MarkVCID Plasma Endothelial Signaling Biomarker Kit

1. Executive summary

We are proposing to validate a fluid biomarker kit that measures plasma-based measures of endothelial signaling. Our underlying model of small vessel disease posits that endothelial dysfunction and subtle breakdown of the blood brain barrier initiate a pathological cascade leading to inflammation, diffusion abnormalities, white matter injury and cognitive dysfunction. Accordingly, levels of endothelial signaling molecules could reflect an early stage in the chain of biological processes that lead to frank cerebrovascular disease. Our preliminary work has offered considerable support for this model. In a sample of 237 adults across the spectrum of typical aging, VCID, and Alzheimer's disease (1-8 study visits, 398 total longitudinal observations), a composite score of key endothelia signaling molecules was directly associated with older age and increased over time. Baseline levels of the endothelial signaling composite were significantly associated with MRI markers of white matter injury (cerebral free water, whole-brain fractional anisotropy, whole-brain mean diffusivity, and these relationships remained significant even after controlling for amyloid PET status. Importantly, when examined in longitudinal models, higher baseline levels of the endothelial signaling composite predicted larger increases in white matter hyperintensities (WMH) and CDR, and greater declines in MMSE.

We propose to carry out two types of validation for this endothelial signaling kit. The first type is methods validation, in other words, how reliably can these molecules be measured across sites. We hypothesize that the same set of samples run at different sites will yield similar results such that an intraclass correlation coefficient will exceed .8 and the coefficient of variation in absolute values will be less than .25. The second type of validation will provide conceptual support for the biomarker. We hypothesize that at each participating site; 1) levels of the endothelial signaling composite will be negatively associated with an executive function composite score generated from item response theory (primary hypothesis), 2) positively associated with cerebrovascular disease (WMH burden), negatively associated with MMSE after controlling for age, sex, and CDR, and positively associated with CDR sum of boxes after controlling for age and sex; and 3) baseline levels of the endothelial signaling composite will be associated with faster longitudinal increases in white matter injury and faster longitudinal decreases in executive function. We anticipate completion of the first set of validation studies within 15-months of initiation and the second set of validation studies within 24-months.

Our primary proposed biomarker category is risk stratification. Certainly, trials that target endothelial dysfunction as an early intervention for cerebrovascular disease could select endothelial signaling as a marker of efficacy, and a trial wanting to select subjects at greatest risk of decline would use the kit for risk stratification. Alternate applications are also possible, of course, and depend entirely on the goal of the clinical trial. As trials increasing target underlying biological mechanisms of disease, for example, the endothelial signaling kit could readily serve as proof of target engagement or even help define relevant disease pathways.

2. Brief description of biomarker kit

Our proposed "Endothelial Signaling Kit" measures three plasma proteins important for endothelium maintenance: vascular endothelial growth factor (VEGF-D), placental growth factor (PlGF), and basic fibroblast growth factor (bFGF). The selected endothelial markers critically regulate angiogenesis, support collateral circulation, promote endothelial cell growth, and signal macrophage migration.

Table 1. Meso Scale Discovery (MSD) V-Plex Angiogenesis Panel

	LLoD (pg/mL)	LLOQ-ULOQ (pg/mL)	Coefficient of Variance (%)* (median, IQR)	Coefficient of Variance <10%*
VEGF-D	4.36	67.1 - 18,800	2.9 (1.4, 5.2)	93.6%
PlGF	0.05	1.5 - 800	2.0 (0.9, 3.5)	99.1%
bFGF	0.09	2.6 - 1,780	2.9 (1.3, 5.4)	92.8%
*based on n=642 time-point measurements (in duplicate)				

These three markers of endothelial health were selected due to their *a priori* theoretical importance and extensive pilot work showing that they can to be reliably captured (Table 1) using the Meso Scale Discovery (MSD) V-Plex platform (most rigorous MSD standard and according to best practices of MarkVCID), and are sensitive to the MRI and cognitive correlates of VCID. To reduce the data to a single score, we calculated a composite measure of plasma VEGF-D, PlGF, and bFGF by log transforming the original values, computing sample-based z-scores, and averaging the three z-scores.

Running this kit requires a MesoScale Discovery machine and lab supplies summarized in Table 2.

Table 2. Lab supplies for endothelial signaling kit
Eppendorf Xplorer 8 channel electronic pipette, 15-300uL
Eppendorf Xplorer single channel electronic pipette, 50-1000uL
Eppendorf Research Plus Pipette, 10-100uL
Eppendorf Research Plus Pipette, 1-10uL
Eppendorf Research Plus Pipette, 1000uL
Drummond Portable Pipet-Aid XL
Pyrex Jar 1L
Ice Tray/ Bucket
Tube Rack
Graduated cylinder
Plate Shaker
MESO QuickPlex SQ 120 imager (MSD, Rockville, MD)
Cold Room
Angiogenesis plate MSD V-plex K15190G
Other Consumables: Eppendorf Plate, Pipette tips, Disposable Serological Pipettes, Paper towels, Falcon Tubes (15mL & 50mL), 1.5mL Eppendorf Tubes, Disposable Reagent Reservoirs

3. Participating sites

Four sites, UCSF, UKy, CHARGE, and UNM, will be analyzing samples. All seven sites have offered to provide samples.

4. Protocol for fluid sample acquisition

All prospective plasma samples will be collected according to MarkVCID fluid best practices determined by the Fluid Biomarker Subcommittee. We will require aliquoted plasma that has not undergone more than one freeze/thaw cycle. Shipping protocols will also follow the subcommittee best practices.

5. Additional data collection required for analysis

All additional data required for analysis are routine parts of standard MarkVCID data collection. We will use Uniform Data Set data as determined by the Clinical Data Subcommittee for our measures of cognition, history and presence of cerebrovascular and cardiac disease and vascular risk factors, and CDR. We also require that all subjects who participate in this validation study have at least one brain MRI with a Fazekas score and that the image has undergone processing using the pipelines that are routinely used at the site to generate WMH volumes.

6. Protocol for fluid biomarker analysis

The protocol for running plasma samples through the MSD platform are very well established with extensive validation data available. We will be using the MSD V-Plex Angiogenesis Panel 1 Kit for humans. In general, MSD V-Plex products are developed under rigorous design control. These are sandwich immunoassays and MSD provides a plate pre-coated with antibodies. Users add the sample and solution containing detection antibodies conjugated with electrochemiluminescent labels over the course of one or more incubation periods. The Angiogenesis kit comes as a 7-spot multiplex kit, and reagents that are supplied with all kits include diluents, blocker, and buffer kits plus wash buffers, plate seals, and controls. Additional materials required to run the assays include appropriately sized tubes, polypropylene microcentrifuge tubes, pipettes, plate washing equipment, microtiter plate shaker, phosphate-suffered saline, adhesive plate seals, and deionized water (see Table 2). Kit lot numbers and adherence to SOP processing will be recorded at each site for MSD plate run. Best practices for running MSD assays are clearly spelled out in the MSD documentation. Specific steps, including shaker speed, incubations times, volumes, and dilution are outlined in Table 3. The ambient plate shaker should be on a lab bench that is bolted to lab flooring and adjacent benches with vibration isolation pads. We are proposing that all samples are run in triplicate. Sensitivity and degree of agreement for our selected signaling molecules are well documented and robust across a range of dilutions. In our preliminary work with this V-Plex, missing data (i.e., insufficient detectability) is very rare, and median coefficients of variance (CV) are below .03. Well over 90% of the samples run thus far have CVs that are lower than 0.1.

Table 3. MSD Angiogenesis Panel procedures
Diluent #7 is thawed, plates brought to RT, samples, calibrators and controls thawed on ice.
Angiogenesis panel plate is blocked with Blcoker A (150µl per well) and plate shaken at 700rpm for 1 hour at RT
75 ul of each sample is transferred to the polypropylene 96 well plate
Angiogenesis Plate is washed 3X with 150µL wash buffer/well
Calibrators, controls and samples are transferred to MSD plate
Angiogenesis Panel-Calibrators and Controls 50µL per well; samples -25µL diluent 7 +25µL samples
Plates are incubated overnight at 4°C while shaking at 700rpm (modification of MSD protocol)
Bring the plate to RT
Start thawing diluent 11 at RT
Break for 1 hour
Prepare the Detection antibody cocktail
Antibody diluent -Diluent 11
Add 60µl of each Ab in 2580µl Diluent 11
Wash MSD plate 3X with 150µL wash buffer/well
Add 25ul of detection antibody cocktail per well. Incubate the plate at RT for 2 hr with shaking at 700rpm.
Wash Angiogenesis Panel 3X with 150µL wash buffer/well. Add 150µL 2X Red buffer T/well and read the plate.

Although the protocol for running the MSD V-Plex Angiogenesis panel is well detailed and the process yields reliability data, we are proposing a training period to ensure optimal reliability of this kit within MarkVCID. UCSF will provide eight identical plasma samples to each participating site. Lab techs at those sites will these plasma samples using their MSD Quick Plex SQ120 and send their data to the Coordinating Center. UCSF will review and analyze these data to measure measurement reliability and interrater reliability. Any lab where >10% of CVs exceed .15 or whose absolute values are discrepant with the other sites (>25%) will be reviewed and additional practice samples will be carried out if necessary.

After completion of the training, the four sites will carry out the larger reliability experiment. The Coordinating Center will assist with identifying 96 plasma samples (5 aliquots each), with one aliquot from each sample sent to UCSF, UKy, CHARGE, and UNM for analysis. Sample size was based on achieving power of greater than .80 to detect an ICC of greater than .8. Analyses will be run in triplicate, requiring four V-Plex Angiogenesis kits per site. Upon completion, each site will digitally upload the values of each analyte in each well to the Coordinating Center for data storage and distribution. Although the V-Plex Angiogenesis kits yield values for seven different analytes, our kit relies on VEGF-D, PlGF, and bFGF. Cross-site variation in the distribution of obtained values will be analyzed by Bland-Altman analysis and cross-site correlation matrices to ensure cross-site reliability.

7. Step-by-step analytic plan

Aim 1a. Methods validation (reliability across sites): We will first compute the CVs for each sample at each site. As samples are run in triplicate, there will be four CVs per sample (three dyadic comparisons and one overall). If all four CVs are under .2, the value for that analyte will be the mean of the three wells. If the measurement from one well is an outlier (CV with the other two wells >.20), the value for that analyte will be the mean of the other two wells. If the individual and overall CVs >.20, the value for that analyte will be coded as missing. Although the distributions of these values tend to be normal, we address the issue of outlier values by carrying out a log transformation of the original values and then generate z scores using the mean and standard deviation of a large reference sample at UCSF/UCD. The value of the endothelial signaling composite is the mean of the z-scores.

The resulting dataset will have five values (one from each site) for each sample on the endothelial signaling composite and the z-scores for VEGF-D, PlGF, and bFGF. The reliability of these measurements will be estimated using Intraclass correlation coefficients (ICC). Mixed effects regression models will be used to estimate the ICC by treating the five values for each sample as repeated measures.

Aim 1b. Methods validation (reliability within subjects): An important step in determining whether these assays can serve as a biomarker is demonstrating that plasma levels in a given subjects do not vary dramatically over short periods of time. We proposed that each participating site collects three blood draws on 10 subjects at 3 timepoints at least 5 days apart from one another, and completed within 30 days. All 30 samples at each site will be run in duplicate simultaneously on the same plate and mean values and CVs calculated for each subject at each timepoint and the reliability of these measurements will be estimated using Intraclass correlation coefficients.

Aim 2. Conceptual validation: Further validation for the endothelial kit will come from replicating early results from UCSF at the other participating sites. In cross-sectional analyses, we found that the endothelial signaling composite was significantly associated with MRI markers of white matter injury (cerebral free water, whole-brain fractional anisotropy, whole-brain mean diffusivity, and these relationships remained significant even after controlling for amyloid PET status. In longitudinal analyses, higher baseline levels of the endothelial signaling composite predicted larger increases in WMH and CDR, and greater declines in MMSE.

We propose to replicate and expand these findings at UKy, CHARGE, and UNM. Each site has existing cohorts with multiple timepoints in either archival form or prospectively followed, with stored plasma, UDS measures, Fazekas scores, and locally obtained MRI metrics reflecting degree of WMH burden. Each site will study 96 subjects (half with CDR of 0, half with CDR of .5 or 1) reflecting a range of WMH burden. Cross-sectional analyses will use the first time point in which there is a plasma sample and all the available clinical and imaging data. We hypothesize that levels of the endothelial signaling composite will be: a) positively associated with cerebrovascular disease (Fazekas score,

WMH burden); b) negatively associated with executive functioning after controlling for age, sex, and CDR; and c) positively associated with CDR sum of boxes after controlling for age and sex.

Our primary cognitive outcome measure for executive function will be an item-response theory (IRT) generated score based on four executive tasks from the UDS 3.0: Trails B (number of correct lines per minute), backward digit span (total score), phonemic fluency (number of correct F-words in one minute), and categoryfluency (number of correct animal responses in one minute). Confirmatory factor analyses support good model fit for these donor scales. IRT scores will be built using baseline UDS data from 3,450 clinically normal subjects. Each measure will be entered into an item responsive analysis using the R *ltm* module (Kramer et al., 2014; Mungas, Reed, Kramer, 2003). The parameters from this analysis will be saved and applied to MarkVCID participants to generate a composite score for each individual at each time point. IRT is an advantageous approach because the composite is invariant to the specific scores that are used. Therefore, the IRT score should produce an unbiased estimate of a participant's executive ability even if different variables are used to generate the scores, making this composite particularly appropriate for longitudinal research. IRT scores will allow the consortium to maximize the sample size while also improving the reliability and robustness of the outcome measure. A provisional version of this metric correlated .84 with an independent measure of executive functioning, supporting the construct validity of the IRT measure.

The longitudinal analyses will require at least one follow-up clinical assessment and MRI at least one year after the baseline blood draw. Using linear mixed models, we hypothesize that higher baseline endothelial signaling composite scores will predict larger increases in WMH and CDR, and greater declines in executive function.

8. Sample size calculation (individual site level)

Methods validation (reliability): With 96 samples each evaluated at the 4 sites, we will have over 80% power to detect an ICC of at least 0.8 assuming a null hypothesis ICC of 0.7.

Conceptual validation: For cross sectional analyses, with n=96, we will have 80% power to detect an association accounting for as little as 8.4% of the variability in the outcome (a correlation as small as 0.29). For analyses of change in cognition, we will have 80% power to detect an additional contribution of as little as 8.4% of the variability in cognitive change, assuming other variables such as age, gender, and CDR account for 2% of the variability (what was observed in our preliminary data from the UH2 phase). Should covariates account for a higher percentage of the variability in cognitive change in this phase, our power will be higher to detect an additional contribution of 8.4% to the variability in the outcome.

9. Plan for longitudinal data collection and analysis

Longitudinal data collection and analyses will proceed in two ways. In our two large legacy cohorts, Rush and CHARGE, the longitudinal data will consist of all timepoints subsequent to the baseline evaluation. Both sites have already collected a significant amount of longitudinal data, with banked plasma, stored MRI scans, and clinical phenotype data. We have already proposed to utilize these data to address longitudinal questions about endothelial signaling biomarker kit using linear mixed models (Section 7). Other sites that are actively enrolling or prospectively following subjects will continue to collect biofluid, imaging, and clinical data using standard MarkVCID protocols. While Section 7 also includes a proposal to utilize these data to address longitudinal questions, we recognize that some sites may not have sufficient amounts of longitudinal data to meaningfully carry out linear mixed models. Accordingly, we stress the importance of ongoing longitudinal data collection to assess several key longitudinal questions, including: a) are there longitudinal changes in endothelial signaling levels; b) do endothelial signaling levels change in tandem with clinical measures (e.g., primarily executive measure, and secondarily in executive function and CDR) and neuroimaging (Fazekas scores, WMH); 3) are there relationships between endothelial signaling and vascular risk factors (e.g., blood pressure, insulin resistance); 4) does the presence of positive AD biomarkers (e.g., amyloid PET, CSF) influence the relationships between endothelial signaling and other key

variables; 5) are there endothelial signaling factors (e.g., batch effects; test-retest consistency) that clinical researchers and trialists need to consider as they incorporate endothelial signaling into their research; and 6) how do our proposed longitudinal relationships with neuroimaging perform with those MarkVCID neuroimaging kits that are sufficiently validated.

10. Plan for reporting outcomes

There is an explicit agreement that each site will share their data with the Coordinating Center to create the best opportunity to advance science. The merging of the endothelial data with the cross-sectional and longitudinal clinical imaging, and other biofluid data will offer an extraordinary opportunity to enhance not just our understanding of VCID but also to better prepare for clinical trials. This would undoubtedly promote analysis, presentation and publication of our results. The results of our work could also entice pharmaceutical company interest in the treatment of VCID, resulting in new clinical trials.

11. Plan for sharing data, samples/images, protocols

The detailed protocols and plan for validation will be shared with all sites. We also plan to share all data with the Coordinating Center so that it can be shared in accordance with protocols and agreements outlined by the MarkVCID consortium.

12. Milestones

We propose three milestones to be accomplished in the first year.

1. The 'reliability across sites' methods validation study based on 40 plasma samples will yield an ICC of greater than .70.
2. The 'reliability between subjects' methods validation study based on 10 subjects will yield an ICC of greater than .70.
3. The conceptual validation study will yield partial correlation coefficients between the endothelial signaling composite and that site's MRI markers of white matter injury and our proposed executive function score of greater than .20 for cross-sectional analyses. For those sites able to carry out longitudinal analyses, baseline endothelial signaling will be significantly associated with an increase in WMH and decline in executive functioning.

References

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