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Plasma Biomarkers of Inflammation Reflect Seizures and Hemorrhagic Activity of Cerebral Cavernous Malformations

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Abstract

The clinical course of cerebral cavernous malformations (CCMs) is highly variable. Based on recent discoveries implicating angiogenic and inflammatory mechanisms, we hypothesized that serum biomarkers might reflect chronic or acute disease activity. This single site prospective observational cohort study included 85 CCM patients, in whom 24 *a priori* chosen plasma biomarkers were quantified and analyzed in relation to established clinical and imaging parameters of disease categorization and severity. We subsequently validated the positive correlations in longitudinal follow-up of 49 subjects. Plasma levels of matrix metalloproteinase-2 and intercellular adhesion molecule 1 were significantly higher ($P=0.02$, $P=0.04$ respectively, FDR corrected), and matrix metalloproteinase-9 was lower ($P=0.04$, FDR corrected) in patients with seizure activity at any time in the past. Vascular endothelial growth factor and endoglin (both $P=0.04$, FDR corrected) plasma levels were lower in patients who had suffered a symptomatic bleed in the prior 3 months. The hierarchical clustering analysis revealed a cluster of 4 plasma inflammatory cytokines (interleukin 2, interferon gamma, tumor necrosis factor alpha and interleukin 1 beta) separating patients into what we designated “high” and “low” inflammatory

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Compliance with Ethical Standards

Disclosures: The authors declare no conflict of interest.

Ethical approval: All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki, and approved by The University of Chicago Institutional Review Board (IRB). The ethical principles guiding the IRB are consistent with The Belmont Report, and comply with the rules and regulations of The Federal Policy for the Protection of Human Subjects (56 FR 28003).

states. The “high” inflammatory state was associated with seizure activity ($P=0.02$) and more than one hemorrhagic event during a patient’s lifetime ($P=0.04$), and with a higher rate of new hemorrhage, lesion growth or new lesion formation ($P<0.05$) during prospective follow-up. Peripheral plasma biomarkers reflect seizure and recent hemorrhagic activity in CCM patients. In addition, 4 clustered inflammatory biomarkers correlate with cumulative disease aggressiveness, and predict future clinical activity.

Keywords

Cerebral Cavernous Malformation; Plasma Biomarker; Neuroinflammation; Hierarchical clustering

Introduction

Cerebral cavernous malformation (CCM) is a common neurovascular pathology affecting 0.5% of the population. It exists in two forms, sporadic/solitary (70–80% of cases) and familial/multifocal (20–30%), with identical vascular lesions consisting of clusters of abnormally thin-walled blood filled sinusoids lined by endothelium [1]. The familial/multifocal CCM is associated with an autosomal dominant Mendelian inheritance with mutations at one of the three documented gene loci (*CCM1/KRIT1*, *CCM2/MGC4607* and *CCM3/PDCD10*) [2]. Lesions in familial and sporadic disease harbor biallelic somatic mutations in the same three genes, indicating a common pathogenic mechanism of lesion genesis [2]. The *CCM* genes encode proteins involved in maintaining endothelial barrier integrity by inhibiting RhoA kinase (ROCK) activation [3]. CCM may arise from increased ROCK activity leading to tight-junction dysregulation between vascular endothelial cells, resulting in a defective blood brain barrier and chronic blood leakage across the lesion with non-heme iron deposition [3–5]. These two phenomena (lesional hyperpermeability and iron deposition) are quantifiable using advanced magnetic resonance imaging (MRI) techniques, and have been shown to correlate with prior disease activity [5, 6].

Many CCMs remain asymptomatic while some lesions exhibit symptoms of seizures or focal hemorrhage, with varying aggressiveness. CCM patients have a highly unpredictable clinical course [1]. It is not known what factors trigger or may predict symptomatic manifestations in individual cases.

Recent experimental evidence in transgenic murine models and biological studies of resected human CCM lesions demonstrate a complex pathobiologic milieu, involving a likely interplay of neuroinflammatory and angiogenesis processes, and the loss of endothelial barrier function [7–11]. B-cell depletion has been shown to blunt lesional development and hemorrhage in murine CCM model [7]. A proinflammatory genotype was recently associated with a more aggressive CCM disease severity in humans [12]. These discoveries have motivated hypotheses regarding the role of inflammatory states in clinical behavior of CCM, and whether these may be reflected in cytokines and other molecules in peripheral plasma.

The use of quantifiable biological markers to predict the behavior and relevant outcome of common hemorrhagic, metabolic or degenerative neuropathologic diseases is gaining considerable momentum in clinical research [13]. The importance of biomarkers in clinical practice was reflected by the FDA/NIH designating a dedicated working group in this expertise [14, 15]. Specific metabolic signatures of biological processes have been proposed with different end points such as diagnosis, severity, etiology classification and prognosis in various neurological diseases including multiple sclerosis, Alzheimer's or Parkinson's disease [13]. Our group had previously associated biomarkers of CCM lesional iron content and vascular permeability assessed by novel MRI techniques with recent clinical activity, and reported limited correlations of chronic disease aggressiveness with plasma Vitamin D and Non-HDL Cholesterol levels [4–6, 16]. We now hypothesized that plasma levels of a broader panel of inflammatory and angiogenic molecules would be associated with various features of recent versus chronic disease activity, and potentially predict future clinical behavior. The putative biomarkers were chosen based on their reported role in pathology of CCM including angiogenesis, inflammation, endothelial cell integrity and permeability, cell adhesion and extracellular matrix remodeling (Supplemental Table 1) [7–11].

Materials and Methods

Patient recruitment

This was a single-site prospective observational cohort study, without planned intervention. From July 2014 to November 2016, 85 patients (mean age = 37.46 ± 17.89 years, range = [4.62 – 75.57]) with confirmed diagnosis of CCM (sporadic or familial form) were enrolled. Patients gave written informed consent to participate in this research, including the collection of blood sample for biomarker studies, in accordance to the Declaration of Helsinki, and approved by The University of Chicago Institutional Review Board (IRB). The ethical principles guiding the IRB are consistent with The Belmont Report, and comply with the rules and regulations of The Federal Policy for the Protection of Human Subjects (56 FR 28003). This was performed in conjunction with routine clinical evaluations or follow-up, and MRI with optimized CCM protocols including dynamic contrast-enhanced quantitative permeability (DCEQP) and quantitative susceptibility mapping (QSM) research protocols described previously [4–6].

As per currently accepted disease categorization, cases were classified as sporadic if they harbored a solitary lesion on the most sensitive susceptibility weighted imaging (SWI) MRI sequences, or a cluster of lesions associated with a developmental venous anomaly (DVA) [4, 6]. They were classified as familial if they harbored multifocal CCM lesions, a family history of CCM in a first-degree blood relative or a mutation genotyped at a CCM gene locus [4, 6]. Patients with partial or complete CCM lesion resection or any prior brain irradiation were not included.

Clinical features and categorization of disease aggressiveness

We hypothesized that a relevant disease biomarker may reflect chronic disease aggressiveness over the patient's lifetime, or acute more recent clinical activity [14, 16]. Based on previous reports by our group and others [16, 17], we prospectively defined

features of chronic CCM disease severity as (1) a history of 2 or more adjudicated clinically overt lesional hemorrhages [6, 18] at any time, (2) age of symptom onset prior to age 18, or (3) high lesion burden in familial cases (more than 5 lesions > 4 mm in diameter on conventional T₂-weighted MRI, or more than 25 lesions on the most sensitive SWI)[4]. Similarly, we defined features of acute disease severity as (1) a lesional growth or a symptomatic hemorrhage, or (2) new lesion formation on comparable MRI sequences within the past 12 months [6].

For each patient enrolled in the study, the aforementioned features were assessed during the clinical visit when the blood sample and imaging was performed. These were reviewed and adjudicated by the senior author with experience in the care of CCM (IAA), blinded to any knowledge about the biomarker levels, and electronically stored in a secure database for subsequent analysis [4–6, 16].

Plasma isolation and biomarkers assessment

All blood samples were collected using standard clinical 10 ml heparinized vacutainer tubes (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The use of heparinized plasma to quantify biological compounds is in agreement with clinical practice and instructions provided by the bio-assay kit manufacturers [19]. Because clinical visits were conducted at various times of the day, we could not require the patients to undergo fasting. However, we focused only on the metabolic compounds not affected by fasting condition [20]. For plasma isolation, 3 mL of heparinized blood were centrifuged at 2,300 rpm at 4°C for 10 minutes. The supernatant plasma was equally aliquoted (200 µl) in 1.7 ml microcentrifuge tubes and stored at –80°C. Eighteen plasma biomarkers were assessed using customized magnetic bead-based multiplex Luminex screening immunoassay kits (R&D Systems, Minneapolis, Minnesota, USA). The measurements were performed using a BioRad BioPlex-100 analyzer (Bio-Rad Laboratories, Hercules, California, USA). Lipid panel was assessed using Roche Cobas 8000 Modular Analyzer (Roche Diagnostics, Risch-Rotkreuz, Switzerland). 25-hydroxyvitamin D [25-(OH) vitamin D] levels were quantified using high-pressure liquid chromatography coupled to mass spectrometry (LC/MS). *For more information on quantification Methods, please refer to the online supplement.*

Statistical methods

The univariate associations between the different plasma biomarker levels and each clinical features of acute and chronic severity were analyzed as continuous variables using an unpaired two samples Student's t-test. To control the proportion of false rejected hypotheses, we performed a false discovery rate (FDR) analysis. Linear combinations of biomarkers with significant associations with clinical features were generated using the Canonical discriminant function analysis. Linear Pearson correlation coefficients, or partial Pearson correlations, were calculated between the computationally estimated imaging biomarkers (permeability or QSM values) and each plasma biomarker separately.

Finally, a multivariate unsupervised hierarchical clustering analysis combined with logistic regressions was performed to determine the association between CCM-related clinical

events and class separation observed on the dendrogram (Group 1 or Group 2) as determined by expression of inflammatory plasma biomarkers. We also performed a principal component analysis (PCA) to assess the relative contributions of the individual biomarkers to the demonstrated association.

All P values were considered to be statistically significant at $P < 0.05$. The statistical analyses were conducted with SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and R statistical framework version 3.1 (R Foundation for Statistical Computing, Vienna, Austria). *For more information on statistical Methods, please refer to the online supplement.*

Results

Demographic and CCM lesions characteristics

Among the 85 CCM patients (49 with solitary/sporadic lesions and 36 with multifocal/familial CCMs), 43 patients presented initially with symptomatic hemorrhage secondary to their CCM, and 19 presented with seizures. Twenty-two patients had initial CCM-related symptoms before 18 years old, and 17 experienced at least two adjudicated clinically overt hemorrhages during their lifetime. Among the familial cohort, 13 patients harbored more than 25 lesions observed using SWI sequence, and 14 harbored more than 5 lesions of minimum 4 mm in diameter observed on T₂-weighted images. There were no significant differences in the sex, age at the time of enrollment or age at symptom onset in sporadic versus familial cohorts (Table 1).

Intercellular adhesion molecule 1 (ICAM1/CD54), metalloproteinase-2 (MMP2) and metalloproteinase-9 (MMP9) are associated with seizures in CCM disease, while vascular endothelial growth factor (VEGF) and endoglin/CD105 (ENG) are associated with recent CCM-related bleeding in univariate analysis

The plasma levels of 7 biomarkers including C-reactive protein (CRP) (t-value=-2.58, $P=0.02$), interleukin 2 (IL2) (t-value=5.19, $P<0.0001$), interleukin 10 (IL10) (t-value=-3.68, $P=0.0004$), MMP2 (t-value=5.56, $P<0.0001$), roundabout guidance receptor 4 (ROBO4) (t-value=2.78, $P=0.0069$), ICAM1/CD54 (t-value=-2.64, $P=0.012$) and interferon gamma (IFN γ) (t-value=2.43, $P=0.018$) were affected by a batch effect, while the others 17 biomarkers were unaffected. We independently considered the correlations between the 24 plasma biomarkers and each respective features of chronic and acute disease aggressiveness for our primary hypothesis testing. After correction for batch effect where appropriate, patients experiencing seizures showed a higher MMP2 and ICAM1/CD54 ($P=0.02$ and $P=0.04$ respectively, FDR corrected) along with a lower MMP9 ($P=0.04$, FDR corrected) plasma levels (Fig. 1). The receiver operating characteristic (ROC) analysis using the combination of plasma levels of MMP2, ICAM1/CD54 and MMP9 (Supplemental equation 1) was able to differentiate patients experiencing seizures from others with a sensitivity of 91% and a specificity of 60% (area under curves (AUC) =0.79, $P<0.0001$).

Moreover, patients who suffered of a symptomatic bleed within the preceding 3 months had lower VEGF and ENG ($P=0.04$, FDR corrected; for both) plasma levels (Fig. 2). The ROC analysis using the combination of plasma levels of VEGF and ENG (Supplemental equation

2) distinguished patients who had a CCM-related bleed within the past 3 months from others with a sensitivity of 100% and a specificity of 81% (AUC=0.91, $P=0.03$).

No intercorrelation between MMP2, MMP9, ICAM/CD54 and between VEGF, ENG were observed (Supplemental Table 2). Supplemental Table 3 reports all the P values of the comparison between the 24-plasma biomarkers and the relevant validated CCM-related features. We did not identify any potential confounders affecting the level of any of the 24 biomarkers such as age of enrollment, solitary/sporadic or multifocal/familial, genotype (sporadic, *CCMI*, 2 or 3) or sex (male or female).

IL10, chemokine ligand 2 (CCL2/MCP1) and ROBO4 are correlated with iron deposition assessed *in vivo* using QSM

Of the 85 CCM patients enrolled in the study, 70 had DCEQP (41 solitary/sporadic cases and 29 multifocal/familial) and 60 had QSM (32 solitary/sporadic cases and 28 with multifocal/familial) MRI protocols concurrently with blood sample collection. We first considered all CCM subjects, and then distinguished the *stable* from *unstable* patients in correlations between plasma and imaging biomarkers [6, 18]. We also identified lesions that recently bled and correlated their lesional permeability and QSM values with the plasma biomarkers levels.

Among the 70 patients who had a DCEQP (vascular permeability) sequence, 22 were defined as *unstable* and 14 lesions (accounting for 14 patients) experienced a hemorrhage within the prior 12 months and had satisfactory DCEQP data acquisition. There were no significant correlations between the 24-plasma biomarkers and lesional or background brain permeability values in sporadic or familial cases. This result suggests that the plasma biomarkers do not reflect lesional or background brain permeability in CCM patients.

Among the 60 patients who had QSM (iron deposition) sequence, 33 were defined as *unstable* and 16 lesions (accounting for 16 patients) that experienced a clinically symptomatic hemorrhagic event within the prior 12 months and had satisfactory QSM data acquisition. After correction for batch effect when necessary, there were higher levels of IL10 ($r=-0.61$, $P=0.02$), CCL2/MCP1 ($r=-0.60$, $P=0.02$) and ROBO4 ($r=-0.53$, $P=0.05$) in association with lower mean lesional QSM, in the lesions that recently bled (Fig. 3). There were no significant correlations between these biomarkers and lesional iron in *stable* lesions, or in the combined cohort of *stable* and *unstable* lesions.

4-Clustered inflammatory biomarkers are associated with CCM-related hemorrhages and seizures

Hierarchical clustering of plasma markers revealed a distinct two-class separation within the resulting dendrogram (Fig. 4). Patients present in the left branch of the hierarchical tree (Group 1, $N = 25$; in yellow in Fig. 4) were associated with lower plasma levels of 5 inflammatory biomarkers: IL2, IFN γ , tumor necrosis factor alpha (TNF α), interleukin 1 beta (IL1 β) and interleukin 6 (IL6). Conversely, patients present in the right branch (Group 2, $N = 57$; in brown in Fig. 4) were found to be associated with higher plasma levels of these 5-clustered inflammatory biomarkers.

Further analysis showed that 4 of the 5-clustered biomarkers previously identified, IL2, IFN γ , TNF α and IL1 β plasma values were strongly significantly higher (all, $P<0.001$) in patients present in Group 2 as compared to Group 1 (Supplemental Table 4). Logistic regression model including features of chronic disease severity showed significant independent associations between higher plasma levels of IL2, IFN γ , TNF α and IL1 β and prior hemorrhage, more than one hemorrhage, and the presence of seizures in the patient's lifetime, but not lesion burden (Table 2). A second logistic regression addressing more recent lesion behavior demonstrated that higher levels of IL2, IFN γ , TNF α and IL1 β were not associated with a recent hemorrhage (within 3, 6 or 12 months), recent lesion growth or new lesion formation. Finally, the ROC analysis using the combination of plasma levels of IL2, IFN γ , TNF α and IL1 β (Supplemental equation 3) distinguished patients who experienced more than 1 hemorrhage and/or seizures during a patient's lifetime from others patients with a sensitivity of 65% and specificity of 69% (AUC=0.67, $p=0.02$) (Supplemental Fig. 1).

Validation cohort with higher incidence of events in the group with higher plasma levels of IL2, IFN γ , TNF α and IL1 β

Of the 85 subjects included in the hierarchical clustering analysis, 49 patients (33 in Group 2 and 16 in Group 1) were prospectively followed-up after the initial blood sample was drawn. Subjects in the Group 2 experienced a total of 10 events (bleed or lesional growth; 14 events when including new lesion formation) during 32.0 patient-years of follow-up (rate: 0.312, 95% CI: [0.150–0.574]). One event (bleed or lesional growth; 2 events when including new lesion formation) during 21.6 patient-years of follow-up (rate: 0.05, 95% CI: [0.001–0.258]) occurred in Group 1. Group 2 had a higher incidence rate of new events ($p=0.03$ and $p=0.02$ when including new lesion formation, relative risk: 6.74 and 4.72 respectively). There was no difference in the proportion of sporadic/familial cases in the 2 groups.

Discussion

We evaluated herein 24 plasma biomarkers individually in relation to clinically relevant parameters of CCM disease activity. We replicated previous results on the association between chronic disease severity and vitamin D and non-HDL cholesterol levels in a larger cohort of CCM patients [16]. Five additional plasma biomarkers including ICAM1/CD54, MMP2, MMP9, VEGF and ENG showed significant correlation with disease activity in univariate correlations.

ICAM1/CD54, MMP2 and -9 showed a significant association with prior seizure activity. ICAM1/CD54 plasma levels were higher in CCM patients demonstrating seizure activity. An up-regulation of this endothelial molecule expressed as part of the leukocyte-endothelial adhesion mechanism has already been associated with epilepsy [21, 22]. Using a murine model, Fabene et al. reported a synergistic reduction of leukocyte-endothelial interactions and blood-brain barrier permeability after blockage of ICAM1/CD54 resulting in fewer epileptic episodes [21].

MMP2 and -9 belong to the matrix metalloproteinase family associated with various neurological diseases including multiple sclerosis, stroke, Alzheimer's disease and epileptic

seizures [23–25]. In the brain, MMPs are involved in the remodeling of the cellular microenvironment by regulating numerous biological mechanisms including blood-brain barrier permeability [23, 26, 27]. Increased endothelial expression of MMP2 and -9 have been previously reported in CCM lesions [28], and when upregulated these are known to cause a blood brain barrier disruption by degrading tight junction proteins [23, 26, 27, 29]. MMP2 and -9 have also been reported to be associated with epileptic seizures [24, 25, 30, 31]. We cannot determine if our biomarker associations reflect seizure activity per se, or specifically in association with CCM disease, since we did not examine patients with seizure activity alone. Interestingly, MMP2 and -9 peripheral plasma levels of CCM patients in our study showed an opposite effect compared to some of the previously reported literature on seizures [24, 31]. The association between plasma MMPs level and seizures in CCM patients merits further investigation, particularly in relation to factors not examined in our study, including seizure severity and treatment, and older versus more recent seizure activity. Further investigation should also address the timeline between the seizure episodes and sample collection in order to better understand the association of MMPs activity and seizures.

We also showed lower levels of two angiogenic molecules ENG and VEGF in plasma from patients who experienced a recent symptomatic CCM hemorrhage within the prior 3 months. A homozygosity for a 6-base pair insertion allele in exon 7 of the endoglin protein-coding gene has previously been associated with a higher risk of sporadic intracerebral hemorrhage [32]. Moreover, mutations in the *ENG* gene resulting in functionally null alleles have been reported to be associated with hereditary hemorrhagic telangiectasia, a disease involving vascular dysmorphism [33, 34]. Up-regulated VEGF in cultured CCM endothelial cells compared to control cells has been reported [35], and has been demonstrated to worsen the blood-brain barrier permeability and progression of CCM lesions in murine model [3]. Clinical studies have also reported increased VEGF plasma levels during the 3 months following an ischemic stroke [36, 37]. However, Jung et al. described an increase, followed by a decrease, of serum VEGF level after a recent bleeding [38], while Mairui et al. did not find any difference in VEGF lesional levels in progressive unstable CCM lesions compared to stable lesions [39]. Lower concentrations of VEGF and ENG observed in correlation with recent CCM hemorrhage are consistent with the modulatory effect of VEGF in the hemorrhagic brain [36], and should generate hypotheses for future research. As with seizures, we cannot determine if our biomarker associations reflect hemorrhage per se, or specifically in association with CCM disease, since we did not examine patients with hemorrhage alone.

Lesions that recently bled showed an inverse correlation between IL10 and CCL2/MCP1 levels and lesional iron content as assessed by QSM [6]. IL10 is an anti-inflammatory molecule, with an established role in limiting and controlling acute and chronic inflammatory processes [40, 41]. CCL2/MCP1 is a chemotactic protein involved in recruiting monocytes that may play a role in iron removal by phagocytosis [42, 43]. A process of lesional iron clearance was already suggested by the significant decrease of QSM values during longitudinal follow-up of CCM patients [6]. Our results herein suggest that anti-inflammatory processes may contribute this clearance system of lesional iron.

In CCM lesions that recently bled, we also observed lower plasma ROBO4 levels in cases with higher lesional QSM. ROBO4 is expressed by vascular endothelial cells and dynamically maintains vascular network stability, during pathological angiogenesis and inflammation processes [44, 45]. Silencing the *CCM1* gene has been associated with a down-regulation of *ROBO4* gene expression [9]. Our result is consistent with an effect of ROBO4 on enhanced vascular leak after CCM hemorrhage.

Multivariate analysis revealed clustering of 4 inflammatory cytokines, IL2, IFN γ , TNF α and IL1 β . The plasma levels of these inflammatory molecules were significantly higher in patients with a history of one and more than one adjudicated bleeding as compared to those with no previous hemorrhage. Moreover, we showed that heightened levels of these 4 clustered inflammatory biomarkers in plasma were unlikely to have arisen from a hemorrhage within the previous 12 months. Hence, we suggest that this clustering of higher baseline inflammatory cytokines appear to reflect greater propensity to hemorrhagic events over time, rather than recent hemorrhage activity. These findings are consistent with our previous association of low Vitamin D and non-HDL cholesterol levels with chronic but not recent disease aggressiveness [16], and with evidence that polymorphisms in inflammatory genes are also associated with greater CCM disease severity [12]. It is likely that these biomarkers reflect a chronic pro-inflammatory state in the affected host, modulating disease aggressiveness over time. Indeed, cases where these 4 clustered inflammatory biomarkers are elevated were more likely to manifest new lesion hemorrhage or growth during prospective follow-up.

The correlations herein do not imply a specific causality related to CCM disease. We hope that these results will motivate other correlations with epilepsy and brain hemorrhage beyond CCM. We also cannot claim that these biomarkers contribute information beyond that derived by clinical examination or imaging of individual patients. However, they generate useful hypotheses about mechanism of disease severity, and a potential role in predicting future disease behavior.

While this represents the largest study to date involving CCM patients and peripheral biomarkers, the sample size did not allow further analyses beyond those presented herein. The batch effect correction generated arbitrary units that could not be extended directly into clinical practice. Future studies will assess respective differences in biomarker plasma levels in relation to the mean of a control group or the reference group with stable clinical activity, and these would be more applicable clinically. With limited follow-up to date, the predictive value of each biomarker was not assessed herein. This is being evaluated prospectively as the cohort logs additional longitudinal follow-up. However, we are encouraged by the early predictive significance which emerged when assessing the 4 clustered pro-inflammatory biomarkers in limited follow-up to date. Finally, some of the chosen biomarkers were selected based on their documented functions and speculative pathogenic role in CCM, rather than on direct experimental data. Correlations with epilepsy were categorical, and did not query severity, treatment or time course of seizures. Recent investigations have highlighted additional key molecules involved in the genesis and progression of CCM disease [11, 12, 46], which should be explored as putative biomarkers of disease activity in future research.

This exploratory study is the first to demonstrate that peripheral levels of inflammatory and angiogenic molecules may be used as potential susceptibility/risk biomarkers in CCM disease, according to the FDA/NIH BEST criteria (<https://www.ncbi.nlm.nih.gov/books/NBK326791/>). Our results provide hypothesis-generating clinical research for future studies or trials to understand and examine the physiopathologic functions of these plasma biomarkers in CCM disease and extend their role as potential diagnostic, prognostic, predictive and/or response biomarkers in follow-up cohorts of patients [14, 15].

Summary

We identified a number of inflammatory and angiogenic plasma parameters in correlation with various facets of CCM clinical behavior. Our results support an extensive literature on the influence of central and systemic inflammatory processes in the etiology and progression of cardio- and cerebrovascular, neurodegenerative diseases, such as atherosclerosis, Alzheimer's or Parkinson's disease [47, 48]. Our mathematical approaches to examine multiple independent, and potentially co-dependent or clustered parameters of neuroinflammation in correlation with CCM clinical behavior may be useful to study other pathologies with a postulated inflammatory role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. MMP2, MMP9 and ICAM1/CD54 plasma levels are associated with seizures in CCM patients

(A) MMP2 and (B) ICAM1/CD54 levels were significantly higher (after correction for batch effect, $P=0.02$ and $P=0.04$ respectively, FDR corrected), (C) while levels of MMP9 were lower ($P=0.04$, FDR corrected) in patients with seizures compared to those without seizures (respectively, $N=22$ and $N=63$). (D) The receiver operating characteristic (ROC) curve generated using the combination of plasma levels of MMP2, ICAM/CD54 and MMP9 distinguished the patients experiencing seizures from others [area under curve (AUC)=0.79, $P<0.0001$] with a sensitivity of 91% and specificity of 60%. * $P<0.05$.

Fig. 2. VEGF and ENG plasma levels are lower after CCM-related bleeding within 3 months
 Patients who experienced a CCM-related hemorrhage within the past 3 months (N=5) had lower (A) VEGF and (B) ENG levels ($P=0.04$, FDR corrected, for both) compared to patients without bleeds (N=80). (C) The ROC curve generated using the combination of plasma levels of VEGF and ENG was able to differentiate patients who had a hemorrhage within the past 3 months from others [area under curve (AUC)=0.91, $P=0.03$] with a sensitivity of 100% and specificity of 81%. * $P<0.05$.

Fig. 3. IL10, CCL2/MCP1 and ROBO4 plasma biomarkers are negatively correlated with lesional iron deposition in recent CCM-related bleeding
 (A) IL10 ($r=-0.61$, $P=0.02$), (B) CCL2/MCP1 ($r=-0.60$, $P=0.02$) and (C) ROBO4 ($r=-0.53$, $P=0.05$; after batch correction) plasma levels were negatively correlated with iron deposition values of lesions that bled within the past 12 months.

Fig. 4. Unsupervised hierarchical clustering of the 24-plasma biomarkers

The dendrogram (tree-structured graph above) distinguishes two groups of patients (Group 1 and Group 2) based on 5 biomarkers: IL2, IFN γ , TNF α , IL1 β and IL6. Patients (Group 1, N = 25; in yellow) at the left branch of the dendrogram had lower plasma levels of 5-clustered inflammatory biomarkers, than patients (Group 2, N = 57; in brown) at the right branch.

* $P < 0.05$.

Table 1

Clinical and lesion features of CCM subjects

	Solitary/Sporadic		Multifocal/Familial	
	Male	Female	Male	Female
Total subjects	18	31	12	24
Mean age in years (SEM)	38.28 (4.19)	43.27 (2.66)	36.59 (5.78)	29.78 (3.82)
Mean age at first symptoms (SEM)	28.47 (4.76)	36.4 (2.93)	24.15 (5.82)	23.17 (4.46)
Mean # T2 lesions [*] > 4 mm in size per patient (SEM)	1 (0.0)	1 (0.0)	10.75 (5.04)	8.46 (2.17)
Mean # SWI lesions [†] per patient(SEM)	1 (0.0)	1 (0.0)	30 (10.28)	29.25 (7.32)
Symptoms[‡]				
Prior hemorrhage	11	16	5	14
Prior seizures	4	8	4	6
Asymptomatic/Non-specific	3	7	3	4
Aggressiveness parameters[‡]				
Symptoms onset 18 years	6	2	3	11
>1 hemorrhages	4	6	2	5
>25 SWI lesions	NA	NA	5	8
>5 T ₂ -weighted lesions	NA	NA	4	10
Hemorrhage within 3 months	2	3	0	0
Hemorrhage within 1 year	6	9	0	3
Lesion growth within 1 year	1	5	1	4
New lesion within 1 year	NA	NA	2	6

SEM indicates standard error of the mean; NA, not applicable.

^{*} Mean # T2 lesions = mean number of lesions identified on T₂-weighted imaging.[†] Mean # SWI lesions = mean number of lesions identified on (the more sensitive) susceptibility weighted imaging.[‡] number of subjects in each category.

Table 2

Logistic regression analysis of the association between the levels of the 4-clustered plasma inflammatory biomarkers and parameters of chronic CCM disease severity*

Variable		<i>p-value</i>	Odds Ratio (95% CI)
Total hemorrhages	One	0.08	2.79 (0.91, 9.26)
	More than one	0.04	4.81 (1.17, 25.82)
Seizures		0.02	4.91 (1.38, 23.83)
Number of T ₂ [†] lesions > 4mm in size		0.39	1.05 (0.95, 1.22)
Number of SWI [‡] lesions		0.51	0.99 (0.95, 1.03)

T₂ indicates T₂-weighted; SWI, susceptibility weighted imaging.

* Age of symptom onset prior to 18 was not considered in this multivariate analysis of disease severity, in order to include asymptomatic patients in the analysis.