Official Title: CCMs among HIspanic Population Study group (CHIPS)

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Protocol/Research Activity Plan

A. Review of prior work in the area

Genetic contributions to major forms of cerebral cavernous malformations (CCMs) have been recognized for a long time, however, many causative factors for cerebral cavernous malformations have yet to be determined. CCMs were initially identified as a genetic defect among Hispanic patients [1] and, to date, remain a major neurological disorder among Hispanic populations with the highest prevalence compared to other ethnic groups [2-4]. Despite these overwhelming statistics, only three loci have been identified in CCM patients (CCM1-3)[2, 5-11], with many patients still harboring unidentified mutations [12]. The proposed research will be focused on the elucidation of causative factors and diagnostic biomarkers for cerebral cavernous malformations by the **C**CMs among **HI**spanic **P**opulation **S**tudy group (**CHIPS**), which is composed of several basic scientists, neurologists, and neurosurgeons.

B. Specific Objectives

The objective of this research is to identify new genetic loci and validate novel serum biomarkers we recently identified for CCMs and other types of hemorrhage strokes in humans. The purpose of the study is to identify and validate the causative factors and prognostic biomarkers for hemorrhagic strokes in humans. Recently, a team led by our research co-PI has made some breakthrough findings involving new serum biomarkers for predicting CCMs hemorrhagic events (TTU has filled provisional patent). Our data (from IACUC 18004-submitted and in peer review) demonstrated that there is a correlation between serum levels of Progesterone (PRG) and the initiation of hemorrhagic strokes in Ccms mutant mice. Furthermore, four more biomarkers (Serpin A6, IL-12, IL-6, and Albumin) are all associated with PRG homeostasis/biogenesis in serum, and also correlate well with hemorrhagic events in Ccms mutant mice. This research is especially significant for this region which contains the highest percentage of Hispanics in the nation (>80% of the entire population), recognizing El Paso as the epicenter for CCMs by stroke clinicians around the world. As such, this proposed project has regional relevance and importance in rural health disparities on the U.S.-Mexico border region.

C. Hypothesis

We hypothesize that there are additional causative genetic loci contributions to the pathogenesis of human cerebral cavernous malformations and conformation of effective serum biomarkers for diagnostic and pragmatic use will be an essential tool to prevent hemorrhagic CCMs in humans. We will use our collected patient samples from local Hispanic populations for next-generation-sequencing (NGS) and biomarker assays.

D. Study Design

Patient recruitment and clinical data acquisition

Clinical and translational research will be structured to assess potential biomarkers and heritable factors for hemorrhagic CCMs. Up to 900 CCM Patients (between both Standard of Care and Research only samples) will be identified from participating clinical co-PI (Principle investigators) practices to investigate the risk factors and hemorrhage incidence rates among the Hispanic population. All patients will be asked to invite their relatives for the possibility of participation in this study by providing a flyer to the subject to give to their relatives, and if they are interested, the relative may contact the research PI or Clinical co-PI's to participate in this study and provide consent. Non-English speaking participants will be provided all documents in Spanish, and will also be consented, in English or Spanish. Demographic details of patients will be recorded as well. Potential subjects will be identified using the following criteria:

1. Having a positive medical history associated with CCMs, or stroke/epilepsy
2. Having a family member with a positive medical history associated with CCMs, or stroke/epilepsy

Clinical co-PIs and co-investigators will describe in detail the clinical presentations associated with CCMs, or stroke/epilepsy, and the study procedures for an initial screening. If the potential subject agrees to participate in the initial screening, informed consent and/or assent will be obtained by the Clinical co-PIs or co-investigators and/or approved research personnel working on the study. Consenting will occur in the PFS and Laboratory suite located at University Medical Center of El Paso, 1st floor UMC main building, 4815 Alameda Ave, El Paso, TX 79905. Alternatively, if this location is not available for consenting patients, we will consent patients in private, closed-door conference rooms that can be reserved for CONSENTING ONLY. These rooms are located in MSBI rooms #2106, 3106 and 4106 located at 5001 El Paso Drive, El Paso, TX 79905. No other research procedures pertaining to this study will occur in these rooms other than consenting patients. Once consent has been obtained, information gathered from the initial screening will be cross-examined with electronic and paper medical records to confirm their charted medical history. All patients who do not meet eligibility criteria will be excluded from participating in the study. After study criteria have been met, all subjects will have blood taken from one of the arm veins, as part of standard of care, with an additional one and a half tablespoons (2 vials, one for serum collection and one for plasma) being drawn for this study for genetic testing and validation analysis by research co-PI. All samples provided will be coded for confidentiality and privacy. The biological molecules (nucleic acid and proteins) obtained from the sample will be frozen and stored for future use. Completion of the questionnaire and the blood draw should take less than 30 minutes of the subject's time and will be completed in one visit. A blood draw is normally a routine part of the standard of care, for diagnosing and initial evaluation of stroke/epilepsy and CCM diagnosis.

In the unique circumstances where blood draws are not required for standard of care, one-and-a-half tablespoons of blood (2 vials, one for serum collection and one for plasma) will be extracted for genetic testing and validation analysis by research co-PI for this study with the following modifications from the standard procedure.

1. Up to 900 CCM Patients (between both Standard of Care and Research only samples), will initially be asked if they would like to participate in this study if they meet the inclusion criteria.
2. Primary care physicians on this protocol (Drs. Vellipuram, Prospero-Ponce, Gavito, Gupte, Lavezo, Brower) will be able to perform an electronic medical search of their patients to identify patients that were previously diagnosed with CCMs in the past 10 years (1/1/2011-1/1/2021). Primary Care physicians may ask currently approved research personnel to assist them with this task. Medical records will only be assessed to confirm ICD9/10 codes for their patients to confirm eligibility requirements, and to obtain electronic mail addresses, phone numbers, and/or mailing address to contact/present their patients with the currently approved IRB recruitment flyer/letter so they can decide if they would like to participate.
3. Any Patients that qualify to participate upon a medical search review by their primary care physician, would still be contacted and presented with our IRB approved recruitment flyer so that they can decide on whether they would like to participate. If these patients are interested in participating, they will be consented, as normal, and blood draw performed, as normal.
4. Alternatively, patients may contact either Dr. Johnathan Abou-Fadel or Dr. Jun Zhang to see if they qualify to participate in the study (based off the inclusion criteria) from posted notices of solicitation and recruitment flyers posted at the following websites:

1. https://www.angioma.org/biomarker-development-project-in-el-paso-seeking-participants/
2. https://elpaso.ttuhsc.edu/research/ccm-survey.aspx
3. https://elpaso.ttuhsc.edu/research/ccm-survey-spanish.aspx

5. Patients participating in blood draw for research purposes only that were not recruited involving a UMC/TTUHSCEP physician may provide their medical records to either Dr. Johnathan Abou-Fadel or Dr. Jun Zhang at their discretion to help make the analysis of their blood donation more meaningful in terms of clinical symptoms associated with the scope of this study. All medical records will be stored according to the same HIPAA compliance and regulations as if those records were obtained from their direct PCP.
6. If the participant agrees to provide medical records, additional demographic information that will be obtained includes affected individuals, age of onset, ethnicity, gender, type of stroke, date of birth, treatments received. An assessment of the patient's current condition will be made by asking if the patient shows any signs of gait apraxia, cognitive deficiency, and seizures. Specific questions will be asked in order to evaluate physical, social, cognitive, and overall health.
7. Consent/blood draws will be in the PFS and Laboratory suite located at University Medical Center of El Paso, 1st floor UMC main building, 4815 Alameda Ave, El Paso, TX 79905. Alternatively, if this location is not available for consenting patients, we will consent patients in private, closed-door conference rooms that can be reserved for CONSENTING ONLY. These rooms are located in MSBI rooms #2106, 3106 and 4106 located at 5001 El Paso Drive, El Paso, TX 79905. No other research procedures pertaining to this study will occur in these rooms other than consenting patients
8. Patients will be walked over to the PFS and Laboratory suite by Dr. Johnathan Abou-Fadel or Dr. Jun Zhang where informed consent and/or assent will be conducted (if consenting was not already completed in MSBI 2106, 3106 or 4106) by either Dr. Johnathan Abou-Fadel or Dr. Jun Zhang, followed by blood draw for research purposes only which will be handled by a phlebotomists working in the PFS and laboratory suite.
9. The PFS and Laboratory suite is both a research lab as well as having equipped stations where phlebotomy work can be safely performed. Having one site for both processes eases our workflow and limits the number of coordinators that need to be added to this study by using different locations for each process. As stated in the procedure section for "blood draw for research purposes only" in the study application, the patients/subjects will be personally escorted by Dr. Johnathan Abou-Fadel or Dr. Jun Zhang and will stay with the subjects until all assents/consents have been performed as well as completion of the blood draws. Once complete, Dr. Johnathan Abou-Fadel or Dr. Jun Zhang will transport the samples back to the lab where they will be processed, and Biological molecules obtained from this sample (Nucleic acid and proteins) will be stored in MSBI in our -80 freezers until ready for analysis. All samples provided will be coded for confidentiality and privacy. Completion of the questionnaire and the blood draw should take less than 30 minutes of the subject's time and will be completed in one visit.

A small group of subjects will be identified as high-risk for hemorrhage due to CCMs from the stroke/epilepsy clinics. If any imaging modality is performed to assess their CCMs, these will be

included in the research analyses. All safety precautions and confidentiality to the findings from the blood samples will be upheld at all times during the study.

For screening and enrollment purposes, we define hemorrhage from CCMs as evident from brain imaging. Whether it may cause neurologic deficits or not, the subject shall be included as part of the study, once they are diagnosed to have one or may cavernous malformations. Given the high morbidity and mortality of the disease, demographic information, age at the time of treatment, type of treatment, and treatment dates will be obtained for all individuals. All clinical data will be de-identified with numerical coding in accordance with personal health information (PHI) regulations and will be stored using Excel software with encryption and security codes on a PC in the **C**CMs among **Hi**spanic **P**opulation **S**tudy group (CHIPS). Confidentiality of protected PHI in compliance with the Health Insurance Portability and Accountability Act (HIPAA) will be ensured by utilizing text encryption, password protection, and limited personnel involvement.

Both sporadic and familial cases of CCMs will be recruited for this study. If the patient has either a first, second, or third-degree relative with similar clinical conditions, then we will consider this patient as a familial stroke case. The demographic information that will be obtained includes affected individuals, age of onset, ethnicity, gender, type of stroke, date of birth, treatments received. An assessment of the patient's current condition will be made by asking if the patient shows any signs of gait apraxia, cognitive deficiency, and seizures. Specific questions will be asked to evaluate physical, social, cognitive, and overall health. Responses from mothers of the patients will be acquired if the patient is a child to maintain consistency. If the patient is a child, we will also ask if the patient has experienced any recent increased frequency of headaches, nausea, and vomiting, or engorgement of the scalp veins. We ask these latter questions because some of these patients might have been lost to follow-up before and we will redirect them to the attending neurologist's and neurosurgeon's office for scheduling of clinic appointments. For patients, or their family representative, who are unable to provide an adequate medical history, the patient's electronic and paper medical records will be used to supplement incomplete data parameters. All screened patients for whom data could not be completely confirmed by interview, electronic or paper medical records will be excluded from further analyses. Willing candidates with severe anemia or active allergies will be excluded from this study.

Eligibility Criteria/Subject Population

- Inclusion Criteria
  1. 10 to 89 years of age
  2. Medical history meets the criteria for the International Classification of Diseases diagnosis codes 10 (ICD-10) for cerebral cavernous malformations (CCMs) (Q28.3 or D18.02) or stroke/epilepsy.
  3. Medical history meets the criteria for the International Classification of Diseases diagnosis codes 9 (ICD-9) for cerebral cavernous malformations (CCMs) (228.02) or stroke/epilepsy.

OR

  1. Has a family member whose medical history meets the criteria for the *International Classification of Diseases* diagnosis codes for cerebral cavernous malformations (CCMs) (Q28.3 or D18.02 or 228.02) or stroke/epilepsy.
  2. Has a relatively complete medical history that can be accurately confirmed by an interview or electronic and/or paper medical records
  3. Hispanic and non-Hispanic Americans

- Exclusion Criteria
  1. 9 years of age or younger; 90 years of age or older
  2. Has no medical history or no clear clinical description that cannot be accurately confirmed by an interview or electronic and/or paper medical records

Blood collection, DNA and protein preparation

Venipuncture will be performed for participants with Vacutainer™ (Bectin-Dickenson, NJ) to collect blood specimens using one 3-4 ml Gold tube (serum) and one 3-4 ml blue/black (lavender) tube (plasma) for anticoagulated whole blood. Genomic DNA will be obtained from blue/black (lavender) tube through a standard procedure of separation of white blood cells from red cells by low-speed centrifugation, lysis of white cells, and precipitation of DNAs as the standard protocol describes [4]; red cells will be spun down by high-speed centrifugation in the remaining supernatant, and the remaining supernatant will be plasma used for protein analysis, along with collected serum samples. These samples (Nucleic acids and proteins) will be frozen and stored for future use. DNA and protein preparation will be performed by research co-PI (Principle investigator, JZ) for the next stage of genomic and proteomic analysis.

Acquisition of Control samples:

1. 1000 control Biospecimens (blood collection tubes with various amounts of blood), from the UMC-EP regional lab located in the parking garage at University Medical Center of El Paso, 4800 Alberta Ave, El Paso, TX 79905, will be collected after all standard of care testing has been run and before the biospecimens are to be discarded.

2. In order for a sample to be categorized as a "healthy control", the biospecimen sample (serum or plasma) must display normal established levels for at least 2/4 common laboratory tests (depending on what was ordered by the PCP) run on human blood to determine any signs of infection or inflammation. These tests include CBC (with or without differentials, depending on what was ordered), ESR test, CRP test, and CMP test. We will require, at minimum that both ESR and CRP have normal results as the baseline for determining if a sample can be considered a "healthy control". Depending on what additional tests are ordered for the patient, then CBC and CMP may also be evaluated and must show normal healthy levels (if ordered). Therefore, the 3 following possibilities may exist when the lab is screening samples.

   (1) If CBC or CMP was not ordered for the patient, then, at minimum, ESR and CRP must demonstrate normal levels, allowing this sample to qualify as a healthy control.

   (2) If a patient's PCP has ESR, CRP and CBC ordered, then all three of these tests must show normal healthy levels for this sample to qualify as a healthy control.

   (3) If a patient's PCP had all 4 tests ordered (ESR, CRP, CBC and CMP) then all 4 panels must show normal healthy levels for the sample to qualify as a healthy control.

3. The biospecimens (blood tubes with various volumes, depending on standard of care laboratory tests performed) will be de-identified by Dr. Johnathan Abou-Fadel or Dr. Jun Zhang before leaving the PFS laboratory suite.

4. Information about biospecimens will be recorded in such a manner that the identity of the human subjects cannot readily be ascertained directly or through identifiers linked to the subjects, and we will not contact the subjects, or attempt to re-identify subjects.

5. The only information that will be collected is age (xx yrs), Sex (M/F), and ethnicity (White/Hispanic/Asian/African American, etc) so that it may be used for age/sex/ethnicity matching of our CCM patient samples.

6. Dr. Johnathan Abou-Fadel or Dr. Jun Zhang will transport the samples back to the lab where they will be processed, and Biological molecules obtained from this sample (Nucleic acid and proteins) will be stored in MSBI in our -80 freezers until ready for analysis.

Next-generation sequencing (NGS)

Most of our collected stroke (CCMs) families are small nuclear families, consisting of two or more affected siblings which resemble the genetic basis of common and complex genetic traits. Updated results suggest that rare variants may account for the "missing" heritability. Such rare variants may have a large effect as genetic risk factors for complex genetic diseases. Therefore, current challenges will be to define the genetic basis of 'missing' heritability.

Next-generation sequencing (NGS) technologies will enable us to identify all the causative variants including "rare variants". It is anticipated that whole-genome sequencing (or exome sequencing) will make significant contributions to our understanding of the genetic etiologies that contribute to complex human disease [13]. In this proposal, we hypothesize that due to the heterogeneous nature of CCMs, some causative alleles for CCMs are rare genetic variants located in the unknown genetic loci; furthermore, a common pathophysiological event during hemorrhagic initiation makes predicting hemorrhagic events possible with shared biomarkers. Although the genetic basis for the stroke is likely to be heterogeneous, there are certainly some genetic overlaps among different families. Therefore, the whole-exome sequencing at TTUHSC with Agilent Sure Select Human All Exon kit will be used to sequence serum samples to identify the causative genetic variants for CCMs/stroke/epilepsy.

Using this new NGS technology at TTUHSC genomic core, we have successfully generated the whole-genome sequencing (exome) data and performed analysis for three unrelated patients in our other genetic projects, which built a solid foundation for our future success of this proposed project. Bioinformatics analysis of the NGS data would be finished by the end of 2025.

There are implications related to knowledge of possible genetic information that may be obtained as a result of this study. These include knowledge of genetic mutations which may be shared with the patient, as well as significant risk factors for those who test positive for these gene mutations. Because of the potential of a genetic basis for genetic inheritance of CCMs, resources have been provided in a separate document for additional genetic screening, if desired, as well as resources for genetic counselling. All additional resources are the subject's financial responsibility and are not associated with this study.

Serum/plasma biomarker discovery
Working with our Ccms mutant animal models, we have discovered some very promising serum biomarkers for prediction of the occurrence of stroke hemorrhagic events in Ccms mouse models. With our collected serum and plasma samples from CCMs patients, we will validate our candidate biomarkers found in our animal models and continue our investigation for novel biomarkers with proteomic approaches. Our goal is to discover and define a new set of biomarkers to predict the early stage of stroke and hemorrhagic events in CCM patients that will lead to effective prevention and better treatment for this devastating health condition. Our findings will also help deepen our scientific understanding of these human diseases.

Data Collection Methods and Exposure Assessment and management
Clinical data will be collected through in-person structured interviews, directed by clinical co-PIs (Drs. Brower, Gupta and Vellipuram). All first-degree relatives will be coded. For each relative, subjects will be asked about questions listed in the questionnaire. Clinical data will be stored using Excel software on a PC in the CHIPS. Personal identifiers are removed from these data for implementing HIPAA rules and further reformatted into ASCII files for the preliminary descriptive and genetic analysis. There are three PCs located in research suit 242 with double door security. The research co-PI (JZ) has gained expertise during the current next-generation sequencing project; he has performed NGS raw data generation and acquiring, reference-based assembly, and genetic variant detection process. Working with our IT division, we have outlined an effective NGS protocol to tackle the huge data volume problem during NGS data acquisition, data analysis, and management within our system. A postdoctoral fellow researcher (Dr. Johnathan Abou-Fadel) has extensive bioinformatics analysis training background and a strong publication record, and will further enhance our expertise on genomic and proteomic analysis.

Statistical Analysis Strategy
The statistical analysis for a genetic mutation in the Hispanic population will be performed using SAS software with support from the Division of Biostatistics & Epidemiology at Texas Tech University Health Sciences Center in El Paso, Texas. To assess the familial aggregation of stroke among Hispanics, Odds ratios (OR) and their 95% confidence intervals (CI) will be calculated using stratified data analysis and unconditional logistic regression. Odds Ratio (OR) for risk of disease in first-degree relatives of an affected person to risks of disease in first degree relatives of an unaffected person. Let OR = ratio of the disease odds for first-degree relatives of an affected person to the disease odds for first-degree relatives of an unaffected person. This is the familial risk ratio for first degree relatives and is a measure of the strength of familial aggregation of the disease. Cox proportional hazard models will be used to estimate hazard ratios (HR) and 95% confidence intervals (CI) estimating the relative risk of stroke according to the exposure of interest. The cumulative incidence of stroke among first-degree relatives will also be estimated. All models will be adjusted for matching variables including age and gender. All p-values will be two-sided, with the significance level cutoff at $p<0.05$.

7. Research Team of CCMs among **Hi**spanic **P**opulation **S**tudy group (**CHIPS**)

The Research Team of **C**CMs among **Hi**spanic **P**opulation **S**tudy group (CHIPS) is consist of both research and clinical co- Principle Investigators (**co-PIs**), co-investigators, research and clinical coordinators, and other staff team members.

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