Circulating biomarkers in familial cerebral cavernous malformation

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Summary

Background Cerebral Cavernous Malformation (CCM) is a rare cerebrovascular disease, characterized by the presence of multiple vascular malformations that may result in intracerebral hemorrhages (ICHs), seizure(s), or focal neurological deficits (FND). Familial CCM (fCCM) is due to loss of function mutations in one of the three independent genes *KRIT1* (*CCM1*), *Malcavernin* (*CCM2*), or *Programmed Cell death 10* (*PDCD10/CCM3*). The aim of this study was to identify plasma protein biomarkers of fCCM to assess the severity of the disease and predict its progression.

Methods Here, we have investigated plasma samples derived from n = 71 symptomatic fCCM patients (40 female/31 male) and n = 17 healthy donors (HD) (9 female/8 male) of the Phase 1/2 Treat_CCM trial, using multiplexed protein profiling approaches.

Findings Biomarkers as sCD14 ($p = 0.00409$), LBP ($p = 0.02911$), CXCL4 ($p = 0.038$), ICAM-1 ($p = 0.02013$), ANG2 ($p = 0.026$), CCL5 ($p = 0.00403$), THBS1 ($p = 0.0043$), CRP ($p = 0.0092$), and HDL ($p = 0.027$), were significantly different in fCCM compared to HDs. Of note, sENG ($p = 0.011$), THBS1 ($p = 0.011$) and CXCL4 ($p = 0.011$), were correlated to CCM genotype. sROBO4 ($p = 0.014$), TM ($p = 0.026$) and CRP ($p = 0.040$) were able to predict incident adverse clinical events, such as ICH, FND or seizure. GDF-15, FLT3L, CXCL9, FGF-21 and CDCP1, were identified as predictors of the formation of new MRI-detectable lesions over 2-year follow-up. Furthermore, the functional relevance of *ang2, thbs1, robo4* and *cdcp1* markers was validated by zebrafish pre-clinical model of fCCM.

Interpretation Overall, our study identifies a set of biochemical parameters to predict CCM progression, suggesting biological interpretations and potential therapeutic approaches to CCM disease.

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The Treat_CCM trial is registered at EudraCT (2017-003595-30) and ClinicalTrials.gov (NCT03589014) and has been closed by December 2021.

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Research in context

Evidence before this study

We searched PubMed from inception to 20 July 2023, for "cerebral cavernous malformations and biomarkers" without language restriction. We found three articles by citation matching, published in a period 2014–2018. Moreover, we have extended our research using the search terms "cerebral cavernous malformations biomarkers": 113 publications were retrieved in a period 1995–2023, focused on different type of biomarkers such as miRNAs, proteins, metabolites and imaging criteria. Regarding protein plasma biomarkers, different key studies have pointed out that blood levels of proteins involved in inflammation and angiogenesis were significantly deregulated in patients affected by cavernous angiomas symptomatic hemorrhage (CASH). Although certain biomarkers of inflammation are well documented in CCM with a cavernous angiomas symptomatic hemorrhage (CASH), there is still a significant gap in knowledge regarding inflammatory circulating biomarkers with predictive and prognostic role in CCM.

To our knowledge, the evaluation of the role of circulating biomarkers in a on a non-selected cohort of patients affected by familial cavernous malformation (fCCM), followed up to two years, and on matched healthy controls, is not yet documented.

Added value of this study

This is an exploratory study analysing circulating biomarkers over two years, to characterize CCM patients and to define subjects prone to clinical events, such as of symptomatic intracerebral hemorrhage (ICH), focal neurological deficit (FND) and epileptic seizures, in a on a non-selected cohort of patients affected by familial cavernous malformation (fCCM). In details, we found that: *i)* patients with fCCM have higher levels of circulating biomarkers that are associated with inflammation and angiogenesis in comparison with healthy donors; *ii)* there is a panel of biomarkers able to subclassify patients by CCM genotype; *iii)* there is a panel of proteins useful to predict adverse clinical events; *iv)* the formation of new-MRI detectable lesions over a two year follow-up is detectable by a pool of proteins. Furthermore, we also tested candidate genes in CCM models in zebrafish to provide further evidence for an evolutionary-conservation of particular biomarkers, which lends further importance to their relevance. Finally, deep proteomics profiling of plasma samples was performed using multiple platforms and high-plex proteomics innovative technologies as the Proximity Extension Assay (PEA).

Implications of all the available evidence

Altogether, the findings from other studies and the present analysis, suggest that peripheral levels of inflammatory and angiogenic molecules may be used as potential diagnostic and predictive biomarkers in CCM disease, paving the way for the identification of actionable targets and overall promoting an improvement in CCM clinical management.

Introduction

Cerebral Cavernous Malformations (CCMs), also known as cavernomas, are capillary-venous malformations, characterized by mulberry-like lesions located in the central nervous system as brain and spinal cord.[1,2] CCM lesions are leaky and prone to rupture, leading to epileptic seizures, intracerebral hemorrhages (ICHs) and focal neurological deficits (FNDs). This neurovascular disease occurs in sporadic and familial forms. The familial form of CCM (fCCM) is inherited in an autosomal dominant manner with an overall prevalence of 1:10,000, and is characterized by multifocal lesions that increase in number and size during a patients' lifetime.[3] In contrast, sporadic CCM, with a prevalence of 1:100–1:200, occurs in the majority of cases as a single vascular lesion. FCCM has been demonstrated to be caused by germline heterozygous loss of function mutations in one of three CCM proteins, that form a trimeric complex linked to endothelial cell (EC) adherens junctions. This CCM signaling complex comprises *CCM1 (KRIT1)*, *CCM2(OSM)* and *CCM3(PDCD10)*.[3–5] Remarkably, PDCD10/CCM3 is a multifaceted gene that is functional in the canonical CCM1 and CCM2 complex and in other biological processes such as the regulation of cell cycle, neuronal cell migration and tumorigenesis.[6] In murine CCM models as well as in human patients, ECs lining the cavernomas, present with an endothelial-to-mesenchymal transition (EndMT) phenotype,[7] characterized by a loss of EC junctions, increased proliferation, and an acquisition of mesenchymal-like properties. Cavernomas arise when a loss of *CCM* genes causes a few endothelial progenitor cells to undergo an uncontrolled clonal expansion, breakdown of mural cell association and recruitment of healthy neighboring ECs.[8,9]

At present, the available curative treatment to this disease is limited to surgical resection, while the role of radiosurgery is debated. Surgery is an invasive approach and can carry significant complications, particularly when the lesion is located in deep brain structures like the brainstem. Hence, an effective pharmacological therapy for this disease is urgently missing.[2,10,11] Treatment of CCM patients and preclinical studies with different murine CCM models have pointed at propranolol, a non-selective β-adrenergic receptor blocker, in reducing and stabilizing vascular lesions, which reduced the risk of intracerebral hemorrhages.[12–14] Recently, we have conducted, a randomized, open-label, blinded-endpoint phase 1/2 pilot trial, entitled "Treat_CCM". We found that propranolol was safe and well-tolerated in familial CCM patients. It suggested that propranolol might be beneficial for reducing the incidence of clinical events.[15,16]

Currently, the diagnosis of CCM severity and progression is mainly based on MRI. Because of the recurring nature of this pathology, patients may live with high levels of anxiety and would benefit from measurable plasma biomarkers to help guide therapeutic decision making and predict clinical outcomes. However, the low prevalence of the disease and the incidence of CCM-related clinical adverse events consistently reported in the range of 2–4%,[11,16–18] markedly limits the accuracy of prediction.

Plasma biomarkers would facilitate the surveillance of CCM disease and complement MRI-based diagnostics. So far, only few published studies addressed the clinical value of circulating biomarkers in CCM.[19,20] This highlighted the need to further investigate biomarkers in human liquid biopsies from patients.[19,21,22]

We have explored circulating peptides using targeted and untargeted approaches in parallel. Firstly, based upon systematic review of the literature, 17 circulating biomarker candidates were identified as proteomic signatures of CCM for analysis: roundabout guidance receptor 4 (ROBO4), thrombospondin 1 (THBS1), platelet factor 4/PF4 (CXCL-4), thrombomodulin (TM), pentraxin 3 (PTX3), cluster of differentiation 14 (sCD14), vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM1/CD54), endoglin/CD105 (ENG), lipopolysaccharide binding protein (LBP)' chemokine (C–C motif) ligand 5 (CCL5), C reactive protein (CRP), angiopoietin-2 (ANG2), tissue factor, total cholesterol, HDL (high-density lipoprotein) cholesterol and triglycerides. In addition, a large number of peptides were assayed in plasma by Olink protein biomarker panels, based on a proximity extension assay technology (PEA). Given that, zebrafish are being seen as a powerful model of genetic human diseases with increasing utility in translational research, we also tested candidate genes in CCM models in zebrafish to provide further evidence for an evolutionary-conservation of particular biomarkers, which lends further importance to their relevance. We find that a combination of plasma biomarkers could improve the prediction of CCM clinical events.

Materials and methods

Study design

Treat_CCM was a phase 1/2, randomized, open-label clinical trial conducted in six Italian hospitals[15,16] (*Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico (Milano), Fondazione Policlinico Universitario A. Gemelli (Roma), ASST Grande Ospedale Metropolitano Niguarda (Milano), Fondazione IRCCS Istituto Neurologico Carlo Besta (Milano), Fondazione IRCCS Casa Sollievo della Sofferenza (San Giovanni Rotondo), IRCCS Centro Neurolesi "Bonino Pulejo", Messina*). The study protocol and clinical trial have been published,[15,16] EudraCT, 2017-003595-30, and ClinicalTrials.gov, NCT03589014. In short, patients with symptomatic familial CCM aged ≥18 years were randomized 2:1 to propranolol (20 up to 320 mg daily) on top of standard care or standard care alone for two years (Supplementary Table S1). Patients underwent brain MRI and clinical visit at baseline, 12 and 24 months. New occurrence of symptomatic intracerebral hemorrhage (ICH) or focal neurological deficit (FND) were evaluated as primary outcomes of the study.

Ethics statement

The Treat_CCM trial is registered with EudraCT, 2017-003595-30, and ClinicalTrials.gov, NCT03589014. Written informed consent was obtained from all participants and the study was approved by local research ethics committees for each study site in accordance with the Declaration of Helsinki. The study was approved by AIFA (Agenzia Italiana del Farmaco, AIFA/SC/P/19831).

Cohort of study

The current work is based on an analysis of peripheral blood samples from n = 71 patients with fCCM collected at baseline and 2 years of follow-up. In addition, 17 samples from healthy controls, balanced for age and sex, were collected at the Neurology Department of Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico-Milano (Italy) (Supplementary Table S2). After collection, blood samples were centrifuged within 60 min at 4 °C for 15 min at 2000×*g*, EDTA plasma was stored at −70 °C in a certified ISO 9001:2015 biobank (SATURNE, Institute for Pharmacological Research Mario Negri IRCCS, Milano, Italy), until described analyses were performed.

Cytokine measurements

Quantification of soluble inflammatory mediators in the plasma (ELLA)

Cytokines were assayed in plasma by the multi-analyte microfluidic ProteinSimple Ella detection system

(product no. 600-100, ProteinSimple, Bio-Techne, MN, USA), by adding 50 µL of each sample and running chip within cartridges in triplicate following the manufacturer's instructions. Briefly, plasma samples were thawed on ice and then diluted 1:50 (ANG2, ENG, CCL5 and THBS1; kit #174393), 1:200 (VCAM-1 and ICAM-1; kit: #174379) and 1:2000 (sCD14, LBP; kit: 174383). Simple Plex Runner Software and the Simple Plex Explorer software were used for analysis.

Quantification of soluble inflammatory mediators in plasma (Luminex)

Custom panels sROBO4 (1:2; Kit# L141254), TM (1:2), CXCL4 (1:200; Kit# L141255) and Tissue Factor (1:2; Kit# L141254) were quantified, according to manufacturer recommendations, using a bead-based multiplexed ELISA assay (R&D System Inc., MN) on a Luminex 200 platform (Luminex, TX) available at the Istituto Europeo di Oncologia, Milan (Italy). Details of the assay are reported in the Supplement.

Quantification of soluble PTX3 in plasma (ELISA)

PTX3 plasma levels were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) developed in Humanitas Research Hospital, in according to previously described protocol.[23] Details of the assay are reported in the Supplement.

Olink multiplex proteomics assay

Olink® assay and clinical chemistry assays are described in the Supplement. The Inflammation (*#95302* Olink Proteomics, Sweden) and Cardiovascular III (*#95611* Olink Proteomics, Sweden) Olink® Target 96 protein biomarker panels were used to quantify proteins by the Proximity Extension Assay (PEA) technology, which use qPCR to measure the relative changes in protein expression. The expression data is normalized by Olink® using inter-plate controls as reported in (https://olink.com/application/data-normalization-and-standardization/) and output as "Normalized Protein Expression" (NPX) in Log2 scale.

Clinical chemistry

Plasma samples were analysed on Cobas pro analyser (Roche Diagnostics) in the Hospital PIO XI Laboratory, Desio, Italy. Total cholesterol, HDL cholesterol and triglycerides were analysed by enzymatic-colorimetric methods. CRP was assayed using immunoturbidimetric method.

Statistical analysis

Differences in biomarker concentration between groups of patients were assessed with Kruskal–Wallis test and adjusted for FDR. The correlations between circulating biomarkers were analysed with Spearman's rank correlation coefficient. The discriminatory value of each biomarker with clinical variables was assessed on the basis of the area under the curve (AUC) of ROC (receiver operating characteristic curve) analysis. Olink® Target 96 protein biomarkers of the Inflammation and Cardiovascular III panels were applied for identification of predictive protein biomarkers for CCM progression. OLINK Target 96 protein biomarkers of the Inflammation and Cardiovascular III panels were applied for identification of predictive protein biomarkers for CCM progression. We leveraged the information from two time points, baseline and 2-years follow-up, and hypothesized that predictive protein biomarkers were differentially expressed at both time points. A 2-fold identification strategy, therefore, was designed for predictive protein biomarker discovery. In first phase of biomarker discovery, we compared two groups between at least and less than five new CCM lesion at both baseline and 2-year follow-up respectively, the protein biomarkers which were differentially expressed (p value < 0.05) at both time points were taken as predictors in the subsequently predictive modelling. Logistic regression modelling was then applied to develop a predictive model for discrimination between at least and less than five new CCM lesions, and to identify a predictive protein biomarker or a predictive protein biomarker panel consisting of a small number of predictors using baseline data. To jointly evaluate the prediction performance, the following cross-validation strategy was applied: the samples were randomly divided into one training set (80% of samples) to train the model and one test set (20% of samples) used for prediction performance assessment. This process was repeated for 10 random partitions of the samples into training and test sets. The final prediction accuracy was calculated as the percentage of correctly classified samples across the 10 cross-validation rounds. For best predictor(s) selection, we firstly identified significant predictor variable(s) ($p \leq 0.05$) associated outcome in multiple logistic regression model. Then, we fit logistic regression models sequentially for at least one significant predictor and each possible combination of all other predictors with it, and prediction performance were evaluated using cross-validation as mentioned above. The final model with best variable(s) was the one which gave the highest predictive accuracy and sensitivity. Analysis was performed in the R software (version 4.2.1). AUCs under the ROC curves were calculated using the pROC package (version 1.18.0) and violinplot were produced using ggplot2 package (version: 3.4.0).

Transcriptomic analysis of zebrafish models of CCM

A previously published ccm2 mutant zebrafish dataset[24] was data mined for misregulation of the identified biomarkers. The dataset is based on *ccm2*^{m201} zebrafish whole heart tissue at 72hpf. For identification of enriched biological processes, a functional annotation clustering analysis using DAVID was performed.[25,26] Terms and genes were considered significant with $p < 0.05$.

Role of funders
This study is independent from funding in terms of study design, data collection, experimental workflow, interpretation and writing manuscript.

Results

Baseline and two years patients' characteristics
Overall, 71 patients with genetically confirmed fCCM participated in the Treat_CCM trial[15,16] and were enrolled and randomly assigned to receive propranolol plus standard care (n = 48; intervention group) or standard care alone (n = 23; control group)[16] (Supplementary Table S1). The mean age of participants was 45.5 years (SD 14.9) and 40 (56%) were female. Patients had a history of CCM-related recurrent headache (n = 54, 76%), intracerebral hemorrhage (n = 46, 65%), focal neurological deficit (n = 34, 48%), and epileptic seizures (n = 30, 42%), see Table 1. The median duration of follow-up was 764 days (IQR 736–808).

Patients with fCCM have higher levels of circulating biomarkers that are associated with inflammation and angiogenesis
To gain insight into the proteome landscape of CCM, high-throughput automated ELISA assays were performed on plasma samples. We compared such samples that had been collected at the Treat_CCM clinical trial at baseline (T0) and after 2 years (T2). Our study is schematized in Fig. 1a.

We found no differences in the baseline characteristics due to study treatment. In addition, there was no effect of propranolol treatment on biomarker concentrations after 2 years of follow-up, allowing us to study the 71 patients as a single cohort, independent of the treatment. The measured levels of plasma biomarkers are listed in Table 2. Concentrations of circulating biomarkers in patients and healthy controls at baseline are shown in Table 3. Interestingly, molecules involved both in inflammation and angiogenesis, such as sCD14 ($p = 0.004$), LBP ($p = 0.029$), ICAM-1 ($p = 0.020$), CCL5 ($p = 0.004$), THBS1 ($p = 0.004$) and CRP ($p = 0.009$) were significantly higher in fCCM patients in comparison with healthy donors. On the other hand, ANG-2 ($p = 0.026$) was higher in the healthy control group (Fig. 1b). As can be seen in the heat-map (Fig. 1c), most of the candidate biomarkers were independent from each other. At baseline, a strong correlation was found for sCD14 and LBP (r = 0.77, 95% CI: 0.65–0.85) and for THBS1 with CXCL4 (r = 0.60, 95% CI: 0.41–0.74) and CCL5 (r = 0.85, 95% CI: 0.77–0.91). In addition, a correlation was also found between ICAM-1 and VCAM-1 (r = 0.52, 95% CI: 0.32–0.68) and CCL5 and CXCL4 (r = 0.55, 95% CI: 0.34–0.70).

These findings were consistent with previous reports on increased angiogenesis signaling and inflammation associated with CCM.[27] In zebrafish *ccm2*^{m201} mutants, expression levels of *ang2* mRNA were increased compared with wild-type controls (Supplementary Table S3).[24,28]

Clinical parameters	fCCM N = 71
Age (year)	45.5 ± 14.9
Sex (female)	40 (56%)
BMI (kg/m^2)	24.0 ± 3.6
Systolic blood pressure (mmHg)	122 ± 14
Diastolic blood pressure (mmHg)	79 ± 8
Heart rate (bpm)	71 ± 12
Intra cerebral hemorrhages	46 (65%)
Focal Neurological deficit	34 (48%)
Seizure	30 (42%)
Headache	54 (76%)
Cutaneous angiomas	25 (35%)
Mutation	
KRIT1/CCM1	54 (76%)
MGC4607/OSM/CCM2	12 (17%)
PDCD10/CCM3	5 (7%)
Hypertension N (%)	15 (21%)
Diabetes N (%)	1 (1%)
Hypercholesterolemia N (%)	10 (14%)
Ischemic heart disease N (%)	1 (1%)
Antiepileptic treatment	33 (47%)
NSAID treatment	1 (1%)
Antihypertensive treatment	16 (23%)
Antidepressant treatment	9 (13%)

Table 1: **Baseline characteristics of 71 patients with fCCM included in the analysis.**

Expression levels of THBS1, CXCL4 and ENG by CCM genotype
We wondered whether the biomarkers were differentially expressed according to the genetic background of fCCM patients. Among 71 patients of this cohort, 76% carried a mutation in *CCM1*, 17% in *CCM2* and 7% in *CCM3*. Despite the long-standing notion that a loss of any single one of the three proteins CCM1, CCM2 or CCM3 results in the development of CCM, we found that several biomarkers strictly correlated with the CCM genotype. CCM2 patients were characterized by lower levels of THBS1 ($p = 0.011$), CXCL4 ($p = 0.011$) and sENG ($p = 0.011$) (Table 4 and Fig. 2a–c). Of note, a downregulation of *thbs1b*, *thbs2b*, and *thbs3a/b* gene expression was also detected in *ccm2*^{m201} mutant zebrafish (Supplementary Table S3). Patients with high blood levels of THBS1 tended also to have high levels of CXCL4 (Fig. 1b). This suggested that inflammation and matrix mechano-transduction is involved in the process of CCM formation. Most importantly, these findings might indicate that specific signaling pathways are disregulated in distinct fCCM genotypes.

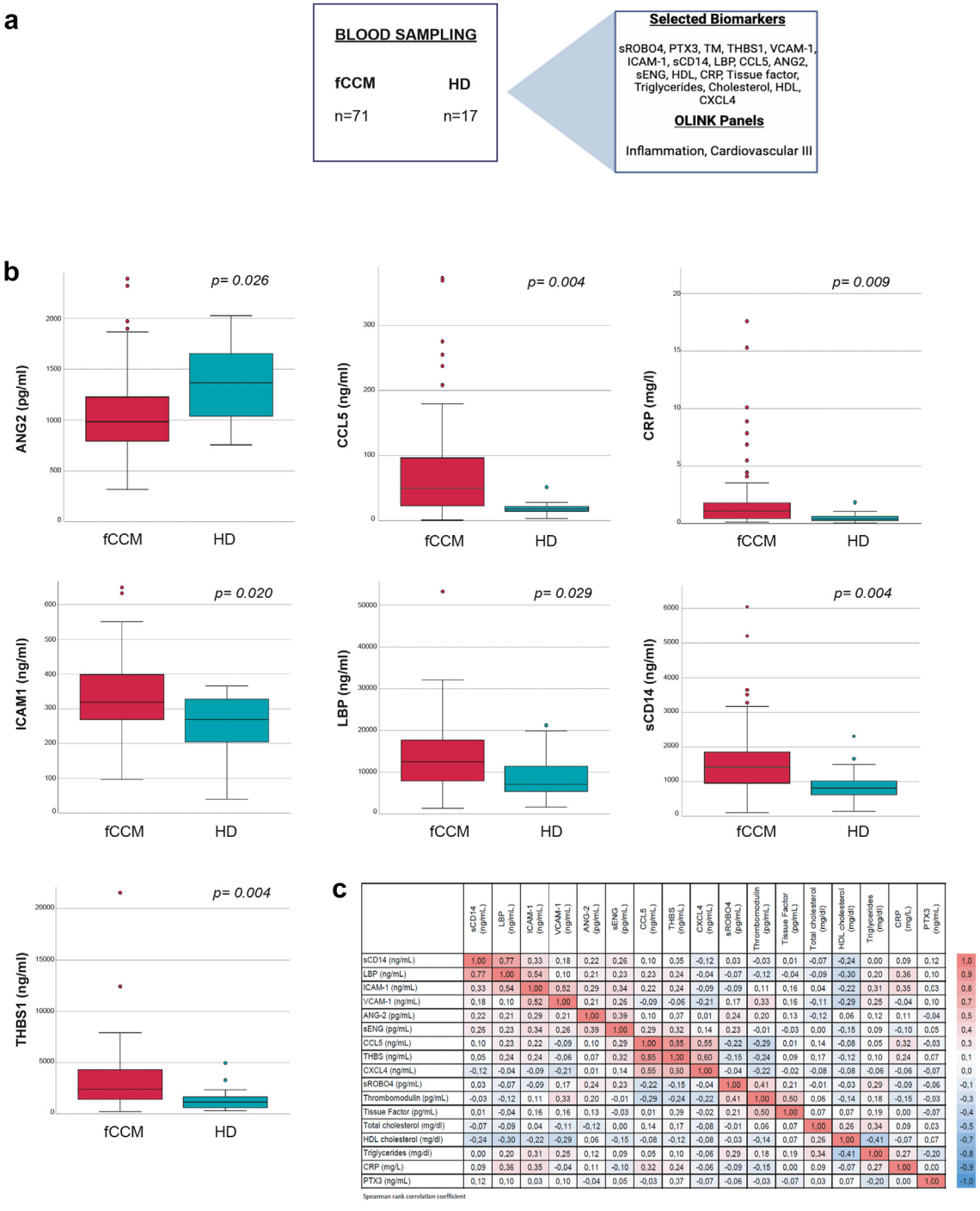

Fig. 1: **Workflow, sample information and expression of inflammatory proteins in fCCM patients (a)** A schema depicting the workflow of this study. 71 fCCM patients were enroled in Treat_CCM clinical trial. In addition, 17 healthy subjects were enrolled. Selected biomarkers and circulating proteins of Inflammation and Cardiovascular III Olink biomarker panels were analysed. **(b)** Box and whisker plots (box represent the interquartile range and outliers are 1.5 box lengths rom median) of the concentrations of plasma biomarkers. Among the 17 plasma molecules (n = 3 technical replicates), fCCM patients showed upper plasma levels of CCL5, CRP, ICAM1, LBP, sCD14, THBS1 and a lower level of ANG2. fCCM are represented by red box, and HDs by green box. p-values were calculated by means of Kruskall–Wallis test and account for false discovery rate (FDR). CCL5, Chemokine (C–C motif) ligand 5; CRP, C reactive protein; ICAM1, intercellular adhesion molecule 1; LBP, lipopolysaccharide binding protein; sCD14, cluster of differentiation 14; THBS1, thrombospondin1; ANG2, angiopoietin2. **(c)** Colored heatmap of the pair-wise Spearman's rank correlation coefficients computed for circulating plasma molecules. The colors refer to the correlation coefficient direction and magnitude, ranging from −1 (blue) to 1 (red).

Biomarker	Visit	N	Mean	Std dev	Median	Q1	Q3	Min	Max	Sensitivity	Assay range
sCD14 (ng/mL)	BL	71	1623	1016	1418	910	1864	107	6048	16.9 pg/mL	16.9–4130 pg/mL
	24 M	68	1920	923	1842	1235	2414	310	4605		
LBP (ng/mL)	BL	71	14,093	8565	12,457	7816	17,690	1306	53,279	98.3 pg/mL	98.3–150,000 pg/mL
	24 M	68	17,534	10,371	15,477	11,485	21,339	2407	66,179		
ICAM-1 (ng/mL)	BL	71	335	112	319	268	399	97	649	4.1 pg/mL	4.1–15,630 pg/mL
	24 M	68	365	178	354	268	437	44	923		
VCAM-1 (ng/mL)	BL	71	678	227	659	528	855	240	1154	53.7 pg/mL	137–83,490 pg/mL
	24 M	68	762	355	719	536	966	97	1687		
ANG2 (pg/mL)	BL	71	1061	415	984	789	1234	318	2390	9.91 pg/mL	9.91–15,124 pg/mL
	24 M	68	1128	505	1076	795	1478	111	2341		
sENG (pg/mL)	BL	71	3468	811	3454	2957	3787	1578	6374	15.4 pg/mL	15.4–147,150 pg/mL
	24 M	67	3506	1246	3662	2664	3972	1002	7486		
CCL5 (ng/mL)	BL	71	75.5	80.5	49.7	21.8	96.5	1.0	372.4	0.296 pg/mL	0.68–2600 pg/mL
	24 M	68	48.3	47.3	34.3	14.4	64.6	2.3	237.9		
THBS1 (ng/mL)	BL	71	3253	3090	2411	1421	4328	248	21,568	91.7 pg/mL	498–303,880 pg/mL
	24 M	68	3071	3068	2343	1222	3614	153	17,044		
CXCL4 (ng/mL)	BL	64	4305	4628	2997	1937	5102	296	31,047	25.2 pg/mL	370–90,000 pg/mL
	24 M	62	4351	3628	3357	1947	5582	17	18,747		
sROBO4 (pg/mL)	BL	71	41.9	36.5	28.8	18.7	50.5	3.6	191.6	1.5 pg/mL	53.5–13,000 pg/mL
	24 M	67	51.5	30.5	43.5	31.8	66.7	7.9	162.4		
TM (pg/mL)	BL	71	4620	1388	4374	3595	5561	2184	8229	7.06 pg/mL	86.4–21,000 pg/mL
	24 M	68	5365	1741	5235	3978	6568	2176	10,993		
Tissue Factor (pg/mL)	BL	71	26.9	8.5	24.3	19.8	32.2	9.8	61.9	0.237 pg/mL	4.94–1200 pg/mL
	24 M	68	40.4	18.8	35.8	29.1	47.3	12.02	123.9		
Cholesterol (mg/dL)	BL	71	181.7	31.8	179.0	161.5	201.0	112.0	271.0	3.86 mg/dL	3.86–800 mg/dL
	24 M	68	182.9	33.5	186.0	156.2	211.2	104.0	255.0		
HDL (mg/dL)	BL	71	54.9	15.4	53.6	42.9	61.8	30.7	103.0	3.09 mg/dL	3.09–150 mg/dL
	24 M	68	58.6	59.8	49.7	42.1	59.9	29.2	532.0		
Triglycerides (mg/dL)	BL	71	102.9	47.3	87.5	72.5	135.2	31.0	245.0	8.85 mg/dL	8.82–885 mg/dL
	24 M	68	118.9	54.3	104.8	80.5	156.5	39.0	315.0		
CRP (mg/L)	BL	71	1.7	3.2	0.8	0.4	1.7	0.04	17.6	0.3 mg/L	06–350 mg/L
	24 M	68	1.9	2.7	0.9	0.4	1.7	0.1	14.4		
PTX3 (ng/mL)	BL	71	2.9	1.6	2.7	1.8	3.7	0.8	9.55		
	24 M	68	2.9	1.9	2.3	1.8	4.0?	0.7	11.7	0.1 ng/mL	0.075–2.4 ng/mL

Table 2: **Targeted circulating biomarkers at baseline and 24 months.**

Blood levels of ROBO4, TM and CRP can predict adverse clinical events

Circulating blood biomarkers would be particularly useful when providing a means to predict disease progression and severity. To systematically assess such possibility, we precisely monitored incident adverse CCM-related clinical events that occurred among the patient cohort during the 24-month trial period. We considered the occurrence of new symptomatic ICH or FND as the primary outcome, while epileptic seizures were assessed as the secondary clinical outcome. These events were validated by an independent Event Committee blind to study treatment and patients' identity. During that trial period, clinical outcomes linked to CCM occurred in 7 out of 71 fCCM patients (n = 2 ICH; n = 2 FND; n = 3 seizures). Next, we investigated whether circulating biomarkers could be correlated with incident CCM-related adverse clinical outcomes. We found that the baseline plasma levels of sROBO4 ($p = 0.014$) and TM ($p = 0.026$) were higher in subjects who experienced clinical lesion events during the 24 months trial period. It will be an important question for future research whether this correlation is due to an involvement in endothelial cell dysfunction. Interestingly, sRobo4 homolog is also upregulated in *ccm2*^{m201} mutant zebrafish (Supplementary Table S3). We also identified the blood biomarker CRP ($p = 0.040$) to have reduced levels in patients who experienced CCM-related clinical events over two years (Table 5). The AUC values for ROC curve analysis were: 0.89 [0.79–0.98], 0.81 [0.69–0.94] and 0.85 [0.72–0.98] for sROBO4, CRP and TM, respectively (Fig. 3a–c).

	Familial	Healthy donors	p^a	p^b
	N = 71	N = 17		
sCD14 (ng/mL)	1418 [910–1864]	806 [575–1017]	0.0007	0.004
LBP (ng/mL)	12,492 [7816–17737]	7076 [5387–11566]	0.012	0.029
ICAM-1 (ng/mL)	335 ± 112 319 [268–399]	255 ± 87 266 [200–328]	0.006	0.020
VCAM-1 (ng/mL)	678 ± 227 659 [528–854]	563 ± 255 521 [372–669]	0.043	0.073
ANG2 (pg/mL)	984 [789–1234]	1367 [1016–1654]	0.009	0.026
sENG (pg/mL)	3454 [2957–3787]	3753 [3350–4029]	0.108	0.153
CCL5 (ng/mL)	49.7 [21.8–99.3]	15.9 [11.1–22.1]	0.0004	0.004
THBS1 (ng/mL)	2411 [1422–4328]	952 [445–1682]	0.0006	0.004
CXCL4 (ng/mL)	2997 [1917–5215]	1986 [1161–2930]	0.039	0.073
sROBO4 (pg/mL)	28.8 [18.7–50.5]	37.6 [27.8–55.6]	0.151	0.198
TM (pg/mL)	4620 ± 1388 4375 [3595–5562]	4844 ± 1099 4676 [3983–5579]	0.377	0.401
Tissue Factor (pg/mL)	24.3 [19.8–32.2]	27.3 [24.5–31.5]	0.313	0.355
Cholesterol (mg/dL)	182 ± 32 178 [160–202]	186 ± 30 186 [162–201]	0.627	0.627
HDL (mg/dL)	53 [43–62]	64 [51–83]	0.028	0.060
Triglycerides (mg/dL)	88 [73–136]	82 [55–98]	0.245	0.298
CRP (mg/L)	1.07 [0.42–1.83]	0.42 [0.24–0.74]	0.002	0.009
PTX3 (ng/mL)	2.8 [1.9–3.9]	2.1 [1.3–3.6]	0.078	0.121

Data reported as median [IQR]. [a]p-value for Kruskall–Wallis test. [b]p-value adjusted for FDR.

Table 3: **Baseline biomarker concentrations.**

Levels of circulating biomarkers can predict the formation of new MRI-detectable lesions during a 2-year follow-up

To assess whether circulating biomarker levels had a predictive value with respect to lesion formation, we monitored their development via MRI during the 2-year follow-up among 67 patients with complete MRI data. During that trial period, at least five new CCM lesions were detected in 32 participants. The other 35 patients developed less than five brain lesions each during a period of 2 years. The two circulating biomarkers PTX3 and THBS1 had a tendency to be expressed at lower levels in patients who developed more than 5 lesions; however, these results were not statistically significant after correction for multiple testing (Table 6).

	Mutations			p^a	p^b
	CCM1 (N = 54)	CCM2 (N = 12)	CCM3 (N = 5)		
sCD14 (ng/mL)	1440 [900–2148]	1341 [925–1671]	1416 [900–1833]	0.796	0.851
LBP (ng/mL)	12,996 [7676–18026]	11,541 [6880–16417]	15,672 [8773–19130]	0.677	0.851
ICAM-1 (ng/mL)	349 ± 115 339 [270–415]	294 ± 105 297 [217–348]	289 ± 30 286 [263–315]	0.159	0.338
VCAM-1 (ng/mL)	700 ± 234 673 [561–859]	636 ± 221 704 [428–784]	548 ± 49 527 [515–595]	0.232	0.394
ANG2 (pg/mL)	999 [781–1283]	910 [693–1201]	959 [938–1093]	0.718	0.851
sENG (pg/mL)	3546 [3165–3899]	2639 [2449–2994]	3457 [3103–3935]	0.0008	0.011
CCL5 (ng/mL)	59.9 [25.5–100.4]	18.1 [7.4–44.9]	55.2 [34.1–212.5]	0.020	0.085
THBS1 (ng/mL)	3312 [1507–4408]	1103 [486–1844]	4261 [2662–6631]	0.002	0.011
CXCL4 (ng/mL)	3068 [2058–5359]	1505 [497–3419]	8930 [4230–11840]	0.002	0.011
sROBO4 (pg/mL)	29.3 [18.6–51.1]	23.0 [16.5–47.2]	27.2 [21.5–72.9]	0.748	0.851
TM (pg/mL)	4459 ± 1368 4312 [3383–5433]	5434 ± 1097 5399 [4298–6307]	4413 ± 1796 3595 [3112–6123]	0.072	0.204
Tissue Factor (pg/mL)	24.5 [19.4–32.4]	22.7 [21.7–31.3]	24.3 [21.7–36.4]	0.827	0.851
Cholesterol (mg/dL)	180 ± 32 178 [159–200]	195 ± 28 197 [169–209]	164 ± 27 160 [143–188]	0.117	0.284
HDL (mg/dL)	53 [43–62]	54 [42–77]	52 [44–68]	0.851	0.851
Triglycerides (mg/dL)	90 [74–142]	95 [67–141]	75 [53–87]	0.231	0.394
CRP (mg/L)	0.86 [0.41–1.76]	1.26 [0.67–2.03]	1.27 [0.66–6.04]	0.485	0.750
PTX3 (ng/mL)	2.67 [1.98–3.89]	2.00 [1.18–3.46]	3.70 [3.14–4.18]	0.036	0.122

Data reported as median [IQR]. [a]p-value for Kruskall–Wallis test. [b]p-value adjusted for FDR.

Table 4: **Baseline biomarker concentrations by CCM mutation.**

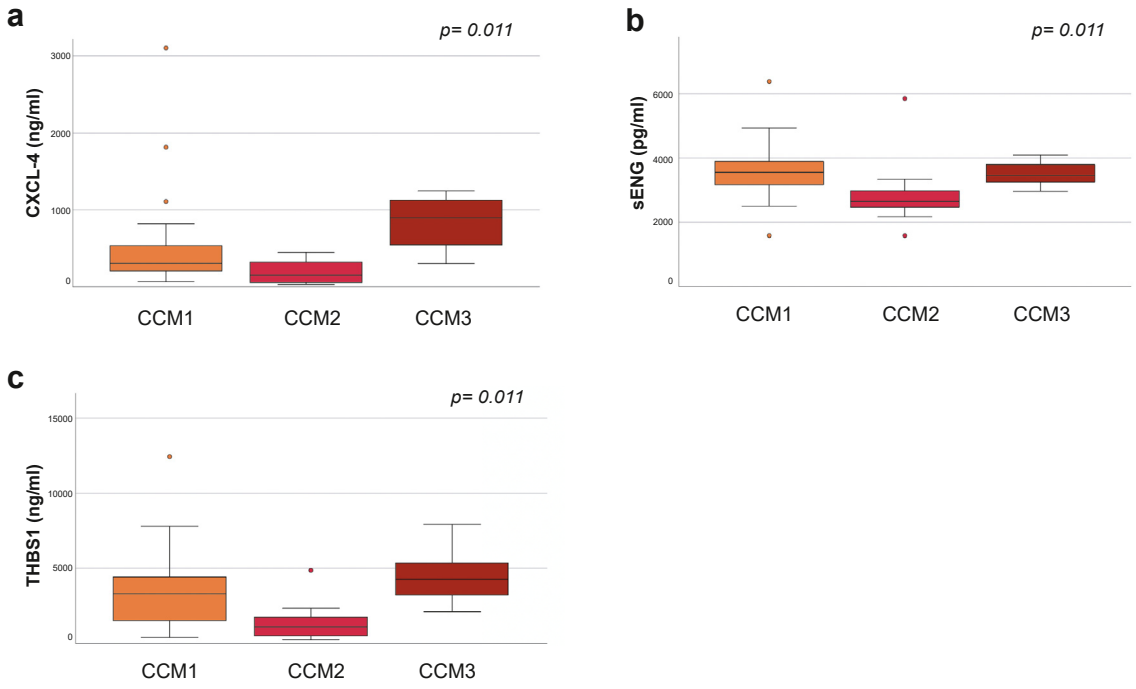

***Fig. 2:* Expression levels of biomarkers to stratify patients according to genotype.** Box and whisker plots (box represent the interquartile range and outliers are 1.5 box lengths from median) of the concentrations of plasma CXCL4 (a), sENG (b) and THBS1 (c) markers for each fCCM genetic group (n = 3 technical replicates). The central black lines show the median values, regions above and below these lines show the upper and lower quartiles, respectively. CCM1 is represented by orange box, CCM2 by light red and CCM3 by dark red boxes. The p-values were calculated by means of Kruskall–Wallis test and account for false discovery rate. CXCL4, chemokine (C-X-C motif) ligand 4; sENG, soluble endogline; THBS1, thrombospondin1.

	ICH, FND or seizure		p^a	p^b
	Yes (N = 7)	No (N = 64)		
sCD14 (ng/mL)	1213 [1105–1416]	1462 [872–2072]	0.335	0.539
LBP (ng/mL)	8461 [6571–9951]	13,939 [8014–18758]	0.023	0.098
ICAM-1 (ng/mL)	292 ± 61 269 [258–374]	340 ± 115 328 [274–412]	0.196	0.383
VCAM-1 (ng/mL)	816 ± 171 914 [607–964]	663 ± 228 640 [518–802]	0.054	0.167
ANG2 (pg/mL)	1034 [897–1184]	972 [772–1234]	0.589	0.715
sENG (pg/mL)	3787 [3327–4823]	3450 [2934–3765]	0.203	0.383
CCL5 (ng/mL)	44 [14–67]	53 [24–103]	0.385	0.545
THBS1 (ng/mL)	3210 [1001–4471]	2372 [1451–4321]	0.923	0.923
CXCL4 (ng/mL)	3901 [2266–10014]	2983 [1872–4675]	0.349	0.539
sROBO4 (pg/mL)	80 [50–151]	27 [18–46]	0.0008	0.014
TM (pg/mL)	6203 ± 1331 6357 [4900–7347]	4447 ± 1290 4262 [3415–5406]	0.003	0.026
Tissue Factor (pg/mL)	35 [22–42]	24 [20–32]	0.171	0.383
Cholesterol (mg/dL)	180 ± 38 160 [158–205]	182 ± 31 179 [163–201]	0.809	0.860
HDL (mg/dL)	49 [46–77]	53 [43–62]	0.671	0.760
Triglycerides (mg/dL)	82 [51–148]	88 [73–136]	0.469	0.613
CRP (mg/L)	0.40 [0.17–0.48]	1.17 [0.50–2.07]	0.007	0.040
PTX3 (ng/mL)	3.4 [2.6–5.2]	2.5 [1.9–3.7]	0.059	0.167

Data reported as median [IQR]. [a]p-value for Kruskall–Wallis test. [b]p-value adjusted for FDR.

***Table 5:* Baseline biomarker concentration by ICH, FND or seizure events during follow-up.**

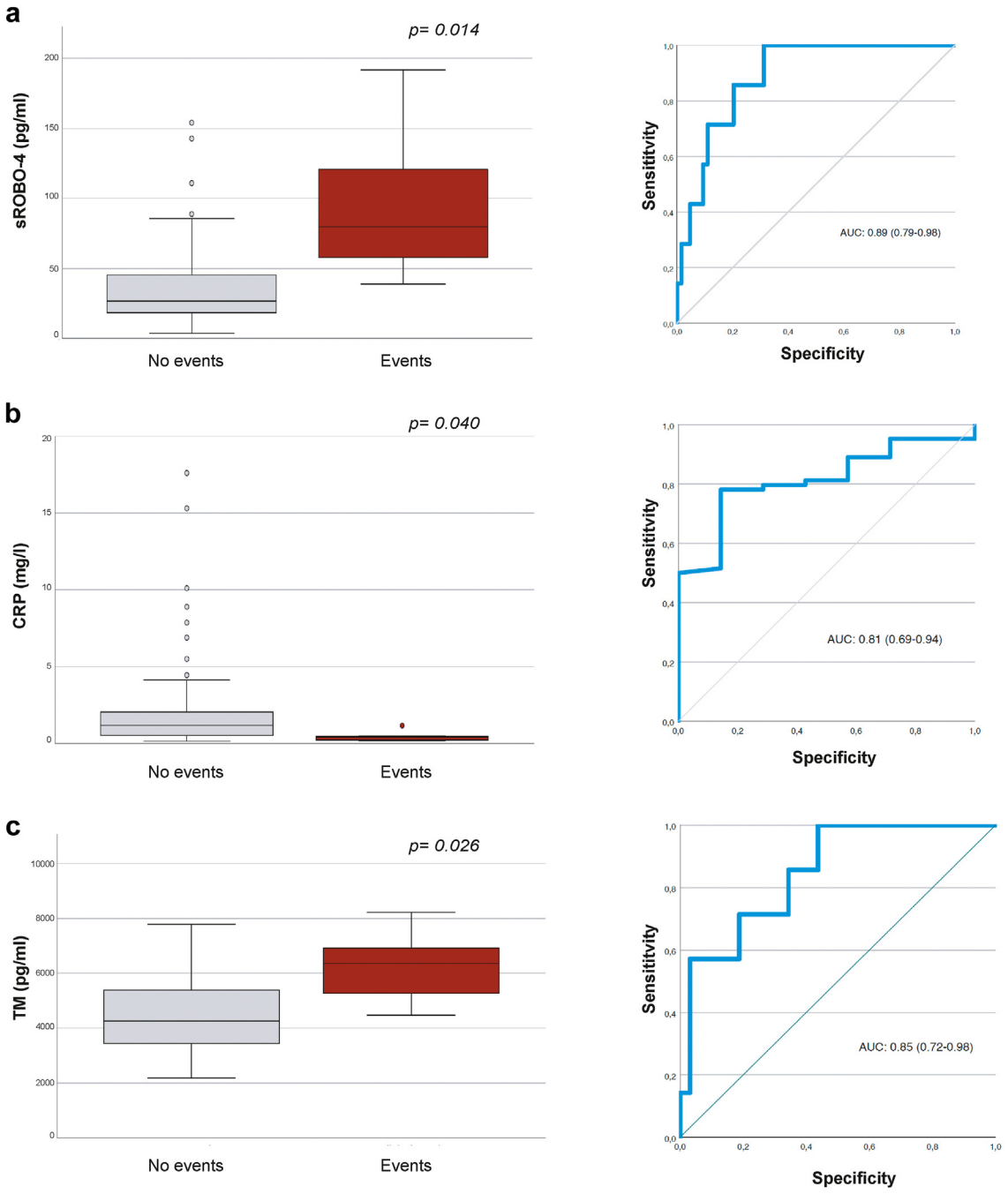

***Fig. 3*: Plasma concentrations of sROBO4, TM and CRP by incident adverse clinical events and ROC curve analyses.** Box and whisker plots (box represent the interquartile range and outliers are 1.5 box lengths from median) of the plasma concentrations of (a) sROBO4, (b) CRP and (c) TM in patients who had confirmed incident adverse CCM-related clinical events that occurred during the 2-years trial period (n = 3 technical replicates). The AUC ROC curve analyses of the differentiation between the participants who experienced clinical events versus patients without any clinical outcomes. The p-values were calculated by means of Kruskall Wallis test and account for false discovery rate (FDR).

To facilitate the identification of predictive molecular biomarkers for prognostic applications, we performed an exploratory study using a Proximity Extension Assay (PEA) approach on the Olink® Target 96 protein biomarker panels: Inflammation and Cardiovascular III. We constructed a logistic regression model to predict

	De novo lesions		p^a	p^b
	less than 5 new lesions n = 35	at least 5 lesions n = 32		
sCD14 (ng/mL)	1421 [1064–1864]	1396 [857–2261]	0.960	0.960
LBP (ng/mL)	13,501 [9564–19906]	11,588 [7224–16918]	0.380	0.618
ICAM-1 (ng/mL)	313 ± 97 301 [258–374]	362 ± 128 359 [254–448]	0.117	0.398
VCAM-1 (ng/mL)	643 ± 223 610 [497–792]	704 ± 232 676 [542–848]	0.400	0.618
ANG2 (pg/mL)	959 [786–1215]	1027 [773–1365]	0.498	0.644
sENG (pg/mL)	3476 [3091–3787]	3450 [2746–3839]	0.530	0.644
CCL5 (ng/mL)	60 [24–119]	39 [20–66]	0.085	0.398
THBS1 (ng/mL)	3794 [1558–4770]	1938 [1286–4554]	0.024	0.204
CXCL4 (ng/mL)	3637 [2266–5714]	2542 [1817–4554]	0.097	0.398
sROBO4 (pg/mL)	27 [19–47]	29 [18–54]	0.739	0.838
TM (pg/mL)	4407 ± 1331 4187 [3223–5610]	4735 ± 1315 4690 [4130–5505]	0.253	0.614
Tissue Factor (pg/mL)	23 [20–31]	26 [20–34]	0.350	0.618
Cholesterol (mg/dL)	183 ± 37 186 [160–204]	182 ± 28 178 [163–199]	0.821	0.872
HDL (mg/dL)	50 [40–68]	55 [44–62]	0.315	0.618
Triglycerides (mg/dL)	84 [65–143]	96 [78–133]	0.214	0.606
CRP (mg/L)	1.07 [0.33–2.51]	1.14 [0.55–1.82]	0.455	0.644
PTX3 (ng/mL)	3.24 [2.30–4.29]	2.25 [1.86–3.06]	0.009	0.153

Data reported as median [IQR]. [a]p-value for Kruskall–Wallis test. [b]p-value adjusted for FDR.

Table 6: **Baseline biomarker concentration by development of new lesions during follow up.**

the CCM progression (classification of patients with at least five new CCM or less than five lesions over 2 years of trial period). Cross-validation was applied to evaluate model performance and optimize model parameters; for the cardiovascular panel, the average sensitivity was estimated to 64.1% (95% CI: 50.0%–78.5%), specificity to 80.3% (95% CI: 71.3%–89.0%) and the AUC value was 0.75 (95% CI: 0.66–0.83) from ROC analysis with growth differentiation factor 15 (GDF-15) emerging as best predictor in the model (Fig. 4a, Supplementary Table S4). We further compared GDF-15 protein expression level at 2-year follow-up with the expression level at baseline for patients developing at least 5 new lesions and less than 5 lesions, respectively, it showed statistically significant for patients developing at least 5 new lesions using paired t-test (p = 0.006, log2 fold-change = 0.19 (95% CI: 0.06–0.32)). It is not significant for patients developing less than 5 lesions over two years (p = 0.83).

For the inflammation panel, Fms-related tyrosine kinase ligand 3 (FLT3L), chemokine ligand 9 (CXCL9), fibroblast growth factor 21 (FGF-21) and cub domain-containing protein 1 (CDCP1) were selected as a panel of four predictors (Fig. 4b). The average sensitivity of this panel of four protein biomarkers was 74.1% (95% CI: 68.0%–81.5%), the specificity 81.8% (95% CI: 76.3%–87.1%) and AUC was 0.80 (95% CI: 0.72–0.88) (Fig. 4c, Supplementary Table S5, Supplementary Fig. 1A–D: ROC curve of individual protein marker). We further compared the protein expression level of FLT3L, FGF-21, CXCL9 and CDCP1 at 2-year follow-up with their expression at baseline for patients developing at least 5 new lesions and less than 5 lesions, respectively. It showed that FLT3L (p = 0.01, log2 fold-change = 0.20 (95% CI: 0.05–0.34)) and CDCP1 (p = 0.03, log2 foldchange = 0.20 (95% CI: 0.02–0.38)) are statistically significant for patients developing at least 5 new lesions. FGF-21 showed significance (p = 0.05, log2 foldchange = 0.47, (95% CI: 0.00–0.94)) for patients with less than 5 new lesions. Other proteins showed no statistical significance (data not shown). Elevated expression levels of the zebrafish homolog *cdcp1* was also detected in $ccm2^{m201}$ mutants (Supplementary Table S3).

The correlations between FLT3L, CXCL9, FGF-21 and CDCP1 was accessed by Spearman's correlation and confirmed that the four protein biomarkers were weakly correlated ($r \leq 0.36$ 95% CI: 0.12–0.56 between FLT3L and CDCP1). Hence, the combination of these proteins has a good power in the prediction of new lesion formation.

Discussion

It would be highly desirable to have easily accessible biomarkers to predict the progression of CCM disease and to uncover new aspects of CCM biology. This information would hopefully help in improving the clinical management of CCM patients and in finding the most appropriate pharmacological treatment for CCM disease. To this end, we systematically examined candidate inflammation- and angiogenesis-associated proteins as potential circulating blood biomarkers of fCCM patients enrolled in the Treat_CCM clinical trial.

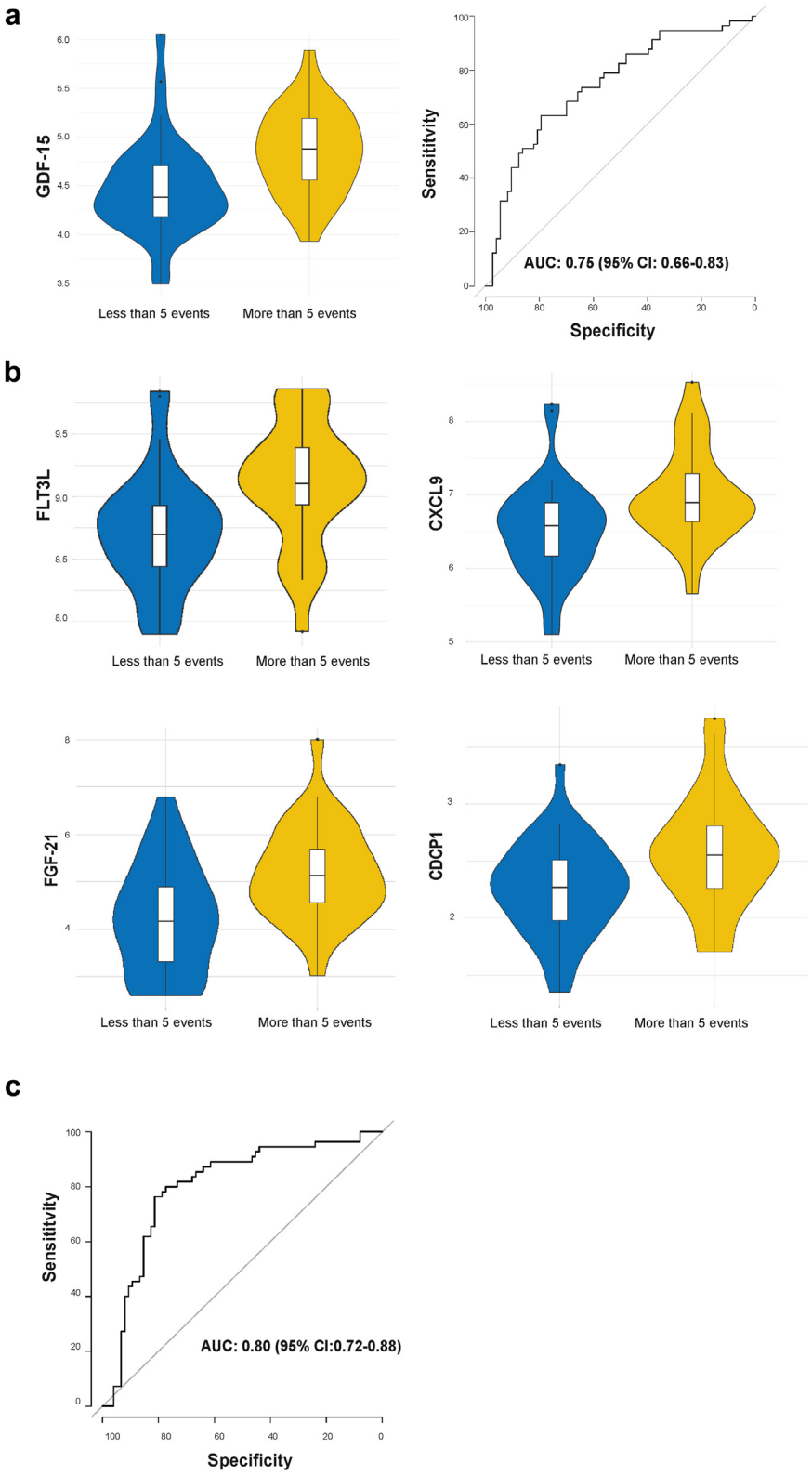

Fig. 4: **Plasma proteomic predictors of new MRI-detectable lesions**. (a) Violin plot of normalized protein expression (NPX) of GDF-15 for patients with more than 5 new CCM-MRI lesions and less than 5 lesions over 2 years of trial period; and ROC curve analysis for predictive model

In this study, the trend towards a clinical benefit with propranolol observed in the clinical trial[15,16] was not reflected by a significant modification in any of the tested biomarkers. This was possibly due to the small sample size and the possibility that other mechanisms of action of propranolol not mirrored by the peptides assayed are involved. This result allowed us to analyse the propranolol-treated and untreated patients as a single population. Therefore, in this study, we leveraged plasma biomarker measurement to characterize CCM patients at diagnosis and to define subjects prone to clinical events, such as ICH, FND and epileptic seizures.[19–22,29] Although certain biomarkers of inflammation are well documented in CCM patients with a cavernous angiomas symptomatic hemorrhage (CASH)[19,21,22,30,31] and in a homogeneous group of Hispanic fCCM patients and *Ccm* mutant mice,[32] as well as a specific panel of CCM etiological blood biomarkers associated with BBB disruption is pinpointed in *Ccm1-3* mouse models recently,[33–35] there is still a significant gap in knowledge regarding inflammatory circulating biomarkers with predictive and prognostic role in CCM.

Here, we have highlighted that blood levels of proteins involved in inflammation and angiogenesis, such as sCD14, LBP, CXCL4, ICAM-1, CCL5, THBS1 and CRP, were significantly higher in fCCM patients in comparison with healthy donors. The high blood levels of these circulating biomarkers pinpoint that the marked inflammatory and pro-angiogenic activation that contributes to the onset and progression of CCM[21,22,27] has a strong prognostic and diagnostic value. In comparison to another study by the team of Awad, which measured plasma biomarkers on CASH patients,[27,31] we focused on a non-selected cohort of fCCM patients and on balanced healthy controls. Awad and colleagues reported that low circulating levels of sCD14 was one of diagnostic and prognostic CASH biomarker. In our study, we found that blood levels of sCD14 and LBP were significantly higher in fCCM patients in comparison with healthy donors, which is in tune with the other studies that reported an involvement of inflammatory processes at CCM lesion sites.[36–38] Of note, our analysis revealed that blood levels of ANG2 and HDL were higher in the healthy control group. Whether this points to a protective role of these proteins is an interesting finding for future studies. This finding is rather surprising, as increased exocytosis of ANG2 has been shown to contribute to CCM in a preclinical mouse model of CCM3.[39] Even though the clinical manifestations of fCCM are highly heterogeneous, CCM3 patients have a more aggressive form of the disease with an earlier onset of intracerebral hemorrhages and multiple angiomas associated with the CCM phenotype as compared with CCM1 and CCM2.

The high blood levels of sENG, THBS1[30] and CXCL4 in CCM1 and CCM3 patients suggest that the inflammation and angiogenesis processes associated with CCM can have a diagnostic value. Surprisingly, we found that the incidence of CCM-related adverse events was similar between CCM1 and CCM2. Yet, CCM2 patients showed lower levels of all these circulating blood proteins. The potential of biomarker screening in CCM was further exemplified by our discovery that disease progression in patients who experienced clinical lesional activity such as ICH, FND or epilepsy during the 24 months following the initial blood sample, was predictable based on blood levels of proteins involved in vascular stability during pathological angiogenesis and inflammation processes, such as sROBO4 and TM.[31] Whether this indicates any functional relevance of these proteins in endothelial cell dysfunction and bleeding disorders remains to be tested. It should be noted that TM together with vWF has been described to contribute to vascular lesion and homeostasis heterogeneity as found in pre-clinical models of CCM3.[36] Our results are consistent with elevated levels of sROBO4 and TM during enhanced vascular leakage and seizure episodes, by tightly regulating expression of tight junction proteins. This finding also suggests that their expression levels in blood already rise before patients present with adverse clinical symptoms. The link between sROBO4 and incident hemorrhagic clinical events, confirms a previous study, which highlighted its overexpression in patients who experienced symptomatic hemorrhagic expansion within one year from blood draw.[21,27,31] However, we observed a decreased plasma level of CRP, and this shows a different trend from the other inflammatory markers found to be increased in CCM. Although not statistically significant after correction for multiple testing, we also observed that PTX3 and THBS1 had a tendency to be expressed at lower levels in patients who developed more than 5 lesions; the interplay between PTX3 and THBS1 that was demonstrated in synaptogenesis, will be further investigated in angiogenesis and CCM development.[40,41]

Furthermore, our targeted analysis using the proximity extension assay, revealed new biomarkers that might be of relevance in CCM such as GDF-15,[42]

using GDF-15 (derived from OLINK® cardiovascular panel) as a predictor to discriminate between patients with more than 5 new CCM-MRI lesions or less than 5 lesions over 2 years trial period. (b) Violin plot of normalized protein expression (NPX) of FLT3L, CXCL9, FGF-21 and CDCP1 for patients with more than 5 new CCM-MRI lesions and less than 5 lesions over 2 years of trial period. (c) ROC curve analysis for predictive CCM progression using a panel of predictors, FLT3L, CXCL9, FGF-21 and CDCP1 (derived from OLINK® inflammation panel) to discriminate between patients with more than 5 new CCM-MRI lesions or less than 5 lesions over 2 years trial period.

FLT3L,[43] CDCP1,[44] FGF-21[45,46] and CXCL9.[47] These six biomarkers possibly reflect the local accumulation of immune cells and point to vascular dysfunction, cell remodeling, oxidative stress, altered calcium signaling and inflammation in CCM lesions, suggesting their clinically role in future prospective studies.

The methodological strength of the present study is that it is based on plasma samples from all 71 patients included in the clinical trial Treat_CCM in six different Italian centers, the largest prospective trial in CCM to date. This reduces the potential bias associated to single-center studies. A major limitation is the sample size of patients, 71, and that of healthy donors, 17. The power was modest for predicting the endpoint of ≥5 new CCM lesions, and even more for incident adverse CCM-related clinical events: sROBO4, TM and CRP were significant even after FDR correction and the power was 98% for sROBO4, 88% for TM, and 16% for CRP. There are two draw backs of the low statistical power: (1) any analysis by subgroups suffers more markedly the inadequacy of power, and (2) analyses adjusted by several meaningful covariates are unlikely to reduce potential confounding. Last, propranolol did not change concentrations of any biomarker over 2-year treatment, this is not unexpected since several examples exist in the literature.[48–50]

In summary, this exploratory study analysing circulating biomarkers over 2 years suggests that peripheral levels of inflammatory and angiogenic molecules may be used as potential diagnostic and predictive biomarkers in CCM disease. Comparative analyses with zebrafish transcriptomic data suggest that there is conservation between patients and disease models, even in different tissues as compared in this study. Further analysis on protein level at later developmental stages when the zebrafish immune system is fully developed, will provide a clearer picture of the level of conservation. Follow-up experiments are urgently needed to validate the potential role of biomarkers in preclinical CCM disease models. Here, we provide evidence that this will be feasible in the near future.

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PEA data analysis: Y.S.
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All authors read and approved the final version of the manuscript. RL supervised the study and had final responsibility for the decision to submit for publication. R.L., F.L. and J.M.T.A.M. verified all data. Y.S. verified raw PEA data. All authors had full access to all the data and accept responsibility for the decision to submit for publication.

Data sharing statement

The data are stored at the Department of Cardiovascular Medicine, Mario Negri Institute for Pharmacological Research in Milan, Italy. Deidentified individual participant data and the data dictionary, study protocol, and informed consent forms will be made available for scientific purposes upon formal request and consequent approval of the proposal by the Steering Committee after publication. Requests should be sent to roberto.latini@marionegri.it.

The codes for circulating biomarkers will be made available for scientific purposes upon request to jennifer.meessen@marionegri.it and to ying.sun@igp.uu.se and peetra.magnusson@igp.uu.se for PEA analysis.

Declaration of interests

B.B is inventor of patents on PTX3 and obtains royalties on PTX3-related reagents.

N.B. serves on Advisory Board and Speakers Bureau for Celgene and Janssen, and on Speakers Bureau for Takeda, Amgen.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104914.

References

1 Cox EM, Bambakidis NC, Cohen ML. Pathology of cavernous malformations. *Handb Clin Neurol*. 2017;143:267–277.
2 Kivelev J, Niemelä M, Hernesniemi J. Treatment strategies in cavernomas of the brain and spine. *J Clin Neurosci*. 2012;19:491–497.
3 Fischer A, Zalvide J, Faurobert E, Albiges-Rizo C, Tournier-Lasserve E. Cerebral cavernous malformations: from CCM genes to endothelial cell homeostasis. *Trends Mol Med*. 2013;19:302–308.
4 Labauge P, Denier C, Bergametti F, Tournier-Lasserve E. Genetics of cavernous angiomas. *Lancet Neurol*. 2007;6:237–244.
5 Lampugnani MG, Malinverno M, Dejana E, Rudini N. Endothelial cell disease: emerging knowledge from cerebral cavernous malformations. *Curr Opin Hematol*. 2017;24:256–264.
6 Valentino M, Dejana E, Malinverno M. The multifaceted PDCD10/CCM3 gene. *Genes Dis*. 2021;8:798.
7 Maddaluno L, Rudini N, Cuttano R, et al. EndMT contributes to the onset and progression of cerebral cavernous malformations. *Nature*. 2013;498:492–496.
8 Detter MR, Snellings DA, Marchuk DA. Cerebral cavernous malformations develop through clonal expansion of mutant endothelial cells. *Circ Res*. 2018;123:1143.
9 Malinverno M, Maderna C, Abu Taha A, et al. Endothelial cell clonal expansion in the development of cerebral cavernous malformations. *Nat Commun*. 2019;10. https://doi.org/10.1038/S41467-019-10707-X.
10 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.
11 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.
12 Prey S, Voisard JJ, Delarue A, et al. Safety of propranolol therapy for severe infantile hemangioma. *JAMA*. 2016;315:413–415.
13 Léauté-Labrèze C, Hoeger P, Mazereeuw-Hautier J, et al. A randomized, controlled trial of oral propranolol in infantile hemangioma. *N Engl J Med*. 2015;372:735–746.
14 Oldenburg J, Malinverno M, Globisch MA, et al. Propranolol reduces the development of lesions and rescues barrier function in cerebral cavernous malformations: a preclinical study. *Stroke*. 2021;52:1418–1427.
15 Lanfranconi S, Scola E, Bertani GA, et al. Propranolol for familial cerebral cavernous malformation (Treat_CCM): study protocol for a randomized controlled pilot trial. *Trials*. 2020;21:1–10.
16 Lanfranconi S, Scola E, Meessen JMTA, et al. Safety and efficacy of propranolol for treatment of familial cerebral cavernous malformations (Treat_CCM): a randomised, open-label, blinded-endpoint, phase 2 pilot trial. *Lancet Neurol*. 2023;22:35–44.
17 Weinsheimer S, Nelson J, Abla AA, et al. Intracranial hemorrhage rate and lesion burden in patients with familial cerebral cavernous malformation. *J Am Heart Assoc*. 2023;12:e027572.
18 Santos AN, Rauschenbach L, Gull HH, et al. Natural course of cerebral and spinal cavernous malformations: a complete ten-year follow-up study. *Sci Rep*. 2023;13:1–7.
19 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.
20 Srinath A, Xie B, Li Y, et al. Plasma metabolites with mechanistic and clinical links to the neurovascular disease cavernous angioma. *Commun Med*. 2023;3. https://doi.org/10.1038/S43856-023-00265-1.
21 Girard R, Zeineddine HA, Koskimäki J, et al. Plasma biomarkers of inflammation and angiogenesis predict cerebral cavernous malformation symptomatic hemorrhage or lesional growth. *Circ Res*. 2018;122:1716–1721.
22 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.
23 Knoflach M, Kiechl S, Mantovani A, et al. Pentraxin-3 as a marker of advanced atherosclerosis results from the bruneck, ARMY and ARFY studies. *PLoS One*. 2012;7. https://doi.org/10.1371/JOURNAL.PONE.0031474.
24 Renz M, Otten C, Faurobert E, et al. Regulation of β1 integrin-Klf2-mediated angiogenesis by CCM proteins. *Dev Cell*. 2015;32:181–190.
25 Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2008;4:44–57.
26 Huber W, Von Heydebreck A, Sültmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18(Suppl 1). https://doi.org/10.1093/BIOINFORMATICS/18.SUPPL_1.S96.
27 Snellings DA, Hong CC, Ren AA, et al. Cerebral cavernous malformation: from mechanism to therapy. *Circ Res*. 2021;129:195–215.
28 Grdseloff N, Boulday G, Rödel CJ, et al. Impaired retinoic acid signaling in cerebral cavernous malformations. *Sci Rep*. 2023;13:1–11.
29 Lopez-Ramirez MA, Pham A, Girard R, et al. Cerebral cavernous malformations form an anticoagulant vascular domain in humans and mice. *Blood*. 2019;133:193–204.
30 Lopez-Ramirez MA, Fonseca G, Zeineddine HA, et al. Thrombospondin1 (TSP1) replacement prevents cerebral cavernous malformations. *J Exp Med*. 2017;214:3331–3346.
31 Lyne SB, Girard R, Koskimäki J, et al. Biomarkers of cavernous angioma with symptomatic hemorrhage. *JCI Insight*. 2019;4. https://doi.org/10.1172/JCI.INSIGHT.128577.
32 Croft J, Grajeda B, Abou-Fadel JS, et al. Blood prognostic biomarker signatures for hemorrhagic cerebral cavernous malformations (CCMs). *bioRxiv*. 2023. https://doi.org/10.1101/2023.07.01.547354.
33 Gnanasekaran R, Aickareth J, Hawwar M, Sanchez N, Croft J, Zhang J. CmPn/CmP signaling networks in the maintenance of the blood vessel barrier. *J Pers Med*. 2023;13. https://doi.org/10.3390/JPM13050751.
34 Abou-Fadel J, Jiang X, Padarti A, et al. mPR-specific actions influence maintenance of the blood–brain barrier (BBB). *Int J Mol Sci*. 2022;23. https://doi.org/10.3390/IJMS23179684/S1.
35 Zhang J, Croft J, Le A. Familial CCM genes might not Be main drivers for pathogenesis of sporadic CCMs-genetic similarity between cancers and vascular malformations. *J Pers Med*. 2023;13. https://doi.org/10.3390/JPM13040673.
36 Globisch MA, Onyeogaziri FC, Jauhiainen S, et al. Immunothrombosis and vascular heterogeneity in cerebral cavernous malformation. *Blood*. 2022;140:2154–2169.
37 Yau ACY, Globisch MA, Onyeogaziri FC, et al. Inflammation and neutrophil extracellular traps in cerebral cavernous malformation. *Cell Mol Life Sci*. 2022;79:206.
38 Lai CC, Nelsen B, Frias-Anaya E, et al. Neuroinflammation plays a critical role in cerebral cavernous malformation disease. *Circ Res*. 2022;131:909–925.
39 Zhou HJ, Qin L, Zhang H, et al. Endothelial exocytosis of angiopoietin-2 resulting from CCM3 deficiency contributes to cerebral cavernous malformation. *Nat Med*. 2016;22:1033–1042.
40 Fossati G, Pozzi D, Canzi A, et al. Pentraxin 3 regulates synaptic function by inducing AMPA receptor clustering via ECM remodeling and β1-integrin. *EMBO J*. 2019;38. https://doi.org/10.15252/EMBJ.201899529.
41 Mantovani A, Garlanda C. Humoral innate immunity and acute-phase proteins. *N Engl J Med*. 2023;388:439–452.
42 Roth P, Junker M, Tritschler I, et al. GDF-15 contributes to proliferation and immune escape of malignant gliomas. *Clin Cancer Res*. 2010;16:3851–3859.
43 Choi JH, Cheong C, Dandamudi DB, et al. Flt3 signaling-dependent dendritic cells protect against atherosclerosis. *Immunity*. 2011;35:819–831.
44 Tschernig T, Pabst R, Kasper M, El-Hadi M, Singh B. Expression of caveolin-1 and podocalyxin in rat lungs challenged with 2-kDa macrophage-activating lipopeptide and Flt3L. *Cell Tissue Res*. 2014;356:207–216.
45 Lun Y, Borjini N, Miura NN, Ohno N, Singer NG, Lin F. CDCP1 on dendritic cells contributes to the development of a model of kawasaki disease. *J Immunol*. 2021;206:2819–2827.
46 Yang N, Zhang Y, Huang Y, et al. FGF21 at physiological concentrations regulates vascular endothelial cell function through multiple pathways. *Biochim Biophys Acta, Mol Basis Dis*. 2022;1868. https://doi.org/10.1016/J.BBADIS.2022.166558.
47 Winford E, Davis S, Pennypacker K. Human brain microvascular endothelial cells produce CXCL9 after IFN gamma stimulation through JAK2/STAT1 Activation. *FASEB J*. 2019;33:500.1.
48 Collins R, Armitage J, Parish S, Sleight P, Peto R, Heart Protection Study Collaborative Group. Effects of cholesterol-lowering with simvastatin on stroke and other major vascular events in 20 536 people with cerebrovascular disease or other high-risk conditions. *Lancet*. 2004;363:757–767.
49 Simes RJ, Hunt D, Kirby A, et al. Long-term effectiveness and safety of pravastatin in 9014 patients with coronary heart disease and average cholesterol concentrations: the LIPID trial follow-up. *Lancet*. 2002;359:1379–1387.
50 Cunningham JW, Myhre PL. NT-proBNP response to heart failure therapies: an imperfect surrogate. *J Am Coll Cardiol*. 2021;78:1333–1336.