Supplemental Material

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

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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±SD=39.54±18.53 years, range=[4.62-75.02]) with a blood sample and a clinical follow-up visit within 1 year (± 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±SD=40.00±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 T_2-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 µl) 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-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), soluble matrix metalloproteinase-2 (MMP2) and -9 (MMP9), tumor necrosis factor alpha (TNFα), 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 (IFNγ) 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 IFNγ ($p=0.018$) 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-1β.[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-1β 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-1β and IL-6.

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

In order to detect potential confounders, we tested whether the plasma levels of sCD14, sROBO4, VEGF, IL-1β 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 T_2-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, T_2-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, IFNγ, TNFα and IL-1β) 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 ($u=0.36$, $p=0.36$) between the inflammatory state ("high" or "low") and the combined plasma values including sCD14, sROBO4, VEGF and IL-1β. 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-1β 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-1β 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]).

Online Table I. CCM Patient Demographics and Lesion/Disease Features

Patient characteristics	Group 1 Algorithm definition		Group 2 Algorithm testing			
	Stable	Hemorrhagic expansion	Stable	Hemorrhagic expansion	Familial Cases without New Lesion Formation	Familial Cases with New Lesion Formation
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 T_2-weighted lesion counts, respectively) and new lesion formation (p=0.0001 and 0.01 for differences in SWI and T_2-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) ± SD	39.54±18.53	40.00±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 ± SD	14.72±28.11	9.45±20.63
Mean # of T_2-weighted lesions ± SD	4.90±9.47	3.82±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-1β - IL-2 - IL-6 - IL-8 - IL-10	- Inflammation - Immune response - Angiogenesis	Girard et al. (2017) [9]	Increased baseline of IL-2, IL-1ß plasma levels is associated with unfavorable course of CCM disease.
IFNγ	- Endothelial permeability - Inflammation	Girard et al. (2017) [9]	CCM patients with increased plasma levels of IFNγ are more prone to experience an aggressive clinical course during their lifetime.
Tumor necrosis factors - TNFα - TNF RI	- Inflammation	Girard et al. (2017) [9]	Increased baseline of TNFα 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-1β	-0.36	-0.87*	-0.06	1.00	
VEGF	0.08	0.22	0.17	-0.19	1.00

* denotes statistical significance $p<0.05$.

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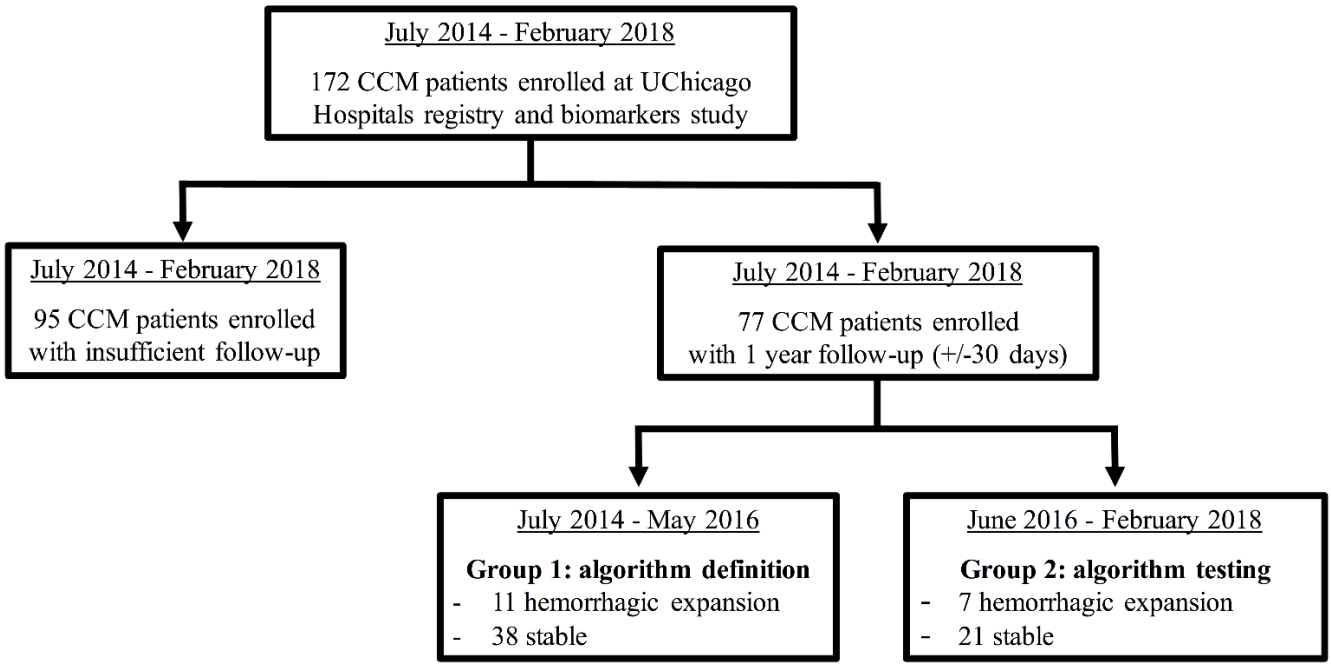
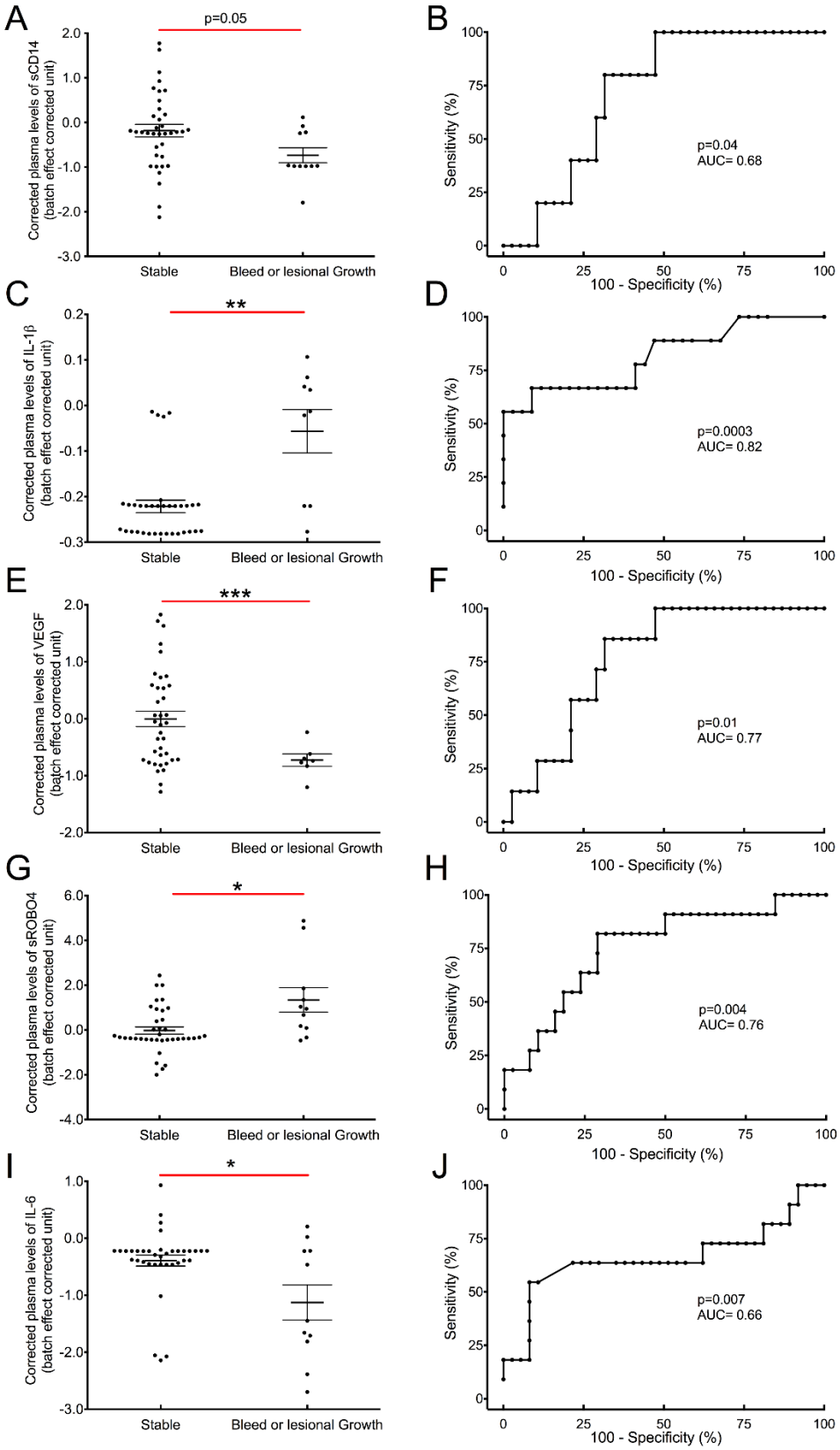
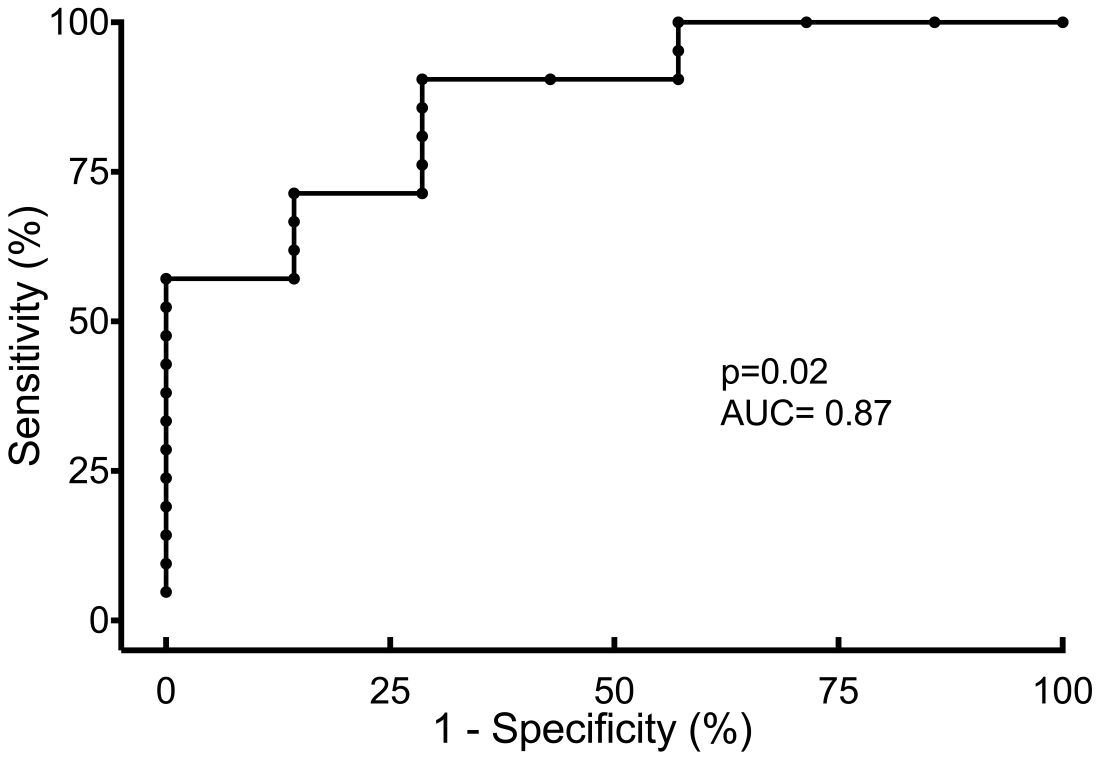
Online Figures



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 (± 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-1β (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.