports that identified PI3K and mTOR signaling in CCM regulation of autophagy<sup>113</sup> and in blinded studies of potential CCM signaling mechanisms<sup>114</sup> and identify PI3K-mTOR signaling as a critical downstream effector mechanism in CCM disease and reveal a compound molecular and genetic pathogenesis similar to cancer in which the CCM complex plays the role of a vascular growth suppressor while PIK3CA plays the role of a vascular growth oncogene.

**Other pathways implicated in CCM pathogenesis**—In addition to the pathways described above, studies have implicated Notch<sup>115–117</sup>, WNT<sup>118</sup> and TGF $\beta$  signaling<sup>72, 73</sup> in various stages of CCM disease. We have not discussed them in detail because their roles of these pathways are less investigated and their precise contributions less defined.

**Future signaling directions.** There remain many outstanding questions regarding the signaling events that culminate in CCM disease. Why do CCMs arise specifically in the central nervous system? Does this reflect a specific environmental factor, e.g. the presence of perivascular versican or some other aspect of the neurovascular unit, or is it possible that they arise elsewhere but only come to clinical attention in the brain. How does the CCM complex negatively regulate MEKK3? Is MEKK3 an STK24/25 substrate? Are there still unidentified molecular players? Could CCM loss of function mutations contribute to other vascular malformations, e.g., those strongly associated with increased PI3K-mTOR signaling? Finally, are the vascular changes conferred by CCM loss of function reversible? The recent strides made in understanding CCM pathogenesis have shed great light on the mechanism of this disease and raised as many questions as they have answered.

### **CM signaling pathways: Mechanisms underlying hemorrhage**

**Why do CCM lesions bleed?**—Bleeding is a major source of morbidity in CCM, yet the molecular and cellular events that underlie acute or chronic hemorrhage in this disease are unclear. CCMs are dynamic lesions that can form, enlarge, regress, or behave aggressively,

producing repetitive hemorrhages<sup>49, 119, 120</sup>. Hemorrhagic strokes are a significant cause of disability in patients living with CCMs<sup>121–124</sup> and a combination of CCM enlargement and bleeding can cause focal neurological deficits, headaches, epileptic seizures, and occasionally death<sup>123, 124</sup>. Natural history studies and MRI analysis have identified that CCM location and past hemorrhage as significant risk factors for bleeding and subsequent clinical sequelae.<sup>123, 125–127</sup> Factors such as gender, lesion number, and size are not conclusively related to bleeding risk<sup>127, 128</sup>. Studies also estimate that symptomatic CCM hemorrhages rates are higher for familial CCMs than for sporadic CCMs<sup>123, 124, 129, 130</sup>. In addition, patients with *PDCD10* mutations are more likely to present significant CCM hemorrhages earlier in life<sup>24–26</sup>. Although the annual symptomatic hemorrhage rate varies largely among different studies from 0.25 to 22.9% per patient-year,<sup>131, 132</sup> it is thought that all CCM harbor occult bleeding<sup>123, 132</sup> because a hemosiderin halo is the hallmark of CCMs on MRI. Here we summarize some of the potential mechanisms that may lead to bleeding in CCM (Figure 4.)

**Loss of cell-cell junctions as a cause of CCM hemorrhage**—Electron microscopic and immune histochemical analyses have documented that disruption of the endothelial barrier by disassembling of inter-brain endothelial junctions in CCMs<sup>104, 133, 134</sup>. This loss of cell-cell junctions could trigger the recurring micro-hemorrhages that cause hemosiderin deposition and the propensity for seizures<sup>119</sup>. Moreover, this mechanism is also supported by results in CCM mouse models. The expression and assembly of claudin5, ZO1, and VE-cadherin at the endothelial junctions are dramatically altered in dilated and hemorrhagic CCM lesions but normal in perilesional vessels<sup>72, 104, 105, 121</sup>. In addition, in familial CCM3 disease, there is a profound change in the tight junctions in brain endothelial cells that could affect the underlying severity of CCM3 disease<sup>134</sup> and early-onset in brain hemorrhage<sup>26</sup>. Importantly CCM endothelium also exhibits combination an increase in cytoskeleton contractility in low flow areas<sup>98, 99, 101, 102, 127</sup> that predispose to rupture leading to hemorrhagic stroke<sup>134, 135</sup>. Furthermore, mice harboring CCMs experience acute hemorrhage when injected with a single sub-lethal dose of LPS<sup>136</sup>, which could mimic the important effects of gut microbiome and gut barrier integrity on CCM exacerbation<sup>89</sup> described above. Together these data suggest that CCM hemorrhages may result from unstable endothelial cell-cell contacts and that LPS-induced complete disassembly of endothelial cell-cell junctions or frank endothelial cell death can greatly exacerbate bleeding<sup>137–139</sup>.

**CCMs form an anti-thrombotic vascular domain in the brain**—Normal brain vasculature maintains restricted levels of thrombomodulin (TM) and endothelial protein C receptor (EPCR) compared to systemic blood vessels<sup>140, 141</sup> suggesting that the brain is a pro-hemostatic environment. A genome-wide transcriptome analysis of the acute effects of inactivation of *Krit1* or *Pdcd10* in murine brain endothelial cells (BMEC) found increased levels of *Thbd* mRNA, which encodes the natural anticoagulant receptor, TM.<sup>104, 142</sup> 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 EPCR,<sup>143, 144</sup> the mRNA of which is also increased with loss of *Krit1* or *Pdcd10* in BMECs.<sup>104, 121, 142, 145</sup> Because APC is a potent natural anticoagulant,

that inactivates the coagulation factor Va and factor VIIIa, high levels of TM have been associated with a bleeding disorder<sup>146, 147</sup> and used as a biomarker of endothelial cell dysfunction,<sup>148</sup> these data suggested that increased TM and EPCR could create a local bleeding diathesis in CCM<sup>121</sup>.

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<sup>104</sup>. Elevated levels of TM in plasma are associated with endothelial dysfunction<sup>148, 149</sup> and bleeding disorders.<sup>146–148</sup> Moreover, human CCM endothelium displays increased levels of TM and EPCR mRNA and protein<sup>104</sup>. As discussed above, the increased TM can reflect an increased formation of APC in lesions which, in turn, leads to anticoagulant activity<sup>143</sup> that facilitates bleeding. 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.<sup>150, 151</sup>

APC induces cytoprotective effects on endothelial cells and neurons that are dependent on protease activated receptor (PAR) 1, PAR3, and EPCR<sup>152, 153</sup>. 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.<sup>154</sup> 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 (Figure 4). Several anticoagulant-specific strategies targeting the protein C pathway have been proposed for control of bleeding in hemophilia patients.<sup>150, 151</sup> 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 central nervous system (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.<sup>123, 132</sup> 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.<sup>144, 155</sup>

The dramatic elevation of TM and EPCR in CCM endothelium<sup>121, 145</sup> may also serve a protective anti-thrombotic function. The low flow environment of CCM may be conducive to thrombosis, which, by obstructing outflow, could lead to lesion expansion or frank rupture. Indeed, a recent retrospective analysis suggested that CCM patients who received anti-thrombotic therapy experienced less morbidity<sup>156</sup>. In this scenario, the activation of Protein C by CCM endothelium may serve a protective role. Resistance to APC anti-coagulant activity, due to Factor V Leiden, is one of the commonest thrombophilias<sup>157</sup> and CCM patients with this mutation may be at enhanced risk for thrombosis within CCM and at much greater likelihood to be treated with an anti-thrombotic. Indeed, pre-screening CCM patients for thrombophilia may be helpful in guiding future studies of the effect of anti-thrombotics on CCM.

The studies described above show that the potential consequences of the enhanced APC generation in CCM are manifold (Figure 4). The diverse effects of Protein C as an anticoagulant and as a cytoprotectant emphasize the need for studies to analyze these

complexities and to evaluate strategies targeting the different functions of the protein C system in CCM. Studies that develop approaches to junction stabilization<sup>99, 101, 158</sup> and the APC pathway can point to new therapies. Conversely, as the number of potentially new therapeutic interventions in CCMs grows,<sup>73, 89, 104, 117, 135</sup> the identification of markers to identify patients with elevated risk of hemorrhage becomes imperative. Indeed, recent studies<sup>137, 159</sup> that showed that levels of inflammatory and angiogenic molecules in peripheral blood plasma could predict CCM behavior have already begun. The work reviewed here also suggests that a thorough understanding of the hemostatic balance, and Protein C pathway in particular, can contribute to the management of CNS hemorrhage in CCM.

### Translational Roadmap: Biomarkers and Trial Readiness

**Biomarkers and Clinical Features of CCM disease**—In CCM disease, several primary sequelae are considered strongly in clinical management, including symptomatic lesional growth and hemorrhage, and lesion burden (Figure 5).<sup>160</sup> While the annual risk of a first symptomatic hemorrhage is quite low (0.08% per patient year), after an initial bleeding event, the risk of a subsequent bleed increases more than ten-fold.<sup>160</sup> The clinically adjudicated definition of Cavernous Angioma with Symptomatic Hemorrhage (CASH) requires imaging evidence of bleeding or growth with directly attributable symptoms. Bleeding may be clinically silent or misdiagnosed, and other neurological symptoms such as headache or seizure can occur without an overt, new bleed.<sup>160, 161</sup> Seizures and focal neurologic deficits can also be caused by lesion growth or by cumulative, chronic bleeding. Lesional growth may occur because of proliferation of new caverns, or as a result of expansion of one or more caverns (intralesional bleeding). Hemorrhage may also leak beyond the confines of the lesion. Lesional expansion and symptomatic hemorrhage are clinically relevant in familial disease and also in the more common sporadic lesions, where similar somatic mutations have been identified. There is a need for sensitive and specific diagnostic and prognostic biomarkers of CASH lesions.<sup>162</sup> These biomarkers may help reveal which patients bled or may suffer a hemorrhagic stroke in the near future and therefore need more urgent or risky surgical interventions. High risk lesions may also be selectively targeted by emerging therapies. A goal of lesion stabilization may be pursued, or ultimately a more ambitious goal of lesion regression.

Lesion burden is relevant in familial disease, where lesions develop stochastically during a patient's lifetime throughout the brain and spinal cord, and in sporadic cases, where new lesions can develop in association with a pre-existing developmental venous anomaly. Lesion burden can be tracked using susceptibility magnetic resonance imaging (MRI) sequences revealing the earliest development of punctate lesions, well before any clinical manifestations. An increase in the number of CCMs increases the chances of subsequent related lesional growth and SH (Figure 5).

Mechanistic research and the transcriptome of human and mouse CCM lesions have clarified several features of CCM disease (Table 1), and disease models have been adapted to better examine various features of lesion genesis, maturation and growth, intralesional and extralesional hemorrhage.<sup>97, 136</sup> Therapeutic targets may be better suited for one aspect

or another among disease manifestations, including a potential promise of combination therapies.

**Imaging in Preclinical Models and in CCM Patients**—Imaging modalities, such as MRI and Computerized Tomography (CT), have been used clinically as standard for diagnosis of CCMs (Figure 6 A and B). In preclinical studies, MRI was originally lauded as an opportunity to study lesions at near histologic resolution at various stages of development.<sup>163, 164</sup> However, high field murine MRI scans are expensive and time consuming, preventing high-throughput application.<sup>163</sup> Micro-CT instruments (using paleo-CT instruments initially developed for fossil imaging) have been effectively purposed for high-throughput assessment of lesion burden and volume while complementing histology in *ex-vivo* pre-clinical studies in CCM mouse models.<sup>165</sup> This diffusible iodine-based, contrast-enhanced, micro-CT protocol was originally developed to study the consequences of endothelial TLR4/CD14 signaling<sup>89</sup> and the influence of the gut microbiome on CCMs pathogenesis.<sup>27</sup> Later, the lesional burden, estimated using micro-CT, has been used to monitor not only the efficacy of candidate therapeutics,<sup>97, 104, 166</sup> but also genetic manipulation in preclinical mouse models of CCM disease.<sup>103, 136</sup>

Increased vascular permeability is a hallmark of CCM disease physiopathology, mediating hemorrhage and non-heme iron accumulation,<sup>99, 101, 167, 168</sup> In CCM patients, two *in-vivo* imaging biomarkers have therefore been proposed to assess lesional and background brain permeability (i.e., Dynamic Contrast Enhanced Quantitative Perfusion [DCEQP]) and iron accumulation (i.e., Quantitative Susceptibility Mapping [QSM]) (Figure 6 C and D).<sup>126, 167–171</sup> DCEQP has been used to assess vascular properties of brain disorders, allowing estimation of perfusion, blood volume and blood-brain barrier permeability.<sup>169, 172</sup> In fact, background brain permeability was confirmed to be greater in familial CCM disease, consistent with heterozygous germ line mutations in brain vascular bed.<sup>167</sup> Further, longitudinal studies have reported an increased permeability within CCM lesions proper, after an SH, and in background brain parenchyma preceding new lesion formation.<sup>126</sup> Taken together, these results support the use of DCEQP as diagnostic and monitoring biomarkers for endothelial hyperpermeability of CCMs.

QSM is a gradient echo-generated imaging modality that has been used to assess brain iron deposition in cerebrovascular disorders.<sup>173, 174</sup> In surgically excised CCMs, QSM measurements have correlated precisely with iron concentrations measured by mass spectroscopy.<sup>170</sup> No significant differences were observed in lesional iron deposition between sporadic and familial CCMs, nor in stable CCM lesions over time.<sup>171</sup> The lesional QSM values increase consistently after a hemorrhagic event, suggesting that QSM can be used as a monitoring biomarker of CASH.<sup>126, 175</sup>

**Circulating Plasma Biomarkers in Mice and Humans**—CCMs demonstrate a complex pathophysiological milieu, involving a dynamic interplay between inflammation, angiogenesis, and the loss of endothelial barrier function.<sup>137, 159, 166, 176, 177</sup> Preliminary studies have reported associations between the clinical course of CCM disease and systemic circulating compounds, suggesting they could act as peripheral blood biomarkers.<sup>104, 121, 137, 159, 178, 179</sup> A weighted combination of 4 of the plasma levels

of these candidates biomarkers, including sCD14, VEGF, CRP, and IL-10, was able to distinguish CASH patients with 76% sensitivity and 80% specificity thereby acting as a possible diagnostic biomarker.<sup>137, 179</sup> In the same way, a prognostic biomarker of CASH, including the weighted plasma levels of sCD14, VEGF, IL-1B, and sROBO-4, was also able to predict an SH within the following year with 86% sensitivity and 88% specificity.<sup>137, 179</sup> These are being optimized and validated as much needed diagnostic and prognostic biomarkers of CASH. These preliminary studies were the first insight into the development of diagnostic and prognostic biomarkers of CASH. They have since paved the way for testing new candidate biomarker molecules emerging from mechanistic studies.<sup>162</sup>

**Transcriptomic identification and validation of biomarkers.:** The transcriptome of lesional neuro-vascular units (NVUs) has served as a reference to validate mechanistic hypotheses related to the pathogenesis of CCMs, and also to identify candidate biomarkers.<sup>142</sup> In humans, the transcriptome of CASH NVUs identified several DEGs related to 5 of the 6 circulating proteins composing the prognostic and diagnostic biomarkers, and therefore provided evidence that these markers are related to hemorrhagic CAs.<sup>142</sup> In preclinical murine models, some of the mechanistic findings were then translated and validated in human patients. Lopez et al. (2019) showed increased levels of vascular Thrombomodulin (TM), an anticoagulant receptor, following an upregulation of transcription factors KLF2 and KLF4 in *Ccm1*<sup>-/-</sup> or *Ccm3*<sup>-/-</sup> BMECs and confirmed that *Thbd* was also dysregulated in human lesional NVUs.<sup>121</sup> In addition, the plasma levels of TM were also increased in CCM patients, suggesting that plasma soluble TM may be used as a marker for hemorrhagic risk.<sup>121</sup> These results provide compelling evidence that the transcriptomes can validate mechanistic discoveries, and also identify novel candidate biomarkers (Table 1).

**Micro-RNAs (miRNAs).:** In addition to plasma proteins, circulating micro-RNAs (miRNAs) have been suggested as possible mechanistic biomarkers. In CCM disease, 13 differently expressed (DE) miRNAs were identified in the plasma of CASH patients.<sup>179</sup> These 13 DE miRNAs showed 16 putative gene interactions in the coding sequence (CDS) region of mRNA within the transcriptomes of human CCM NVUs. Of interest, further analyses showed that *IL-10RA* is the putative target of *miT-185-5*, one of these 13 DE miRNAs and decreased plasma levels of IL-10 have been reported in CCM subjects that experienced recent SH.<sup>179</sup> While this is just the start of the vast utility of miRNAs clinically, further initiatives have been proposed to integrate these plasma DE miRNAs with plasma proteins to complement and/or increase the sensitivity/specificity of prognostic and diagnostic biomarkers of CASH.<sup>162</sup> Preliminary ML simulations showed that the diagnostic biomarker of CASH was improved when plasma levels of DE miRNAs were included in the model.<sup>162</sup> Further clinical and research utility of miRNAs may arise from their application as diagnostic and monitoring biomarkers of disease genotype.

**Trial Readiness and Multisite Therapeutic Initiatives—**While the development of clinically useful biomarkers as smart blood tests is important for the diagnosis, prognosis, and monitoring of the disease, current treatment of CCMs remains neurosurgical intervention and removal or ablation of a symptomatic lesion. Such intervention is



associated with high cost and significant risks, particularly if the lesion involves critical brain regions.<sup>160</sup> Neurosurgical intervention will not be necessary if a symptomatic lesion is stabilized, and its growth or hemorrhage are prevented. Therapies may also be targeted at preventing lesion development or growth, and potentially even to induce lesion regression. Therefore, pharmacotherapeutics are urgently needed in this disease, particularly given the wealth of emerging mechanistic information as described above. Several preclinical studies have already reported the effects of repurposed or novel drugs on CCM development and hemorrhage (Table 1).<sup>71, 97, 104, 166, 176, 180, 181</sup>

Potential obstacles have been identified related to accurate estimation of clinical case prevalence and the projected enrollment and event rates needed to drive clinical trials, harmonization of clinical and imaging data collection across clinical sites, and the analytic validation of risk and monitoring biomarkers that may be deployed in clinical trials. And there is a need to characterize precise event rates to monitor the impact of therapies. The CASH Trial Readiness (TR) initiative has set forth a protocol for foundational data collection and has been followed by an Exploratory Proof of Concept Trial of high dose atorvastatin as a ROCK inhibitor versus placebo.<sup>161, 182</sup> The latter trial is planned based on effect size of differences in lesional iron deposition (assessed using QSM) and lesional permeability (assessed using DCEQP).<sup>182</sup>

Another recent initiative assembles three CCM Centers of Excellence in order to optimize and validate diagnostic and prognostic biomarkers of CASH.<sup>162</sup> As a part of this project, the aim will be to test whether individual and combined levels of candidate plasma proteins and miRNAs are associated with the diagnosis and prognosis of CASH. The project will also assess whether changes in imaging monitoring biomarkers after CASH are reflected in plasma molecules. Analyses will be done on possible confounders, such as age, hemorrhagic microangiopathy, and seizures, in the context of clinical use of these biomarkers to determine generalizability in the population.

Much mechanistic information has come to light in the past decade, to guide the therapeutic roadmap.<sup>176, 183, 184</sup> CCM pathogenesis is a continuum from lesion development, to lesion growth, acute and chronic bleeding, with interactions including germ line mutations, somatic mutations, a permissive microbiome,<sup>90</sup> pro-inflammatory genotypes and a defined immune response. It may be difficult to ascertain which aspect of the disease is being targeted by a drug in clinical trials, and optimal dose response considerations are required. Other determinations include how long to treat a patient, at what stage of the illness, and what therapeutic risk is justified and acceptable. This requires very focused mechanistic studies in preclinical models and rigorous translational paradigms. Biomarker driven trials offer advantages at Phase I and II stages to compare relative effects of various therapies, dose calibration and drug combinations, before committing a limited patient population to a Phase III trial with best chances at success.

Finally, there should be considerations beyond systemic pharmacotherapies. Other options may be pursued in the context of the previously articulated therapeutic goals, such as local drug therapy through an injected or endovascularly delivered viral vector, and laser or ultrasound lesion ablation. The development of multiple treatment options could aid in

personalized patient treatment plans based on complexity of disease and comorbidities and calibrated risks.

**Closing Remarks**—The past two decades have seen a remarkable convergence of human genetic studies, genetic studies in fish and mice, and molecular and cellular insights in the field of CCM disease. These studies have shed light on the endothelial cell molecular and genetic responses to loss of CCM function, identified environmental conditions that strongly influence the cellular response and clinical disease, and, most recently, yielded updated genetic insights that support a cancer-like mechanism for the most aggressive CCM lesions. With a more sophisticated understanding of the disease mechanism in hand, the field is poised for successful translation of these findings to the creation of new, non-surgical therapies for CCM disease.

A final question that merits consideration is why so much progress has been made with CCM disease. CCM disease has benefited from the convergence of a number of scientific and medical approaches that provided synergy between multiple investigators to rapidly advance the field. Human genetics has played a major role, first defining the CCM complex through classic positional cloning studies of familial disease and then providing additional mechanistic insights through more advanced genomic sequencing techniques. Vertebrate models have also contributed important mechanistic insights, as expected, but the identification of a faithful neonatal mouse model that was adopted by a large number of investigators in the field has played a major role in moving the field forward because it enabled studies in many laboratories to be directly compared and integrated. As efforts to bring these insights to patients intensify, it will be important to remember these lessons and perform translational and clinical studies in a similarly integrated and efficient manner

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## NONSTANDARD ABBREVIATIONS AND ACRONYMS

<b>CCM</b>	cerebral cavernous malformation
<b>LOF</b>	loss of function
<b>GOF</b>	gain of function
<b>GNB</b>	gram negative bacteria
<b>BMEC</b>	murine brain endothelial cells
<b>CASH</b>	cavernous angioma with symptomatic hemorrhage
<b>DCEQP</b>	dynamic contrast enhanced quantitative perfusion
<b>QSM</b>	quantitative susceptibility mapping

NVU neuro-vascular unit

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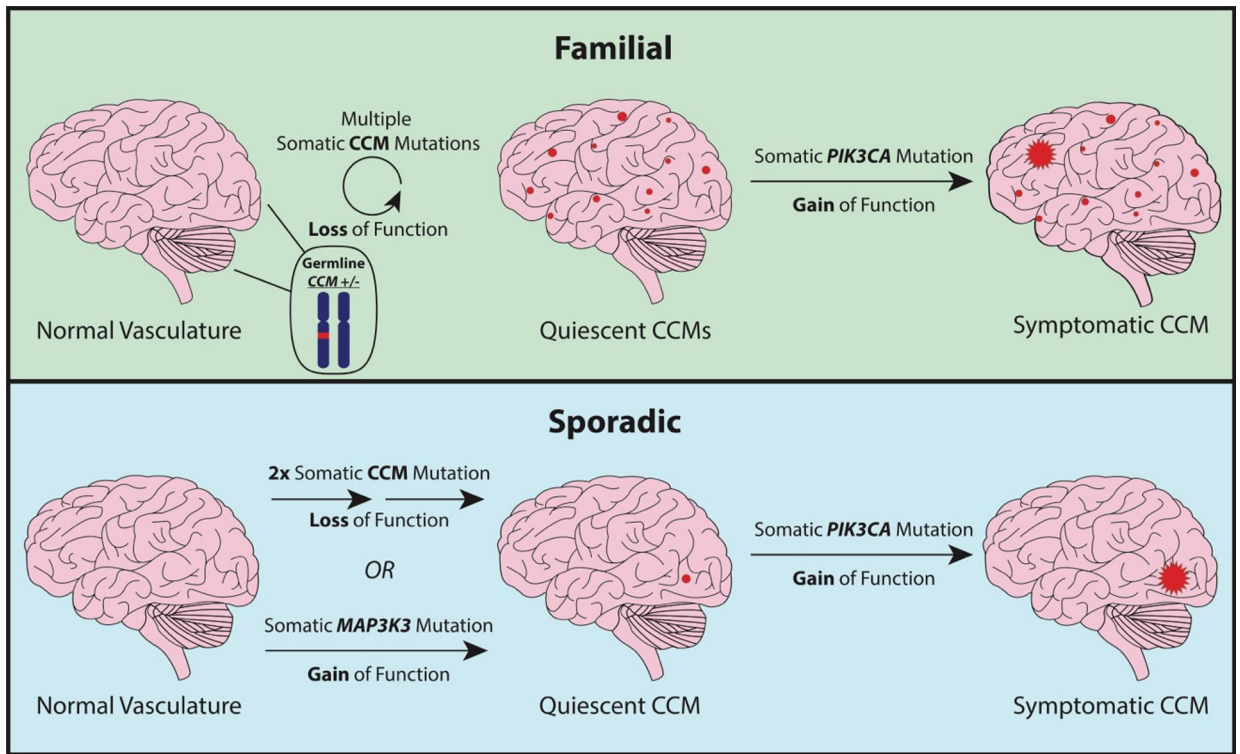
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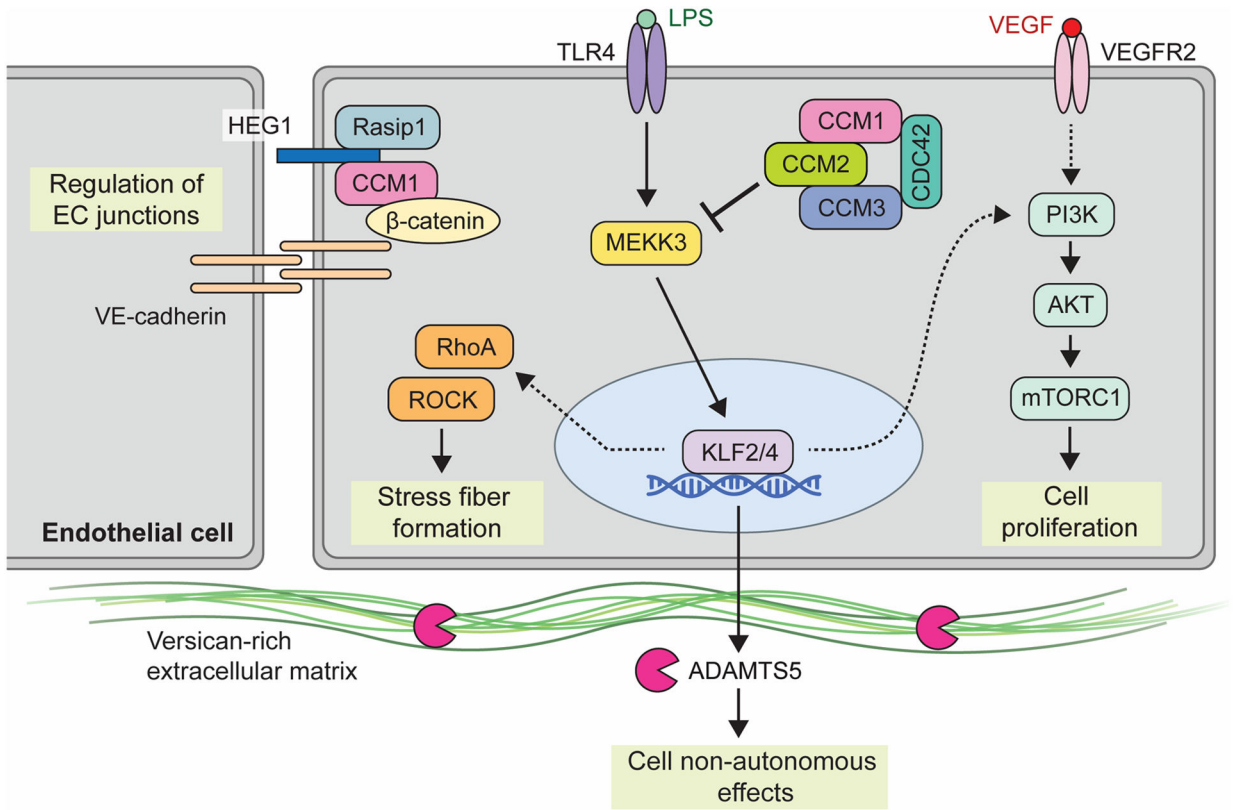
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**Figure 1. Somatic mutations driving CCM progression.**

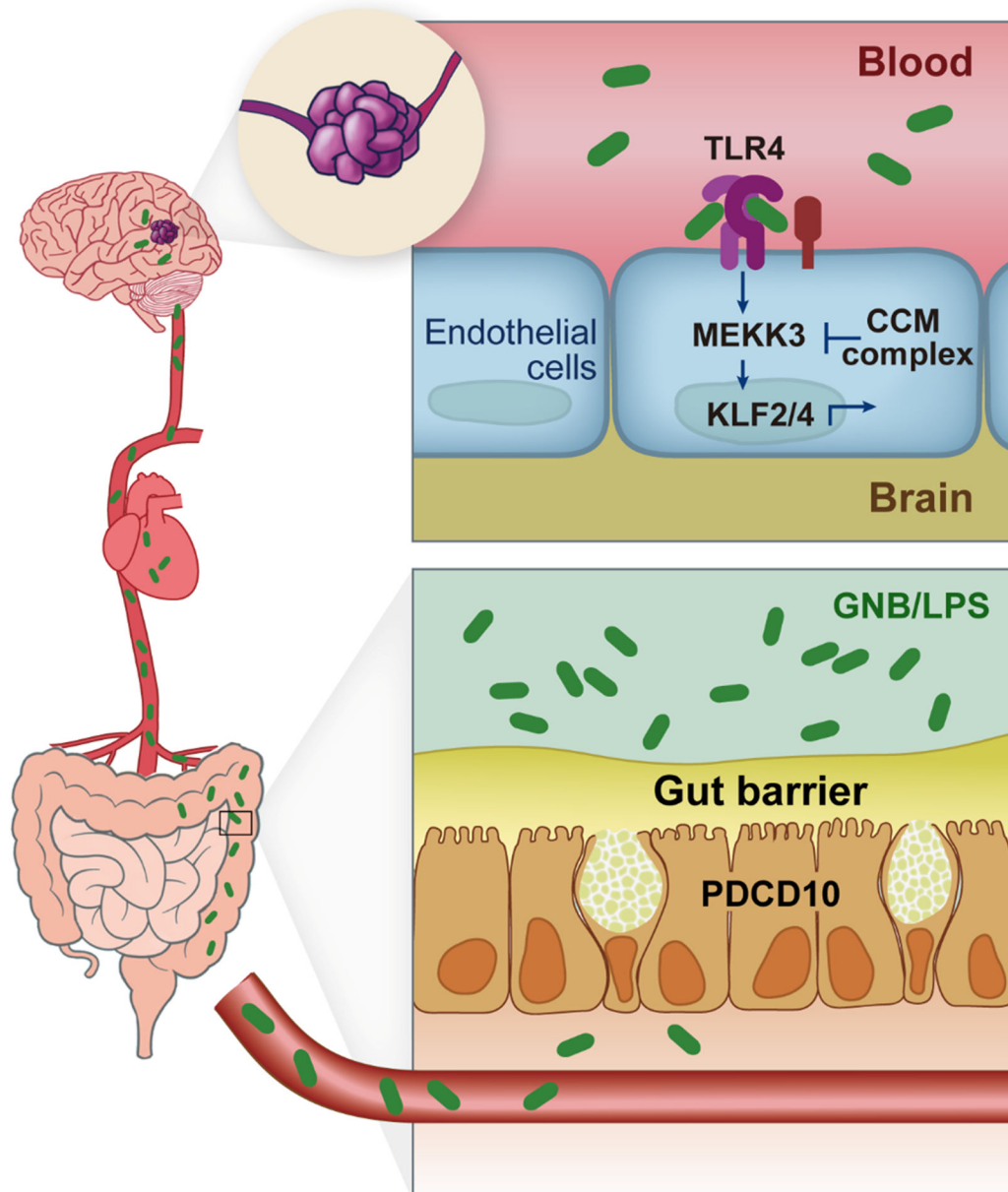
In familial CCM (top), lesion formation is initiated by a somatic mutation in a CCM gene resulting in biallelic loss of function which may occur multiple times resulting in the formation of multiple quiescent CCMs. A subset of these CCMs acquire a somatic gain of function mutation in *PIK3CA* which fuels lesion growth. Sporadic CCM formation (bottom) requires *either* two somatic CCM mutations to occur in the same cell resulting in biallelic loss of function or a single gain of function somatic mutation in *MAP3K3*. A CCM at this stage may or may not acquire a somatic gain of function in *PIK3CA* which would fuel lesion growth. The order in which the mutations occur is not yet known. This figure shows the CCM/*MAP3K3* mutations as occurring first, however the *PIK3CA* mutation may occur first in some or all cases.



**Figure 2. Molecular signaling pathways involved in CCM lesion formation.**

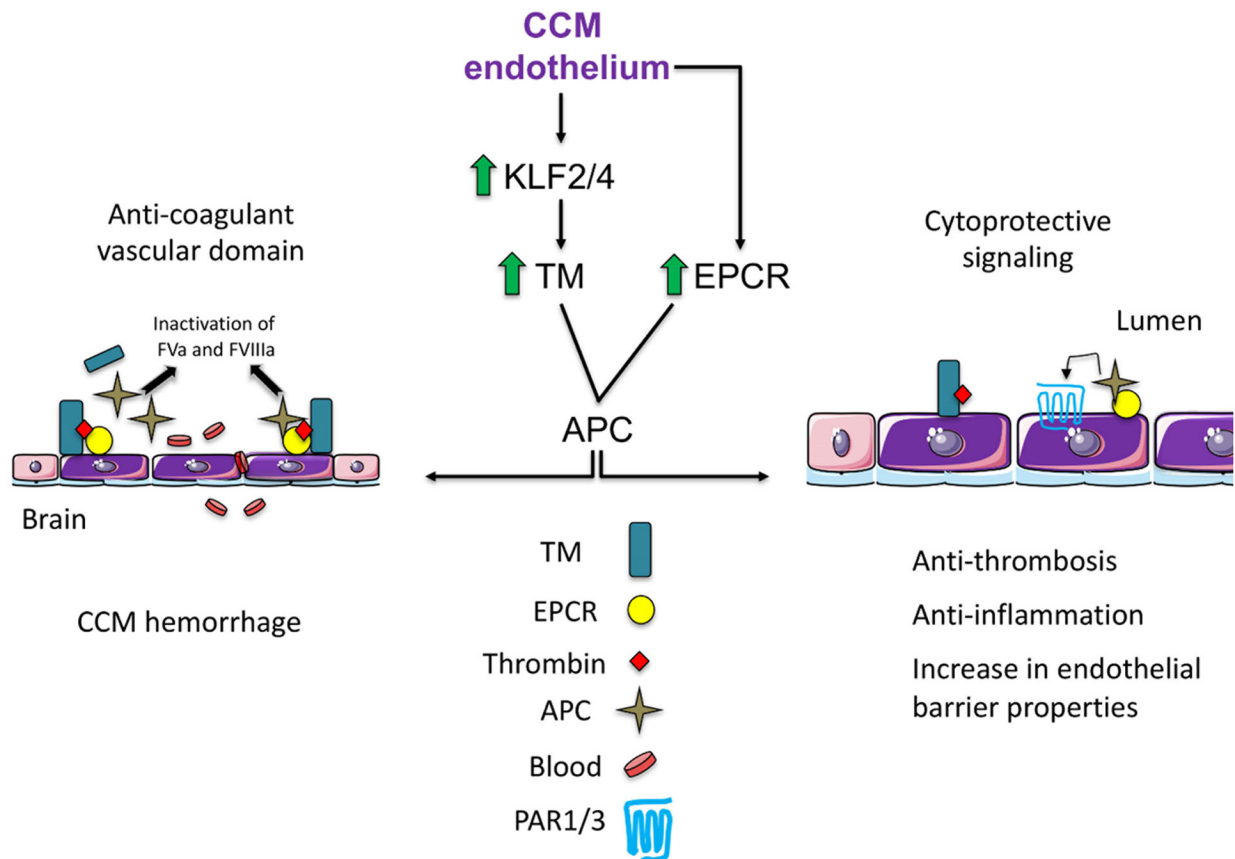
Shown is a partial summary of the various upstream inputs and downstream effectors implicated in CCM signaling. The heterotrimeric CCM complex, consisting of CCM1 (KRIT1), CCM2, and CCM3 (PDCD10), is an inhibitor of MAP3K3 Kinase, MEKK3. Inflammatory signals via TLR4 serve as the upstream input into MEKK3 signaling, as may shear forces associated with blood flow (not shown). Loss of CCM and subsequent activation of MEKK3 leads to upregulation of transcription factors KLF2 and KLF4. Downstream KLF2/4 transcriptional targets include both cell autonomous pathways through PI3K signaling and RHO/ROCK signaling, as well as cell non-autonomous effects via metalloprotease ADAMTS5 and extracellular matrix cleavage. VEGF is shown as a possible distinct input for PI3K-mTOR signaling while HEG1 has been associated with regulation of endothelial cell junctions. Of note, numerous other pathways have been found to be affected by loss of CCM function. This diagram focuses on those that are presently best understood and those that have been investigated using mouse and human genetic studies and/or are presently targeted therapeutically.





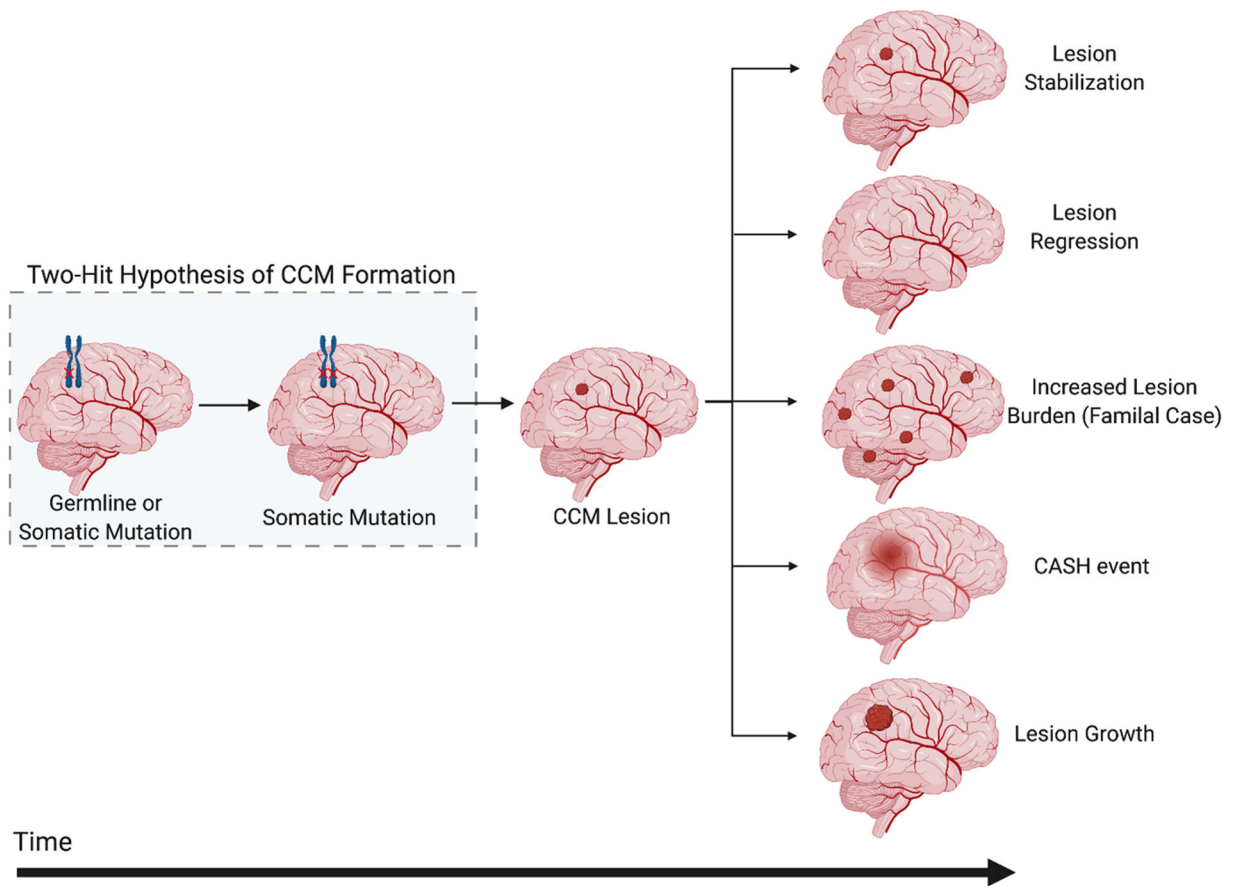
**Figure 3. Gut-brain axis of CCM disease.**

The gut microbiome and gut barrier are drivers of cavernoma formation. Gram-negative bacteria (GNB) and GNB-derived lipopolysaccharide (LPS) translocate across the mucosal and epithelial gut barrier into blood stream. LPS activates Toll-like Receptor 4 (TLR4) on brain endothelial cells and serves as an inflammatory upstream input into the MEKK3-KLF2/4 signaling pathway. PDCD10, unlike KRIT1 and CCM2, is required for the secretion of mucus by intestinal goblet cells. Germline loss of one PDCD10 allele reduces the gut barrier, thereby increasing translocation of LPS to the blood and accelerating the growth of CCM lesions in the brain. Adapted from Tang et al.<sup>27, 89</sup>



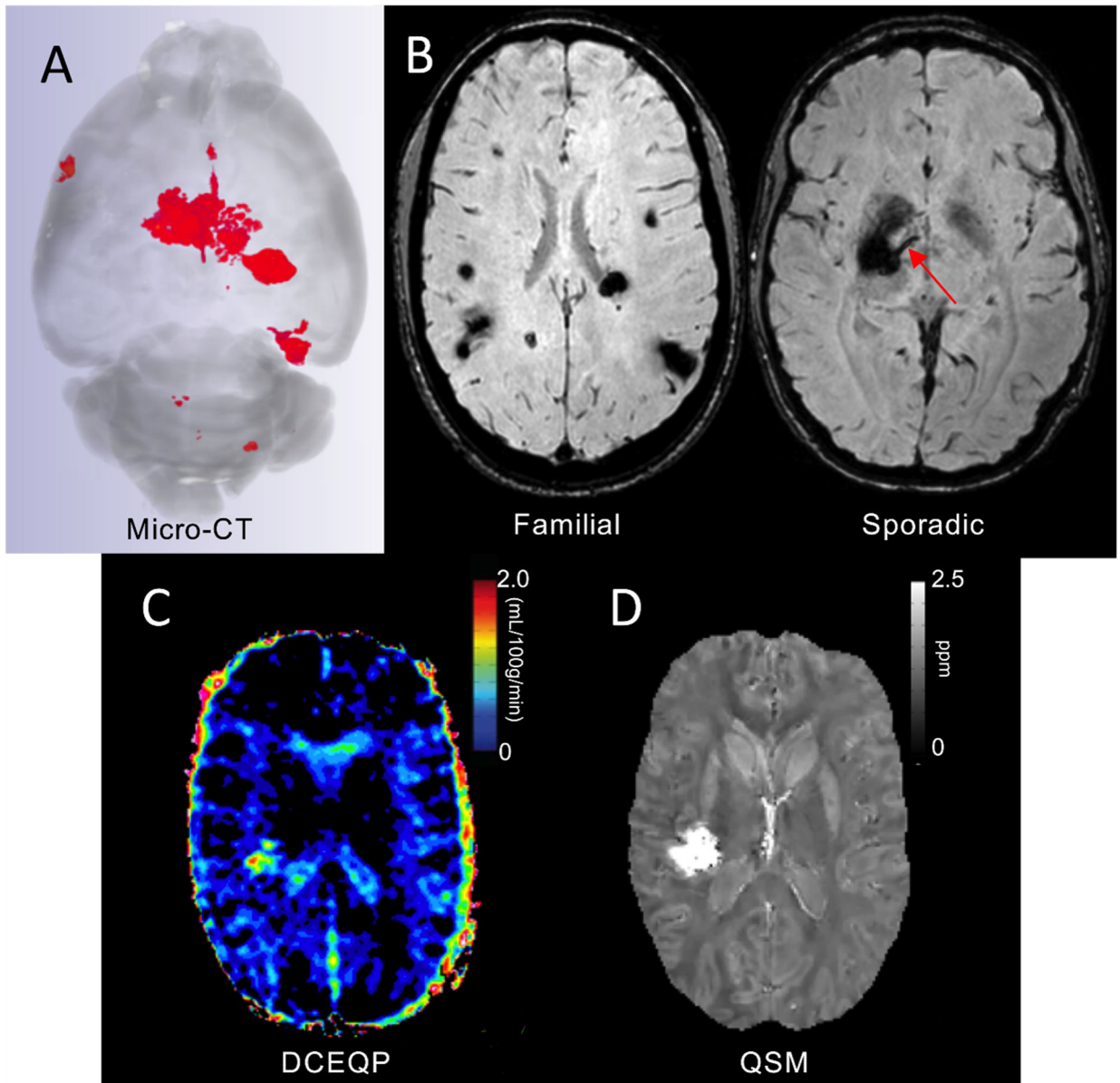
**Figure 4. Mechanism of increase in TM and EPCR in CCM.**

CCM endothelium is associated with locally elevated expression of anticoagulant endothelial receptors TM and EPCR. TM upregulation is due to upregulation of KLF2 and KLF4 transcription factors. Increased levels of vascular TM and EPCR result in enhanced APC and the anticoagulation cascade by inactivation of FVa and FVIIIa, thus contributing to an increase in lesion bleeding (Anti-coagulant vascular domain). Proposed cytoprotective signaling in CCMs, retention of APC to endothelial receptor EPCR allows activation of PAR1/3 and subsequent cytoprotective signaling in CCM endothelium. TM-thrombin complex reduces fibrin generation, and TM exerts anti-inflammatory properties. TM=thrombomodulin, EPCR= endothelial protein C, APC=activated protein C. Adapted from Lopez-Ramirez et al.<sup>121</sup> and Mosnier et al.<sup>152</sup>



**Figure 5. Progression of CCM Lesion Pathogenesis.**

The pathogenesis of CCMs begins with an inherited or somatic mutation, followed by somatic mutations resulting in lesion genesis and growth. The natural history of CCM disease is thought to lead to five clinically relevant outcomes (1) lesion stabilization, (2) lesion regression, (3) increased lesion burden, (4) symptomatic hemorrhage (SH), and/or (5) lesional growth (shown from top to bottom). CASH, Cavernous Angioma with Symptomatic Hemorrhage.



**Figure 6. Imaging Biomarkers of CCM.**

(A). Micro-CT is used to assess lesion burden, volume, and stage *ex-vivo* in murine brains.

(B). Susceptibility weighted MRI assesses lesion burden in familial cases, and the presence

of an associated developmental venous anomaly (red arrow) in sporadic cases. (C). Dynamic

contrast enhanced quantitative perfusion (DCEQP) MRI assesses the vascular permeability

of CCM lesions and background brain. (D). Quantitative susceptibility mapping (QSM) MRI

assesses iron content in CCM lesions.

**Table 1.**

Candidate circulating plasma and imaging biomarkers and potential CCM therapies.

Mechanisms of disease	Plasma circulating biomarkers	Validating preclinical models	Candidate therapeutics	Lesion Pathogenesis	Development status
MAPK Signaling, PI3K-mTOR Signaling, microbiome mechanisms <sup>27, 43, 51, 52, 71, 89, 90, 103, 137</sup>	ADAMTS4 ADAMTS5 TLR4 LPB (LPS) CD14	murine/ human	BIX02189 (anti-MEK5) XMD17-109 (anti-ERK5) Rapamycin (mTOR inhibitor) TLR4 inhibitors	Lesion burden Bleeding	Preclinical
Angiogenesis <sup>106, 137, 162, 179</sup>	VEGF Angiopoietin 1-2 ROBO4 Thrombospondin 1	murine/ human	Bevacizumab Semaxanib TSP1 replacement	Lesion growth Bleeding	Preclinical
Inflammatory processes, autophagy, focused immune response <sup>137, 159, 176, 178, 179, 184</sup>	25-OH Vitamin D CRP Endoglin IL-1 $\beta$ IL-10	murine/ human	Vitamin D3 Tempol Anti-BR3 antibody	Lesion burden Bleeding	Preclinical
Coagulation domains and thrombin-endothelial interactions <sup>104, 121, 162</sup>	Thrombomodulin	murine/ human	Potential biomarker	Bleeding	Preclinical
Rheologic mechanisms <sup>97, 121</sup>	Blood pressure pro-thrombotic states	zebrafish/ murine/ human	Propranolol $\beta$ 1 receptor antagonists	Lesion growth	Preclinical Phase I-IIA
Endothelial-mesenchymal transition <sup>97</sup>	TGF- $\beta$	murine	Exisulind Propranolol	Lesion burden	Preclinical
Endothelial permeability (RhoA/ROCK) <sup>166, 180-182</sup>	pMLC pMBS Leukocytes	murine/ human	Fasudil BA1049 Atorvastatin	Lesion burden Bleeding	Preclinical Phase I-IIA

MAPK = Mitogen Activated Protein Kinase; RhoA = Ras homolog gene family member A; ROCK = Rho-associated protein kinase; ADAMTS4 = ADAM Metallopeptidase With Thrombospondin Type 1 Motif 4; ADAMTS5 = ADAM Metallopeptidase With Thrombospondin Type 1 Motif 5; TLR4 = Toll-Like Receptor 4; LPB = Lipopolysaccharide Binding Protein; LPS = Lipopolysaccharide; CD14 = Cluster of Differentiation 14; VEGF = Vascular Endothelial Growth Factor; ROBO4 = Roundabout Homolog 4; TSP1 = Thrombospondin 1; CRP = C-Reactive Protein; IL-1 $\beta$  = Interleukin-1 $\beta$ ; IL-10 = Interleukin-10; TGF- $\beta$  = Transforming Growth Factor- $\beta$ ; pMLC = phosphorylated Myosin Light Chain; pMBS = phosphorylated Myosin Binding Subunit; MEK5 = dual specificity mitogen-activated protein kinase 5; ERK = extracellular signal-regulated kinase; BR3 = BlyS receptor 3/BAFF-R