MarkVCID OCTA Retinal Vessel Skeleton Density (VSD) Biomarker Kit Protocol

1. **Executive Summary**

The MarkVCID is a collaborative effort to identify meaningful biomarkers for use in clinical trials of interventions in cerebral small vessel disease (SVD). Cross-sectional epidemiologic and histopathologic data suggest that clinically detectable retinopathy in cerebral SVD is accompanied or preceded by sub-clinical changes in capillary density on a more global level in the retina and CNS. Our preliminary data demonstrate significant associations between a measure of retinal capillary density called vessel skeleton density (VSD) and measures of cognition, learning and memory as well as neuroimaging. Specifically, VSD shows significant associations with the Montreal Cognitive Assessment (MoCA), Clinical Dementia Ratings (CDR) sum of boxes, Spanish English Verbal Learning Task Recall (SEVLT), Cerebrovascular Reactivity (CVR) and global cerebral blood flow. These findings strongly suggest that VSD may serve as a biomarker of capillary-level change in SVD and could be among the earliest changes that are detectable in the disease. In this proposal, we will test the primary hypothesis that there is a significant and positive cross-sectional association between MoCA and VSD after controlling for age, gender, and level of education. This will be assessed on a site-specific basis. Exploratory outcomes will include association of VSD with composite measures of executive function as well as longitudinal analyses of VSD with measures of cognition, executive function and neuroimaging. We will cross-validate VSD with the consensus clinical and cognitive MarkVCID data available at each site. Aggregate analysis of multisite data will be used to confirm and further explore site-specific findings. Repeatability of the VSD metric will be carefully assessed among raters and devices at each site and among all sites. Longitudinal follow-up at 12 and 24 months will be performed. In addition, a limited number of cadaver eyes from MarkVCID participants may become available for histopathologic correlation of VSD measures in the retina. At the end of the UH3 phase, we will have a validated retinal VSD biomarker of cerebral SVD susceptibility that will be ready for use in clinical trials.

Our proposed timeline for validation of the VSD biomarker:

- Months 1-3 (11/1/19-1/31/20): Set up OCTA data acquisition and VSD analysis software at all participating sites. Conduct training, quality control and repeatability analyses.
- Months 4-11 (2/1/20 – 10/31/20): Sites collect prospective OCTA data from MarkVCID cohort
- Month 12 (11/1/20): Collect data from the available UH3 sample for interim analysis.
- Months 12-24 (11/1/20-10/31/21): Continue the collection of data.
- Month 24 (11/1/21-11/30/21): Perform site-specific data analysis to validate the VSD biomarker. In addition, data from all sites will be analyzed in aggregate as a multisite cohort by the proposing site (USC).
- Months 0-24: Collect cadaver specimens for histopathologic analysis

2. Biomarker Kit Overview

Our proposed biomarker is Vessel Skeleton Density (VSD), a measure of retinal capillary density, as assessed by OCTA. OCTA is a completely non-invasive, low-risk, FDA approved, low-cost procedure.[1]

<u>Applications to Clinical Trial:</u>

Our primary goal in this UH3 proposal is to develop VSD as a biomarker of susceptibility/risk, as defined by the FDA-NIH Biomarker Working Group, for cerebral SVD. It is important to note that the use of VSD in this context is limited to subjects without any subjective visual complaints or vision-threatening disease.

Primary Biomarker Category (*susceptibility/risk biomarker)*: We propose that baseline VSD is an ideal *susceptibility/risk biomarker* for prevalent and incident vascular cognitive impairment as assessed by association with MoCA.

Kit Components

All sites participating in the MarkVCID consortium that intend to perform VSD analysis will have a commercially available OCTA device. Any site that does not have access to an OCTA device or believes that an additional device will facilitate recruitment of subjects may opt to lease an OCTA device for the duration of the project. The VSD biomarker kit is comprised of written "Data Acquisition Instructions" explaining the OCTA scanning/data acquisition protocol and data analysis methods as well as video training files. A software file containing an executable script that can be implemented on OCTA data from commercially available devices will also be included. Both files can be transferred via email. With the written instructions and software component, each site will be able to fully and independently carry out all necessary experiments and analyses, and prepare results to report back to the consortium. Dr. Kashani and his staff will visit sites to help demonstrate and implement the written protocol if necessary. This site visit will typically be 1-2 days and will involve training the site staff on OCTA scanning protocol (including quality control), data management and storage as well as pilot data analysis of several subjects. Dr. Kashani has already visited both Johns Hopkins and UCSF. Staff at UCSF have visited USC and have been trained in OCTA data acquisition and quality control. The VSD assessment method has been published and has no IP restrictions on its use.

Kit Rationale

There is growing evidence that changes in the retinal vessels correlate with vascular changes elsewhere in the central nervous system (CNS) indicative of SVD.[2,3] Clinical and histopathologic data demonstrate significant and reproducible correlations between large caliber retinal vascular features and markers of SVD such as white matter lesions (WML),[4,5] lacunar infarcts[4,6] and microbleeds.[2,3] In addition, microvascular changes at the level of the retinal capillaries, or "retinopathy," correlates with cognitive performance[7,8] and cerebral atrophy[9] independent of vascular risk factors such as diabetes mellitus (DM) or hypertension (HTN). In the context of these studies, "retinopathy" is essentially end-stage capillary disease that is likely preceded by a significant period of subclinical capillary loss or damage. Histopathology[10,11] and research studies using non-clinical, non-FDA approved devices[12] have demonstrated similar changes in retinal capillary density in asymptomatic subjects with mild HTN and DM.

We hypothesize that clinically detectable retinopathy is preceded or accompanied by sub-clinical changes in capillary density on a more global level in the retina and CNS and that this subclinical change is a biomarker for cognitive decline. We propose to use Optical Coherence Tomography Angiography (OCTA),[1] a novel, non-invasive, FDA approved, low cost, and rapid method of assessing retinal capillary density in subjects with significant risk for cerebral SVD. Our group and others have described the use of OCTA to quantify clinically relevant changes in vessel skeleton density (VSD), a measure of retinal capillary density, in several retinal vascular diseases, including DM and HTN, both well-known risk factors for SVD.[1] In addition, OCTA has been used to describe decrease in capillary density in CADASIL, a hereditary form of SVD.[13]

Preliminary Data

In the UH2 phase, we implemented OCTA imaging at three locations (USC, UCSF and Johns Hopkins) with one additional site (Rush) in the process of receiving OCTA equipment to start data acquisition. We demonstrated that VSD has an ICC between 0.75-0.85 in subjects with known risk factors for SVD. Our previous publications[14] and our preliminary data from 45 subjects in the USC UH2 phase cohort demonstrate that a significant change in VSD from 0.011 to 0.015 units is detectable between healthy subjects and those with vascular risk factors such as DM or HTN. Univariate analysis of relevant UH2 preliminary data including correlations of VSD with CDR, MoCA, SEVLT Delayed Recall, global CBF using pCASL as well as CVR are presented in Figure 1.

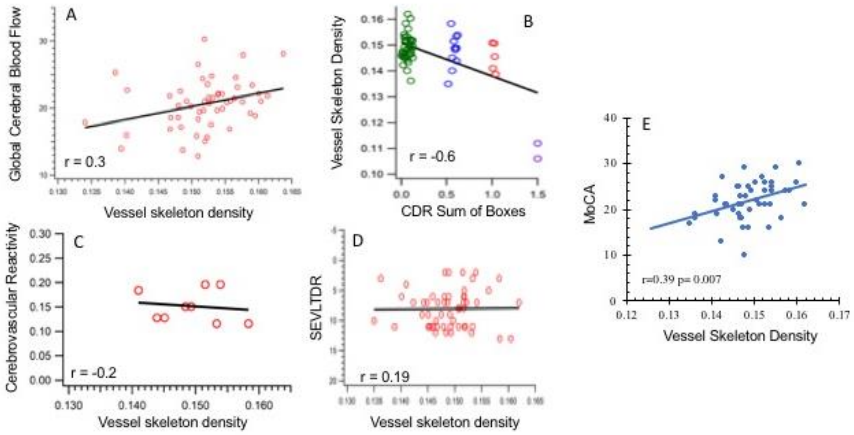


Figure 1. Univariate analyses of cognitive and cerebral variables with vessel skeleton density (VSD) from UH2 data. (A) Univariate correlation of global cerebral blood flow from pCASL measurements with VSD (Data from USC and UCSF combined). (B) Univariate correlation of Clinical Dementia Rating Sum of Boxes score with VSD (USC Data only). (C) Univariate correlation of cerebrovascular reactivity with VSD (USC Data only). (D) Univariate correlation of Spanish English Verbal Learning and Memory Test Delayed Recall and VSD (USC Data only). (E) Univariate correlation of MoCA with VSD (USC Data only). The solid lines are the regression fits to the data.

Multivariate analyses of these data controlling for age, gender and correlation between two eyes from a single subject demonstrate significant associations with VSD and are summarized in Table 1. Notably, our multivariable model demonstrates significant positive associations between VSD and MoCA. Similar findings are demonstrated independently in the UCSF pilot data. In a multivariable model including neuropsychological parameters we have also detected a significant correlation between VSD and tests of verbal memory and learning called the Spanish English Verbal Learning Task, Delayed Recall (SEVLT,DR; β = -95.3 and p = 0.0034). Delayed recall of an arbitrary word list is a sensitive measure of hippocampal-based episodic memory. Impaired performance on similar tests have also been demonstrated in subjects with retinopathy in population-based studies.[3] As most subjects in the UH2 cohort are non-demented (global CDR scores of less than 1), our data suggest that VSD may be a sensitive marker for preclinical disease. These cognitive and neuropsychological correlations are supported by significant correlation between VSD and cerebrovascular reactivity (CVR; β = 1.7 and p = 0.037).

Table 1. Summary Results from UH2 for VSD Assessment

GEE Multivariate Model Outcome (Dependent Variable)	Beta (β)	P-Value
CDR Sum of Boxes	-15.7	0.047
Global CDR	-3.7	0.3
USC MoCA	118	0.05
UCSF MoCA	157	0.08
USC + USCF MoCA	189	0.0022
Spanish English Verbal Learning Task, Immediate Recall (SEVLT, IR)	-21.2	0.5
Spanish English Verbal Learning Task, Delayed Recall (SEVLT, DR)	-95.3	0.0034
Cerebrovascular Reactivity	1.7	0.037

Vessel Skeleton Density (VSD)	Number of Eyes SB>0 / SB=0	ODDs SB>0.5 : SB=0	
Lower 1/3	13/15	9 : 10	p = 0.14
Middle 1/3	9/19	5 : 10	p=0.04
Upper 1/3	7/22	3 : 10	p = 0.25

Table 2. Odds of impaired cognition based on tertiles of VSD distribution from USC pilot data. The raw odds (second column) were normalized to 10 for viewing purposes only (third column). The p-values reported were derived from chi square comparisons of the proportions of subjects with SOB>0 in each VSD category.

In order to further determine if VSD correlates with impaired cognition we also assessed the odds of having a CDR-SB score greater than 0 for every tertile of VSD in our pilot data (Table 2). This analysis showed that there is a significantly increased odds of

having a CDR-SB score greater than 0 between subjects in the top and bottom tertile of the VSD distribution. These strongly suggest that VSD may be a fast, economical, completely non-invasive and clinically feasible biomarker for assessing risk of cognitive decline from cerebral SVD.

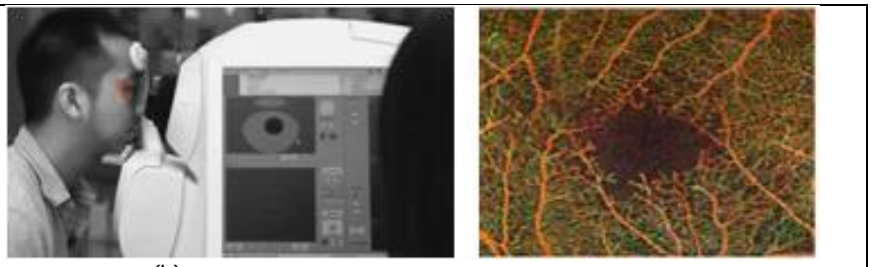
3. Participating Sites and Recruitment Goals

Table 3. Summary of Minimum Data Collection Goals for UH3 Across All Sites

UH3 Subjects	USC	UCSF	JHU	Rush	UTHSCSA
Total Proposed	120	73	60	60	60
# Normal	60	58	20	39	20
# MCI/Dementia	60	15	40	21	40
# with Longitudinal F/U	50	50	50	50	50
Follow-up Time	12 and 24 months				

USC, UCSF, JHU, UTHSCSA and Rush have indicated their participation in the cross-site testing of the VSD biomarker for the UH3 phase of the study. Each site has agreed to recruit at least 60 subjects for the UH3 phase of the study (Table 3). We encourage sites to have as close to a 1:1 recruitment ratio as possible (1 subjects with at least minimal cognitive impairment (CDR-SB > 0) for every cognitively normal subject (CDR-SB = 0) that is recruited). Assuming ~15% subject attrition during follow up, we anticipate that 250 participants (50 per site) will be available with longitudinal data and over 300 subjects will be studied across all sites with cross-sectional baseline data. For the UH2 phase, subjects at USC were recruited from the community-based Los Angeles Latino Eye Study in which there is a high prevalence of cardio- and cerebrovascular risk factors though participants are generally cognitively intact. Recruitment of cognitively impaired subjects at USC will be facilitated by co-enrollment of subjects from Dr. Ringman and Dr. Kashani's newly funded R01 (R01AG062007) in which subjects undergo the identical clinical and ophthalmological assessment and longitudinal follow-up. The various sites of MarkVCID will recruit an ethnically diverse cohort including Hispanics, African Americans, and Non-Hispanic Whites.

4. Protocol for Optical Coherence Tomography (OCTA) Acquisition



(b)

Figure 2: Illustration of OCTA scanning procedure and OCTA scan result **(a)** Picture of a subject positioned for scanning on an OCTA device. **(b)** Illustration of OCTA data from a 3x3mm scan centered on the fovea demonstrating layer specific capillaries in the superficial (red), deep (green) and overlay (yellow).

OCTA at each site will be performed on a commercially available OCTA device. OCTA data acquisition will be performed via a standardized and written protocol that has already been piloted at UCSF and JHU. All sites may lease additional OCTA units for the duration of the proposal to facilitate the completion of the OCTA Biomarker aims. OCTA imaging will be performed on both eyes of each subject. To assess the impact of pupil dilation for OCTA analysis, the protocol requires that the right eye of each subject be dilated and the left eye remain undilated. The right eye will be first topically anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution. For dilation, 1-2 drops of 2.5% phenylephrine and 1% tropicamide will be applied. Dilation takes approximately 10-15 minutes. Each eye is then scanned at least 4 times using the 3x3mm^2 Angiography scan acquisition settings to ensure a good quality scan is available for analysis. In addition, scans of the optic disc will be obtained for each eye using the Optic Disc cube acquisition setting. Additional scans may be acquired at the discretion of the site technicians if needed. All scans should be acquired with signal strength of 8 or higher. Each scan takes approximately 1 minute depending on subject cooperation. Subjects are scanned in a seated position as shown in Figure 2. In all cases imaging should be performed with room lights off and door closed to minimize ambient lighting and maximize mydriasis of the non-dilated eye. Data acquisition for both eyes is expected to take approximately 30-45 minutes. Another 5-10 minutes is

required for quality control at the time of data acquisition.

5. Additional Data (Clinical/Cognitive/Pathology etc) Required for Analysis

MarkVCID clinical and cognitive data will be used for cross-validation of the VSD biomarker. We hypothesize that VSD will be associated with clinical diagnosis of MCI and dementia. Because USCF is already one of the consortium sites for the OCTA biomarker we plan on using the WMH pipeline available from that site for exploratory cross-validation with WMH. As Table 1 shows, VSD demonstrates a significant correlation with CVR in our UH2 data. Both USC and JHU are already performing CVR measurements and will be able to provide CVR data for cross-validation in the UH3 phase. We recommend that any other site performing OCTA also perform CVR measurements, if possible, at their site.

6. Protocol for Image Analysis (VSD Determination)

OCTA data are exported as bitmap (BMP) files directly from the OCTA device for quantification of VSD measurements. VSD analysis must be performed using semi-automated custom software that will be provided by the lead site (USC) and in collaboration with Dr. Ruikang Wang from the University of Washington, Seattle. The custom script is automated except for user input that is required at one step for assessment of background noise. This step requires minimal training and takes approximately 10-20 seconds to perform. VSD output is in arbitrary units representing a skeletonized vessel density that is independent of vessel caliber. VSD quantification of one subject's data is anticipated to take about 5 minutes. Dr. Kashani and his staff will train users in the use of the semi-automated software for VSD analysis. In addition, a video training file will be provided. The native file format data on the OCTA device can be exported and analyzed at USC for quality control and data analysis purposes. Specifically, images will be evaluated for signal-to-noise and motion artifacts that may adversely impact VSD analysis. Specific criteria for quality control of the OCTA images will be provided to each site in the written instructions provided as part of the kit. All the data will be stored at the Coordinating center for the consortium.

7. Step-by-Step Analytic Plan

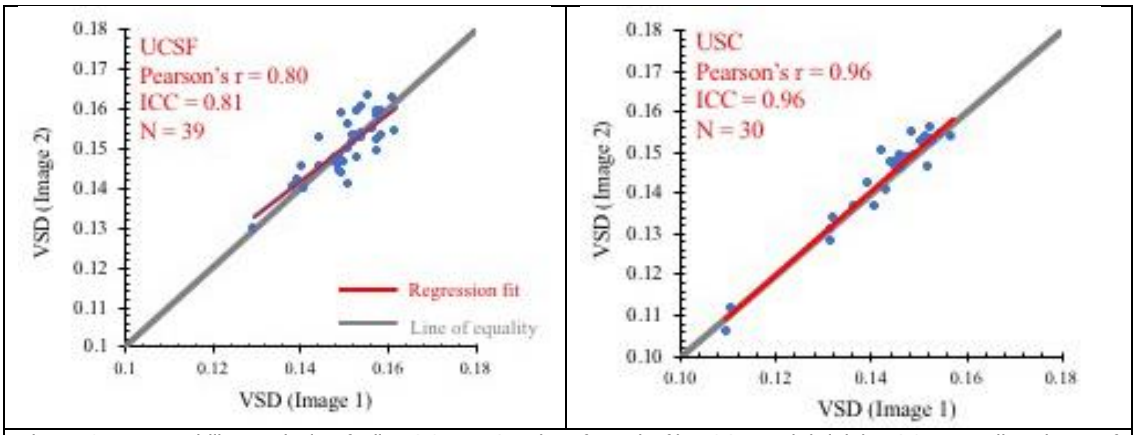


Figure 3. Repeatability analysis of pilot OCTA VSD data from (Left) UCSF and (Right) USC over pilot phase of MarkVCID based on training plan implementation and harmonized quality control of all data using same parameters. Data show the correlation between two VSD measurements on the same subject within the same day. ICC values are also shown. Data from USC was obtained with dilation and data from UCSF was obtained without dilation.

a) At each site, test-retest using separate OCTA scans on the same 6 individuals will be performed to assess between- and within-subject standard deviation, and intra-observer standard error of measurement (SEM). The scan protocol for the test-retest subproject is: two separate OCTA scans on the same individual on the same day will be acquired within two weeks (14 calendar days) of another scan of that same individual. The patient should be repositioned between successive scans at the multi-acquisition scan session. ICC values will be calculated for VSD values and the sample

size of 6 allows us to detect ICC of 0.81 or greater, with an alpha of 0.05 and a minimum power of 80% (PASS v19.02, NCSS, LLC).[15] Based on our most recent preliminary data the ICC for VSD is 0.81 and 0.96 from UCSF and USC, respectively (Figure 3). Therefore, the designed test-retest plan is expected to have sufficient power to measure repeatability of VSD at each individual site. In addition, repeatability of VSD will be assessed in the same 6 subjects on a separate day but within 2 weeks (14 calendar days) of baseline. Repeatability analysis will be performed separately for VSD analyses from both dilated and nondilated eyes of the same subject to determine the impact of dilation on the VSD scores. Each site must achieve a minimum ICC of 0.81 for data acquisition and VSD analysis of 6 subjects on the same day and within 2 weeks (14 calendar days). The Bland and Altman method will also be used to assess the distribution of measurement errors.

b) Inter-rater reliability showing that different raters can calculate the same result for VSD when analyzing the same scan will also be assessed. At least one independent rater from each site (total of 5 or more raters) will use the same software package to process the same baseline OCTA scans from the same 20 subjects. ICC values will be calculated for VSD values from all independent raters. Since the software for VSD calculation is semi-automated with minimal user input we expect that there will not be a rater dependence on VSD results. Our previous experience suggests that ICC values for interrater reliability are >0.9.[14] We will also ask the raters to repeat the assessment of the same 20 subjects on a second day. For a better estimate of the intra- and inter-observer variability that may occur in more generalized settings, random effect models, which treat both the subject effect and the observer effect as random effects, will also be performed to estimate intra- and inter-observer reliability simultaneously.

c) Our primary outcome will examine the continuous association between VSD and MoCA scores at each MarkVCID site. This association will be corrected for age, gender and education level. Descriptive statistics of VSD and MoCA will be summarized by median, mean, standard deviation and 95% confidence intervals for each site. Generalized estimating equation or mixed models will also be considered to include VSD data from both eyes of the same participants, control for correlation between eyes of the same subject, and account for observer differences. For analyses of the combined data from all sites, individual participant data will be analyzed in a hierarchical model that accounts for clustering of participants within sites. Both participant-level and relevant site-level factors will be included[16]. If there is a significant heterogeneity across studies in the association between VSD and MCI/dementia, we will conduct sensitivity analyses and mixed-effects meta-regression[17], to identify the sources of the heterogeneity.

d) Our secondary outcome will be to determine the cross-sectional odds of cognitive impairment (CDR-SB > 0) corresponding to baseline retinal vessel skeleton density (VSD) at each site. In the context of VCID, the CDR-SB is preferable to the global CDR score in light of its greater dynamic range and increased applicability across dementia subtypes (e.g. its lack of disproportionate weighting of memory impairment). The CDR-SB score provides a validated composite clinical measure of functional impairment, encompassing multiple cognitive and behavioral domains, with well-characterized correlation to disease progression in mild and mild-moderate Alzheimer's disease.[18] Although less is known about rate of change in CDR-SB for vascular dementia, it has been used to quantify the impact of vascular risk factors, such as hypertension, on progression of cognitive decline in subjects with Alzheimer's Disease.[19] Descriptive statistics of VSD and CDR-SB will be summarized by median, mean, standard deviation and 95% confidence intervals for each site. We will use multivariable models to determine the magnitude and significance of the association between VSD and cognitive impairment with adjustment for covariates including age, gender, and medical comorbidity.

e) As part of our exploratory analyses, we will look at (1) the above primary and secondary measures among the entire multisite MarkVCID consortium of 300+ subjects and (2) longitudinal association of baseline VSD with odds of worsening over 24 months in well-accepted measures of cognition such as CDR-SB and MoCA. Test version that differ across sites (e.g. the SEVLT and CVLT) will be compared using z-scores of subjects calculated from group means as well as Item Response Theory (IRT) derived values. Separate analyses will be performed for the dilated and undilated eye to determine if pharmacologic dilation has any impact on the analyses.

f) Quality Control (QC). In order to ensure adequate quality and appropriateness of OCTA data collected and analyses performed, the OCTA protocol will incorporate several QC steps. Subjects will be asked

to complete a screening questionnaire to exclude subjects with confounding ocular conditions including cataracts, glaucoma, advanced forms of macular degeneration and diabetic retinopathy. All subjects will have the right eye pharmacologically dilated and the left eye will remain undilated. Each subject will have scans performed on both eyes in quadruplicate. Only scans with signal strength 8 or higher will be used in the analysis. Data and data analyses will be reviewed on a weekly basis from each site during the first few months to ensure the protocol is being successfully implemented at all sites.

8. Sample Size Calculation

For our primary outcome examining the continuous association between VSD and MoCA, each MarkVCID site will be able to detect a significant correlation of the same approximate magnitude (0.25-0.36) as our preliminary data with 80% power. Aggregate analysis of the data from all sites will be powered to detect a correlation of at least 0.145. For our secondary outcome examining the association between baseline VSD and odds of CDR-SB>0, the predicted recruitment sample at each site also suggests that we will have 80% power to detect an odds ratio of cognitive impairment between 1.76-2.39 for 1 standard deviation difference in VSD depending on the individual site. Table 4 summarizes the power calculations for the primary (VSD and MoCA) and secondary outcomes (VSD and odds of CDR-SB>0).

Table 4 also illustrates the minimum detectable difference in VSD between normal and MCI/dementia that can be detected with 80% power (α=0.05, 2-sided) for the expected sample size at each site and all sites combined. The statistical estimates show that we will have power to detect a baseline difference of ~0.0035 VSD units in the combined cross-site cohort and ~0.010 VSD units within each site's cohort. This is within the range of VSD change that occurs in known retinal vascular diseases like diabetic retinopathy (0.011-0.015). We expect the difference in VSD associated with MCI/dementia to be equal to or greater than these levels. Our study power can be further improved if VSD data from both eyes of the same person are used for analysis using generalized estimating equation or mixed models.

Table 4. Summary of Statistical Power for Primary and Secondary Outcomes Based on UH3 Recruitment Goals Per Site and Across Sites

	Minimum Expected Sample size (N)		Minimum Detectable (80% power) Odds for CDR-SB > 0	Minimum Detectable (80% power) partial Correlation coefficient for association of VSD and MoCA	Detectable Difference (80% power) in VSD between Normal and Dementia
Site	Normal	MCI or Dementia			
USC	60	60	1.76	0.256	0.0062
UCSF	58	15	2.39	0.328	0.0099
JHU	20	40	2.39	0.361	0.0094
Rush	39	21	2.37	0.361	0.0093
UTHSCSA	20	40	2.39	0.361	0.0094
All sites combined	197	176	1.37	0.145	0.0035

9. Plan for Longitudinal Data Collection and Analysis

Each site has agreed to recruit at least 60 subjects for the prospective phase of the study. We anticipate a 15% attrition rate over the duration of the study resulting in a minimum of 50 subjects at each site for the longitudinal analysis. Here we present our preliminary plans for longitudinal analysis of VSD with change in measures of cognition including MoCA and CDR-SB.

To assess how baseline VSD level is associated with the change in participants' MoCA score between visits, we will use multivariable mixed effects Poisson regressions to model the number of errors on MoCA (i.e. 30 minus MoCA score). Interaction between baseline VSD level and study visit will be

included to measure rate ratios for change in the number of errors on the MoCA. Subject effects will be treated as random effect and a random intercept will also be included. All models will also control for fixed effects of age, sex, education, and other potentially relevant covariates. In addition, we will examine the proportion of participants with a change in MoCA beyond normal intrasubject variation. It has been reported that the normative intrasubject standard deviation in MoCA score are 1.51±1.07 for ages 60-69, 1.69±0.91 for ages 70-79, 1.98±1.09 for ages 80-89, and 1.88±1.16 for ages 90-99.[20] An equal-to or greater-than 2-point decline in MoCA may be considered significant.[21] Thus, we will also define worsening in MoCA score as a 2-point or more decline. Multivariable logistic regression will be used to assess how baseline VSD level may be associated with a higher risk of ≥ 2-point decline in MoCA score. VSD levels will be analyzed as a continuous variable and also categorized by tertiles of the sample distribution.

Furthermore, we will also assess how baseline VSD level may be associated with other measures of cognition, especially the CDR-SB score. The mean change in CDR-SB score has been estimated to be approximately 1.5 in 12 months and 2.7 in 18 months for individuals with a global CDR of 0.5.[18] Therefore we propose to use an increment of 0.5-points or more in CDR-SB score as our measure of longitudinal clinical decline. Conservatively, we assume that 20% of the study population will experience a 0.5-point or more increment in CDR-SB score over 12 months and 30% over 18 months or more.[22] Therefore, the site-specific sample size of 50 or more will give us 80% power to detect a minimum odds ratio of 2.67 at 18+ months for 1 standard deviation difference in VSD. In addition, an aggregate sample size of 250 subjects will give us 80% power to detect a minimum odds ratio of 1.52 at 18+ months for 1 standard deviation difference in VSD. Our study power will be further improved if the study participants are followed for a longer period of time, e.g. 48 months, for more substantial changes in CDR-SB. Multivariable logistic regression will be performed to assess the predictive relationship between baseline VSD level and future risk of worsening in CDR-SB, after adjustment for age, gender, and other relevant covariates. Worsening in CDR-SB score will be defined as 0.5-point or more increment change from baseline and 1-point change in MoCA.

10. Plan for Reporting Outcomes

An interim report will be generated ~12 months into the UH3 phase with the data that is available at that time point. We do not anticipate that statistical analysis at this time point will be informative due to the incomplete sample size at each individual site. However, multisite aggregate data will be analyzed. We anticipate that the majority of data will be available within 22 months for statistical analysis at each site and across all sites. Relevant publications will be based on contributions from each of the consortium sites with approval from the consortium's Publications Subcommittee.

11. Plan for Sharing Data, Samples/Images and Protocols

No IP issues are anticipated. OCTA devices are FDA approved and commercially available. The VSD analysis methods are also published[14,23] and the software will be made available to consortium sites and interested researchers. De-identified data from the UH3 will be stored in the consortium's data management system, and shared based on policies established by and with approval from the Data Sharing Subcommittee. Each site will be sharing three types of datafiles: 1. Bitmap exported images, 2. Deidentified Native/DICOM files, and 3. TIFF files and Excel file from analysis software. In addition to those files, each site will be reporting deidentified subject data via a CRF form which includes the following variable: signal strength, subject history of eye care visits, glasses or contact information, and history of cataract surgery. The de-identified samples and clinical data could be used for research in any type of disease or genetic factors, not just vascular contributions to cognitive impairment and dementia. There is a risk that someone could use information from the deidentified data to identify the subject from which it was derived. However, any user of this sample must agree not to use it for that purpose.

12. Addendum: Plan for Histopathologic Analysis

Our preliminary data strongly suggests that a decrease in retinal capillary density, as measured by VSD, is correlated with cognitive decline and vascular changes occurring in the brain. In order to explore the pathophysiological basis of decreased VSD, we propose to collect cadaver eyes, as they become available, from subjects who are enrolled in the MarkVCID study and who have undergone all the standardized consensus testing as well as OCTA imaging for VSD. In collaboration with Dr. David Hinton at USC, these eyes will be used for immunohistochemistry and transmission electron microscopy corresponding to the region of VSD assessment to determine the cellular basis of decreased VSD. Endothelial and pericyte loss are implicated in cerebrovascular disease[24] and retinal vascular disease.[25,26] We hypothesize that decreased VSD is caused by abnormal function or loss of vascular tissue elements such as capillary pericytes, endothelial cells or changes in the basement membrane. Decreased capillary density or abnormal capillary function has been demonstrated in two prototypical retinopathies including diabetic retinopathy[1,14] and hypertensive retinopathy[27] as well as cerebral small vessel disease.[24] For example, in diabetic retinopathy, histopathology from human subjects and primate models has demonstrated thickened capillary basement membranes, loss of pericytes and endothelial cells that lead to subclinical ischemia.[25] Endothelial dysfunction in subjects with hypertension impairs retinal autoregulation and also causes ischemia.[27,28] The MarkVCID study presents an opportunity to demonstrate the potentially unique pathophysiologic basis of decreased VSD in subjects at high risk for cerebral SVD and in whom detailed *in vivo* cognitive, neuroimaging and retinal capillary density assessments have been made. Therefore, we will perform experiments to confirm the quantitative findings from our VSD measurements and also to qualitatively determine if there are gross changes in cellular structure of retinal capillaries.

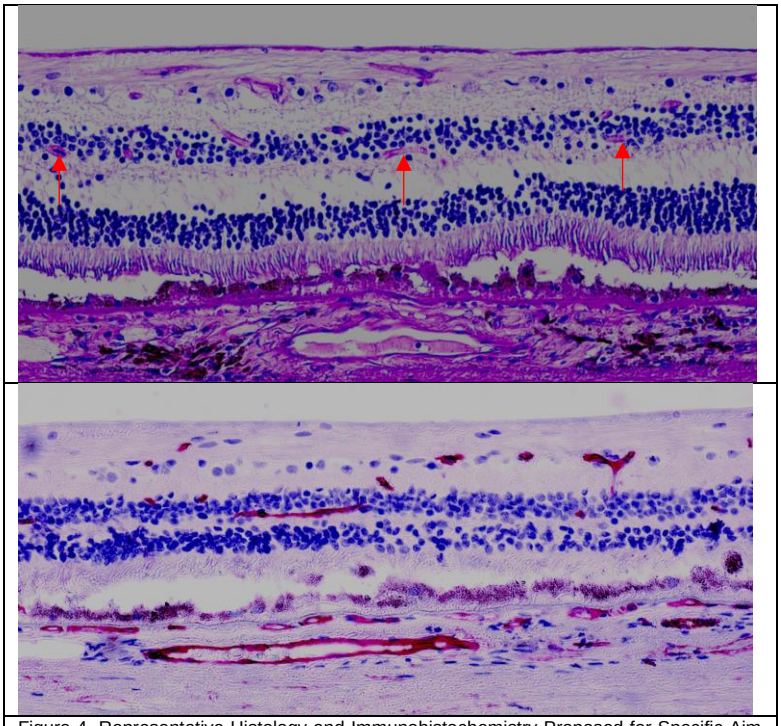


Figure 4. Representative Histology and Immunohistochemistry Proposed for Specific Aim 2. (**Top**) Hematoxylin and Eosin with Periodic Acid Schiff staining of retina cross-section from a non-MarkVCID study subject at USC. Tubular structures in the retina represent basement membrane of capillaries (red). (**Bottom**) Hematoxylin and Eosin staining with immunohistochemistry for CD34 in red. Retinal and choroidal vessels are visible. Different section than top panel. Data from human cadaver eye that was collected, fixed, and processed by Dr. Kashani and Dr. Hinton's group at USC.

We propose to assess the relative density of retinal capillaries containing pericytes and endothelial cells in serially sectioned macular regions corresponding to regions of VSD analysis from subjects in the MarkVCID consortium. Macular regions corresponding to the region of VSD analysis from OCTA will be grossly dissected and isolated for serial sectioning. Standard Hematoxylin and Eosin staining will be performed on every 10th section. Immunohisto-chemistry of endothelial cells will be performed using the pan-endothelial marker CD31[29] and CD34 with commercially available antibodies that have been used successfully by our group in the past (Figure 4). Immunohistochemistry of pericytes will be performed using PDGFRβ antibodies. We also propose glial fibrillary acidic protein (GFAP) to assess for reactive astrocytes/Muller cells. Capillary basement membrane thickness will be assessed with Periodic Acid Schiff staining (Figure 4). Collectively, these studies will provide qualitative cross-validation of the VSD metric as well as histopathologic data to guide the clinicopathologic correlation of decreased VSD in human retina.

We anticipate that only a limited number of cadaver eyes will become available during the course of the MarkVCID from participating sites (USC, UCSF, JHU and Rush). We propose to collect and preserve up to 10 eyes per year (from up to 5 subjects enrolled) in the MarkVCID prospective cohort and who undergo VSD assessment. We have identified regional tissue banks accredited by the Eye Bank Association of America (https://restoresight.org/) that serve each of the MarkVCID consortium sites participating in the VSD assessment. We will engage the professional services of these organizations to harvest cadaver eyes as they become available for subjects who provide consent at study enrollment or at time of death through family members. Dr. Kashani's group has previously obtained tissue from eye and tissue banks in California and Florida for analogous studies requiring tissue pathology. As an example, Dr. Kashani has discussed this plan with and obtained a quote for services from the Illinois Eye and Tissue Bank (Eversight). The costs for these activities will be covered by the Brightfocus Foundation supplement. All tissue collection activities will be performed under an IRB approved amendment. Both USC and Rush MarkVCID sites already have other ongoing studies with collection of postmortem human tissue and do not anticipate difficulty in obtaining IRB approval. Dr. Kashani will help supervise other MarkVCID sites for obtaining IRB approval and coordinating logistics for tissue collection.

All eyes will be professionally collected and fixed immediately (within 4-8 hours) using a standardized formalin fixation method that has previously been successfully used by Dr. Kashani at USC. Eyes will be shipped to USC where Dr. David Hinton (Board Certified Neuropathologist) and Dr. Kashani will perform gross dissection of the eyes and isolate the macular region that was used for OCTA imaging and VSD measurement. Dr. Kashani and USC staff have performed similar procedures in the past (Figure 4). Gross pathology will be photographed and documented. Optic nerves and peripheral retina will be embedded and saved for future analysis. Since the MarkVCID cohort includes both normal as well as affected subjects we may not need to collect age- and gender matched controls from outside the study. However, if age- and gender- matched controls are not available through the MarkVCID due to the limited time frame during which tissue may become available we will engage the eye and tissue banks to identify and collect age-, gender- and comorbidity- matched specimens. This process has been implemented before by Dr. Kashani's group to obtain appropriate tissue for analysis and is feasible due to the high volume of eye tissue that becomes available through the eye banks.

Embedding and sectioning of the eyes will be performed in the Department of Ophthalmology histology Core that has been used by Dr. Kashani and Dr. Hinton for other ongoing histopathology studies in human eyes. Immunohistochemistry will be performed at the CLIA certified USC Pathology Immunohistochemistry Core. We will process a small number of eyes (up to 3) for transmission electron microscopy (TEM) to evaluate ultrastructural details about the vascular alterations identified by light microscopy/immunohistochemistry. Transmission electron microscopy will be performed at the USC Cell and Tissue Imaging Core directed by Dr. Hinton. These data will be invaluable to propose further studies that may be relevant to understanding the pathologic changes occurring in the retina in SVD.

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