Original Contribution

Cytochrome P450 and matrix metalloproteinase genetic modifiers of disease severity in Cerebral Cavernous Malformation type 1

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A B S T R A C T

Background: Familial Cerebral Cavernous Malformation type 1 (CCM1) is an autosomal dominant disease caused by mutations in the Krev Interaction Trapped 1 (KRIT1/CCM1) gene, and characterized by multiple brain lesions. CCM lesions manifest across a range of different phenotypes, including wide differences in lesion number, size and susceptibility to intracerebral hemorrhage (ICH). Oxidative stress plays an important role in cerebrovascular disease pathogenesis, raising the possibility that inter-individual variability in genes related to oxidative stress may contribute to the phenotypic differences observed in CCM1 disease. Here, we investigated whether candidate oxidative stress-related cytochrome P450 (CYP) and matrix metalloproteinase (MMP) genetic markers grouped by superfamilies, families or genes, or analyzed individually influence the severity of CCM1 disease.

Methods: Clinical assessment and cerebral susceptibility-weighted magnetic resonance imaging (SWI) were performed to determine total and large (≥5 mm in diameter) lesion counts as well as ICH in 188 Hispanic CCM1 patients harboring the founder KRIT1/CCM1 ‘common Hispanic mutation’ (CCM1–CHM). Samples were genotyped on the Affymetrix Axiom Genome-Wide LAT1 Human Array. We analyzed 1,122 genetic markers (both single nucleotide polymorphisms (SNPs) and insertion/deletions) grouped by CYP and MMP superfamily, family or gene for association with total or large lesion count and ICH adjusted for age at enrollment and gender. Genetic markers bearing the associations were then analyzed individually.

Results: The CYP superfamily showed a trend toward association with total lesion count (P=0.057) and large lesion count (P=0.088) in contrast to the MMP superfamily. The CYP4 and CYP8 families were associated with either large lesion count or total lesion count (P=0.011 and 0.007, respectively). CYP4F12 rs11085971, CYP8A1 rs5628, CYP4F12 rs11085971, and MMP3 rs117153070 single SNPs, mainly bearing the above-mentioned associations, were also individually associated with CCM1 disease severity.

Conclusions: Overall, our candidate oxidative stress-related genetic markers set approach outlined CYP and MMP families and identified suggestive SNPs that may impact the severity of CCM1 disease, including the development of numerous and large CCM lesions and ICH. These novel genetic risk factors of
1. Background

Cerebral Cavernous Malformation (CCM) is a major cerebrovascular disease consisting of closely clustered, abnormally dilated and leaky capillary malformations, which can be single or multiple (even hundreds), ranging in size from a few millimeters to a few centimeters [1–4]. Despite their high prevalence in the general population (0.3–0.5%), only approximately 30% of people with CCM lesions will eventually develop clinical symptoms, including headaches, focal neurological deficits, seizure, stroke, and fatal intracerebral hemorrhage (ICH) [5]. Symptomatic disease usually occurs between the 2nd and 5th decades of life, although it has been described in all age groups, including young children [3].

Although advances have been made toward understanding the natural history and pathogenic mechanisms of CCM disease, the clinical behavior in individual patients, including the development of numerous and large symptomatic lesions and the risk of serious complications such as ICH, is highly unpredictable [6]. Identification of modifiable risk factors of prognostic value associated with clinical severity of CCM disease is therefore needed to ultimately provide better options for disease prevention and treatment.

CCM disease may arise sporadically or can be inherited as an autosomal dominant condition with incomplete penetrance and variable expressivity. The familial form has been linked to loss-of-function mutations in any of three known CCM genes (KRIT1/CCM1, MGC4607/CCM2 and PDCD10/CCM3), and is characterized by the presence of multiple CCM lesions whose number, size and susceptibility to ICH vary widely even among carriers of the same gene mutation [7,8]. The origin of this variability is still largely unknown, although it is likely that multiple factors contribute to CCM disease severity, including genetic, environmental and lifestyle risk factors. Consistently, we have recently reported the influence of cardiovascular risk factors [7], and inflammatory and immune response genetic factors [8] on the severity of familial CCM1 disease in a cohort of Hispanic CCM patients, all carrying the founder KRIT1/CCM1 Q455X ‘Common Hispanic Mutation’ (CCM1–CHM).

Comprehensive studies in CCM animal models have revealed a causal link between loss-of-function of CCM genes and the development of vascular malformations mimicking human CCM lesions [9–13]. However, experiments in inducible, endothelial-specific knockout mouse models have clearly demonstrated that lesion development is highly restricted, both spatially and temporally, despite the pan-endothelial deletion of CCM genes, suggesting that the homozygous loss of CCM genes is not fully sufficient to induce CCM lesions, and raising the possibility that additional events occurring locally within the neurovascular microenvironment, including stress events, are necessary to trigger CCM lesion formation [14–16]. Among the microenvironmental stress events that might account for a sort of environmental second hit, triggering CCM lesion formation, there is oxidative stress. This may occur as a consequence of an endogenous imbalance between molecular mechanisms of production and scavenging of reactive oxygen species (ROS), as well as by exogenous oxidative insults, including cell exposure to xenobiotics, ionizing radiations, or pro-oxidant factors that may be released locally following inflammatory responses, impaired neurovascular coupling, and ischemia-reperfusion events [17,18]. Remarkably, there is now a wealth of evidence indicating that oxidative stress is a major cause of vascular remodeling and neurovascular unit (NVU) dysfunction associated with cerebrovascular diseases [17,19,20]. Indeed, oxidative stress has been clearly implicated in major molecular and cellular dysfunctions related to CCM disease, including destabilization of VE-cadherin-mediated endothelial cell-cell junctions and blood–brain barrier (BBB) integrity, increased β1 integrin activation, reduced cells’ ability to maintain a quiescent state, and increased vascular permeability and angiogenic activity [18,19,21–23], suggesting that it might significantly contribute in driving the onset and progression of CCM disease. Consistently, considerable evidence from both in vitro and in vivo studies demonstrates that CCM proteins, including KRIT1/CCM1, CCM2 and PDCD10/CCM3, are implicated in the modulation of various redox-sensitive signaling pathways and cellular responses to oxidative stress [16,22,24–29]. In particular, original findings indicated that KRIT1 is involved in the maintenance of intracellular ROS homeostasis through the modulation of master regulators of cellular responses to oxidative stress, including FoxO1 and SOD2, which prevent the accumulation of superoxide anions produced at the mitochondrial electron transport chain and consequent mitochondrial and cellular dysfunctions [24]. Since then, accumulating evidence has provided strong support for a role of oxidative stress in CCM pathogenesis, demonstrating that CCM proteins play an important role in limiting pro-oxidant and pro-inflammatory pathways and mechanisms, including c-Jun-dependent pathways [22] and defective autophagic removal of dysfunctional mitochondria [28]. Furthermore, there is also evidence that compounds endowed with antioxidant properties are effective in rescuing major disease phenotypes associated with loss-of-function of CCM genes [16,22,24,26,27], including decreased endothelial barrier function and increased lesion burden in a mouse model of CCM disease [16].

While pointing to a novel pathogenic mechanism whereby the loss-of-function of CCM proteins sensitize vascular cells to local oxidative stress events, these findings raised also the possibility that inter-individual variability in susceptibility to oxidative stress may contribute to CCM disease pathogenesis [29].

Notably, several genes encoding proteins involved in ROS metabolism and vascular responses to oxidative stress are characterized by single nucleotide polymorphisms (SNPs) that confer substantial inter-individual variability in susceptibility to various oxidative stress–related pathologies, including vascular diseases [30–33], suggesting that inter-individual variation in polymorphisms of genes related to oxidative stress responses might contribute to the phenotypic differences characterizing the familial form of CCM disease.

In particular, among several polymorphic candidate genes related to oxidative stress that might be associated with CCM disease development and severity, we focused our analysis on the highly polymorphic cytochrome P450 (CYP) and matrix metalloproteinase (MMP) genes. Indeed, genetic variants in these genes have been shown to be significant indicators for susceptibility to the most severe forms of well-established oxidative stress–related diseases [34,35].

The human CYP superfamily comprises 57 genes encoding for monoxygenases performing a variety of oxidation reactions implicated in important physiopathological processes [36]. High levels of CYP activity are associated with increased oxygen radical formation and cellular oxidative stress, which can damage cellular macromolecules, including proteins, lipids and DNA [37,38]. Importantly, whereas most CYP enzymes exhibit a large variability in
expression and activity among tissues, among individuals of a population and in a given individual under various conditions [39], compelling evidence supports the expression of these enzymes by BBB endothelial cells and astrocytes associated with cerebral microvessels [40–43]. Indeed, it has been demonstrated that variations in the expression and activity of CYPs can influence the local regulation of cerebral blood flow and vascular homeostasis, and contribute to cerebrovascular diseases [44,45]. Consistently, genetic polymorphisms in several CYPs have been associated with inter-individual differences in susceptibility to various vascular and neurological disorders [39,46–48].

The human matrix metalloproteinase (MMP) superfamily comprises 24 genes encoding proteolytic enzymes that degrade various components of the extracellular matrix (ECM) in both physiological and pathological processes, mediating ECM remodeling and supplying bioactive molecules that impact cellular function. In vascular tissues, including cerebral microvessels, the expression and activity of MMPs are regulated by major drivers of vascular remodeling, such as ROS [49] and oxidative stress [50,51], and may contribute to oxidative stress-mediated vascular dysfunctions and BBB breakdown in cerebrovascular diseases [52,53]. Indeed, once activated, MMP enzymes mediate the proteolytic degradation of endothelial basal lamina components and the release of ECM-bound latent bioactive molecules, including pro-angiogenic growth factors and cytokines such as VEGF and TGF-β1, thus playing a pivotal role in vascular remodeling and BBB rupture induced by various physiological stimuli and pathological stresses, and contributing to oxidative stress-related signaling events [49,54–56]. Consistently, polymorphisms in some MMP genes are important genetic risk factors influencing inter-individual differences in susceptibility to the onset and/or severity of various vascular diseases, including cerebrovascular diseases [57–62].

Overall, our analysis of polymorphic candidate genes related to oxidative stress identified suggestive SNPs in CYP and MMP genes that showed a statistically significant association with phenotypic markers of CCM1 disease severity, indicating that variation in these genes may contribute to inter-individual differences in susceptibility to CCM disease.

2. Methods

2.1. Study population

The study sample comprised 188 CCM1 subjects, all confirmed carriers of the KRIT1/CCM1–CHM mutation by genetic testing as previously described [7], and with both genotype and phenotype data available. Subjects were recruited through the Brain Vascular Malformation Consortium (BVMC) study at the University of New Mexico (UNM) and the Angioma Alliance patient advocacy group’s DNA & Tissue Bank study as previously described [8]. The study was approved by the local institutional review boards at UNM, University of California, San Francisco (UCSF), and Quorum IRB (Angioma Alliance), and by the National Institutes of Neurological Disorders and Stroke (NINDS). Written informed consent was obtained from all participants.

2.2. Clinical characteristics

Clinical assessment included information on characteristics of presenting symptoms leading to CCM diagnosis and classified as acute cerebral hemorrhages, seizures, focal neurological symptoms, and headaches using standardized guidelines [63]. Cerebral magnetic resonance imaging (MRI) was performed for all patients at study enrollment using a volume T1-weighted magnetization prepared rapid gradient echo acquisition (MPRAGE, 1-mm slice reconstruction) and axial T2-weighted turbo spin echo (TSE T2), T2 gradient-recalled echo (GRE), susceptibility-weighted imaging (SWI), and FLAIR sequences. Lesion counting was based on current evaluation of axial SWI as previously described, and lesion size was measured on TSE T2 images [7]. Three phenotypes were used as markers of CCM1 disease severity: (1) total lesion count, (2) large lesion count (> 5 mm), and (3) history of ICH at baseline. In our sample, 30.3% of CCM1–CHM subjects had a history of ICH and 90% had multiple lesions on MRI as previously reported [8], with total lesions mean ± SD of 60.1 ± 115.1 (range from 0 to 713) and large lesions mean ± SD of 4.9 ± 8.7 (range from 0 to 104).

2.3. Genotyping and quality control

Blood or saliva samples were collected and genomic DNA was extracted using standard protocols. DNA samples were genotyped at the UCSF Genomics Core Facility using the Affymetrix Axiom® Genome-Wide LAT 1 (Axiom GW LAT) Human Array [64], as previously described [8]. This array is designed to provide optimized genome-wide coverage of both common and rare variants in populations with West African, European, and Native American ancestries and consists in a total of 817,810 genetic markers, including 813,551 SNPs and 4259 insertion/deletions (Indels). All samples had a genotyping call rate of 97% or greater, and the two populations with West African, European, and Native American ancestry were classed into 18 and 6 families, respectively, according to the sex discordance and Mendelian errors and cryptic relatedness, were applied to genotype data using PLINK software (v1.07) before data analysis. Neither sex discordance nor Mendelian errors were identified [8].

2.4. Candidate genes and genetic markers selection

Candidate genes were selected for association studies on the basis of their involvement in vascular responses to oxidative stress and potential influence on major phenotypic markers of familial CCM1 disease severity, including total lesion burden, total large lesion burden, and history of ICH at baseline, as suggested by their physiopathological functions and impact on cerebrovascular diseases [31,43,44,53,65].

As a preliminary approach, candidate SNPs in multiple biologically relevant oxidative stress-related genes, including those implicated in modulating vascular homeostasis and remodeling in response to oxidative stress, were screened as putative genetic modifiers of CCM disease severity in our homogeneous cohort of Hispanic CCM1–CHM subjects, using the Axiom GW LAT Array as previously described [8].

Based on the outcomes of our preliminary screening (data not shown), we focused on the in-depth investigation of the two following gene superfamilies:

1) CYP superfamily consisting of 57 genes, divided among 18 families, encoding monooxygenase enzymes involved in the metabolism of xenobiotics and endobiotics, and the production of bioactive oxidation metabolites and reactive oxygen species that may affect the vasculature.

2) MMP superfamily consisting of 24 genes, divided among 6 families, encoding proteolytic enzymes that degrade the ECM scaffold, including the vascular basal lamina, enabling vascular remodeling in response to various physiological stimuli and pathological stresses.

The complete list of the members of CYP and MMP superfamilies is given in Supplementary Table 1. CYP and MMP proteins were classified into 18 and 6 families, respectively, according to their structures, substrate specificity and cellular localization [39,66]. For example, MMP families include: Collagenases (MMP-1,
-8, -13), Gelatinases (MMP-2, -9), Stromelysins (MMP-3, -10, -11, -12), Matrilysins (MMP-7, -26), membrane type MMPs (MMP-14, -15, -16, -17, -24, -25) and other MMPs (MMP-19, -20, -21, -27, -28) (Supplementary Table 1).

Gene loci were defined as ± 5 kb upstream and downstream of the sequence using UCSC Genome Browser Assembly Feb. 2009 (GRCh37/hg19). The following cut-off points were used: genotype call rate < 98%, a minor allele frequency (MAF) < 1%, or deviation from Hardy-Weinberg equilibrium (P < 0.001). We included 1122 genetic markers (1116 SNPs and 6 Indels) that fulfilled the above-mentioned criteria and extracted the genotypes from the Axiom GW LAT Human Array.

2.5. Statistical analysis

Residuals of log-transformed total or large lesion count were obtained after adjustment for age at enrollment and gender (R v2.10.1 software). To analyze the sets of genetic markers grouped by superfamily, family, or gene, we used the genetic markers “set-based test” in PLINK v1.07 which takes account of the linkage disequilibrium (LD) between the genetic markers and corrects P-values for the multiple markers tested within a set using 1,000 permutations. In addition to this correction for multiple genetic markers tested, we considered statistically significant associations with P-values ≤ 0.017 (0.05/3 phenotypes tested). The best SNP bearing each association between CYP or MMP families or genes and CCM1 disease severity was selected for further association analyses: (1) linear regression to assess association between residuals of log-total or large lesion count and single SNP (assuming an additive genetic model, i.e., 0, 1, or 2 copies of the minor allele) using STATA 12.1 statistical software (StataCorp, College Station, Tex., USA), and (2) logistic regression to assess association between ICH and single SNP, adjusting for age at enrollment and gender. All analyses accounted for clustering within families by using robust standard errors, except for MMP3 rs117153070. Since ICH analysis produced an infinite estimated odds ratio (OR) for MMP3 rs117153070, we calculated a one-sided 95% confidence interval (CI) based on the profile likelihood, and derived a p-value from the likelihood ratio test comparing models with and without the polymorphism. To ensure familial clustering could be disregarded for this specific analysis, we tested the effect of family with a mixed-effects logistic regression model.

3. Results

3.1. Association of CYP or MMP superfamilies with CCM1 disease severity

Growing evidence suggests that oxidative stress plays an important role in CCM disease pathogenesis [16,22,24]. Moreover, it is known that polymorphic genes related to oxidative stress and implicated in modulating vascular homeostasis and remodeling, including CYP and MMP genes encoding major enzymatic players of redox metabolism and ECM remodeling, are characterized by SNPs that confer greater susceptibility to vascular diseases [39,47,48,61,67–69], thus representing potential genetic modifiers of CCM disease severity.

In this light, we tested the possibility that genetic inter-individual variability in CYP and MMP genes influences markers of familial CCM1 disease severity, including total lesion burden, total large lesion burden, and history of hemorrhage at baseline. To this end, we first assessed the impact of the whole CYP and MMP superfamilies on CCM1 disease severity by performing a genetic marker set association analysis. The CYP superfamily, including

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Association of CYP or MMP superfamilies and families with phenotypic markers of CCM1 disease severity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic markers set</td>
<td>N genetic markers</td>
</tr>
<tr>
<td>CYP</td>
<td>765</td>
</tr>
<tr>
<td>CYP1</td>
<td>16</td>
</tr>
<tr>
<td>CYP2</td>
<td>176</td>
</tr>
<tr>
<td>CYP3</td>
<td>39</td>
</tr>
<tr>
<td>CYP4</td>
<td>169</td>
</tr>
<tr>
<td>CYP5</td>
<td>80</td>
</tr>
<tr>
<td>CYP7</td>
<td>21</td>
</tr>
<tr>
<td>CYP8</td>
<td>47</td>
</tr>
<tr>
<td>CYP11</td>
<td>29</td>
</tr>
<tr>
<td>CYP17</td>
<td>5</td>
</tr>
<tr>
<td>CYP19</td>
<td>51</td>
</tr>
<tr>
<td>CYP20</td>
<td>11</td>
</tr>
<tr>
<td>CYP21</td>
<td>1</td>
</tr>
<tr>
<td>CYP24</td>
<td>27</td>
</tr>
<tr>
<td>CYP26</td>
<td>25</td>
</tr>
<tr>
<td>CYP27</td>
<td>24</td>
</tr>
<tr>
<td>CYP44</td>
<td>47</td>
</tr>
<tr>
<td>CYP46</td>
<td>19</td>
</tr>
<tr>
<td>CYP51</td>
<td>4</td>
</tr>
</tbody>
</table>

P-values are corrected for the multiple markers within a set (taking account of the LD between these genetic markers). P-values in bold are considered statistically significant (P ≤ 0.017).

765 genetic markers, showed a trend toward association with total lesion count (P = 0.057) and large lesion count (P = 0.088) after correction for multiple markers tested (Table 1). In contrast, the MMP superfamily, including 357 genetic markers, was not associated with phenotypic markers of disease severity.

3.2. Association of CYP or MMP families or genes with CCM1 disease severity

We then evaluated whether the CYP and MMP families were associated with phenotypic markers of disease severity by grouping genetic markers by families (Supplementary Table 1). For CYP or MMP families showing a significant or borderline association with CCM1 disease severity, we performed additional association analyses by grouping markers by genes. Results are presented in Table 2.

The CYP4 family was associated with large lesion count (P = 0.014) (Table 1), and, among the 12 genes belonging to the CYP4 family, the strongest association was exhibited by the CYP4F2 followed by CYP4F11 and CYP4F12 genes (Table 2). CYP4F2, CYP4F8 and CYP4Z1 genes were also modestly associated with total lesion count (Table 2). The CYP8 family was associated with total lesion count (P = 0.014) (Table 1), and, of the two CYP8 genes analyzed, CYP8A1 encoding the prostaglandin I2 (prostacyclin) synthase (PTGIS) was significantly associated with total lesion count (P = 0.013) (Table 2). In addition, two families (CYP46 and MMP Stromelysins) were significantly associated with ICH (P = 0.011 and 0.007, respectively), with CYP46A1 and MMP3 genes bearing these associations (Tables 1–2). Moreover, CYP19, CYP24, CYP27 and CYP51 families were modestly associated...
Table 2
CYP or MMP families associated with phenotypic markers of CCM1 disease severity and association of related genes.

<table>
<thead>
<tr>
<th>Family/Gene</th>
<th>SNPs bearing associations or were individually associated with lesion counts or ICH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family CYP4</td>
<td>CYP4A1, CYP4A2, CYP4B1, CYP4F2, CYP4F8, CYP4F9, CYP4F11, CYP4F12, CYP4F22, CYP4F21, CYP4X1, CYP4Z1</td>
</tr>
<tr>
<td>Family CYP24</td>
<td>CYP24A1</td>
</tr>
<tr>
<td>Family Stromelysins</td>
<td>MMP3, MMP10, MMP11, MMP12</td>
</tr>
</tbody>
</table>

P-values are corrected for the multiple markers within a set (taking account of the LD between these genetic markers). P-values in bold are considered statistically significant (P < 0.017).

3.3. Genetic markers bearing associations between CYP or MMP families and genes and CCM1 disease severity

We then focused on the genetic markers bearing associations of CYP or MMP families and genes with CCM1 disease severity. As a note, none of the six Indels tested explained the observed associations or were individually associated with lesion counts or ICH (Supplementary Table 2).

However, we found that an intronic SNP (rs11085971) of CYP4F12 was mainly driving the association of CYP4 family and CYP4F12 gene with large lesion count, and was consistently individually associated with this marker of disease severity (P < 0.001) (Table 3). Further, CYP4F2 rs3093088, CYP4F8 rs28669833 and CYP4F11 rs12610962, bearing associations of CYP4F2, CYP4F8 and CYP4F11 genes, respectively, were individually associated with at least one disease severity phenotype (Table 3).

A synonymous SNP (rs5628, Leu256Leu) of CYP8A1 was mainly bearing the association of CYP8 family and CYP8A1 gene with total lesion count. Analyzed individually, this SNP was associated with both total lesion and large lesion counts, as the minor allele carriers of rs5628 had 79% more total lesions (P = 0.013) and 40% more large lesions (P = 0.006) at baseline, in comparison to non-carriers (Table 3).

Additional SNPs were found to be individually associated with at least one disease severity phenotype, consistently with associations reported for CYP or MMP families and genes (Table 3). The CYP27B1 rs1048691 and CYP51A1 rs2049900 polymorphisms were associated with smaller total lesion counts, while the CYP27A1 rs645163 and CYP46A1 rs10151332 were associated with greater large lesion counts and ICH (Table 3). Further, CYP19A1 rs72727199 was significantly associated with ICH (P = 0.002), while the CYP19 family or CYP19A1 gene was modestly associated with this phenotype (P = 0.035) (Tables 2 and 3).

Finally, an intronic SNP (rs117153070) of MMP3, which was mainly driving the association of Stromelysin family and MMP3 gene with ICH, was strongly associated with ICH in all the minor allele carriers of rs117153070 had a history of ICH (Table 3). Thus, the results of the individual SNP analyses supported the findings obtained with the family and gene sets.

Table 3
SNPs bearing associations of CYP or MMP families and genes with CCM1 disease severity.

<table>
<thead>
<tr>
<th>Family</th>
<th>Gene</th>
<th>SNP</th>
<th>MAF</th>
<th>Variant function class</th>
<th>Total lesion count</th>
<th>Large lesion count</th>
<th>ICH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP4</td>
<td>CYP4F2</td>
<td>rs3093088</td>
<td>0.13</td>
<td>Upstream</td>
<td>0.62</td>
<td>0.39</td>
<td>0.99</td>
</tr>
<tr>
<td>CYP4F8</td>
<td>rs28669833</td>
<td>0.14</td>
<td>Intron</td>
<td>1.50</td>
<td>1.11</td>
<td>2.02</td>
<td>0.009</td>
</tr>
<tr>
<td>CYP4F11</td>
<td>rs12610962</td>
<td>0.46</td>
<td>3'UTR</td>
<td>1.18</td>
<td>0.93</td>
<td>1.50</td>
<td>0.16</td>
</tr>
<tr>
<td>CYP4F12</td>
<td>rs11085971</td>
<td>0.03</td>
<td>Intron</td>
<td>0.60</td>
<td>0.29</td>
<td>1.26</td>
<td>0.18</td>
</tr>
<tr>
<td>CYP4Z1</td>
<td>rs1502924</td>
<td>0.11</td>
<td>Intron</td>
<td>1.61</td>
<td>0.98</td>
<td>2.65</td>
<td>0.059</td>
</tr>
<tr>
<td>CYP8</td>
<td>rs10151332</td>
<td>0.19</td>
<td>Synonymous (L256L)</td>
<td>1.40</td>
<td>1.13</td>
<td>2.84</td>
<td>0.007</td>
</tr>
<tr>
<td>CYP19</td>
<td>CYP19A1</td>
<td>rs72727199</td>
<td>0.12</td>
<td>Intron</td>
<td>0.93</td>
<td>0.67</td>
<td>1.29</td>
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<tr>
<td>CYP24</td>
<td>CYP24A1</td>
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<td>3'UTR</td>
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<td>0.11</td>
<td>0.99</td>
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<tr>
<td>CYP27</td>
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<td>rs645163</td>
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<td>0.69</td>
<td>1.50</td>
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<tr>
<td>CYP46</td>
<td>CYP46A1</td>
<td>rs10151332</td>
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<td>Intron</td>
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<td>0.73</td>
<td>1.25</td>
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<tr>
<td>CYP51</td>
<td>CYP51A1</td>
<td>rs2049900</td>
<td>0.07</td>
<td>Intron</td>
<td>0.57</td>
<td>0.39</td>
<td>0.84</td>
</tr>
</tbody>
</table>

*The OR cannot be determined, because 100% (6/6) of heterozygous carriers have a history of ICH, compared to 70% of patients all without ICH. We show a one-sided 95% confidence interval (CI) based on the profile likelihood and a p-value from the likelihood ratio test comparing models with and without the polymorphism. A mixed-effects logistic regression model found no estimated family effect; hence, the model was not adjusted for familial clustering.

Table gives odds ratio (OR) or proportional increase (PI) or decrease if less than 1) in either total or large lesion count, along with 95% confidence intervals as well as P-values. P-values in bold are considered statistically significant (P < 0.017).
The allele distribution of these SNPs individually associated with CCM1 severity in Hispanic CCM1–CHM subjects were then compared with those available in general and specific Latin populations, using the public databases from dbSNP (SNP database) (http://www.ncbi.nlm.nih.gov/), 1000 Genomes Project global population and HapMap MEX (Mexican Ancestry in Los Angeles) Hispanic population. No significant differences were found in allele frequencies of SNPs reported in Table 3 in comparison to general populations (data not shown).

3.4. Function of significantly associated CYPs and MMPs

To gain insight into potential regulatory mechanisms through which these CYP or MMP genes and variants might impact CCM1 disease severity, we applied complementary methods, including in silico analyses and extensive manual review of the literature. Results are summarized in Supplementary Table 3.

We first asked whether the encoded proteins might be grouped into specific categories according to their functions and substrates using the DAVID Gene Functional Classification Tool [70]. We found that the most significantly associated CYPs can be grouped into specific categories by both their endogenous substrates and bioactive products. Indeed, they are all monoxygenases that play key roles in the metabolism of important endogenous compounds in the brain, including arachidonic acid (AA), AA derivatives, cholesterol, steroid hormones and vitamin D3, contributing to the production of important bioactive metabolites, such as eicosanoids, oxysterols, estrogens and active vitamin D3, which act as signaling molecules in redox-related pathways and may regulate MMP activity has been clearly implicated in focal degradation of the vascular extracellular matrix underlying the onset and progression of cerebrovascular diseases [53,92,93]. Furthermore, increased endothelial expression of MMPs has been previously suggested to contribute to the formation, enlargement and rupture of human CCM lesions by affecting the stability of the vascular extracellular matrix scaffold [94].

We then asked whether specific redox-related pathways were affected by the CCM disease-associated CYP or MMP genes and SNPs using the NCBI BioSystems database [95] and Reactome, a curated pathway database (http://www.reactome.org/), as well as a comprehensive review of the literature.

All CYPs are involved in biological oxidations, including oxidative metabolism of endogenous and exogenous compounds by monoxygenase activity. Moreover, CYP enzymes are among the oxidoreductases that are potential sources of superoxide anions in mammalian cells [38]. Indeed, compelling evidence indicates that poor coupling of the CYP catalytic cycle may result in continuous ROS production and consequent formation of lipid peroxidation products, such as 4-hydroxynonenal (HNE), which in turn may activate the redox-sensitive JNK/c-Jun signaling pathway, leading to cellular oxidative stress [38,96]. Furthermore, CYP enzymes may also influence redox-related pathways modulated by some of their bioactive metabolic products, including eicosanoids, oxysterols, steroid hormones and vitamin D3 [37,39,48,97]. Intriguingly, whereas there is clear evidence that some CYP enzymes may act as either regulators or targets of redox-sensitive pathways, including the JNK/c-Jun pathway [98–100] (Supplementary Table 3), we have previously demonstrated that the JNK/c-Jun pathway is influenced by KRIT1 loss-of-function [22], suggesting a potential functional connection between CYP and KRIT1 proteins.

On the other hand, there is consistent evidence that also MMP enzymes may act as either regulators or targets of oxidative stress-related signaling events [49,54,55]. In particular, MMP3 has been involved in distinct redox-sensitive pathways, including PKC, PI3K/AKT and ERK1/2 pathways [56] (Supplementary Table 3).

Importantly, none of the CCM disease-associated SNPs was previously reported to affect specific redox related pathways. Most of these SNPs were in non-coding regions of the candidate genes major CYP enzymes involved in cholesterol metabolism in the brain. In particular, CYP51A1 is involved in cholesterol synthesis, whereas CYP46A1 and CYP27A1 catalyze the conversion of excess cholesterol into the oxysterols 24(S)-hydroxysterol (24-OHC) and 27-hydroxysterol (27-OHC), respectively [82,83]. Notably, local excess levels of biologically active oxysterols, such as 24-OHC and 27-OHC, may trigger and sustain local oxidative stress reactions leading to vascular dysfunctions and cerebrovascular diseases [84–86].

CYP27A1 is also involved in the metabolism of vitamin D3, along with CYP27B1. Indeed, these two CYP enzymes catalyze the two key hydroxylation steps that convert the inactive form of vitamin D3 (cholecalciferol) to calcifediol (25-hydroxyvitamin D3) and calcitriol (1,25-dihydroxyvitamin D3 or active vitamin D3), respectively [87,88]. Notably, vitamin D3 is endowed with antioxidant properties in the endothelium, which may prevent peripheral vascular diseases [89,90]. Consistently and importantly, vitamin D3 supplement has been recently demonstrated to decrease vascular oxidative stress and CCM lesion burden in a mouse model of CCM disease [16].

Matrix metalloproteinase-3 (MMP-3), also known as stromelysin-1, is a broad-spectrum extracellular matrix degrading enzyme that acts on collagen types II, III, IV, IX, and X, proteoglycans, fibronectin, laminin, and elastin [91]. In addition, MMP-3 can also activate other MMPs such as MMP-1, MMP-7, and MMP-9, rendering MMP-3 crucial in extracellular matrix remodeling both in physiological and pathological processes (Supplementary Table 3). Notably, MMP activity has been clearly implicated in focal degradation of the vascular extracellular matrix underlying the onset and progression of cerebrovascular diseases [53,92,93]. Furthermore, increased endothelial expression of MMPs has been previously suggested to contribute to the formation, enlargement and rupture of human CCM lesions by affecting the stability of the vascular extracellular matrix scaffold [94].

Specifically, members of the CYP4F subfamily have been involved in the metabolism of vitamins (K1 and E), xenobiotics, including drugs, and endobiotics, including polyunsaturated fatty acids (PUFAs) and their derivatives [72]. In particular, there is clear evidence that CYP4F enzymes catalyze the ω-hydroxylation of arachidonic acid and eicosanoids, such as prostaglandins (PG), leukotrienes (LT) and epoxyeicosatrienoic acids (EET), which act as autocrine and paracrine factors in various biological processes affecting the vasculature, including the modulation of ROS formation, inflammatory responses, and vascular tone, permeability and remodeling [43,72–74].

CYP8A1 encodes the prostaglandin I2 (PGI2) synthase, which catalyzes the conversion of prostaglandin H2 (PGH2) to PGI2 (prostacyclin), a potent vasodilator involved in local regulation of cerebral blood flow and vascular homeostasis [75–77]. Remarkably, recent evidence demonstrates a role of prostacyclin in KRIT1-dependent vascular endothelial cell barrier regulation [78].

CYP19A1 (also called aromatase or estrogen synthase) is expressed in major cellular components of the NVU, such as astrocytes and endothelial cells, and catalyzes the key aromatization step that converts androgens into estrogens, thus playing a role in local regulation of BBB permeability and cerebrovascular homeostasis mediated by sex steroid hormones [79,80]. In particular, estrogens are known to favor vasculoprotection from oxidative stress by inducing the upregulation of distinct proteins that exert anti-oxidant and anti-inflammatory functions and contribute to cerebrovascular homeostasis, including Mn superoxide dismutase (SOD2) and prostacyclin synthase (CYP8A1) [79,81].

CYP51A1 (lanosterol 14α-demethylase), CYP46A1 (cholesterol 24-hydroxylase) and CYP27A1 (sterol 27-hydroxylase) are the
environmental and/or other genetic factors [7,8]. Consistently, in reasons, even among family members of similar ages harboring genes or analyzed individually in CYP and MMP genetic markers grouped by superfamilies, families, 4. Discussion coding variants.

For the rs5628, rs72272199, rs645163, rs1048691, rs2049900 and rs117153070 variants in up to 23 species was observed for the rs5628, rs72272199, rs645163, rs1048691, rs2049900 and rs117153070 variants in in silico tools, such as Ensembl Phylogenetic Context [104]. We found that a high evolutionary conservation among species was observed for the rs5628, rs72272199, rs645163, rs1048691, rs2049900 and rs117153070 variants in up to 23 vertebrates, including mammalian, reptile, and bird classes. Further functional investigations using relevant human tissues and cells are required to robustly assess the regulatory role of these non-coding variants.

To evaluate the potential importance of these non-coding variants in gene regulation, we searched for phylogenetic conservation in KRIT1-dependent vascular endothelial cell barrier regulation [78].

The association between CYP19A1 rs72272199 and ICH is consistent with the important role of CYP19A1 (estrogen synthase) in the local modulation of BBB permeability and cerebrovascular homeostasis mediated by sex steroid hormones [79,80]. Indeed, CYP19A1 is expressed at the NVU and catalyzes a key step in the biosynthesis of estrogens, which are known to favor vasoprotection from oxidative stress by increasing cerebrovascular levels of proteins endowed with antioxidant and anti-inflammatory properties, including SOD2 and prostacyclin synthase (CYP19A1) [79,81]. Intriguingly, whereas there is evidence that deficiencies in estrogen-mediated regulation of cerebrovascular homeostasis may contribute to an increased risk of cerebral aneurysm pathogenesis and rupture [110,111], it has been reported that risk of recurrent ICH due to CCM is greater for women than men [63], suggesting that cerebrovascular levels of sex hormones may have a role in the variable expression of CCM disease.

The association of CYP46A1 rs10151332 and CYP27A1 rs645163 polymorphisms with both large CCM lesion count and susceptibility to ICH is consistent with a substantial body of evidence indicating that variations in CYPs involved in cholesterol metabolism in the brain, such as the cholesterol 24-hydroxylase CYP46A1 and the sterol 27-hydroxylase CYP27A1, can lead to local excess levels of biologically active 24-OHC and 27-OHC oxysterols [84,85,112–115]. Indeed, local accumulation of these cholesterol oxidation derivatives can trigger and sustain local oxidative stress and inflammatory reactions that contribute to distinct human disorders, including cerebrovascular diseases [83,86,116]. Accordingly and importantly, markers of cholesterol metabolism in the brain, including the 24-OHC and 27-OHC oxysterols, showed strong associations with cerebrovascular diseases [86].

CYP27A1 is also involved in the metabolism of vitamin D3 along with CYP27B1, acting as vitamin D3 25-hydroxylase and 25-hydroxyvitamin D3 1α–hydroxylase, respectively [87,88,117]. Consistent with a potential impact on CCM disease severity of genetic variations in CYP enzymes involved in vitamin D3 metabolism, including the identified CYP27A1 rs645163 and CYP27B1 rs1048691 SNPs, recent in vitro and in vivo studies have demonstrated the effectiveness of vitamin D3 in decreasing vascular oxidative stress and CCM lesion burden in a mouse model of CCM disease [16]. Accordingly, vitamin D3 is endowed with antioxidant properties in the endothelium, and may prevent peripheral vascular diseases [89,90].
In this study, we also identified an association between the MMP3 rs171753070 polymorphism and ICH, which is in line with previous studies reporting associations between MMPs and ICH in human [65]. Accordingly, MMP-related genetic variations, including SNPs, may contribute to heterogeneity in vascular remodeling and consequent presentation and natural history of cerebrovascular diseases [53,93]. Indeed, there is compelling evidence that polymorphisms in MMP genes, including MMP3, may influence inter-individual differences in susceptibility to the onset and severity of cerebral aneurysms [57], brain arteriovenous malformations [58–60], ischemic white matter injury [118], and Moyamoya disease [62]. Furthermore and consistent with a potential impact of MMP genetic variations on CCM disease, increased endothelial expression of MMPs has been previously observed in human CCM lesions and suggested to contribute to their formation, enlargement and rupture by affecting the stability of the vascular extracellular matrix scaffold [94]. In addition, whereas there is evidence that polymorphisms in MMP genes may influence cerebral small vessel disease associated with white matter lesions [68], an increased frequency of white matter lesions among familial cases of CCM disease has been recently reported [119].

Some of the identified SNPs were concomitantly associated with two phenotypic markers of CCM disease severity, including CYP8A1 rs5628, which was associated with both total and large number of CCM lesions, and CYP4F11 rs12610962, CYP27A1 rs645163 and CYP46A1 rs10151332, which were associated with large lesion count and susceptibility to ICH phenotypes. However, no significant concomitant association with all three phenotypic markers of CCM disease severity was observed, suggesting that multiple genetic risk factors contribute to the distinct most severe phenotypes of the disease.

The study was centered on an ethnically homogeneous cohort of 188 Hispanic CCM patients carrying the founder KRIT1/CCM1–CHM mutation, which avoided confounding factors due to differences in population genetic structure and disease-causing mutation. Furthermore, it was based on a genetic marker set association analysis, which is more powerful than testing each genetic marker individually [120,121]. Despite some limitations, including the limited ethnic group and sample size of our CCM1 cohort and the fact that our selection of common genetic markers cannot exclude the possibility for rare functional variants in these families to influence the severity of CCM1 disease, our study has the important value of opening a novel avenue for future basic and translational research in the field of CCM disease. Indeed, our experimental outcomes prompt future replication studies with independent and random cohorts of CCM subjects to verify whether the genetic modifiers of CCM1 disease severity identified in our KRIT1/CCM1–CHM cohort are replicated in subjects affected by other forms of CCM disease, including sporadic forms and familial forms linked to different mutations in any CCM gene, as well as across different ethnic groups. The confirmation and extension of our results might lead to the recognition of subsets of CCM patients who are predisposed to develop the most severe disease phenotypes, including ICH, offering the opportunity to prevent these events and related complications, and improve outcomes. Furthermore, our findings pave the way for future basic research investigations aimed at addressing the role of specific CYP enzymes and metabolites in CCM disease pathogenesis and severity, providing a novel useful framework for a better understanding of pathogenic mechanisms, the identification of new diagnostic, prognostic and predictive biomarkers, and the development of specific and effective preventive and therapeutic strategies.

5. Conclusions

This study furthers research into genetic modifiers in CCM disease by studying genetic markers in candidate oxidative stress-related gene families at multiple levels of information: superfamilies, families, and individual genetic markers. Our results suggest that genetic variability within CYP4, CYP8, CYP19, CYP24, CYP27, CYP46, CYP51 and the MMP Stromelysins families may influence the severity of CCM1 disease and could serve as predictors of CCM1 disease outcomes. These findings most probably reflect the important role of the identified CYP and MMP enzymes in vascular homeostasis through the regulation of eicosanoid and cholesterol metabolism, and extracellular matrix remodeling, respectively, suggesting that these biological processes contribute to the heterogeneity in the presentation and natural history of CCM disease.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.01.008.

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