

SHORT COMMUNICATION

Plasma Biomarkers of Inflammation and Angiogenesis Predict Cerebral Cavernous Malformation Symptomatic Hemorrhage or Lesional Growth

Romuald Girard^{1*}, Hussein A. Zeineddine^{1*}, Janne Koskimäki¹, Maged D. Fam¹, Ying Cao¹, Changbin Shi¹, Thomas Moore¹, Rhonda Lightle¹, Agnieszka Stadnik¹, Kiranj Chaudagar¹, Sean Polster¹, Robert Shenkar¹, Ryan Duggan², David Leclerc², Kevin J. Whitehead³, Dean Y. Li³, Issam A. Awad¹

*R.G. and H.A.Z. contributed equally to this manuscript.

¹Section of Neurosurgery, The University of Chicago Medicine and Biological Sciences, Chicago, IL
²Flow Cytometry Facility, The University of Chicago, Chicago, IL; ³Division of Cardiology, and Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT.

Running title: Plasma Predictors in Human CCM Disease



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Address correspondence to:

Dr. Issam A. Awad
Section of Neurosurgery
University of Chicago
5841 S. Maryland
Chicago, IL, 60637
iawad@uchicago.edu

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ABSTRACT

Rationale: The clinical course of cerebral cavernous malformations (CCMs) is highly unpredictable, with few cross-sectional studies correlating pro-inflammatory genotypes and plasma biomarkers with prior disease severity.

Objective: We hypothesize that a panel of 24 candidate plasma biomarkers, with a reported role in the pathophysiology of CCMs, may predict subsequent clinically relevant disease activity.

Methods and Results: Plasma biomarkers were assessed in non-fasting peripheral venous blood collected from consecutive CCM subjects followed for one year after initial sample collection. A first cohort (N=49) was used to define the best model of biomarker level combinations to predict a subsequent symptomatic lesional hemorrhagic expansion within a year following the blood sample. We generated the receiver operating characteristic curves and area under curves (AUC) for each biomarker individually and each weighted linear combination of relevant biomarkers. The best model to predict lesional activity was selected as that minimizing the Akaike Information Criterion (AIC). In this cohort, 11 subjects experienced symptomatic lesional hemorrhagic expansion (5 bleeds and 10 lesional growths) within a year after the blood draw. Subjects had lower soluble cluster of differentiation 14 (sCD14; p=0.05), interleukin-6 (IL-6; p=0.04), vascular endothelial growth factor (VEGF; p=0.0003) levels along with higher plasma levels of interleukin-1 beta (IL-1 β ; p=0.008) and roundabout guidance receptor 4 (sROBO4; p=0.03). Among the 31 weighted linear combinations of these 5 biomarkers, the best model (with the lowest AIC value=25.3) was the weighted linear combination including sCD14, IL-1 β , VEGF and sROBO4, predicting a symptomatic hemorrhagic expansion with a sensitivity of 86% and specificity of 88% (AUC=0.90, p<0.0001). We then validated our best model in the second sequential independent cohort (N=28).

Conclusions: This is the first study reporting a predictive association between plasma biomarkers and subsequent CCM disease clinical activity. This may be applied in clinical prognostication, and stratification of cases in clinical trials.

Keywords:

Cerebral cavernous malformation, plasma biomarker, neuroinflammation, prognostic biomarker, cerebrovascular disease/stroke,

Nonstandard Abbreviations and Acronyms:

AIC	akaike information criterion
AUC	area under the curve
CCM	cerebral cavernous malformations
EC	endothelial cell
IL-1 β	interleukin-1 beta
IL-6	interleukin-6
ROC	receiving operating characteristic
ROCK	rhoA associated kinase
sCD14	soluble cluster of differentiation 14
sROBO4	soluble roundabout guidance receptor 4
VEGF	vascular endothelial growth factor

INTRODUCTION

Cerebral cavernous malformations (CCMs) are a common cerebrovascular pathology predisposing 0.5% of the population, predisposing to a lifetime risk of intracerebral hemorrhage, seizures and progressive focal neurological deficits.^{1,2} A sporadic form of the disease, with solitary lesions, accounts for almost two thirds of cases, while a familial form manifests multifocal lesions developing throughout the patient's lifetime in different regions of the brain. The familial phenotype is associated with an autosomal dominant Mendelian inheritance with germ line mutations at one of the three *CCM* gene loci (*CCM1/KRIT1*, *CCM2/MGC4607* and *CCM3/PDCD10*).³

The lesions harbored in the two forms are histologically indistinguishable and consist of clusters of thin-walled blood filled vascular caverns lined by endothelium, and lacking mature vessel wall angio-architecture.^{4,5} Endothelium of sporadic and familial lesions harbors somatic mutations in one of the three documented *CCM* genes, indicating common pathogenesis mechanisms.⁵ *CCM* genes encode proteins involved in maintaining endothelial barrier integrity by inhibiting RhoA associated kinase (ROCK) activation.⁶ A loss of *CCM* gene function results in increased ROCK activity leading to endothelial cell-cell junction dysregulation and defective vascular permeability.³ Experimental evidence also suggests a complex interplay between vascular permeability, inflammatory, and angiogenic processes in the neuroglial milieu as central features of CCM.⁷

Acute lesional bleeding or proliferative hemorrhagic expansion result in clinically significant sequelae. However, the molecular mechanisms underlying such relevant lesional activity remain unclear, and the behavior of CCMs remains highly variable and unpredictable. Pro-inflammatory gene variants have been associated with higher lesion counts in cases with common familial *CCM1* (Q455X) mutation.^{8,9} Our group has also reported associations of brain vascular permeability,^{1,10} and clustered pro-inflammatory biomarkers in peripheral blood plasma^{7,11} with cumulative CCM disease severity during a patient's life. Yet specific biomarker signatures predicting subsequent clinically relevant disease behavior would be most relevant clinically and have remained elusive. Herein, we studied the prognostic association between the plasma levels of a panel of inflammatory and angiogenic proteins previously related to CCM disease pathobiology,⁷ and the occurrence of a CCM-related clinically significant event in the subsequent year.

METHODS

Because of the sensitive nature of the data collected for this study, requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to the University of Chicago Medicine at Dr. Issam Awad (e-mail iawad@uchicago.edu).

Patients recruitment.

From July 2014 to February 2018, 172 consecutive CCM subjects were evaluated clinically at a single referral center and enrolled in biomarker studies (www.uchospitals.edu/ccm). Of these, 77 CCM subjects (42 sporadic and 35 familial) were followed for 1 year (\pm 30 days) after biomarker collection, and considered as 2 independent cohorts based on their period of enrollment. The first cohort (*Group 1-Algorithm definition*) included 49 patients enrolled and biomarker collected between July 2014 to May 2016. The second cohort entitled *Group 2-Algorithm testing* included an additional 28 patients enrolled between June 2016 and February 2018 (Online Figure I and Online Table I).

All patients gave written informed consent to participate in this research in accordance to the Declaration of Helsinki, and approved by The University of Chicago Institutional Review Board (IRB). The ethical

principles guiding the IRB are consistent with The Belmont Report, and comply with the rules and regulations of The Federal Policy for the Protection of Human Subjects (56 FR 28003).

As per currently accepted disease categorization, cases were classified as sporadic if they harbored a solitary lesion on the most sensitive susceptibility weighted magnetic resonance imaging (MRI) sequences, or a cluster of lesions associated with a developmental venous anomaly. They were classified as familial if they harbored multifocal CCMs, a family history of CCM in a first-degree blood relative or a mutation genotyped at a CCM gene locus. Patients with partial or complete CCM lesion resection or any prior brain irradiation were excluded.^{1, 7, 10}

The best weighted combination of biomarkers to predict subsequent disease activity within 1 year (\pm 30 days) was defined in *Group 1-Algorithm definition* and then tested in *Group 2-Algorithm testing*.

For each patient enrolled in the study, the aforementioned features were assessed during the clinical follow-up visit. 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. *For more methodological details, refer to Supplemental Material.*



RESULTS

Demographic and CCM lesions characteristics.

In the first cohort (*Group 1-Algorithm definition*), 11 of the 49 subjects manifested a hemorrhagic expansion. One subject experienced a new symptomatic hemorrhage from a known CCM per adjudicated criteria,¹² 5 developed lesional growth by >3 mm diameter on T₂-weighted MRI sequences, and 5 had both symptomatic hemorrhage and lesional growth during the subsequent year after biomarker collection.^{2, 10} Thirty-eight subjects (78%) did not experience any change in CCM lesions during the same period and were defined as stable.

In the second cohort (*Group 2-Algorithm testing*), 7 of the 28 subjects experienced hemorrhagic expansion as previously described (2 subjects experienced a new symptomatic hemorrhage, 3 a lesional growth, and 2 both) within one year after biomarker collection.

Among the familial cohort, 8 subjects developed new lesions on the most sensitive MRI susceptibility weighted imaging. Cases with hemorrhagic expansion had higher T₂-weighted ($p=0.001$) and total ($p=0.002$) lesion counts, and non-significant trends toward higher prevalence of brainstem lesion location and recent symptomatic hemorrhage in the prior year. There were no significant differences in the age, gender or mean follow-up time between the stable subjects and the ones who experienced a hemorrhagic expansion within the year after the blood sample. However, the cohort of familial cases that developed new lesions within a year were older than familial patients that did not ($p=0.02$).

Soluble cluster of differentiation 14 (sCD14), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) plasma levels were lower while interleukin-1 beta (IL-1 β) and soluble roundabout guidance receptor 4 (sROBO4) were higher in subjects who experienced clinical lesional activity within the year following the initial blood sample.

After correction for batch effect when appropriate, subjects experiencing a hemorrhagic expansion showed lower plasma levels of sCD14 ($p=0.05$), IL-6 ($p=0.04$) and VEGF ($p=0.0003$), along with higher IL-1 β ($p=0.008$) and sROBO4 ($p=0.03$) plasma levels (Online Figure II). There were no potential

confounders affecting the plasma levels of the 24 biomarkers such as age of enrollment, phenotype (solitary/sporadic or multifocal/familial), genotype (sporadic, *CCMI*, 2 or 3) or sex (male or female). In addition, plasma levels of these biomarkers were not associated with brainstem lesion location or symptomatic bleed within the preceding year, known risk factors of lesional activity.⁴ We did not identify significant association between the formation of new CCMs and the plasma levels of any of the selected biomarkers in the familial cohort.

The combination of sCD14, IL-1 β , VEGF and sROBO4 is the best predictor of a future lesional activity.

The receiving operating characteristic (ROC) curves for sCD14 [area under the curve (AUC)=0.68, $p=0.04$], IL-6 (AUC=0.66, $p=0.007$) showed ‘poor’ accuracy. The accuracies to predict lesional activity were considered as “fair” for sROBO4 (AUC=0.76, $p=0.004$) and VEGF (AUC=0.77, $p=0.01$) while it was “good” for IL-1 β (AUC=0.82, $p=0.0003$) (Online Figure II). Further analysis showed that among the 31-possible weighted linear combinations of these 5 biomarkers, the best model (Equation 1) defined by the lowest Akaike information criterion (AIC) value (AIC=25.3) (Figure 1A) was achieved by combining sCD14, IL-1 β , VEGF and sROBO4 (AUC=0.90, $p<0.0001$).

Equation 1= -0.135 x [sCD14] + 7.73 x [IL-1 β] - 0.775 x [VEGF] + 0.658 x [sROBO4]

The ROC analysis differentiated subjects who experienced a lesional hemorrhagic expansion from stable subjects with a sensitivity of 86% and specificity of 88% (Figure 1B). Further analysis showed that the mean estimated combination value (Equation 1) was 5-fold higher ($p<0.0001$) in subjects who experienced hemorrhagic expansion (mean estimated value= 1.67 ± 1.13) within the following year compared to subjects that remained stable (mean estimated value= -0.39 ± 0.59) (Figure 1C). The mean estimated combination value calculated with the best model was also 3-fold higher ($p<0.01$) in patients of the independent cohort (i.e., *Group 2 - Algorithm testing*) who experienced a subsequent hemorrhagic expansion. In addition, the logistic regression analysis showed that the best linear combination was able to correctly predict the subsequent respective stable or hemorrhagic expansion status in 83.3% and 75.0% of the cases in the independent cohort (Online Table III). This result was also supported by ROC analysis which showed 90% sensitivity and 71% specificity (Online Figure III) of the weighted biomarker equation predicting a hemorrhagic expansion within 1-year (± 30 days) in the independent cohort.

For more results, refer to Supplemental Material.

DISCUSSION

We evaluated the prognostic association between the plasma levels of 24 biomarkers and the occurrence of an impending clinically relevant lesional activity during the subsequent year. The biomarkers were selected based on a systematic literature review of demonstrated associations with relevant disease mechanisms in CCM or brain hemorrhage (Online Table IV).⁷ Results showed that the plasma levels of sCD14, IL-1 β , sROBO4, VEGF and IL-6 were differently expressed in subjects who experienced a CCM-related event in the year following the initial blood sample. We then calculated the AIC, a robust and traditional likelihood-based model-selection, to determine the best linear combination to predict future lesional activity. The best model was achieved by the linear weighted combination including sCD14, IL-1 β , sROBO4 and VEGF.

The influence of pro-inflammatory genotypes and neuro-inflammation in the physiopathology and clinical course of CCM disease has been described in recent years.⁷⁻⁹ Single-nucleotide polymorphisms of *CD14*, *IL1 β* and *IL6* genes have been associated with aggressive phenotypes of hemorrhagic cerebrovascular disease including CCM, brain arteriovenous malformation and aneurysm.^{8, 9, 13} Recently, Tang et al. in collaboration with our group showed that functional gene variants causing increased expression of CD14-anchored membrane glycoprotein is associated with greater lesion counts in familial CCM1 sharing the same mutation (Q455X).⁹ Here we report lower levels of sCD14 as a predictor of subsequent lesional activity. The association between the levels of circulating CD14 and the anchored membrane form remains unclear and may not be correlated.¹⁴ Higher levels of sCD14 may have anti-inflammatory effects by inhibiting lipopolysaccharide-mediated functional responses.¹⁴⁻¹⁶

IL-1 β is a pro-inflammatory cytokine with a role in mediating inflammation-induced angiogenic responses that indirectly regulates the synthesis of pro-angiogenic factors, and facilitates endothelial cell (EC) migration, proliferation and organization into blood vessel-like structures.^{17, 18} IL-1 β has also been shown to increase the endothelial permeability promoting leukocyte transmigration *via* multiple direct and indirect pathways.^{19, 20} We recently reported that an increased permeability assessed by MRI at follow-up correlated with symptomatic lesional hemorrhage or growth.¹⁰ Soluble IL-6 is a multifunctional cytokine mediating pro- and anti-inflammatory processes, as well as regenerative, neural and metabolic pathways.²¹ As with *CD14*, SNP in *IL6R* gene have been correlated with a greater number of CCM lesions.⁸ The roles of sIL-6 versus IL-6 receptor in CCM lesion development and hemorrhage will require further investigation. Our results showing higher IL-1 β and lower IL-6 predicting subsequent CCM clinical activity support the hypothesis that an increased endothelial permeability may occur *via* inflammatory mechanisms, resulting in imminent symptomatic lesional bleeding or expansion.

ROBO4 is an endogenous inhibitor of VEGF signaling expressed by vascular endothelial cells.^{22, 23} This protein has been shown to dynamically maintain vascular network stability, during pathological angiogenesis and pro-inflammatory processes²⁴⁻²⁶ by modulating the expression of tight junction proteins such as zona occludens-1, occludin and claudin-5.²⁷ In CCM disease, lesions harbor structurally defective tight junctions,²⁸ and decreased mRNA expression of occludin, claudin-5, and ZO-1.²⁹ An increase in sROBO4 may reflect pro-inflammatory processes enhancing endothelial permeability, consistent with its prognostic association with CCM bleeding and growth.

VEGF has direct mitogenic effects on ECs, and is a key regulator of angiogenesis.¹⁷ Dysregulation of VEGF expression has been widely studied in CCM disease and has been shown to worsen the blood-brain barrier permeability and promote progression of CCM lesions.^{6, 30} A modulatory effect of VEGF in the hemorrhagic brain has also been described.^{7, 31, 32} However, no difference in lesional VEGF expression was observed in a study comparing unstable versus CCM lesions resected surgically.³³ Here we observed that decreased plasma level of VEGF is a predictor of subsequent lesional hemorrhage or growth in CCM disease. This is somewhat counter-intuitive, and motivates a hypothesis about how lower VEGF plasma levels might reflect an imbalance in vascular integrity, associated with subsequent aggressive lesion behavior.

The combined model including weighted contribution of four biomarkers sCD14, IL-1 β , VEGF and sROBO4 was the best predictor of future lesional activity. The absence of co-correlation among these four protein levels suggest that their relative contribution to CCM lesional activity is independent and additive (Online Table V).

Previous studies had shown that brainstem lesion location and recent symptomatic hemorrhage are predictors of future bleeding risk.⁴ Also, familial cases with aggressive genotype are associated with greater lesion burden and hemorrhagic risk.³ In our study, patients with these risk factors were also more likely to

manifest prospective symptomatic hemorrhage or lesional growth. However, we noted no association of the biomarkers with these or other baseline clinical features of disease, suggesting that the biomarker prognostication was independent of these clinical features. To best explore these interactions, future studies will need to be powered to detect prognostic associations of the weakest biomarker in the smallest relevant disease subgroup.

None of the biomarkers showed association with de novo lesion genesis in familial cases. We had previously shown an association of focal brain vascular hyperpermeability with subsequent lesion genesis in that same brain region.¹⁰ Our study included a small number of familial cases with lesion growth. If confirmed in a larger cohort, it is possible that such focal brain vascular hyperpermeability may not be reflected in our assayed biomarkers.

Our single site study does not exclude referral bias. And cases with biomarker sampling may have undergone lesion resection or otherwise not been available for prospective follow-up at our center. These cases without follow-up did not have significantly different biomarker levels nor clinical features, reassuring us that follow-up bias did not likely impact our results. Beyond larger sample sizes, with sufficient number of cases in relevant subgroups, future multi-site studies will be designed to better control for these potential biases.

Notwithstanding these limitations, this study is the largest to date in this rare disease. And this is the first report of a strong prognostic association of a peripheral blood biomarker with clinical activity in a defined cerebrovascular pathology. We will pursue validation of these results in conjunction with multi-site clinical trial readiness project already underway, funded by the U.S. National Institutes of Health, and in other prospective question-driven studies. These will consider specific clinical scenarios and risk versus benefit of biomarker clinical application.³⁴ In addition, the assay methodologies and batch effect correction have generated arbitrary units that could not be extended directly into clinical practice. Future studies will assess the differences in plasma levels between healthy controls and cohorts of CCM patients with stable and unstable clinical activity, providing reference ranges of biomarker values applicable to positive and negative clinical prognostic risk.

The correlations herein do not imply a specific causality related to CCM disease. But they generate cogent hypotheses about mechanism of disease risk, to be pursued in future laboratory and clinical studies. Finally, the chosen biomarkers were selected based on current knowledge about CCM disease mechanisms.¹⁷ More recent investigations have highlighted additional key molecules involved in pathobiology.^{9, 35} Respective biomarkers related to these novel mechanisms may further refine the predictive power of clinical behavior in this disease.

SUMMARY

Our results support an extensive literature on the influence of inflammatory and angiogenic processes in the pathobiology of CCM disease. They define a mechanistic-based conceptual model to predict short-term future clinical activity based on a panel of inflammatory and angiogenic cytokines. If validated in multi-site studies, this model may play a direct role in selecting patients for aggressive therapies, and in the stratification of cohorts in clinical trials. This same approach may be useful in other cerebrovascular pathologies including hemorrhagic microangiopathy, amyloid angiopathy and aging where similar mechanisms have been postulated.³⁶

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DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

1. Mikati AG, Khanna O, Zhang L, Girard R, Shenkar R, Guo X, Shah A, Larsson HB, Tan H, Li L, Wishnoff MS, Shi C, Christoforidis GA, Awad IA. Vascular permeability in cerebral cavernous malformations. *J Cereb Blood Flow Metab.* 2015;35:1632-1639
2. Akers A, Al-Shahi Salman R, Awad IA, et al. Synopsis of guidelines for the clinical management of cerebral cavernous malformations: Consensus recommendations based on systematic literature review by the angioma alliance scientific advisory board clinical experts panel. *Neurosurgery.* 2017;80:665-680
3. Shenkar R, Shi C, Rebeiz T, et al. Exceptional aggressiveness of cerebral cavernous malformation disease associated with pcdcd10 mutations. *Genet Med.* 2015;17:188-196
4. Al-Shahi Salman R, Hall JM, Horne MA, Moultrie F, Josephson CB, Bhattacharya JJ, Counsell CE, Murray GD, Papanastassiou V, Ritchie V, Roberts RC, Sellar RJ, Warlow CP, Scottish Audit of Intracranial Vascular Malformations (SAIVMs) collaborators. Untreated clinical course of cerebral cavernous malformations: A prospective, population-based cohort study. *Lancet Neurol.* 2012;11:217-224
5. McDonald DA, Shi C, Shenkar R, Gallione CJ, Akers AL, Li S, De Castro N, Berg MJ, Corcoran DL, Awad IA, Marchuk DA. Lesions from patients with sporadic cerebral cavernous malformations harbor somatic mutations in the ccm genes: Evidence for a common biochemical pathway for ccm pathogenesis. *Hum Mol Genet.* 2014;23:4357-4370
6. Whitehead KJ, Chan AC, Navankasattusas S, Koh W, London NR, Ling J, Mayo AH, Drakos SG, Jones CA, Zhu W, Marchuk DA, Davis GE, Li DY. The cerebral cavernous malformation signaling pathway promotes vascular integrity via rho gtpases. *Nat Med.* 2009;15:177-184
7. Girard R, Zeineddine HA, Fam MD, et al. Plasma biomarkers of inflammation reflect seizures and hemorrhagic activity of cerebral cavernous malformations. *Transl Stroke Res.* 2018;9:34-43
8. Choquet H, Pawlikowska L, Nelson J, McCulloch CE, Akers A, Baca B, Khan Y, Hart B, Morrison L, Kim H, Brain Vascular Malformation Consortium S. Polymorphisms in inflammatory and immune response genes associated with cerebral cavernous malformation type 1 severity. *Cerebrovasc Dis.* 2014;38:433-440
9. Tang AT, Choi JP, Kotzin JJ, et al. Endothelial tlr4 and the microbiome drive cerebral cavernous malformations. *Nature.* 2017;545:305-310
10. Girard R, Fam MD, Zeineddine HA, Tan H, Mikati AG, Shi C, Jesselson M, Shenkar R, Wu M, Cao Y, Hobson N, Larsson HB, Christoforidis GA, Awad IA. Vascular permeability and iron deposition biomarkers in longitudinal follow-up of cerebral cavernous malformations. *J Neurosurg.* 2016;127:1-9

11. Girard R, Khanna O, Shenkar R, et al. Peripheral plasma vitamin d and non-hdl cholesterol reflect the severity of cerebral cavernous malformation disease. *Biomark Med.* 2016;10:255-264
12. Al-Shahi Salman R, Berg MJ, Morrison L, Awad IA, Angioma Alliance Scientific Advisory B. Hemorrhage from cavernous malformations of the brain: Definition and reporting standards. Angioma alliance scientific advisory board. *Stroke.* 2008;39:3222-3230
13. Kim H, Hysi PG, Pawlikowska L, Poon A, Burchard EG, Zaroff JG, Sidney S, Ko NU, Achrol AS, Lawton MT, McCulloch CE, Kwok PY, Young WL. Common variants in interleukin-1-beta gene are associated with intracranial hemorrhage and susceptibility to brain arteriovenous malformation. *Cerebrovasc Dis.* 2009;27:176-182
14. Lloyd-Jones KL, Kelly MM, Kubes P. Varying importance of soluble and membrane cd14 in endothelial detection of lipopolysaccharide. *J Immunol.* 2008;181:1446-1453
15. Leveque M, Simonin-Le Jeune K, Jouneau S, Moulis S, Desrues B, Belleguic C, Brinchault G, Le Trionnaire S, Gangneux JP, Dimanche-Boitrel MT, Martin-Chouly C. Soluble cd14 acts as a damp in human macrophages: Origin and involvement in inflammatory cytokine/chemokine production. *FASEB J.* 2017;31:1891-1902
16. Haziot A, Rong GW, Bazil V, Silver J, Goyert SM. Recombinant soluble cd14 inhibits lps-induced tumor necrosis factor-alpha production by cells in whole blood. *J Immunol.* 1994;152:5868-5876
17. Voronov E, Carmi Y, Apte RN. The role il-1 in tumor-mediated angiogenesis. *Front Physiol.* 2014;5:114
18. Carmi Y, Voronov E, Dotan S, Lahat N, Rahat MA, Fogel M, Huszar M, White MR, Dinarello CA, Apte RN. The role of macrophage-derived il-1 in induction and maintenance of angiogenesis. *J Immunol.* 2009;183:4705-4714
19. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. *Neurobiol Dis.* 2010;37:13-25
20. Wang Y, Jin S, Sonobe Y, Cheng Y, Horiuchi H, Parajuli B, Kawanokuchi J, Mizuno T, Takeuchi H, Suzumura A. Interleukin-1beta induces blood-brain barrier disruption by downregulating sonic hedgehog in astrocytes. *PLoS One.* 2014;9:e110024
21. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta.* 2011;1813:878-888
22. Acevedo LM, Weis SM, Cheresch DA. Robo4 counteracts vegf signaling. *Nat Med.* 2008;14:372-373
23. Koch AW, Mathivet T, Larrivee B, et al. Robo4 maintains vessel integrity and inhibits angiogenesis by interacting with unc5b. *Dev Cell.* 2011;20:33-46
24. London NR, Li DY. Robo4-dependent slit signaling stabilizes the vasculature during pathologic angiogenesis and cytokine storm. *Curr Opin Hematol.* 2011;18:186-190
25. Yadav SS, Narayan G. Role of robo4 signalling in developmental and pathological angiogenesis. *Biomed Res Int.* 2014;2014:683025
26. Shirakura K, Ishiba R, Kashio T, Sakai M, Fukushima Y, Yamamoto N, Manabe S, Shigesada N, Tanaka T, Hino N, Aird WC, Doi T, Okada Y. Endothelial robo4 regulates il-6 production by endothelial cells and monocytes via a crosstalk mechanism in inflammation. *Biochem Biophys Res Commun.* 2018;495:801-806
27. Cai H, Liu W, Xue Y, Shang X, Liu J, Li Z, Wang P, Liu L, Hu Y, Liu Y. Roundabout 4 regulates blood-tumor barrier permeability through the modulation of zo-1, occludin, and claudin-5 expression. *J Neuropathol Exp Neurol.* 2015;74:25-37
28. Dejana E, Tournier-Lasserre E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: Molecular basis and pathological implications. *Dev Cell.* 2009;16:209-221
29. Schneider H, Errede M, Ulrich NH, Virgintino D, Frei K, Bertalanffy H. Impairment of tight junctions and glucose transport in endothelial cells of human cerebral cavernous malformations. *J Neuropathol Exp Neurol.* 2011;70:417-429

30. Zhu Y, Wu Q, Fass M, Xu JF, You C, Muller O, Sandalcioglu IE, Zhang JM, Sure U. In vitro characterization of the angiogenic phenotype and genotype of the endothelia derived from sporadic cerebral cavernous malformations. *Neurosurgery*. 2011;69:722-731
31. Matsuo R, Ago T, Kamouchi M, et al. Clinical significance of plasma vegf value in ischemic stroke - research for biomarkers in ischemic stroke (rebios) study. *BMC Neurol*. 2013;13:32-39
32. Jung KH, Chu K, Jeong SW, Park HK, Bae HJ, Yoon BW. Cerebral cavernous malformations with dynamic and progressive course: Correlation study with vascular endothelial growth factor. *Arch Neurol*. 2003;60:1613-1618
33. Maiuri F, Cappabianca P, Gangemi M, De Caro Mdel B, Esposito F, Pettinato G, de Divitiis O, Mignogna C, Strazzullo V, de Divitiis E. Clinical progression and familial occurrence of cerebral cavernous angiomas: The role of angiogenic and growth factors. *Neurosurg Focus*. 2006;21:1-9
34. Leptak C, Menetski JP, Wagner JA, et al. What evidence do we need for biomarker qualification? *Sci Transl Med*. 2017;9
35. Zhou Z, Tang AT, Wong WY, et al. Cerebral cavernous malformations arise from endothelial gain of mekk3-klf2/4 signalling. *Nature*. 2016;532:122-126
36. Poggesi A, Pasi M, Pescini F, Pantoni L, Inzitari D. Circulating biologic markers of endothelial dysfunction in cerebral small vessel disease: A review. *J Cereb Blood Flow Metab*. 2016;36:72-94



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FIGURE LEGEND

Figure 1. The linear combination including sCD14, IL-1 β , VEGF and sROBO4 was the best predictor of future lesional activity within 1 year. (A) Each colored square represents an optimal weighted combination of biomarkers with its associated Akaike Information Criterion (AIC) value. The lowest AIC value (25.3) was achieved with $-0.135 \times [\text{sCD14}] + 7.73 \times [\text{IL-1}\beta] - 0.775 \times [\text{VEGF}] + 0.658 \times [\text{sROBO4}]$. (B) The receiving operating characteristic curve generated for the best linear combination was able to predict a clinical lesional activity within a year (± 30 days) [area under the curve (AUC)=0.90, $p=0.0001$] with a sensitivity of 86% and specificity of 88%. (C) The mean estimated combination value was 5-fold higher in subjects who experienced a hemorrhagic expansion than stable cases ($p<0.0001$).



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NOVELTY AND SIGNIFICANCE

What Is Known?

- Cerebral cavernous malformations (CCMs) is a common neurovascular pathology affecting 0.5% of the worldwide population.
- The clinical course of CCMs is highly unpredictable.
- The central and systemic inflammatory processes influence the etiology and progression of CCMs.

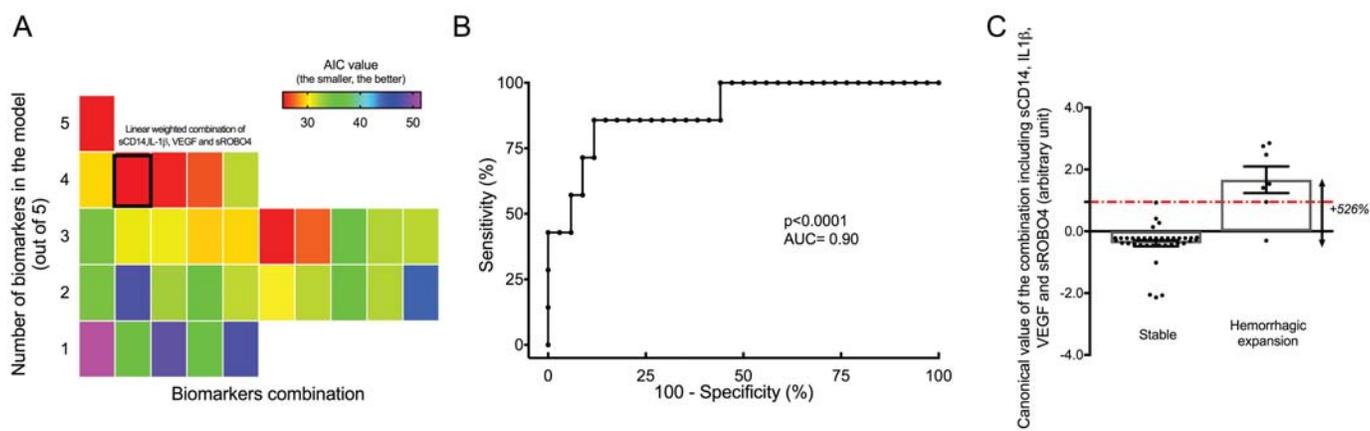
What New Information Does This Article Contribute?

- It is the first study reporting a mathematical model predicting short-term subsequent clinical CCM lesional activity.
- This model may play a direct role in selecting CCM patients for aggressive therapies, and in the stratification of cohorts in clinical trials.
- These results may be critical to define biological targets for therapies.

Herein, we report a predictive association between plasma biomarkers and subsequent lesional clinical activity. Our results support an extensive literature on the influence of inflammatory and angiogenic processes in the pathobiology of CCM disease. They also define a likelihood-based computational model to predict short-term future clinical activity based on a panel of preselected inflammatory and angiogenic cytokines. If validated in multi-site studies, this model may play a direct role in selecting patients for aggressive therapies, and in the stratification of cohorts in clinical trials. The same approach may be useful in other cerebrovascular pathologies including hemorrhagic microangiopathy, amyloid angiopathy and aging, where similar mechanisms have been postulated.

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FIGURE 1



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Supplemental Material

Plasma Biomarkers of Inflammation and Angiogenesis Predict Cerebral Cavernous Malformation Symptomatic Hemorrhage or Lesional Growth

Romuald Girard, PhD*; Hussein A. Zeineddine, MD*; Janne Koskimäki, MD/PhD; Maged D. Fam, MD; Ying Cao, MS; Changbin Shi, MD; Thomas Moore, BS; Rhonda Lightle, BS; Agnieszka Stadnik, MS; Kiranj Chaudagar, PhD; Sean Polster, MD; Robert Shenkar, PhD; Ryan Duggan, BS; David Leclerc, BS; Kevin J. Whitehead, MD; Dean Y. Li, MD; Issam A. Awad, MD

*Drs. Girard and Zeineddine contributed equally.

From Section of Neurosurgery, The University of Chicago Medicine and Biological Sciences, Chicago, IL (R.G, H.A.Z, M.D.F, Y.C, C.S, T.M, R.L, A.S, K.C, R.S, S.P, JK, I.A.A); Flow Cytometry Facility, The University of Chicago, Chicago, IL (R.D, D.L); Division of Cardiology, and Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT (K.J.W, D.Y.L).

Correspondence to: Issam A. Awad,
Section of Neurosurgery
University of Chicago,
5841 S. Maryland,
Chicago,
IL, 60637.
E-mail iawad@uchicago.edu

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Supplemental Methods

Patient recruitment

From July 2014 to February 2018, a total of 77 cerebral cavernous malformation (CCM) patients (mean age \pm SD=39.54 \pm 18.53 years, range=[4.62-75.02]) with a blood sample and a clinical follow-up visit within 1 year (\pm 30 days) were enrolled to study the predictive association between plasma biomarker levels and lesional activity. The 77 patients were divided in two independent cohorts. The first cohort included 49 patients enrolled between July 2014 and May 2016, and was defined as *Group 1-Algorithm definition*. The second cohort included an additional 28 patients enrolled between June 2016 and February 2018, and was defined as *Group 2-Algorithm testing* (**Online Figure I** and **Online Table I**).

In addition, 95 CCM subjects (mean age \pm SD=40.00 \pm 16.03 years, range=[6.61-75.57]) also underwent baseline biomarker studies between July 2014 to February 2018, but were not available for longitudinal follow-up (**Online Figure I**). Among these 95 subjects, 82 patients were lost for follow-up and 13 underwent surgical resection of CCM lesion. Comparison of baseline disease features of cases with and without prospective follow-up are summarized (**Online Table II**).

A written informed consent was obtained for all participants 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).

Based on their clinical data, patients were classified as sporadic if they harbored a sporadic/solitary lesion on the most sensitive susceptibility weighted imaging MRI sequences, or a cluster of lesions associated with a developmental venous anomaly. They were classified as multifocal/familial in the presence of multifocal CCM lesions on MRI, a family history of CCM in a first-degree blood relative or a mutation genotyped at a CCM gene locus.^{1,2} The genotype of familial cases was noted, and non-genotyped cases were characterized as “unknown genotype”. Patients with partial or complete CCM lesion resection or any prior brain irradiation were not included in this study.

Clinical features and categorization of disease aggressiveness

Our CCM patient population is followed through a rigorous radiological and clinical evaluation every 3 to 12 months depending clinical behavior. According to published guidelines on CCM disease, patients who experienced lesional activity (new bleed related symptom or significant lesional growth) were identified based on supporting evidence of both acute and subacute relevant medical symptoms correlated with subacute lesional or extralesional bleed on T₂-weighted images.^{3,4} Patients were categorized as stable if no CCM-attributable clinical event(s) was noted during their clinical/MRI. The clinical lesional events 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.^{1,2,5,6}

Plasma isolation and storage

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 assess biological compounds is in agreement with clinical practice and instructions provided by the bio-assay kit manufacturers.⁷ We focused only on the metabolic compounds not affected by fasting condition,⁸ because clinical visits were conducted at various times of the day and patients did not undergo fasting.

For plasma isolation, 5 mL of heparinized blood were centrifuged (AllegraX-30R, Beckman Coulter, Brea, California, USA) for 10 minutes at 4°C, at 2300 rpm. The supernatant plasma was equally aliquoted (200 μ l) in 1.7 ml microcentrifuge tubes and stored at -80°C.

Systematic literature review for identifying candidate biomarkers

A systematic electronic research was performed in the online bibliographic PubMed database for peer-reviewed articles published between February 15, 2008 and February 15, 2018, using the following key terms (linked to the key words for the condition): (cerebral cavernous malformation [Title/Abstract] OR cerebral vascular malformation [Title/Abstract] OR cerebrovascular malformation [Title/Abstract] OR CCM1 [Title/Abstract] OR CCM2 [Title/Abstract] OR CCM3 [Title/Abstract] OR Krit1 [Title/Abstract] OR PDCD10 [Title/Abstract] OR MGC4607 [Title/Abstract] OR brain permeability [Title/Abstract]) AND (english [Language]) NOT (case report [Publication Type] NOT liver [Title/Abstract] NOT surgery [Title/Abstract] NOT management [Title/Abstract]) NOT treatment [Title/Abstract]). Key words had been selected by the co-authors (RG, HAZ, JK, SP and IA) based on their recurrence in research related to CCM disease.

Seven hundred seventy-five articles were retrieved. The reviewers (RG, HAZ, JK, SP) considered the eligibility criteria for studies to be included by independently assessing titles and abstracts for all retrieved studies. Disagreements were resolved through discussion. Articles were eligible if they were (a) mechanistic or genomic studies on CCM or cerebrovascular malformations in human or murine models; (b) if they reported a candidate biomarker with a soluble form present in blood plasma that can be quantified via high-throughput multiplex Luminex screening immunoassay (R&D Systems) or bioanalyzers available at the Clinical Laboratories core at University of Chicago Hospitals; and (c) if they were published as a full manuscript. Studies reporting prevalence, incidence, natural history, clinical features, epidemiology, surgery or postoperative care, or other therapeutics in CCM or cerebrovascular malformations were excluded. Ultimately, 259 references were considered in support of the recommendations. Only the latest (most recent) published article for each respective biomarker is cited in the **Online Table IV**.

Quantitative assessment of biomarkers

The biomarkers were selected based on systematic literature review of known mechanism of CCM disease and other factors implicated in brain hemorrhage (**Online Table IV**).⁹ Eighteen plasma biomarkers including chemokine ligand 2 (CCL2/MCP1), soluble cluster of differentiation 14 (sCD14), C-reactive protein (CRP), interleukin-8 (IL-8/CXCL-8), interleukin-1 beta (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), soluble matrix metalloproteinase-2 (MMP2) and -9 (MMP9), tumor necrosis factor alpha (TNF α), tumor necrosis factor receptor 1 (TNF-RI), soluble vascular endothelial growth factor (VEGF), soluble vascular cell adhesion protein 1 (sVCAM1), soluble roundabout guidance receptor 4 (sROBO4), soluble intercellular adhesion molecule 1 (sICAM1/CD54), interferon gamma (IFN γ) and soluble endoglin/CD105 (sENG) were assessed using customized magnetic bead-based multiplex Luminex screening immunoassay kits (R&D Systems, Minneapolis, Minnesota, USA), allowing the simultaneous measurements of multiple analytes in a single run.¹⁰ Fifty five plasma samples (from 55 patients) were analyzed using 4 immunoassays kits (batch). The measurements were performed with a BioRad BioPlex-100 analyzer (Bio-Rad Laboratories, Hercules, California, USA) running the BioPlex Manager Software version 5.0.

The plasma samples of the second cohort (i.e., *Group 2-Algorithm testing*) were assessed independently with a distinct customized magnetic bead-based multiplex Luminex screening immunoassay kits (R&D Systems). The measurements were performed using the Luminex 200 System (Luminex Corporation, Austin, Texas, USA) running with xPONENT Software.

In each plate, the plasma samples were loaded in parallel duplicate wells, and then averaged. Fifty beads per region were collected for each well, and a 5-parameter logistic regression analysis was performed to estimate the sample concentration. All the assessments were performed at the Flow Cytometry Core Facility at the University of Chicago.

Clinical laboratory measurements for 25-(OH) vitamin D, lipid panel and C-reactive protein

Plasma aliquots were also analyzed for lipid panel and 25-hydroxyvitamin D [25-(OH) vitamin D] at the University of Chicago Medical Center Phlebotomy and Pre-Clinical Services. Total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein cholesterol levels (in mg/dL)

were quantified on the Roche Cobas 8000 Modular Analyzer (Roche Diagnostics, Risch-Rotkreuz, Switzerland) via enzymatic calorimetry assays.⁵ Non-HDL cholesterol was calculated based on total cholesterol and HDL cholesterol levels using the Friedewald formula. Both values have been previously validated to be reliable when measured in either the fasting or non-fasting state,⁸ obviating any error that could be present using the Friedewald formula to calculate non-HDL cholesterol levels.¹¹

25-(OH) vitamin D levels were quantified using high-pressure liquid chromatography coupled to mass spectrometry (LC/MS).

Statistical methods

As non-biological experimental variation is commonly observed across different multiplexed molecule assays sets,¹² a principal component analysis (PCA) was performed to assess the variability among the different biomarker levels.¹³ The PCA analysis of the 24 plasma biomarker levels showed marked difference in first, third and fourth principal component values, demonstrating that the second multiplexed immunoassay kits acted as a batch effect confounding factor. Seven biomarkers plasma levels namely CRP ($p=0.02$), IL-2 ($p<0.0001$), IL-10 ($p=0.0004$), sMMP2 ($p<0.0001$), sROBO4 ($p=0.0069$), sICAM1/CD54 ($p=0.012$) and IFN γ ($p=0.018$) were affected by a batch effect, the others 17 biomarkers were unaffected.

We first considered independently the predictive associations between the plasma levels of 24 biomarkers and the occurrence of a CCM-related bleed or lesional growth within a year following the initial blood sample. The difference of each of the 24 biomarkers batch corrected levels between the patients who experienced a hemorrhagic expansion within the following 1-year time period (± 30 days) and stable patients was assessed. The plasma levels were considered as continuous variables and compared using an unpaired two samples Student's t-test, assessed with pooled standard deviation or Satterthwaite's method according to the equality of the variance. For each biomarker, a corrected plasma value was considered as an outlier if it deviated more than plus/minus 3 standard deviations away from the mean corrected plasma value.^{14, 15} The cross-correlation between the relevant biomarkers were assessed using a linear Pearson correlation coefficient.

All the 35 possible linear combinations of the 5 biomarkers showing significant associations with lesional activity were processed using the canonical discriminant function analysis.^{16, 17} Receiver operating characteristic (ROC) curves were generated and area under curves (AUC) calculated for each biomarker individually and each linear combination (discriminant score) as well. The optimal cutoff value to determine the best sensitivity and specificity were assessed following Youden index method.¹⁸ The best model to predict hemorrhagic expansion was selected as that minimized the Akaike Information Criterion (AIC), representing parsimonious model offering the best fit to the data with the fewest number of predictors.¹⁹

The best model to predict hemorrhagic expansion was then tested in the independent cohort (i.e., *Group 2-Algorithm testing*) by assessing the difference in the mean estimated combination value between the stable patients and those who experienced a subsequent hemorrhagic expansion. The canonical values were considered as continuous variables and compared using an unpaired two samples Student's t-test. We then performed a logistic model based on the discriminant analysis to predict the probability that a subject will be stable or will experience a hemorrhagic expansion within the following year (± 30 days) using the best linear combination (defined by the *Group 1-Algorithm definition*) and the plasma levels of sCD14, sROBO4, VEGF, IL-1 β .²⁰ Finally, a ROC curve was generated and the AUC was calculated. The optimal cutoff value to determine the best sensitivity and specificity was assessed following Youden index method.

We also probabilistically validated the best linear combination by simulating 1000 stable subjects and 1000 with subsequent hemorrhagic expansion, using a Monte Carlo approach.²¹⁻²³

Statistical analyses were performed using SAS9.4 (SAS Institute Inc., Cary, NC), R (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism4.0 (GraphPad Software Inc., La Jolla, CA). All p values were considered to be statistically significant at * $p<0.05$, ** $p<0.01$, or *** $p<0.001$.

Supplemental Results

Comparison of sCD14, sROBO4, VEGF, IL-1 β and IL-6 in enrolled subjects and those without follow-up

The comparison of the 2 cohorts of CCM patients with and without follow-up did not identify difference in the baseline demographics, genotypic distribution and phenotypic disease features (**Online Table II**). We also did not find difference among these two cohorts in the baseline plasma levels for any of the 5 compounds showing significant association with subsequent lesional activity namely sCD14, sROBO4, VEGF, IL-1 β and IL-6.

Association of sCD14, sROBO4, VEGF, IL-1 β and IL-6 with clinical disease severity

In order to detect potential confounders, we tested whether the plasma levels of sCD14, sROBO4, VEGF, IL-1 β or IL-6 that might be associated with reported features of disease severity.^{2, 5, 9} The results did not show association between the plasma levels of any of these compounds and the phenotype (familial or sporadic), recent CCM-related bleed (Yes or No), the presence of brainstem lesions (Yes or No) or the total number of SWI or T₂-weighted lesions. In addition, we did not observe any significant intercorrelation between the plasma levels of the four biomarkers included in the best combination (**Online Table V**), hence their respective predictive contributions may be considered as independent and non-overlapping. None of the 5 individual plasma biomarkers that had prognostic associations nor the best combination correlated familial disease, T₂-weighted and or total lesion counts, brainstem lesion location or symptomatic hemorrhage in prior year, versus other cases. Hence the prognostic associations could be considered independent of disease features known to influence clinical risk. These were also not different in cases with biomarker sampling who were not available for follow-up.

The combination including plasma levels is not associated with a higher pro-inflammatory state of the hierarchical clustering

We previously reported an association between 4 clustered pro-inflammatory biomarkers (IL-2, IFN γ , TNF α and IL-1 β) and a greater propensity to more CCM-related hemorrhagic events over a patient's lifetime.⁹ This cluster allowed us to segregate the patients as "high" and "low" inflammatory states. Among the 55 patients enrolled in this study, 32 (15 with solitary/sporadic lesions and 17 multifocal/familial CCMs) were classified as "high" inflammatory state and 14 (6 with solitary/sporadic lesions and 8 multifocal/familial CCMs) as "low". Nine patients were not included in this analysis because they were considered as outlier for at least one of the 4 biomarkers constituting the cluster. The point-biserial correlation analysis did not reach any significant association ($r=0.36$, $p=0.36$) between the inflammatory state ("high" or "low") and the combined plasma values including sCD14, sROBO4, VEGF and IL-1 β . The currently applied statistical methodology of testing individual biomarkers, then weighing their combination appears more likely to identify clinically relevant prognostic endpoints, than the hierarchical clustering approach.

The best weighted combination including sCD14, sROBO4, VEGF and IL-1 β distinguished the patients with subsequent hemorrhagic expansion from those remaining stable in a simulated population using a probabilistic (Monte Carlo) approach

We compared the probable plasma levels of sCD14, sROBO4, VEGF and IL-1 β plasma levels in 1000 simulated stable patients and 1000 with subsequent hemorrhagic expansion, assuming normal probability distribution and batch effect corrected values. The best weighted biomarker combination was able to predict an upcoming hemorrhagic expansion in the simulated population with 81% of sensitivity and 90% of specificity (AUC= 0.92, $p<0.0001$; 95% CI=[0.91, 0.93]).

Patient characteristics	Group 1 Algorithm definition		Group 2 Algorithm testing		Familial Cases without New Lesion Formation	Familial Cases with New Lesion Formation
	Stable	Hemorrhagic expansion	Stable	Hemorrhagic expansion		
Sample Size	38	11	21	7	16	8
Mean age (years)±SD	38.74±18.15	29.92±16.26	41.63±19.98	36.29±14.31	29.41±17.95	48.05±13.86 [‡]
Range (years)	4.62-75.02	5.18-56.92	7.13-67.63	13.74-58.28	4.62-63.79	22.23-61.66
Sex						
Male (%)	13(34)	4(36)	4(19)	0(0)	7(44)	3(38)
Female (%)	25(66)	8(64)	17(81)	7(100)	9(56)	5(62)
Genotype						
Familial (%)	17(45)	7(64)	7(33)	2(29)	16 (100)	8(100)
Sporadic (%)	21(55)	4(36)	14(67)	5(71)	NA	NA
Lesion characteristics						
Mean # of SWI lesions ± SD	5.26±7.41	26.64±38.25*	7.95±17.21	20.43±37.98	11.16±8.48	73.0±37.07*
Mean # of T ₂ -weighted lesions ± SD	1.97±1.97	7.1±8.14*	4.0±8.60	6.57±9.62	3.31±2.50	14.38±15.93*
T ₂ -weighted brainstem lesions (%)	13(34)	5(45) [†]	9(43)	2(29)	11(69)	7(88)
Hemorrhagic lesions in prior year (%)	3(8)	2(18) [†]	3(14)	2(29)	0(0)	0(0)
Mean follow-up (days) ± SD	304.82±98.65	297.82±74.97	310±108.26	324.71±84.02	312.44±90.93	330.13±68.55
Ethnicity						
White/Caucasian (%)	30(79)	10(91)	20(95)	6(86)	13(81)	8(100)
African American (%)	1(3)	1(9)	0(0)	1(14)	0	0
Hispanic (%)	4(10)	0	1(5)	0(0)	2(13)	0
Asian (%)	3(8)	0	0(0)	0(0)	1(6)	0

NA = not applicable

* These results are largely accounted by hemorrhagic expansion (p=0.002 and 0.001 for differences in SWI and T₂-weighted lesion counts, respectively) and new lesion formation (p=0.0001 and 0.01 for differences in SWI and T₂-weighted lesion counts, respectively) in familial cases with exceptionally high baseline lesion burden (> 100 SWI lesions). Greater hemorrhage tendency has been previously noted in familial cases harboring exceptionally high lesion burden.²⁴

[†]Higher prevalence of hemorrhage/lesional growth in cases with brainstem lesion location and recent symptomatic hemorrhage is consistent with recognized clinical associations,²⁵ but the trends were not statistically significant (likely in view of small sample size).

None of the other differences between stable and unstable lesions, or those with and without new lesion formation (among familial cases) were significant at p< 0.05.

[‡] Familial cases developing new lesions within a year follow-up were older than the familial patients that did not develop new lesion during the same time period (p=0.02). However, we did not observe association between age and the levels of the preselected plasma biomarkers.

Online Table II. Demographics of the two cohorts with and without follow-up

Patient characteristics*	Patients with 1-year follow-up [Enrolled and analyzed for prognostic questions]	Patients without 1-year follow-up
Sample Size	77	95
Mean age (years) \pm SD	39.54 \pm 18.53	40.00 \pm 16.03
Range (years)	[4.62-75.02]	[6.61-75.57]
Sex		
Male (%)	22 (29)	39 (41)
Female (%)	55 (71)	56 (59)
Genotype		
Familial (%)	35 (45)	40 (42)
Sporadic (%)	42 (55)	55 (58)
Lesion characteristics		
Mean # of SWI lesions \pm SD	14.72 \pm 28.11	9.45 \pm 20.63
Mean # of T ₂ -weighted lesions \pm SD	4.90 \pm 9.47	3.82 \pm 8.86
Presence T ₂ -weighted brainstem lesions (%)	35 (45)	32 (34)
Hemorrhagic lesions in prior year (%)	8 (10)	14 (15)
Ethnicity		
White/Caucasian (%)	66 (86)	75 (79)
African American (%)	3 (4)	13 (14)
Hispanic (%)	5 (6)	4 (4)
Asian (%)	3 (4)	3 (3)

*none of these were significantly different in enrolled patients followed for 1 year, versus those without follow-up

Online Table III. Summary of the number of patients from the logistic model analysis using the best linear combination and predicting the probability of a subject in the independent cohort (i.e., *Group 2-Algorithm testing*) being stable or experiencing a hemorrhagic expansion within the following year (± 30 days).

	Patients predicted to be stable within 1-year time period ± 30 days (%)	Patients predicted to experience hemorrhagic expansion within 1-year time period ± 30 days (%)
Patients clinically stable at 1-year follow-up ± 30 days	20 (83.3)	1 (25.0)
Patients that experienced a hemorrhagic expansion within the 1-year follow-up ± 30 days	4 (16.7)	3 (75.0)

Online Table IV: Selected biomarkers with mechanistic associations reported in CCM or brain hemorrhage diseases

Biomarker	Biological Processes	Publications	Potential Relation to CCM Physiopathology
Interleukins - IL-1 β - IL-2 - IL-6 - IL-8 - IL-10	- Inflammation - Immune response - Angiogenesis	Girard et al. (2017) ⁹	Increased baseline of IL-2, IL-1 β plasma levels is associated with unfavorable course of CCM disease.
IFN γ	- Endothelial permeability - Inflammation	Girard et al. (2017) ⁹	CCM patients with increased plasma levels of IFN γ are more prone to experience an aggressive clinical course during their lifetime.
Tumor necrosis factors - TNF α - TNF RI	- Inflammation	Girard et al. (2017) ⁹	Increased baseline of TNF α is associated with more CCM-related hemorrhagic and seizures events during a patient's lifetime.
C-reactive protein (CRP)	- Inflammation	Gao et al. (2018) ²⁶	CCM3 polymorphism is associated with increased CRP plasma levels.
Matrix metalloproteinases (MMPs) - MMP-2 - MMP-9	- Permeability - Extracellular matrix remodeling	Girard et al. (2017) ⁹	Plasma levels of MMP-2 and MMP-9 were respectively higher and lower in patients with previous seizure activity.
CD14	- Inflammation	Tang et al. (2017) ²⁷	Polymorphisms that increase expression of the gene encoding of CD14-anchored membrane are associated with higher CCM lesion burden in familial CCM disease.
CCL2/MCP-1	- Inflammation	Retta et al. (2016) ²⁸	Oxidative stress is associated to chronic inflammation in hemorrhagic vascular disease
Endoglin	- Angiogenesis - Endothelial permeability - Inflammation	Cunha et al. (2017) ²⁹	Increased expression of ENG is associated to hypersprouting and local increased permeability.
25-OH Vitamin D	- Inflammation	Girard et al. (2016) ⁵	Vitamin D deficiency is associated with a more aggressive clinical course in human CCM disease.

Leukocyte-Endothelial Cell Adhesion - VCAM1 - ICAM1/CD54	- Inflammation - Endothelial permeability	Lampugnani et al. (2017) ³⁰	Increased expression of ICAM-1 and VCAM-1 on endothelial cells is a marker of inflammation.
Lipid Panel: - HDL - Non-HDL - LDL - Triglycerides	- Inflammation	Shenkar et al. (2017) ³¹	Simvastatin decreases ROCK activity in mature CCM lesions in mice.
VEGF	- Angiogenesis - Endothelial permeability	Cunha et al (2017) ²⁹	VEGF is associated to vasculogenesis and endothelial permeability.
ROBO4	- Angiogenesis - Endothelial permeability	Wüstehube et al. (2010) ³²	CCM1 gene expression is associated with a down-regulation of ROBO4 expression.

Updated and modified from Girard et al. (2017).

Online Table V: Matrix correlation among the 5-plasma biomarkers that showed a predictive association with lesional activity

	sCD14	IL-6	sROBO4	IL1- β	VEGF
sCD14	1.00				
IL-6	0.47*	1.00			
sROBO4	-0.19	-0.02	1.00		
IL-1 β	-0.36	-0.87*	-0.06	1.00	
VEGF	0.08	0.22	0.17	-0.19	1.00

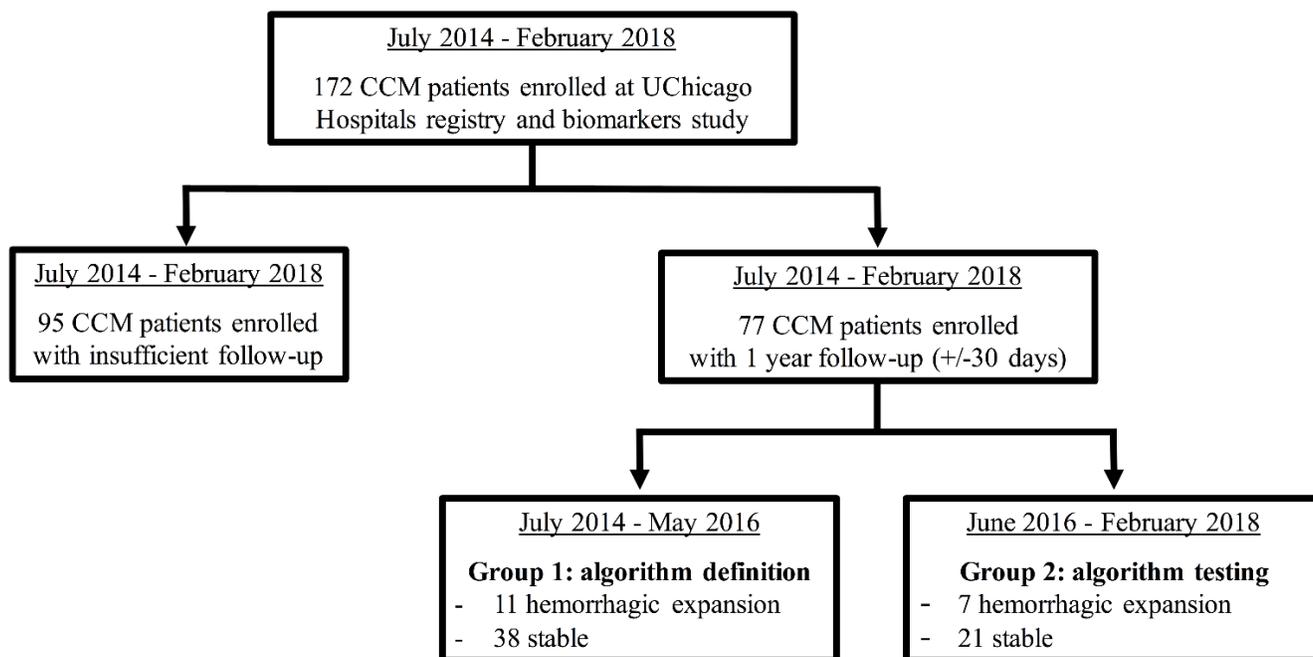
* denotes statistical significance $p < 0.05$.

Supplemental References

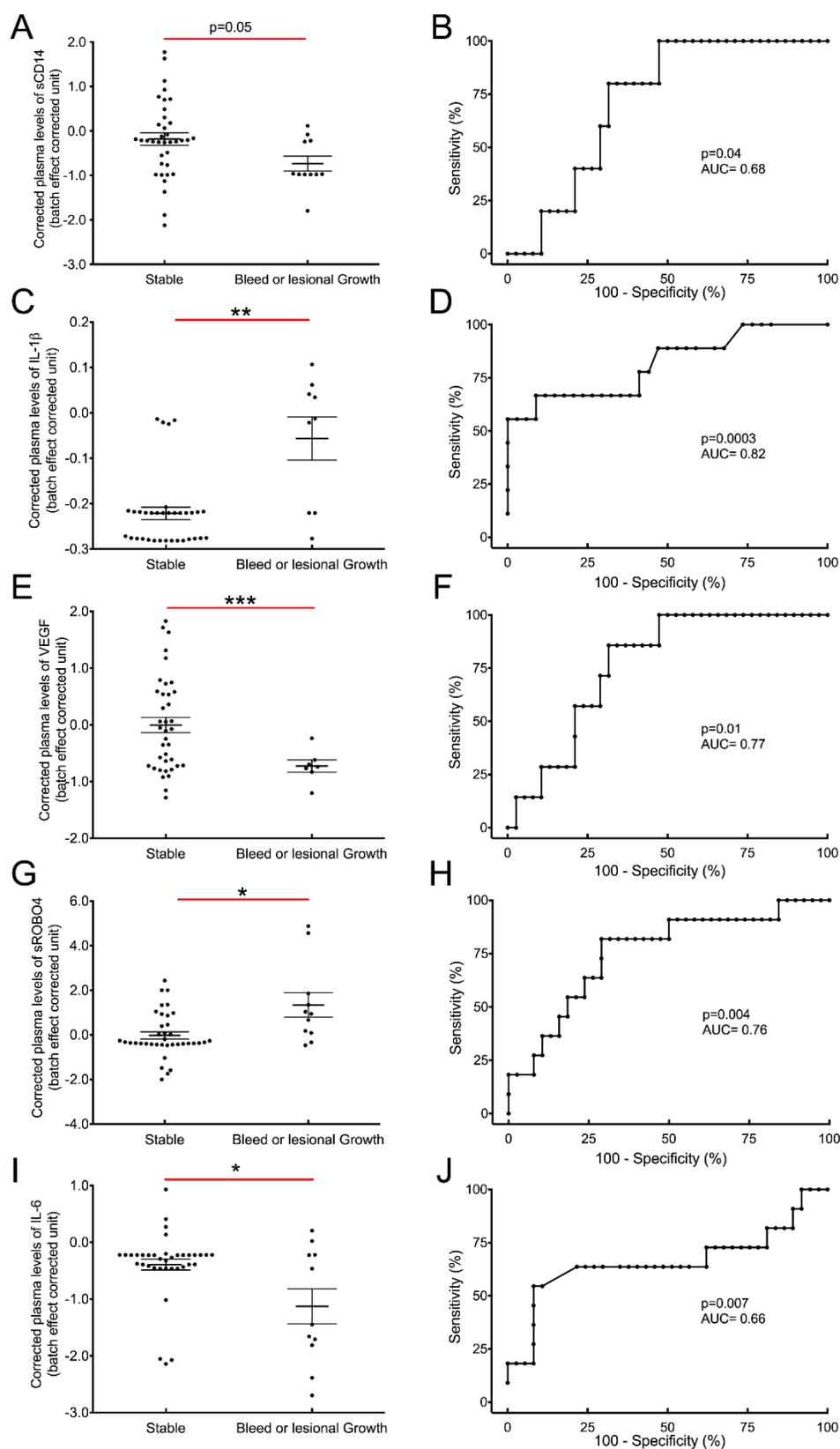
1. Mikati AG, Khanna O, Zhang L, Girard R, Shenkar R, Guo X, Shah A, Larsson HB, Tan H, Li L, Wishnoff MS, Shi C, Christoforidis GA, Awad IA. Vascular permeability in cerebral cavernous malformations. *J Cereb Blood Flow Metab.* 2015;35:1632-1639
2. Girard R, Fam MD, Zeineddine HA, Tan H, Mikati AG, Shi C, Jesselson M, Shenkar R, Wu M, Cao Y, Hobson N, Larsson HB, Christoforidis GA, Awad IA. Vascular permeability and iron deposition biomarkers in longitudinal follow-up of cerebral cavernous malformations. *J Neurosurg.* 2016;127:1-9
3. Al-Shahi Salman R, Berg MJ, Morrison L, Awad IA, Angioma Alliance Scientific Advisory B. Hemorrhage from cavernous malformations of the brain: Definition and reporting standards. Angioma alliance scientific advisory board. *Stroke.* 2008;39:3222-3230
4. Akers A, Al-Shahi Salman R, Awad IA, et al. Synopsis of guidelines for the clinical management of cerebral cavernous malformations: Consensus recommendations based on systematic literature review by the angioma alliance scientific advisory board clinical experts panel. *Neurosurgery.* 2017;80:665-680
5. Girard R, Khanna O, Shenkar R, et al. Peripheral plasma vitamin d and non-hdl cholesterol reflect the severity of cerebral cavernous malformation disease. *Biomark Med.* 2016;10:255-264
6. Tan H, Zhang L, Mikati AG, Girard R, Khanna O, Fam MD, Liu T, Wang Y, Edelman RR, Christoforidis G, Awad IA. Quantitative susceptibility mapping in cerebral cavernous malformations: Clinical correlations. *AJNR Am J Neuroradiol.* 2016;37:1209-1215
7. Zhou X, Fragala MS, McElhaney JE, Kuchel GA. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. *Curr Opin Clin Nutr Metab Care.* 2010;13:541-547
8. Langsted A, Freiberg JJ, Nordestgaard BG. Fasting and nonfasting lipid levels: Influence of normal food intake on lipids, lipoproteins, apolipoproteins, and cardiovascular risk prediction. *Circulation.* 2008;118:2047-2056
9. Girard R, Zeineddine HA, Fam MD, et al. Plasma biomarkers of inflammation reflect seizures and hemorrhagic activity of cerebral cavernous malformations. *Transl Stroke Res.* 2018;9:34-43
10. Fleming BD, Chandrasekaran P, Dillon LA, Dalby E, Suresh R, Sarkar A, El-Sayed NM, Mosser DM. The generation of macrophages with anti-inflammatory activity in the absence of stat6 signaling. *J Leukoc Biol.* 2015;98:395-407
11. Mora S, Rifai N, Buring JE, Ridker PM. Comparison of ldl cholesterol concentrations by friedewald calculation and direct measurement in relation to cardiovascular events in 27,331 women. *Clin Chem.* 2009;55:888-894
12. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics.* 2012;28:882-883
13. Ringner M. What is principal component analysis? *Nat Biotechnol.* 2008;26:303-304
14. Selst MV, Jolicoeur P. A solution to the effect of sample size on outlier elimination. *Q J Exp Psychol.* 1994;47:631-650
15. Davies L, Gather U. The identification of multiple outliers. *J Am Stat Assoc.* 1993;88:782-792

16. Rencher AC. *Methods of multivariate analysis*. Hoboken, New Jersey: Wiley; 2003.
17. Huberty CJ. *Applied discriminant analysis*. Hoboken, New Jersey: Wiley; 1994.
18. Schisterman EF, Perkins NJ, Liu A, Bondell H. Optimal cut-point and its corresponding youden index to discriminate individuals using pooled blood samples. *Epidemiology*. 2005;16:73-81
19. Akaike H. Akaike's information criterion. In: Lovric M, ed. *International encyclopedia of statistical science*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011:25-25.
20. Allison PD. *Logistic regression using SAS: Theory and application*. Cary, North Carolina: SAS Institute; 2012.
21. Robert CP, Casella G. *Monte carlo statistical methods* New York, New York: Springer-Verlag New York, Inc.; 2005.
22. Chen X, Chen X, Xu Y, Yang W, Wu N, Ye H, Yang JY, Hong Q, Xin Y, Yang MQ, Deng Y, Duan S. Association of six cpg-snps in the inflammation-related genes with coronary heart disease. *Hum Genomics*. 2016;10 Suppl 2:21
23. Becerra AZ, Probst CP, Tejani MA, Aquina CT, Gonzalez MG, Hensley BJ, Noyes K, Monson JR, Fleming FJ. Evaluating the prognostic role of elevated preoperative carcinoembryonic antigen levels in colon cancer patients: Results from the national cancer database. *Ann Surg Oncol*. 2016;23:1554-1561
24. Shenkar R, Shi C, Rebeiz T, et al. Exceptional aggressiveness of cerebral cavernous malformation disease associated with pcd10 mutations. *Genet Med*. 2015;17:188-196
25. Horne MA, Flemming KD, Su IC, et al. Clinical course of untreated cerebral cavernous malformations: A meta-analysis of individual patient data. *Lancet Neurol*. 2016;15:166-173
26. Gao Y, Zhao Z, Yang L, et al. Arsenic exposure assists ccm3 genetic polymorphism in elevating blood pressure. *Oncotarget*. 2018;9:4915-4923
27. Tang AT, Choi JP, Kotzin JJ, et al. Endothelial tlr4 and the microbiome drive cerebral cavernous malformations. *Nature*. 2017;545:305-310
28. Retta SF, Glading AJ. Oxidative stress and inflammation in cerebral cavernous malformation disease pathogenesis: Two sides of the same coin. *Int J Biochem Cell Biol*. 2016;81:254-270
29. Cunha SI, Magnusson PU, Dejana E, Lampugnani MG. Deregulated tgf-beta/bmp signaling in vascular malformations. *Circ Res*. 2017;121:981-999
30. Lampugnani MG, Dejana E, Giampietro C. Vascular endothelial (ve)-cadherin, endothelial adherens junctions, and vascular disease. *Cold Spring Harb Perspect Biol*. 2017
31. Shenkar R, Shi C, Austin C, et al. Rhoa kinase inhibition with fasudil versus simvastatin in murine models of cerebral cavernous malformations. *Stroke*. 2017;48:187-194
32. Wustehube J, Bartol A, Liebler SS, Brutsch R, Zhu Y, Felbor U, Sure U, Augustin HG, Fischer A. Cerebral cavernous malformation protein ccm1 inhibits sprouting angiogenesis by activating delta-notch signaling. *Proc Natl Acad Sci U S A*. 2010;107:12640-12645

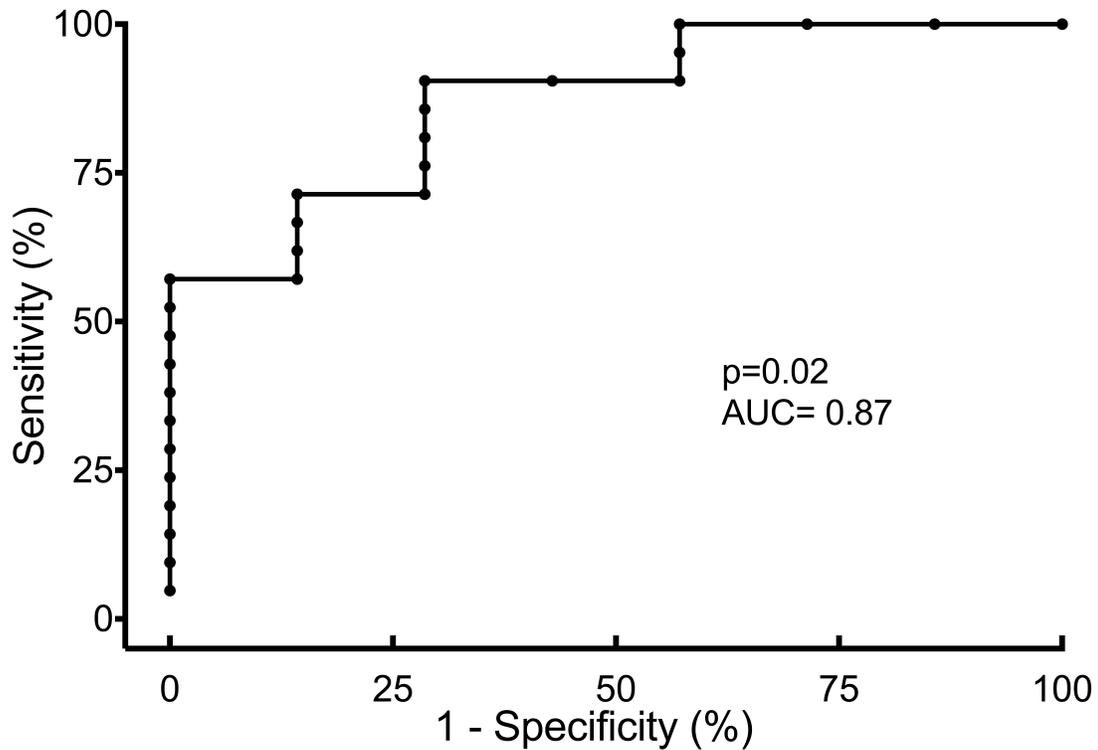
Online Figures



Online Figure I. Consort diagram for patients enrolled in the biomarkers study. One hundred seventy-two CCM patients were enrolled in the plasma biomarkers study from July 2014 and February 2018. Seventy-seven patients with a 1-year follow-up clinical visit (\pm 30 days) were considered as two independent groups based on the period of enrollment. The first cohort defined as *Group 1-Algorithm definition* included 49 patients (11 with subsequent hemorrhagic expansion and 38 remain stable) enrolled between July 2014 and May 2016. The second cohort entitled *Group 2-Algorithm testing* included 28 patients (7 with subsequent hemorrhagic expansion and 21 stable) enrolled between June 2016 and February 2018. Ninety-five CCM subjects were enrolled in biomarker studies at initial consultation between July 2014 to February 2018, but did not undergo imaging or clinical follow-up at our institution after the initial blood sample. These included 82 patients with initial consultation and insufficient follow-up, and 13 patients who underwent CCM lesion resection. Demographic and clinical features of patients with and without follow-up are compared in **Online Table II**.



Online Figure II. Five cytokines were differently expressed in patients who experienced clinically relevant lesional activity within the year following the blood sample. Among the 24 biomarkers, patients who experienced a bleed or a lesional growth within a year after the initial blood sample, showed lower plasma levels of sCD14 ($p=0.05$), IL-6 ($p=0.04$), and VEGF ($p=0.0003$), along with higher IL-1 β ($p=0.008$) and sROBO4 ($p=0.03$) plasma levels.



Online Figure III. The best linear combination was able to predict a clinical lesional activity within a year (± 30 days) in the independent cohort. The receiver operating characteristic curve generated for the best linear combination was able to predict a clinical lesional activity within a year (± 30 days) [area under the curve (AUC)=0.87, $p=0.02$] in the independent cohort (i.e., *Group 2-Algorithm testing*) with 90% sensitivity and 71% specificity.