Supplementary Material

Peripheral Plasma Vitamin D and Non-HDL Cholesterol Reflect the Severity of Cerebral Cavernous Malformation Disease

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SUPPLEMENTARY METHODS

Plasma preparation and leukocyte isolation

For each patient, a 10 mL blood sample was collected into a heparinized vacutainer tube, obtained concomitantly during insertion of an IV cannula required for routine clinical magnetic resonance imaging (MRI) contrast administration. Because MRI was conducted at various times of the day, we could not require the patients to undergo fasting. For plasma isolation, 3 mL of heparinized blood was centrifuged at 2,300 rpm at 4°C for 10 minutes. The supernatant plasma was split into equal aliquots and stored at -80°C for later use.

In a parallel study, 32 CCM patients were recruited for peripheral blood leukocyte ROCK activity between May 2013 and January 2015 based on the same clinical criteria and with identical clinical data collection as the lipid biomarker studies. These included 24 patients who underwent both plasma biomarker and ROCK studies. At the beginning of this project, leukocytes from 8 patients were isolated from the peripheral blood sample using the method described by Liu and Liao [1], amended to allow leukocytes to settle for 30 minutes after an equal volume of 2% dextran in Hank's buffered saline solution was added to the heparinized blood, and lysing the remaining erythrocytes by resuspending the leukocyte pellet in water for 30 seconds. For the subsequent patients, we modified the method of isolating leukocytes by adding 7 mL of blood to 35 mL red blood cell (RBC) lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 1 mM ethylenediamine tetraacetic acid). Samples were gently agitated for 5 minutes, and leukocytes were collected by centrifugation at 300G at 4°C for 6 minutes. The supernatant was discarded; 14 mL of RBC lysis buffer were added and the tube was centrifuged again. After discarding the supernatant, the pellet was re-suspended in M199 medium (Gibco) to achieve a concentration of 5×10^6 cells/mL. Equal 1-mL aliquots of the solution were spun down at 14,000 rpm for 5 minutes. After discarding the supernatant, the pellets were re-suspended in 400 µL M199 medium and fixed by the addition of 400 µL 50% trichloroacetic acid and 50 mM dithiothreitol. The tubes were centrifuged at 14,000 rpm at 4°C for 5 minute and the leukocyte pellets stored at -80°C for subsequent use.

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

Plasma aliquots were sent to the University of Chicago Medical Center Phlebotomy and Pre-Clinical Services for 25-(OH) vitamin D and lipid panel measurements. 25-hydroxyvitamin D [25-(OH) vitamin D] levels were quantified using high-pressure liquid chromatography coupled to mass spectrometry (LC/MS). In our samples, total 25-(OH) vitamin D values were not significantly different with 25-(OH) vitamin D3. Total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol were measured on the Roche Cobas 8000 Modular Analyzer via enzymatic calorimetry assays. C-reactive protein (CRP) was measured using particle-enhanced immunoturbidimetric assay on the same machine platform. Because the patients' labs were obtained in the non-fasting state, we conducted the data analysis using the parameters HDL cholesterol and non-HDL cholesterol. Non-HDL cholesterol was calculated by using the Friedewald formula from total cholesterol and HDL cholesterol. Both have been previously validated to be reliable when measured in either the fasting or non-fasting state [2], obviating any error that could be present using the Friedewald formula to calculate non-HDL cholesterol levels [3]. Total cholesterol, triglycerides and LDL cholesterol levels were not reported because they required fasting.

Leukocyte rho-kinase (ROCK) assay

ROCK activity was determined via the ratio of phosphorylated myosin-binding subunit (pMBS) to the total myosin-binding subunit (tMBS), as measured by a standard immunoblotting protocol, as described by Liu and Liao [1]. Briefly, the leukocyte pellets are re-suspended in 8M urea solution (Sigma) containing 0.2% protease inhibitor (Abcam). A standard positive control was added to each gel to normalize results between different experiments. The 4-15% TGX gels (BioRad) were electrophoresed for 30 minutes at 200V. The proteins were then transferred to a polyvinylidene difluoride membrane at 100V for 2 hours. Blocking was achieved in either 5% milk in tris buffered saline with Tween 20 (TBST, Abcam for tMBS) or 5% bovine serum albumen (Fisher) in TBST (for pMBS). The membranes were incubated overnight at 4°C in either rabbit anti-phospho Thr853-specific myosin-binding subunit primary antibody at 1:1000 dilution, rabbit anti-myosin-binding subunit polyclonal primary antibody (Covance) at 1:1000 dilution or rabbit anti- glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell Signaling) at 1:2000 dilution [1]. After incubation with secondary anti-rabbit IgG-HRP antibody (BioRad) at 1:2500 (for tMBS) and 1:5000 (for pMBS and GAPDH) dilution, membrane bands were visualized using an enhanced chemiluminescence detection kit (BioRad) with equal exposure times for membranes that were run contemporaneously and later treated with either anti-pMBS, anti-tMBS or anti-GAPDH antibodies.

Statistical Methods

The relationship between the biomarker (vitamin D, lipid panel, CRP, ROCK activity) levels and clinical features (number of lesions by susceptibility weighted imaging, number of lesions by T2-weighted MRI, age at symptom onset and hemorrhage) were initially analyzed as continuous variables, assessed using Pearson's or Spearman's correlation coefficient. Multivariate linear regression was applied to control for potential confounders. Next, the differences of the biomarker levels between cohorts with or without chronic disease aggressiveness and between cohorts with or without acute disease aggressiveness were tested using Student's t-test or Wilcoxon Rank Sum test based on the normality of the data. Pre-selected biomarkers with significant findings in predicting chronic aggressive CCM disease course were included in the canonical discriminant function analysis, and a discriminant score was created for each subject to determine their respective status. Receiver operating characteristic (ROC) curves were generated for both individual biomarkers and their combinations (discriminant score). The accuracy of the ROC depends on how well the sample is correctly separated into subjects with and without chronic disease aggressiveness [4]. Precisely, the area under the curve (AUC) measures discrimination, that is, the probability that a randomly chosen subject with chronic disease aggressiveness is accurately ranked with greater suspicion than if randomly chosen without chronic disease aggressiveness. An AUC of 1 represented a perfect ability, while an AUC of 0.5 represented an inadequate ability [5]. CCM chronic disease aggressiveness predictive thresholds (cut off value) of vitamin D, non-HDL cholesterol and their combination were determined by the value that achieved the best sensitivity and specificity together. Statistical analyses were performed using STATA12 (StataCorp LP, College Station, Texas). All p values were considered to be statistically significant at *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$.

SUPPLEMENTARY REFERENCES

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Table 1a. Correlations of demographic features and individual parameters of chronic and acute disease aggressiveness with biomarkers in all CCM subjects.

	Total 25-(OH) vitamin D		Non-HDL cholesterol		HDL cholesterol		RhoA kinase activity		C-reactive protein	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
Demographic features										
Age at symptom onset	0.34	0.04*	0.49	0.002*	0.47	0.004*	0.51	0.02*	0.24	0.17
Season	-0.30	0.05	-0.09	0.56	0.16	0.32	0.11	0.61	0.15	0.34
Gender	-0.17	0.26	0.29	0.06	-0.35	0.02*	-0.23	0.26	-0.05	0.76
Seizures	0.003*	0.99	-0.29	0.06	0.04	0.82	-0.07	0.73	0.04	0.78
Chronic disease aggressiveness										
> 1 hemorrhage	-0.12	0.44	-0.12	0.45	-0.05	0.74	-0.15	0.46	-0.28	0.08
Age at symptoms ≤18 years old	-0.24	0.16	-0.53	0.001*	-0.29	0.08	-0.41	0.07	-0.27	0.12
Acute disease aggressiveness										
Hemorrhage within 1 year	-0.05	0.75	0.03	0.83	0.02	0.91	0.25	0.24	0.28	0.09
Lesional growth within 1 year	-0.21	0.24	-0.25	0.16	-0.03	0.86	0.004	0.99	-0.18	0.32
New lesion within 1 year	-0.15	0.40	0.08	0.66	-0.04	0.84	-0.23	0.35	-0.18	0.33

* denotes significant statistical significance in univariate correlation.

Table 1b. Correlations of demographic features and individual parameters of chronic and acute disease aggressiveness with biomarkers in the sporadic patient cohort.

	Total 25-(OH) vitamin D		Non-HDL cholesterol		HDL cholesterol		RhoA kinase activity		C-reactive protein	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
Demographic features										
Age at symptom onset	0.70	0.003*	0.09	0.74	0.34	0.19	0.56	0.15	-0.02	0.95
Season	0.09	0.70	-0.24	0.31	0.31	0.19	-0.28	0.40	-0.07	0.77
Gender	-0.58	0.008*	0.15	0.53	-0.60	0.005*	-0.28	0.41	-0.02	0.92
Seizures	0.10	0.66	-0.36	0.12	0.22	0.34	-0.20	0.56	0.13	0.60
Chronic disease aggressiveness										
> 1 hemorrhage	-0.14	0.56	-0.08	0.75	-0.08	0.73	-0.10	0.78	-0.33	0.16
Age at symptoms ≤18 years old	-0.54	0.03*	-0.11	0.68	-0.23	0.39	-0.32	0.44	0.02	0.94
Acute disease aggressiveness										
Hemorrhage within 1 year	-0.15	0.52	-0.17	0.47	-0.10	0.68	0.45	0.17	0.43	0.06
Lesional growth within 1 year	0.04	0.89	-0.32	0.30	0.32	0.32	0.43	0.34	-0.17	0.59
New lesion within 1 year	-	-	-	-	-	-	-	-	-	-

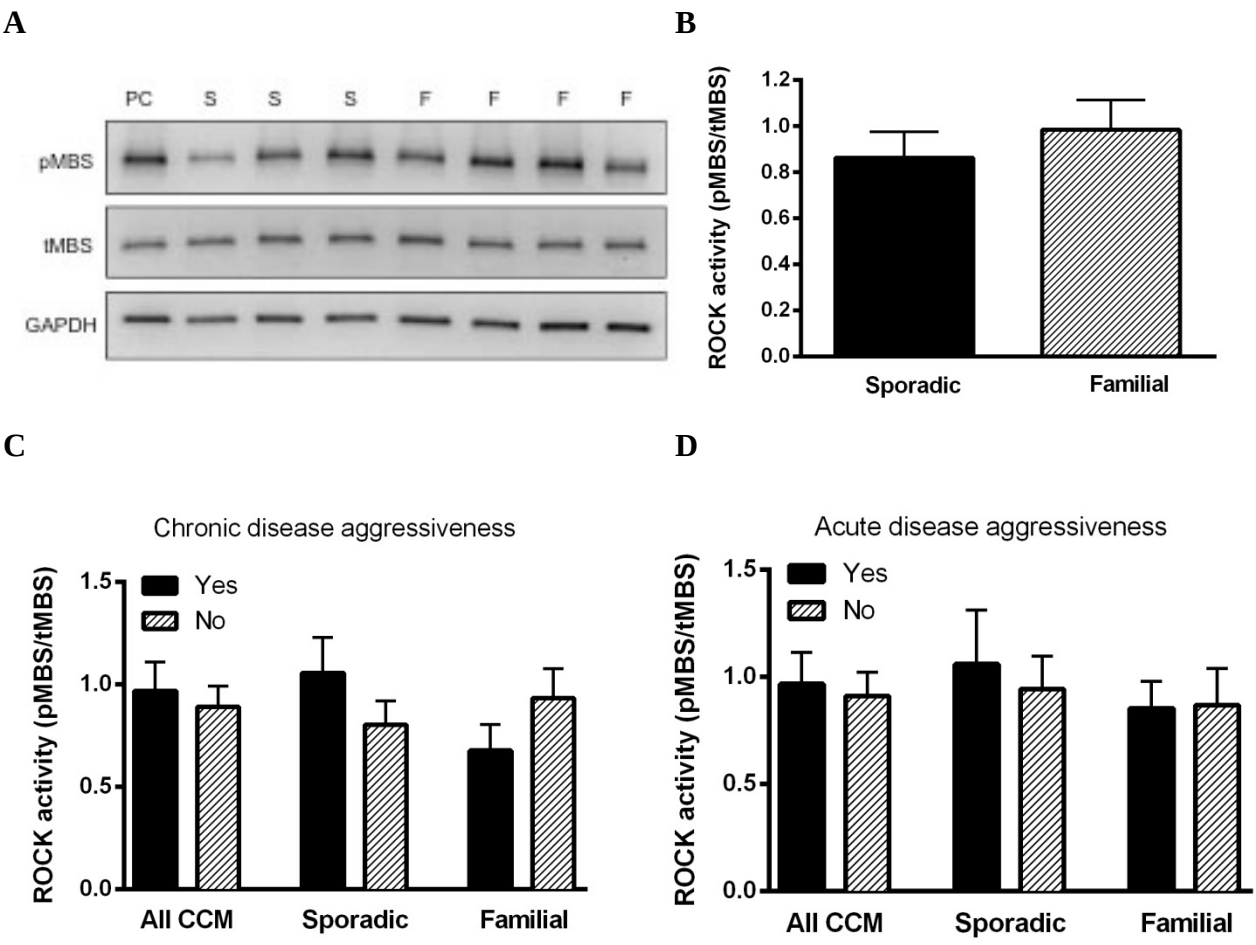
* denotes significant statistical significance in univariate correlation.

Table 1c. Correlations of demographic features and individual parameters of chronic and acute disease aggressiveness with biomarkers in the familial patient cohort.

	Total 25-(OH) vitamin D		Non-HDL cholesterol		HDL cholesterol		RhoA kinase activity		C-reactive protein	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
Demographic features										
Age at symptom onset	0.23	0.33	0.64	0.003*	0.55	0.01	0.41	0.17	0.40	0.10
Season	-0.58	0.004*	0.03	0.90	0.07	0.74	0.60	0.02	0.39	0.08
Gender	0.13	0.56	0.40	0.06	-0.19	0.39	-0.27	0.34	-0.09	0.71
Seizures	-0.06	0.79	-0.34	0.11	-0.30	0.16	-0.16	0.57	-0.16	0.50
Chronic disease aggressiveness										
# of SWI lesions >25	-0.16	0.45	0.10	0.65	0.006	0.98	-0.23	0.44	-0.04	0.87
# of T2-weighted lesions >5	-0.08	0.71	0.06	0.79	0.12	0.57	-0.23	0.44	-0.27	0.23
> 1 hemorrhage	-0.11	0.60	-0.15	0.50	-0.02	0.92	-0.15	0.60	-0.24	0.30
Age at symptoms ≤18 years old	-0.15	0.51	-0.72	0.0004*	-0.25	0.28	-0.36	0.22	-0.48	0.04
Acute disease aggressiveness										
Hemorrhage within 1year	0.09	0.69	0.20	0.37	0.06	0.78	0.15	0.64	-0.15	0.54
Lesional growth within 1 year	-0.28	0.22	-0.19	0.40	-0.09	0.70	-0.11	0.70	-0.18	0.46
New lesion within 1year	-0.16	0.53	0.23	0.35	0.07	0.79	-0.19	0.58	-0.18	0.50

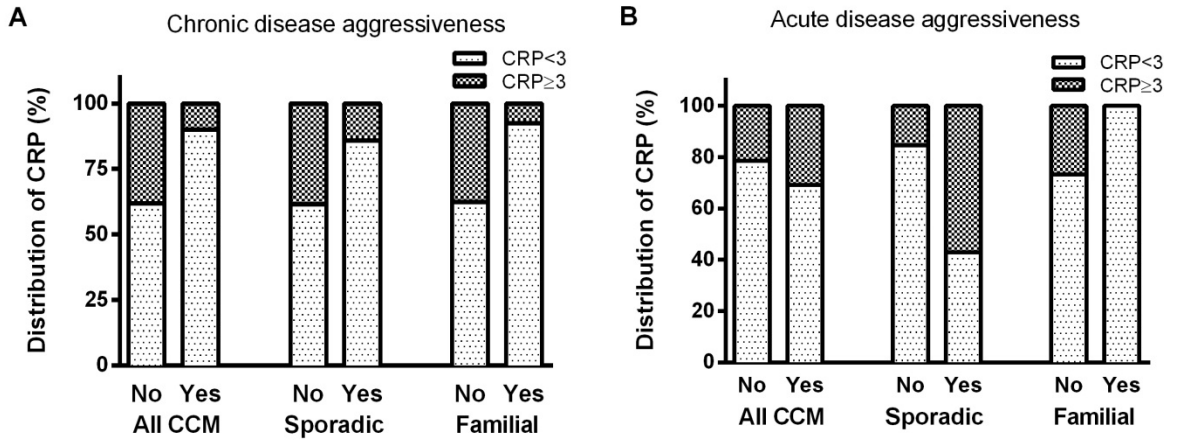
* denotes significant statistical significance in univariate correlation.

Supplementary Figures



Supplementary Figure 1. Peripheral blood leukocyte ROCK activity in CCM subjects with and without chronic or acute disease aggressiveness. (A) Representative immunoblot. (**B)** ROCK phosphorylates myosin-binding subunit. ROCK activity was calculated by using the ratio of pMBS/tMBS normalized to the pMBS/tMBS ratio of the positive control. There was no difference in ROCK activity of peripheral blood leukocytes in familial (N=11) versus sporadic (N=14) CCM patients. (**C)** There was no difference in leukocyte ROCK activity in chronic or no disease aggressiveness in total (N=13, N=12, respectively), familial (N=10, N=4, respectively), except in sporadic (N=3, N=8, respectively) CCM subjects. D. There was no difference in leukocyte ROCK activity in acute or no disease aggressiveness in total (N=9, N=16, respectively), familial (N=5, N=9, respectively) or sporadic (N=4, N=7, respectively) CCM patients.

PC: positive control; S: sporadic patient; F: familial patient; pMBS= phosphorylated myosin binding subunit; tMBS: total phosphorylated myosin binding subunit; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ROCK: Rho kinase; CCM: cerebral cavernous malformation.



Supplementary Figure 2. Plasma CRP levels in CCM subjects with and without chronic or acute disease aggressiveness. (A) There was no statistical difference in CRP values between chronic or no disease aggressiveness in total (N=20, N=21, respectively), familial (N=13, N=8, respectively) or sporadic (N=7, N=13, respectively) CCM subjects. (**B)** There was no difference in CRP values in acute or no disease aggressiveness in total (N=13, N=28, respectively), familial (N=6, N=15, respectively) or sporadic (N=7, N=13, respectively) CCM patients.
CRP: C-reactive protein; CCM: cerebral cavernous malformation.