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A Roadmap for Developing Plasma Diagnostic and Prognostic Biomarkers of Cerebral Cavernous Angioma With Symptomatic Hemorrhage (CASH)

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# A Roadmap for Developing Plasma Diagnostic and Prognostic Biomarkers of Cerebral Cavernous Angioma With Symptomatic Hemorrhage (CASH)

**BACKGROUND:** Cerebral cavernous angioma (CA) is a capillary microangiopathy predisposing more than a million Americans to premature risk of brain hemorrhage. CA with recent symptomatic hemorrhage (SH), most likely to re-bleed with serious clinical sequelae, is the primary focus of therapeutic development. Signaling aberrations in CA include proliferative dysangiogenesis, blood-brain barrier hyperpermeability, inflammatory/immune processes, and anticoagulant vascular domain. Plasma levels of molecules reflecting these mechanisms and measures of vascular permeability and iron deposition on magnetic resonance imaging are biomarkers that have been correlated with CA hemorrhage.

**OBJECTIVE:** To optimize these biomarkers to accurately diagnose cavernous angioma with symptomatic hemorrhage (CASH), prognosticate the risk of future SH, and monitor cases after a bleed and in response to therapy.

**METHODS:** Additional candidate biomarkers, emerging from ongoing mechanistic and differential transcriptome studies, would further enhance the sensitivity and specificity of diagnosis and prediction of CASH. Integrative combinations of levels of plasma proteins and characteristic micro-ribonucleic acids may further strengthen biomarker associations. We will deploy advanced statistical and machine learning approaches for the integration of novel candidate biomarkers, rejecting noncorrelated candidates, and determining the best clustering and weighing of combined biomarker contributions.

**EXPECTED OUTCOMES:** With the expertise of leading CA researchers, this project anticipates the development of future blood tests for the diagnosis and prediction of CASH to clinically advance towards precision medicine.

**DISCUSSION:** The project tests a novel integrational approach of biomarker development in a mechanistically defined cerebrovascular disease with a relevant context of use, with an approach applicable to other neurological diseases with similar pathobiologic features.

**KEY WORDS:** Cavernous angioma with symptomatic hemorrhage (CASH), Cavernous angioma, Cerebral cavernous malformation (CCM), Symptomatic hemorrhage, Biomarkers, Machine learning, Plasma, QSM

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## GENERAL INFORMATION

**Protocol Title (ID#):** Biomarkers of Cerebral Cavernous Angioma With Symptomatic Hemorrhage (CASH) (NIH/NINDS R01NS114552).

**ClinicalTrials.gov Identifier:** NCT04467489  
**Name and Address of the Sponsor/Funding Agency:** NIH/NINDS R01NS114552 (2020-2025)

**ABBREVIATIONS:** CA, cavernous angioma; CASH, cavernous angioma with symptomatic hemorrhage; CCM, Cerebral cavernous malformation; COU, context of use; DCEQP, dynamic contrast enhanced quantitative perfusion; DE, differentially expressed; EC, endothelial cell; HMA, hemorrhagic microangiopathy; miRNAs, micro-ribonucleic acids; MRI, magnetic resonance imaging; NVU, neuro-vascular unit; qPCR, quantitative polymerase chain reaction; QSM, quantitative susceptibility mapping; SH, symptomatic hemorrhage; TR, Trial readiness

Supplemental digital content is available for this article at [www.neurosurgery-online.com](http://www.neurosurgery-online.com).

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## Cerebral Cavernous Angioma and the Problem of Symptomatic Hemorrhage

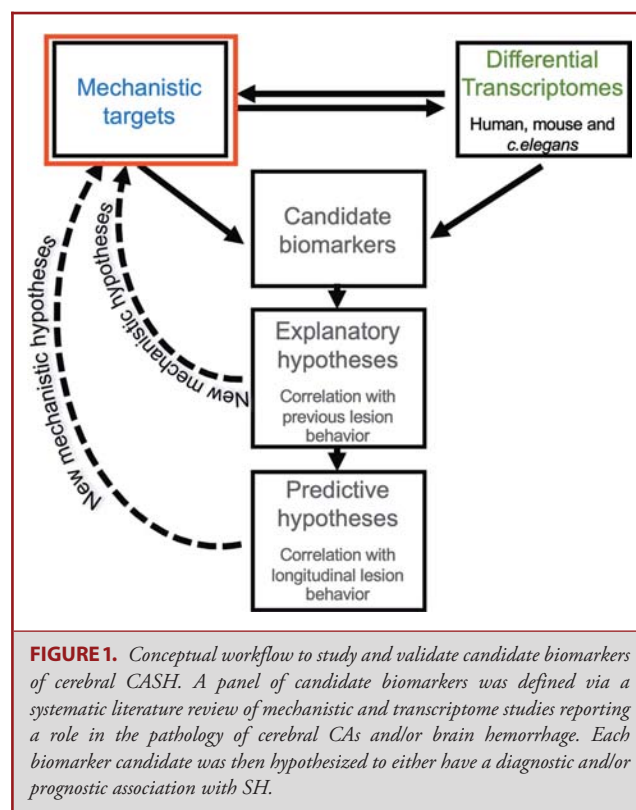
Cerebral cavernous angiomas (CAs) are enlarged, blood-filled capillary spaces, lined by “leaky” endothelium lacking mature angioarchitecture.<sup>1</sup> CA affects >1000 000 Americans, usually sporadic, with a solitary lesion or a cluster of lesions associated with a developmental venous anomaly.<sup>2</sup> The disease is familial in 20% to 30% of cases, exhibiting a Mendelian autosomal dominant inheritance with a heterozygous loss-of-function mutation in 1 of 3 cerebral cavernous malformation (CCM) genes, with multifocal lesions throughout the brain.<sup>3</sup> CAs are histologically identical in familial and sporadic cases, and harbor endothelial cell (EC) somatic mutations in the same 3 genes.<sup>4-6</sup>

The natural history of CAs is generally benign, with <0.5% annual risk of clinically significant bleeding.<sup>7</sup> However, once a lesion has manifested a symptomatic hemorrhage (SH), its untreated course is quite serious, with a 42% rate of recurrent bleeding within 5 yr.<sup>7-9</sup> Cavernous angioma with symptomatic hemorrhage (CASH) is defined as imaging evidence of new lesional bleeding or hemorrhagic growth, in association with directly attributable symptoms.<sup>10</sup> Trial readiness (TR) initiatives are underway, and repurposed drugs are at early stages of clinical evaluation aiming to stabilize the CASH lesion.<sup>11,12</sup> The diagnosis of CASH can be uncertain, particularly when imaging studies are not performed in a timely fashion or are equivocal in relation to specific symptoms. In addition, future CASH remains largely unpredictable.

### Context of Use and Definitions of Biomarkers

Biomarkers are defined with a context of use (COU), which describes their appropriate interpretation and application in medical product, development and regulatory review.<sup>13,14</sup> A *diagnostic biomarker* detects the presence of a specific medical condition; a *prognostic biomarker* assesses the likelihood of progression, regression or recurrence of this medical condition; and a *monitoring biomarker* evaluates the progression or stability of medical conditions, or the effectiveness of an intervention over time.<sup>13</sup>

These biomarker categories are distinct from *predictive biomarkers* (identifying cases with an increased probability to develop an outcome upon exposure to certain medical intervention or an environmental factor), *susceptibility/risk biomarkers* (reflecting a probability of future development of a specific medical condition), or *safety biomarkers* (indicating or predicting toxicity or an adverse effect before or after a medical intervention).



## RATIONALE AND BACKGROUND INFORMATION

### Preliminary Studies: Mechanistic Rationale, Preclinical Studies, and Biomarker Validations

Resected human and murine CAs demonstrate a complex pathobiologic milieu, involving an interplay of inflammation, angiogenesis, loss of EC barrier function and extracellular matrix remodeling.<sup>15-25</sup> These discoveries have motivated exploratory hypotheses of the associations between inflammatory states and the clinical behavior of CAs (Figure 1).<sup>26-34</sup> We queried a broad panel of inflammatory and angiogenic putative biomarkers (Table 1),<sup>35-39</sup> and first reported prognostic associations showing higher plasma levels of 4 biomarkers in cases who suffered a subsequent SH in the year following the plasma sampling.<sup>40</sup> The best weighted combination of the levels of these molecules had an 86% sensitivity and 88% specificity *in predicting a SH in the next year*. Canonical values derived from this best model were 5X higher in patients who suffered a subsequent SH (Figure 2A).<sup>40</sup>

We have also queried the same plasma molecules for diagnostic sensitivity and specificity of SH *in the year prior to plasma sample collection*.<sup>41</sup> The best diagnostic biomarker was a weighted combination of 4 molecules that showed excellent discrimination from healthy controls (Figure 2B). Two of the molecules were common, and two others respectively distinguished the diagnostic and prognostic panels. These results were validated with machine learning (ML) approaches.<sup>41</sup>

**TABLE 1. Panel of Plasma Biomarker Molecules Selected for Pilot Studies, Based on Systematic Literature Review of Associations With CA Disease and Brain Hemorrhage**

Biomarker	Biological processes	References	Potential relation to CA and brain hemorrhagic diseases
25-OH Vitamin D	Inflammation	Girard et al (2016) <sup>26</sup> Gibson et al (2015) <sup>27</sup>	Lower plasma levels of vitamin D in CA patients with a more aggressive disease course. Vitamin D supplementation reduces lesion burden in a preclinical CCM murine model.
CCL2/MCP-1	Inflammation	Retta et al (2016) <sup>28</sup> Keep et al (2014) <sup>29</sup> Luissint et al (2012) <sup>19</sup>	Oxidative stress associated to chronic inflammation in hemorrhagic vascular diseases. Increased CCL2/MCP-1 induces BBB permeability. CCL2/MCP-1 activates the RhoA/ROCK pathway.
C-reactive protein (CRP; binary and continuous)	Inflammation	Gao et al (2018) <sup>30</sup> Hsuchou et al (2012) <sup>20</sup>	CCM3 polymorphism is associated with increased CRP plasma levels. CRP increases BBB permeability.
Endoglin	Angiogenesis Endothelial permeability Inflammation	Chen et al (2009) <sup>36</sup> McAllister et al (1994) <sup>37</sup>	Soluble endoglin induces cerebro-vascular remodeling and may promote sporadic AVM formation. Endoglin gene loss is causative of hereditary hemorrhagic telangiectasia.
Interferon gamma (IFN $\gamma$ )	Endothelial permeability Inflammation	Girard et al (2018) <sup>35</sup> Ng et al (2015) <sup>38</sup>	Increased plasma levels of IFN $\gamma$ in CA patients associated to an aggressive clinical course. IFN $\gamma$ increases endothelial permeability and causes cytoskeletal derangements.
<b>Interleukins:</b> IL-1 $\beta$ IL-1 $\beta$ IL-2 IL-6 IL-8 IL-10	Inflammation Immune response Angiogenesis	Girard et al (2018) <sup>40</sup> Girard et al (2018) <sup>35</sup> Pawlikowska et al (2004) <sup>31</sup>	Lower plasma levels of IL-6 and higher plasma levels of IL-1 $\beta$ in CA subjects who experienced clinical lesional activity within the year following the initial blood sample. Increased plasma levels of IL-2, IL-1 $\beta$ in CA associated to an aggressive clinical course. Homozygosity for IL6-174G allele associated to greater risk of ICH in with AVM.
<b>Leukocyte-EC Adhesion:</b> VCAM1 ICAM1/CD54	Inflammation Endothelial permeability	Lampugnani et al (2018) <sup>21</sup> Robinson et al (1995) <sup>22</sup>	Increased expression of ICAM-1 and VCAM-1 on ECs is a marker of inflammation. Lower expression of ICAM1 and VCAM1 in CA lesions.
<b>Lipid Panel:</b> HDL Non-HDL LDL Triglycerides	Inflammation	Shenkar et al (2017) <sup>23</sup> Girard et al (2016) <sup>26</sup> Whitehead et al (2009) <sup>24</sup>	Simvastatin decreases ROCK activity in mature CCM lesions in mice. Lower levels of non-HDL cholesterol are associated with a more aggressive disease course. Simvastatin rescues CCM phenotype in preclinical murine model.
<b>Matrix metalloproteinases (MMPs):</b> MMP2 MMP9	Permeability ECM remodeling	Girard et al (2018) <sup>35</sup> Bicer et al (2010) <sup>32</sup>	Plasma levels of MMP-2 and MMP-9 were respectively higher and lower in patients with previous seizure activity. Higher expression levels of MMP-2 and MMP-9 may explain CCMs subclinical hemorrhage.
Roundabout4 (ROBO4)	Angiogenesis Endothelial permeability	Girard et al (2018) <sup>40</sup> Wüsthube et al (2010) <sup>17</sup> Jones et al (2008) <sup>39</sup>	Greater plasma levels of ROBO4 in CA subjects who experienced clinical lesional activity within the year following the initial blood sample. CCM1 gene associated with a down-regulation of ROBO4 gene expression. ROBO4 inhibits endothelial hyper-permeability and abnormal angiogenesis.

<b>Biomarker</b>	<b>Biological processes</b>	<b>References</b>	<b>Potential relation to CA and brain hemorrhagic diseases</b>
Soluble cluster of differentiation 14 (sCD14)	Inflammation	Girard et al (2018) <sup>40</sup>	Lower plasma levels of sCD14 in CA subjects who experienced clinical lesional activity within the year following the initial blood sample. Polymorphisms of <i>CD14</i> that codes for the anchored membrane are associated with higher CCM lesion burden in familial CCM disease. <i>CD14</i> polymorphism is associated with an increase susceptibility for high CCM lesions burden.
		Tang et al (2017) <sup>56</sup>	
		Choquet et al (2014) <sup>33</sup>	
<b>Tumor necrosis factors:</b> TNF $\alpha$ TNF RI	Inflammation	Girard et al (2018) <sup>35</sup>  Pawlikowska et al (2004) <sup>31</sup>	Increased TNF $\alpha$ plasma levels in CA patients associated to aggressive disease course. Homozygosity for the TNF $\alpha$ -308 GG genotype is associated with a greater risk of ICH in brain AVM.
Vascular endothelial growth factor (VEGF)	Angiogenesis Endothelial permeability	Girard et al (2018) <sup>40</sup>	Lower plasma levels of VEGF in CA subjects who experienced clinical lesional activity within the year following the initial blood sample. VEGF is associated to vasculogenesis and endothelial permeability. Dynamic changes in VEGF during the clinical course of CCM.
		Cunha et al (2017) <sup>25</sup>	
		Jung et al (2003) <sup>34</sup>	

Arteriovenous malformation (AVM); blood brain barrier (BBB); extracellular matrix (ECM); High-density lipoprotein (HDL); intra-cerebral hemorrhage (ICH); low-density lipoprotein (LDL).

### Micro-Ribonucleic Acids in Peripheral Plasma Reflect CASH Cases

Micro-ribonucleic acids (miRNAs) are small noncoding RNA molecules with roles in mRNA silencing and post-transcriptional inhibition of gene expression.<sup>42</sup> miRNAs, isolated from various body fluids, including plasma,<sup>43</sup> have been shown to rescue endothelial phenotype<sup>44</sup> and to inhibit vasculogenesis.<sup>45</sup> We identified 13 differentially expressed (DE) miRNAs in the plasma of CASH subjects (Table 2).<sup>41</sup> Integrative analysis between those miRNAs and DE genes in lesional CASH neuro-vascular units (NVUs) showed that interleukin-10 receptor subunit alpha is the putative target of *hsa-miR-185-5p*. We had also identified decreased plasma levels of interleukin-10 in CASH subjects.

### Imaging Biomarkers of Vascular Permeability and Iron Accumulation as Monitoring Biomarkers of Hemorrhage in CAs

Our group has implemented novel magnetic resonance imaging (MRI) applications assessing iron deposition in human CAs using quantitative susceptibility mapping (QSM) and dynamic contrast enhanced quantitative perfusion (DCEQP), reflecting postulated vascular hyper-permeability (Figure 3).<sup>46-50</sup> These were not suitable as diagnostic or prognostic biomarkers. Since both measures increased significantly in CAs manifesting interval SH during longitudinal follow-up, with highly sensitive and specific thresholds,<sup>50</sup> they were proposed as monitoring biomarkers of lesional bleeding and responses to therapy.<sup>51</sup>

**TABLE 2. Differently Expressed miRNAs in the Plasma of CASH vs non-CASH Subjects**

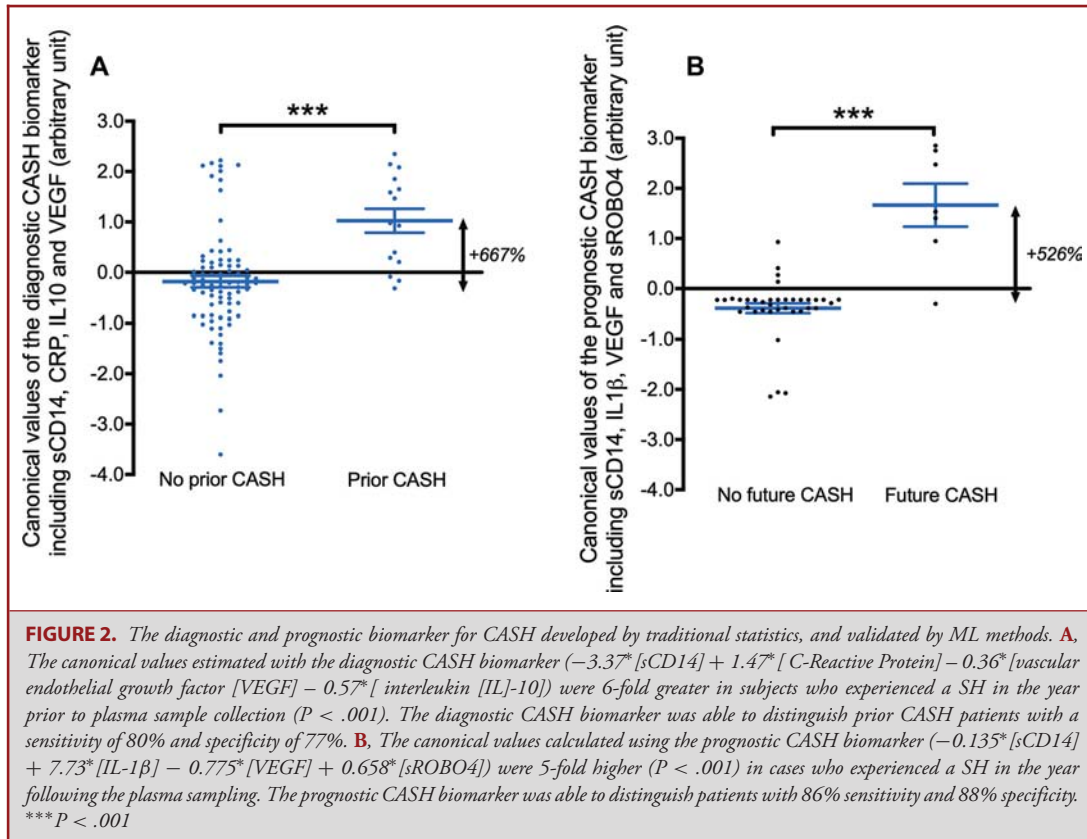
<b>miRNA</b>	<b>Log<sub>2</sub> (fold change)</b>	<b>P value</b>	<b>P value (FDR corrected)</b>
hsa-miR-363-3p	4.275	.000	.003
hsa-miR-486-5p	2.900	.000	.003
hsa-miR-15a-5p	3.490	.000	.003
hsa-miR-25-3p	2.704	.000	.003
hsa-miR-106b-3p	2.975	.000	.006
hsa-miR-16-2-3p	5.263	.000	.007
hsa-miR-183-5p	2.985	.000	.007
hsa-miR-16-5p	2.426	.000	.007
hsa-miR-185-5p	2.813	.000	.012
hsa-miR-501-3p	1.923	.001	.032
hsa-miR-181a-5p	1.183	.002	.036
hsa-miR-532-5p	5.468	.002	.036
hsa-miR-7641-2-3p <sub>novel</sub>	7.484	.002	.037

False discovery rate, FDR

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### Critical Knowledge Gaps

(1) It is unknown if the plasma biomarkers are relevant to disease subgroups. (2) Multisite validation is needed to assess the impact of any analytic and batch errors, and potential referral and enrollment biases. (3) Any approach to identify the best



biomarkers should allow the integration and testing of novel candidates.<sup>17,52-60</sup> (4) It would be important to know if the MRI biomarkers can be reflected by blood tests, facilitating and potentially enhancing the monitoring of CASH lesions. (5) It is important to assess the same biomarkers in non-CA controls, particularly in the aging human brain, with and without hemorrhagic microangiopathy (HMA), and in association with seizures.

## STUDY GOALS AND OBJECTIVES

### Specific Aim 1. Plasma Molecules as Diagnostic and Prognostic Biomarkers of CASH

#### Rationale and Hypotheses to be Tested

Novel candidate molecules (Figure 1, Table 3), and DE plasma miRNAs (Table 2) emerged from recent mechanistic and transcriptome discoveries. Hypotheses include (1) incorporating these novel candidate plasma proteins and miRNAs will enhance the sensitivity and specificity of plasma biomarkers identified in pilot studies, (2) diagnostic and prognostic biomarkers are applicable in relation to sex, age and clinically relevant disease subgroups, and in cases recruited at multiple sites.

#### Objective

To test whether individual and combined levels of candidate plasma proteins and miRNAs can be associated with diagnosis of CASH (cross-sectional) and can predict/prognosticate future SH (longitudinal).

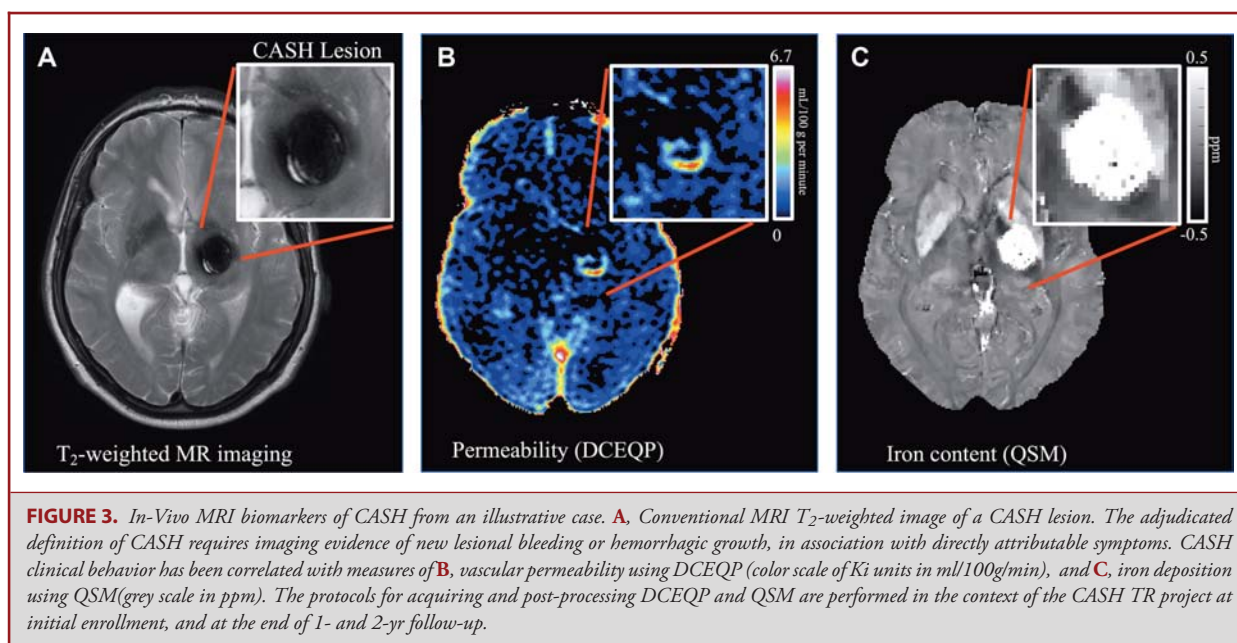
### Specific Aim 2. Imaging vs Plasma as Monitoring Biomarkers in CASH

#### Rationale and Hypotheses to be Tested

Increases in mean lesional QSM iron content and DCEQP permeability were recorded in longitudinal follow-up of CAs, with sensitive/specific thresholds during 1-yr epochs associated with clinically significant SH.<sup>50</sup> We hypothesize that change in the plasma biomarkers of prospective monitoring of CASH cases can reflect or complement changes in the MRI measures during 1-yr epochs.

#### Objective

To assess whether changes in QSM and DCEQP used as monitoring biomarkers after SH are reflected by changes in plasma biomarkers and miRNAs. We leverage imaging biomarkers data already being acquired in the ongoing CASH TR project to correlate with plasma biomarkers being assessed in **Specific Aim 1**.



### Specific Aim 3. Plasma Biomarkers in Non-CA Subjects

#### Rationale and Hypotheses to be Tested

CA, the aging NVU, and potentially HMA share phenotypic signatures and signaling aberrations reflecting blood-brain barrier disruption and hemorrhage.<sup>61-63</sup> Epilepsy also presents a potential confounder with CA biomarkers.<sup>35,64,65</sup> We hypothesize that plasma levels of biomarkers being evaluated in CASH diagnostic and prognostic questions are different in paired comparisons between non-CA young and older subjects, with and without seizures and HMA on MRI.

#### Objective

To assess the plasma biomarkers in non-CA young and older subjects, with and without seizures or HMA, clarify potential confounders in the context of clinical use, and motivate novel hypotheses for broader applications.

## STUDY DESIGN

This is an observational cohort study with no intervention (Figure 4). The inclusion/exclusion criteria for each Specific Aim are presented in **Supplemental Table 1**.

## METHODOLOGY

### Specific Aim 1. Plasma Molecules as Diagnostic and Prognostic Biomarkers of CASH

#### Subjects and Methods

We will recruit 800 consecutive subjects, including 200 from pilot studies whose plasma samples and clinical data are in hand. After Year 2 of the study, a cohort of 500 shall be available for

interim analyses using the proposed biomarker Panel 1, while all 800 subjects will be available after Year 4 to test the enhanced biomarker Panel 2 (Figure 4).

#### Candidate Biomarkers

In all, 12 plasma proteins thus far studied had significant diagnostic or prognostic univariate correlations with SH.<sup>40,41</sup> These 12 will be studied along with 12 novel biomarker candidates that emerged from recent mechanistic discoveries (Table 3).<sup>54,56-58,60,66,67</sup> We will reject non-correlated molecules using TS or less contributive molecules using ML, and add additional candidates in the second half of the study (Panel 2) based on discoveries in the next 2 to 3 yr.

#### Circulating miRNA Discovery and Quantitative Polymerase Chain Reaction Assays

Plasma aliquots from 100 subjects will identify DE miRNAs associated with prior SH (diagnostic), and new bleeding within a year (prognostic) by small RNA-Seq sequencing. The top 5 diagnostic and prognostic miRNAs identified shall be assayed by real time quantitative polymerase chain reaction (qPCR) for plasma levels in the larger cohorts subjected to plasma protein biomarker studies. Plasma qPCR levels of those miRNAs shall be correlated with SH individually, and in integrative combinations with queried plasma proteins.

#### Data Analyses Plan

Plasma biomarker levels shall be correlated with a history of SH in the prior year, as the primary diagnostic outcome, and correlated with new SH occurrence during the subsequent year as the primary prognostic outcome, adjudicated by clinical and imaging review. These shall be assessed at 1 yr of follow-up

**TABLE 3. Proposed Candidate Biomarkers (Panel 1)**

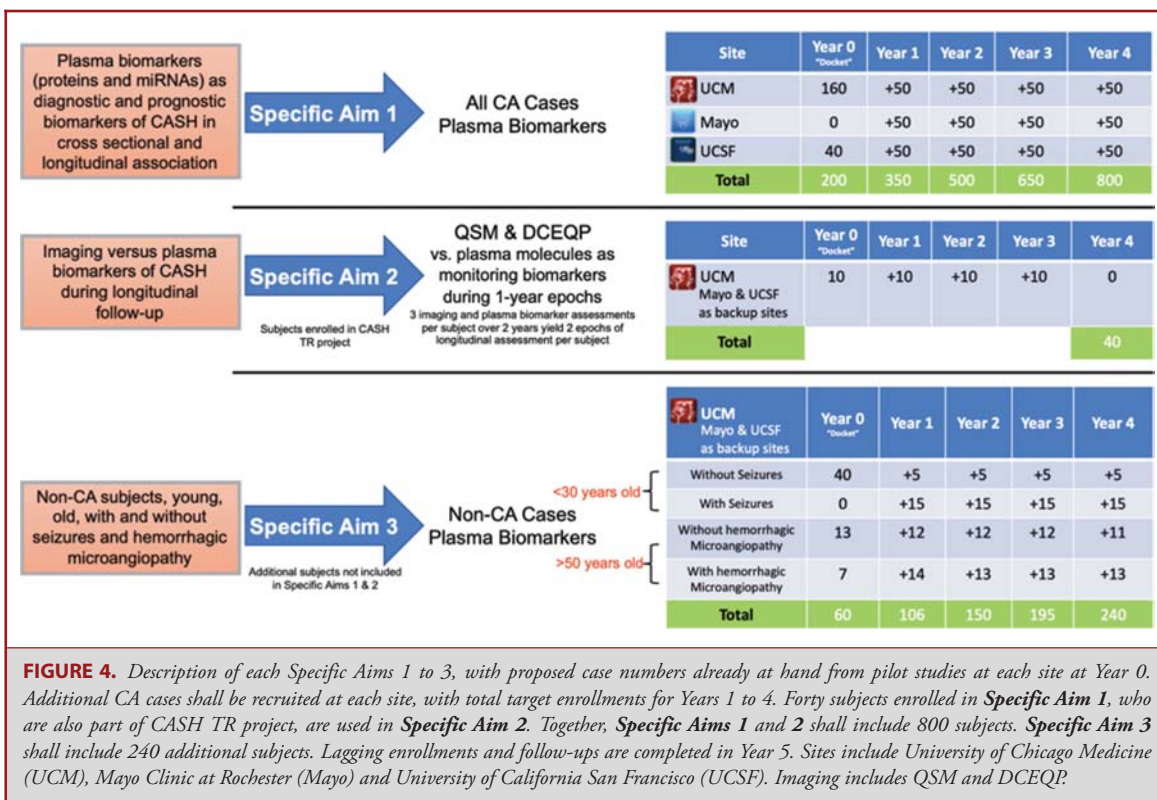
<b>Biomarkers to be assessed by ELISA assays</b>	<b>Mechanistic justification or preliminary validation</b>	<b>Differential transcriptomes (P value, FDR corrected)</b>
C-Reactive Protein (CRP)	Increased CRP plasma levels in CA patients after SH; belongs to the weighted combined diagnostic biomarker. <sup>41</sup>	.001 <sup>a</sup> ( <i>FCGR2B</i> )
Interleukin-1 $\beta$ (IL-1 $\beta$ )	Higher plasma levels of IL-1 $\beta$ in CA subjects who experienced a SH within the year following the initial blood sample; belongs to the weighted combined prognostic biomarker. <sup>40</sup>	.048 <sup>a</sup>
Interleukin-10 (IL-10)	Lower plasma levels of IL-1 $\beta$ in CA patients after SH. <sup>41</sup>	.013 <sup>a</sup>
Roundabout4 (ROBO4)	Lower plasma levels of IL-10 in CA patients after SH; belongs to the weighted combined diagnostic biomarker. <sup>41</sup>	.187 <sup>a</sup>
Soluble cluster of differentiation 14 (sCD14)	Greater plasma levels of ROBO4 in CA subjects who experienced SH within the year following the initial blood sample; belongs to the weighted combined prognostic biomarker. <sup>40</sup>	.025 <sup>a</sup>
Vascular endothelial growth factor (VEGF)	Lower plasma levels of sCD14 in CA patients after SH; belongs to the weighted combined diagnostic biomarker. <sup>41</sup>	.009 <sup>a</sup> ( <i>VEGFA</i> )
ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS4)	Lower plasma levels of VEGF in CA patients after SH; belongs to the weighted combined diagnostic biomarker. <sup>41</sup>	.007 <sup>a</sup> ( <i>FLT1</i> )
ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS5)	Lower plasma levels of VEGF in CA subjects who experienced SH within the year following the initial blood sample; belongs to the weighted combined prognostic biomarker. <sup>40</sup>	$2.3 \times 10^{-4a}$
Angiotensin-1	Endothelial TLR4 and the microbiome drive CAs	$2.1 \times 10^{-3a}$
Angiotensin-2	CAs arise from endothelial gain of MEK3-KLF2/4 signaling. <sup>56,57</sup>	$1.09 \times 10^{-17a}$
Lipopolysaccharide binding protein (LBP)	Endothelial exocytosis of angiotensin-2 resulting from CCM3 deficiency contributes to CAs. <sup>54</sup>	N.A.
<b>Interleukins (IL-)</b>	LBP inhibits LPS bio-activity in-vivo and in-vitro; increased LBP plasma levels in healthy non-CA subjects vs CA cases. <sup>67</sup>	.02 <sup>a</sup>
IL-7	IL-4, -7, -16, -21 and -33 were strong contenders in differential transcriptome of laser capture microdissected human CA neurovascular units. <sup>52</sup>	$2.9 \times 10^{-4a}$
IL-16		0.02 <sup>a</sup>
IL-21		$4.9 \times 10^{-3a}$
IL-33		0.01 <sup>a</sup>
Thrombomodulin	Thrombomodulin increased in human CA lesions and higher plasma levels in CA patients. <sup>60</sup>	$9.64 \times 10^{-14b}$
Thrombospondin-1	Thrombospondin1 replacement prevents CAs. <sup>58</sup>	$3.19 \times 10^{-3b}$
Thrombospondin-2		$5.1 \times 10^{-4a}$
Tissue plasminogen activator (tPA)	Central nervous system hemorrhage in CAs associated with greater expression of endothelial protein C receptor. <sup>60</sup>	N.A.

Cerebral angioma (CA); enzyme-linked immunosorbent assay (ELISA); lipopolysaccharide (LPS); Not Applicable (N.A.).

<sup>a</sup>Data from laser capture microdissected neurovascular units from human resected CA lesions in comparison to human normal brain.

<sup>b</sup>Data from mouse brain microvascular ECs with and without induced *Ccm* gene loss.





during clinical visit, or by follow-up contact and imaging studies if SH is suspected.

Additional analyses for diagnostic and prognostic outcomes shall be conducted in prespecified subgroups of sex, age (<30 and >50 yr), sporadic vs familial genotype, brainstem lesion location, epilepsy (≥1 seizure in the prior year, with/without medications), and site of enrollment. A subgroup analysis of prior SH in the year before enrollment (CASH at enrollment) shall also be conducted for prognostic outcome.

Exploratory analyses shall correlate the biomarkers (individual and combined) with the number of months since SH within the prior year, and the number of months until subsequent SH in the following year.

Plasma samples selected for analyses shall include all SH cases (adjudicated SH within a year prior to enrollment for diagnostic questions, and within 1 yr after enrollment for prognostic questions), and an equal number of plasma samples for biomarker testing among the larger group of non-SH cases in the enrolled cohort, best matching (1:1) SH cases for sex, age, sporadic/familial genotype, epilepsy and brainstem lesion location.

### Specific Aim 2. Imaging vs Plasma as Monitoring Biomarkers in CASH

#### Subjects and Methods

Plasma samples shall be collected from 40 subjects enrolled in the CASH TR project at UCM, in conjunction with their planned imaging

at baseline, at 1- and 2-yr follow-ups (Supplemental Figures 1, 2 and 3).

#### Data Analyses

We shall create a binary variable depending on whether the percent change in mean lesional QSM or DCEQP during 1-yr epochs exceeds previously defined respective thresholds (+5.8% QSM and +39.6% for DCEQP),<sup>50</sup> significantly associated with SH at high sensitivity and specificity. We will then assess the association of the binary variable with changes in plasma protein and miRNA qPCR levels being assessed in Specific Aim 1. We will also have the opportunity to assess the association between the changes in plasma protein and miRNA qPCR levels, and mean lesional QSM or DCEQP as a continuous variable.

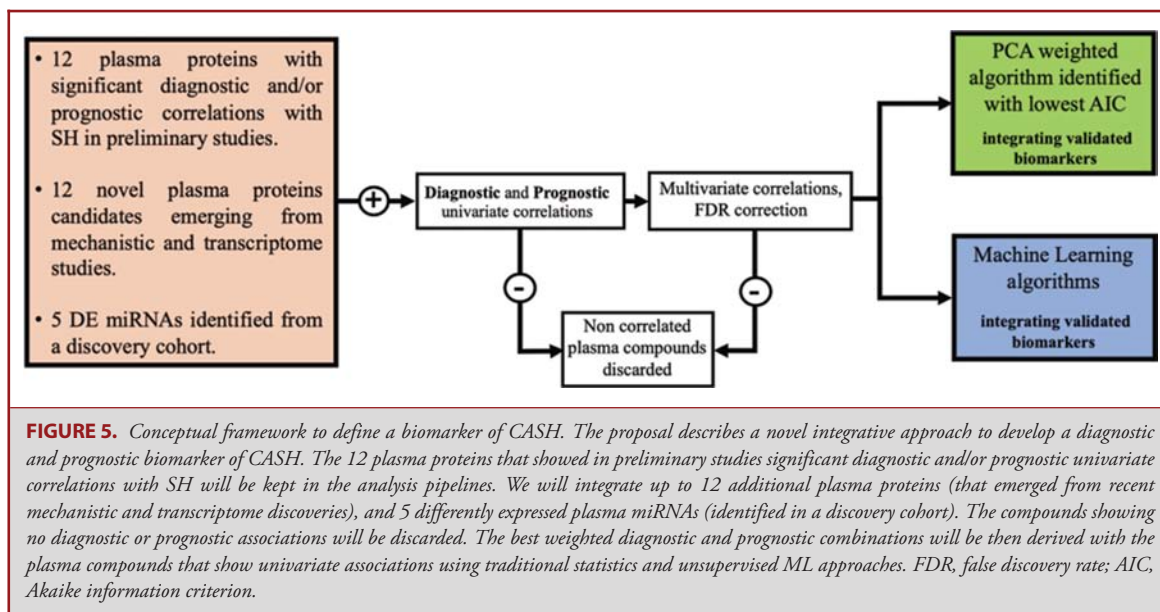
### Specific Aim 3. Plasma Biomarkers in non-CA Subjects

#### Subjects and Methods

At UCM, 240 non-CA subjects shall be recruited, with plasma collection, protein and miRNA assessments as per Specific Aim 1.

#### Data Analyses

The data analysis plan will follow the same procedures in Specific Aim 1 comparing plasma protein and miRNA levels (each separate and combined), between 2 respective cohorts (young/old without HMA, young with/without seizures, and old with/without HMA). Refer to Supplemental Digital Content for more Methods for Specific Aims 1-3.



## DISCUSSION

This proposal describes a novel integrative approach of selecting and combining plasma protein and DE miRNA biomarker candidates, and rejecting noncorrelated biomarkers (Figure 5). Biomarker associations also carry fundamental mechanistic implications, and identifying putative gene targets of miRNAs associated with CASH will generate new hypotheses that can aid in understanding factors impacting SH.

A blood test would be advantageous over more complex and expensive imaging techniques, and would prompt development for a novel COU.

Biomarker links to aging, HMA and seizures in non-CA subjects would identify potential confounders and motivate novel hypotheses about biomarker roles in those settings. Our results may not be generalizable to more or less severe cases than those enrolled, or address other potential confounders. An important practical implication would define normal ranges for the respective biomarkers in non-CA subjects. This will help establish positive and negative explanatory/prognostic thresholds needed for clinical application.

## TRIAL STATUS

The study is expected to commence on July 1, 2020.

## SAFETY CONSIDERATIONS

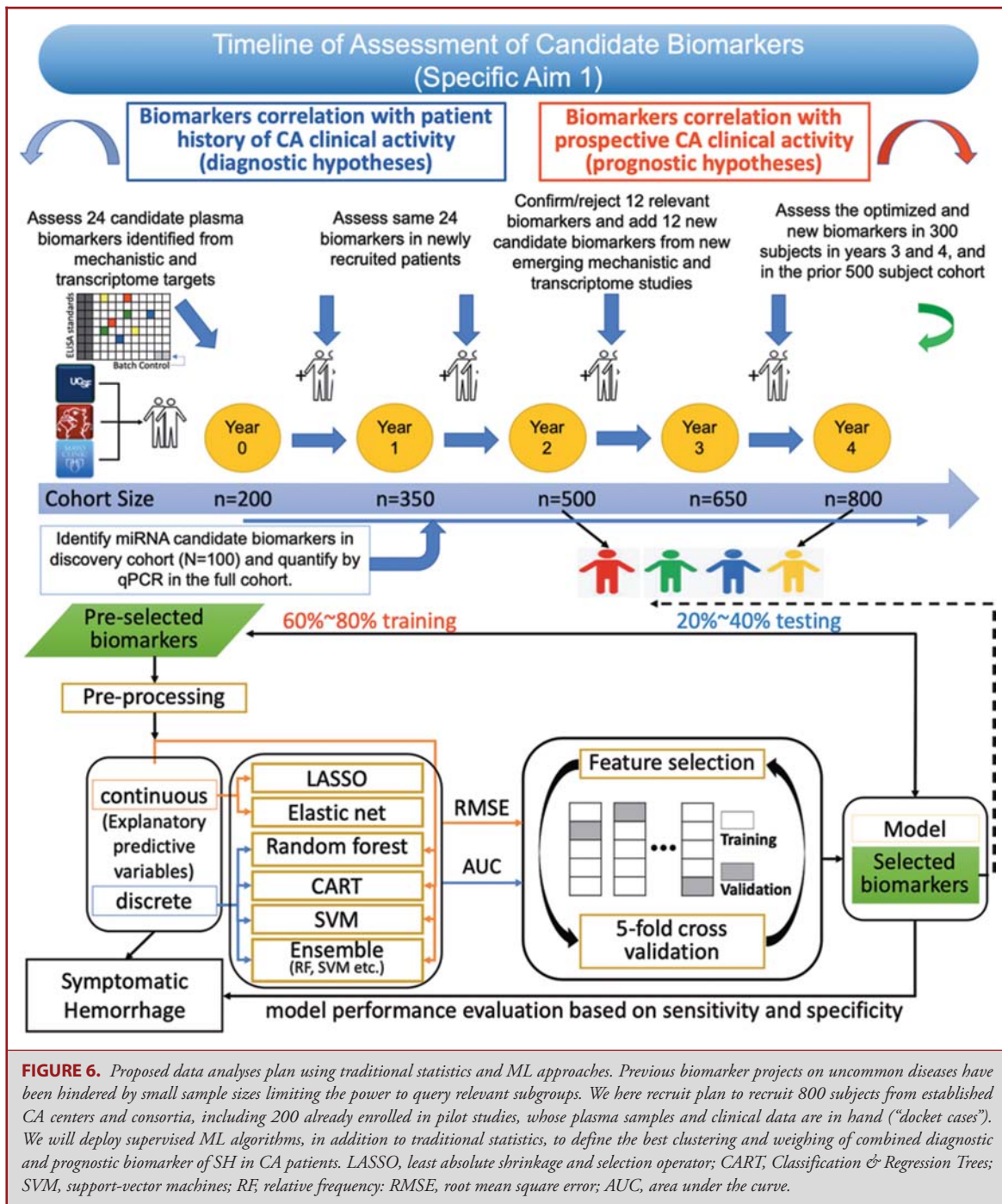
The study involves no more than minimal risks.

## FOLLOW-UP

All 3 sites are committed to yearly follow-up of all CA subjects, per current care standards, including follow-up surveillance imaging if new symptoms arise. For **Specific Aim 1**, 1-yr after enrollment, the medical records are queried for any evidence of new SH (this may be gleaned by telephone contact, with a request of confirmatory MRI report/compact disc, if a clinical visit is not logged). This is also done after a second year of follow-up in cases in **Specific Aim 2**, as also required in the CASH TR project. Follow-up is not required for cases enrolled in **Specific Aim 3**.

## DATA MANAGEMENT AND STATISTICAL ANALYSIS

The ML analysis plan (Figure 6), sample size calculations, as well as ML simulations for each of the three **Specific Aims**, are presented in the **Supplemental Digital Content**. Appropriate and complete statistical modeling and ML implementation of data collected is imperative to the planning, development, and completion of this proposal (**Supplemental Tables 2-5**). The most critical component is the accurate assessment of the enrollment rates of subjects at the three sites, as well as the rates of data missingness and the prevalence of SH in our diagnostic and prognostic cohorts. Estimates of data missingness are smaller in diagnostic correlations, given the simple clinical data elements. We therefore projected that a total sample size of 773 would accommodate the lowest estimates of SH prevalence (15% in the diagnostic samples) and highest estimates of data missingness rate (20% in the diagnostic samples; **Supplemental**



**Tables 2A).** For the more powerful prognostic biomarker, much smaller sample sizes are needed to achieve the same level of significance and statistical power, with prognostic SH rate <10% and data missingness >40% (n = 270; **Supplemental Table 2B).**

**QUALITY ASSURANCE**

UCM shall be the Data Coordinating Center. The investigative teams at UCSF and Mayo shall assist with data adjudication.

## EXPECTED OUTCOMES OF THE STUDY

Our goal is to develop blood tests, potentially reflecting or complementing complex and more expensive imaging assessments, to better diagnose, predict and monitor CASH. Associations of any CASH biomarkers with age, HMA or seizures in non-CA subjects would likely reflect shared mechanisms.

## DURATION OF THE PROJECT

We project enrolling 1040 cases over 4 yr. Completion of lagging enrollments and follow-up data analyses are planned in Year 5.

## PROJECT MANAGEMENT

We propose recruiting a large cohort from leading centers already collaborating in CA research and applying harmonized data elements. UCM includes senior scientists who conducted pilot studies. The investigative teams at UCSF and Mayo have an exceptional track record in CA patient recruitment, clinical data elements and risk stratification. They shall assist with analysis and interpretation.

## ETHICS

UCM will be identified as the governing central Institutional Review Board (IRB). IRB authorization and informed patient consent shall be requested at the three sites to expand ongoing approved biomarker studies.

## Funding

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## Disclosures

The authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article.

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**Supplemental digital content** is available for this article at [www.neurosurgery-online.com](http://www.neurosurgery-online.com).

**Supplemental Digital Content. A Roadmap for Developing Plasma Diagnostic and Prognostic Biomarkers of Cerebral CASH.** This includes Supplemental Methods, Supplemental Statistics and Simulations, as well as 3 Supplemental Figures, 5 Supplemental Tables and Supplemental References.

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