RESEARCH PLAN

Specific Aims. Preeclampsia (PE) is a common pregnancy-related medical problem that is a leading cause of maternal and fetal morbidity and mortality. The global incidence of PE is 5-14% with an associated 46,000 maternal and 500,000 fetal deaths every year. Preeclampsia is characterized by new-onset hypertension often associated with proteinuria after 20-weeks of gestation. Disease manifestations include HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets) syndrome, kidney failure, pulmonary edema, seizures (eclampsia), stroke and cardiovascular complications. Despite it being a common pregnancy complication, the molecular basis for PE is unknown and there is no established treatment other than delivery. In cases that arise before 37-weeks of gestation, the risk of maternal complications from ongoing pregnancy must be weighed against the neonatal risks imposed by preterm delivery. Immunologic maladaptation, in particular, complement activation that disrupts maternal-fetal tolerance leading to placental dysfunction and endothelial injury has been proposed as a pathogenic mechanism but remains unproven. Given the similarity of the pathologic findings in PE to those of atypical hemolytic uremic syndrome (aHUS, a classic immune-mediated kidney disease occurring due to genetic variants in complement proteins), we speculated that overactivity of the complement system, due to underlying genetic variants, defines a subset of patients that develop preterm and recurrent PE. Our data from the first 6 months of the study using whole exome sequencing (WES) on maternal and fetal DNA has identified 14 known pathogenic variants and 49 variants of uncertain significance (VUS) in complement proteins in a cohort of 65 PE patients. Our results have also identified for the first time that 3 of the 14 pathogenic variants and 17 of the 49 VUS are in a set of unique complement regulators called CSMD (CUB and Sushi Multiple Domains) proteins 1, 2 and 3. Mutations in CSMD1 have been previously reported to cause gonadal dysfunction and infertility due to overzealous activation of the complement pathway. While complete loss of function may lead to a severe phenotype like infertility, we hypothesize that partial loss of function due to heterozygous variants in CSMD (like those identified in our cohort) may lead to PE. Given that >95% of patients in our cohort are African Americans (AA), CSMD mutations maybe a distinctive risk factor for the higher incidence of PE in AA. We also hypothesize that majority of the VUS identified in other complement proteins in our patients are also likely pathogenic. We therefore propose the following specific aims:

Aim 1: **Define pathogenicity of VUS in complement proteins in PE.** We have identified 49 VUS in our cohort of high-risk PE patients. 35 of the 49 VUS are exclusively in cases while others overlap with controls. These 35 VUS and those that we will identify over the next 6 months will be assessed for functional consequences in recombinantly expressed proteins, using well-established complement functional assays in our laboratory. Structural analysis of the variants will also be conducted by our collaborators and structural biologists (Pozzi and Perkins). We will begin with 15 VUS in proteins for which we have established assays and subsequently modify the assays to characterize the remaining VUS.These structure-function assays have been previously used to characterize variants in aHUS.

Aim 2: Determine the significance of *CSMD* mutations as a unique risk factor for PE. We have established a collaboration with the Preeclampsia Registry™ which has WES data for 154 PE patients who meet our inclusion criteria (letter attached). An analysis for variants in CSMD proteins will be conducted in this additional cohort using the American College of Medical Genetics (ACMG) guidelines. Contrary to our current cohort, a majority of patients enrolled in the registry are Caucasians. Replication of CSMD variants in the registry will ascertain that they are associated with PE; however, if few or no CSMD variants are identified, then it will establish that it is a specific risk factor for PE in AA.

These Aims will improve scientific knowledge related to the genetic underpinnings of PE through characterization of the complement genetic variants, including defining the role of novel proteins that are significant for reproductive health and in the pathogenesis of PE.

Successful completion of this project will define the clinical phenotype of high-risk PE patients who should undergo genetic sequencing and be treated with anti-complement therapy. Understanding the genetic basis has revolutionized the management of aHUS and can be similarly transformative for PE. Timely diagnosis and initiation of anticomplement therapy will lead to resolution of PE, prevention of premature delivery and avert future risk of kidney disease.

Scope of Work and Relevance to the Mission of the Longer Life Foundation (LLF)

Preeclampsia causes significant maternal and neonatal mortality worldwide[1]. Advances have been made in our understanding of PE, yet the only current "cure" for PE is delivery. Labor is usually induced early to prevent systemic complications (seizures, stroke, liver and kidney failure) in the mother. Although delivery before 37 weeks of pregnancy is generally not recommended, in cases of PE it may be too dangerous for both the baby and the mother to allow the pregnancy to continue. The literature describes a putative role for an overactive complement system in the development of placental dysfunction [2-4]. However, little information is available regarding what causes the complement dysregulation.

Our results now show that complement genetic variants are likely significant drivers of PE risk. We have identified variants in a complement regulator called CSMD that may be an unrecognized risk factor for PE and likely associated with a higher incidence of PE in AA. Corroborating the findings of this novel complement protein by analyzing WES data in an additional cohort of PE patients from the Preeclampsia Registry™ and further defining the functional consequences of these variants will facilitate better understanding of PE mechanisms. Our work will thus establish the role of complement receptors and regulators as "gate keepers" in healthy and preeclamptic pregnancy. Importantly, we will focus on both maternal and fetal genotypes to determine the relative contribution of risk from each. The role of fetal DNA in PE has not been well delineated. This work aligns well with the mission of LLF since it will enable us to identify women who are at risk for developing preterm and recurrent PE and its associated systemic complications. This would facilitate earlier diagnosis and help to determine if complement system modulation is a potential therapeutic target for PE. Identification of novel pharmaceutical targets may be additionally aided by knowing the genetic component of PE. The outcomes of this project could lead to lower maternal and fetal mortality, less dialysis dependency, and improved long-term maternal health.

Significance and Innovation

The complement system is an integral part of innate immunity and likely acts as a regulator of key processes to establish and maintain tolerance during pregnancy[2] (**Fig 1**). Increasing evidence suggests that dysregulation of the complement system breaks down the systemic maternal-fetal tolerance, leading to microthrombi and endothelial damage in PE[5]. This process is analogous to aHUS, a complement-mediated kidney disease, characterized by endothelial injury that occurs due to mutations in complement proteins[6]. Genetic sequencing from our growing cohort of 65 PE patients has identified pathogenic variants and variants of uncertain significance (VUS) in complement proteins. Several of the variants are in a set of complement regulators, CSMD 1,2 and 3.

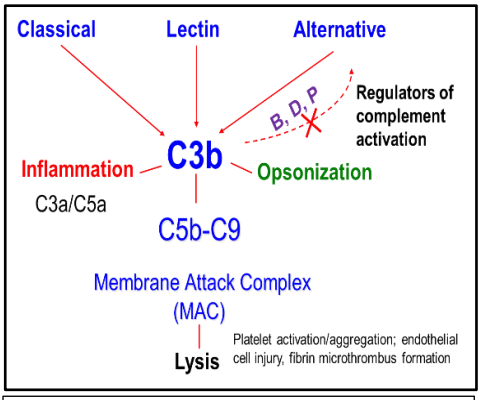

Fig. 1. Schematic of complement activation. There are three complement pathways leading to formation of C3b and MAC (C5b-C9). Complement C3b engages the alternative pathway feedback loop (dashed line), rapidly amplifying the cascade. B, Factor B; D, Factor D; P, Properdin.

CSMD1 is expressed in the placenta and testis[7]. In the testis, increased complement deposition was observed in *CSMD1* knockout rats due to overzealous complement-mediated phagocytosis of developing germ cells leading to defects in fertility and ~25% reduction in the daily sperm production compared to controls. CSMD1 is also highly expressed on oocytes where loss of function has been proposed as the mechanism for oocyte atresia, fewer ovulations, and reduced probability of pregnancy. *CSMD1* knockout females have significantly smaller ovaries by mass when controlling for age. Pups borne of *CSMD1* knockout mothers suffer from significantly higher mortality rates during the neonatal period, with corresponding reduction in the epithelial network of the maternal mammary gland[7].Complete loss-of-function of *CSMD1* leads to a severe and/or systemic phenotype in both humans and mice while partial loss-of-function is buffered by functional redundancy from *CSMD2* and *CSMD3*[8]. However, there is no additional data in the literature about the functional significance of CSMD2 and 3. These data highlight the need for deeper investigation into the role of CSMD in reproductive health.

Our work will establish the role of CSMD variants in the pathogenesis of PE and will particularly define if this is an unrecognized risk factor for PE in AA. To further advance this field, the structural and functional impact of the VUS in complement proteins will be determined. Our study has the potential to determine the genetic architecture of this prevalent and multifaceted disease not explained by known genes. This work will enhance our understanding of biologic pathways and reveal disease mechanisms that will lead to development of new therapies and help to reduce the burden of hypertensive disease in pregnancy. Combining these variants with other risk variants and factors could provide compelling predictors of disease. Importantly, it will lead to defining the subset of patients who are most likely to benefit from anti-complement therapy.

Table1: Inclusion Criteria
Preterm PE <37 weeks
Recurrent PE
HELLP syndrome
PE with acute kidney injury
Pregnancy-associated aHUS

Progress Report

Over the past 6 months, we have obtained maternal blood and placental tissue or cord blood samples for 65 PE patients that met our inclusion criteria (**Tables 1 & 2)** in collaboration with the Women and Infants Health Specimen Consortium (WIHSC) at Washington University School of Medicine (WUSM). 63 of the 65 patients are AA. We have also collected samples for 30 controls matched for gestational age, race and parity. Enrollment is ongoing and we will be able to reach our proposed target of 100 PE patients and at least 50 controls by the end of year 1. For the 65 PE patients and 30 controls, WES has been completed with an initial analysis for 56 complement genes.

Table 2

Year 1	Completed	Ongoing
Aim 1	• Recruitment, whole exome sequencing (WES) and analyses of 65 cases and 30 matched controls. • 14 pathogenic variants and 49 VUS in complement proteins in cases. • 35 of 49 VUS exclusively in cases. • 3 of 14 pathogenic and 17 of 35 VUS in a set of unique complement regulators (CSMD 1,2,3).	• Recruit at least 35 more cases and 20 more controls to reach target of 100 cases and 50 matched controls. • Conduct WES and analyze data on the newly recruited cases and controls.
Aim 2		• Structure-Function analyses for the 9 VUS in complement proteins from the Burwick/Cedars Sinai cohort. Collaboration is ongoing

Preliminary statistical analyses have also been conducted by testing for significance of the variants using logistic regression with Firth's correction for small datasets. We tested for rare variant significance using the optimized SNP-set Kernel Association Test (SKAT-O) and corrected for nominal p-values by Benjamini-Hochberg false-discovery rate. These analyses were conducted by Elisha Roberson, Director, Genomics and Bioinformatics Facility, WUSM, and a consultant for the bioinformatics analysis for this grant (letter attached).

Our results revealed 14 known pathogenic variants in patients with PE (**Fig 2**). The implications of mutations reported as "pathogenic" are clear in their ability to cause dysregulation of complement activation. For example, the mutations in FCN3 likely cause a decrease in serum concentration of ficolins which has been previously reported in association with PE[9].

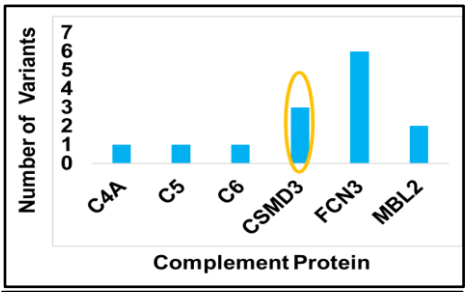

Fig 2: Pathogenic variants in 6 complement proteins identified in patients with PE. CSMD, CUB and Sushi Multiple Domains; FCN3, Ficolin 3; MBL2, Mannose Binding Lectin 2.

Our preliminary analysis also suggests that certain variants may be protective for PE. Focusing on variants rare in all queried databases revealed that the overall dose of CFHR4 variants and the SKAT-O test were significant for protection against PE after multiple testing correction. This finding corresponds with prior evidence that indicates that Factor H-related (FHR) proteins act as antagonists of complement regulator, Factor H (FH), by competing with FH for the binding to complement C3b and human cell surfaces[10]. Therefore, variations in FHRs that lead to low levels of these proteins may be protective. For instance, the lack of *CFHR3* and *CFHR1* due to copy number variation has been reported to be protective against age-related macular degeneration[11]. These

findings will be confirmed further in Year 1 with more comprehensive statistical analyses including evaluation for other protective variants.

As noted in Specific Aim1, we have identified 49 variants of uncertain significance (VUS) in cases, with some patients carrying more than one variant. 35 of the 49 are present exclusively in cases while others overlap with controls (**Table 2 & Fig 3).** The variants in cases are found in proteins that span all three complement pathways as well as the common terminal complex (**Fig 4**). It is remarkable to see the large number of complement variants identified in the cases, however, given that our inclusion criteria comprised of high-risk PE patients (and not all-comers with PE), these data are not surprising and validate our hypothesis that complement is one of the major players in the pathogenesis of PE.

The 35 VUS and others to be identified in cases over year 1 will require further investigation by recombinant protein expression and subsequent functional and structural analysis to determine their clinical significance. We will conduct these analyses in the 2nd year beginning with the 15 variants in proteins highlighted in **blue** since we have established assays and the most expertise in these proteins (**Fig 3 & Table 3)**. The assays will be modified to characterize the variants in the remaining proteins.

Furthermore, of particular interest is the finding that 3 of the 14 pathogenic variants and 17 of the 35 VUS are in a set of unique complement regulators called CSMD (CUB and Sushi Multiple Domains) proteins 1, 2, and 3 (highlighted in **orange** in **Figs 2 & 3**).

CSMD1 is a Type 1 transmembrane protein composed of 14 CUB domains and 28 complement control protein domains (also called sushi domains). Prior work from our collaborators has shown that it is expressed in placenta and testis and is known to inhibit complement activation[7]. CSMD2 and CSMD3 have high (~80%) structural homology to CSMD1 and are also speculated to inhibit complement. According to the Residual Variation Intolerance Score (RVIS), only 0.169% of genes are more intolerant to protein coding changes than *CSMD1*. Consequently, *CSMD1* is depleted for loss-of-function variation in the gnomAD database with only 46 observed variants in ~250k alleles. Rare variant association approach has identified that intronic deletions in *CSMD1* are associated with gonadal dysfunction and single nucleotide variants are associated with early menopause[7]. *CSMD1* loss-of-function is buffered by functional redundancy from *CSMD2* and *CSMD3*[9]. This is an intriguing finding that warrants further investigation. Therefore, we have established a collaboration with the Preeclampsia

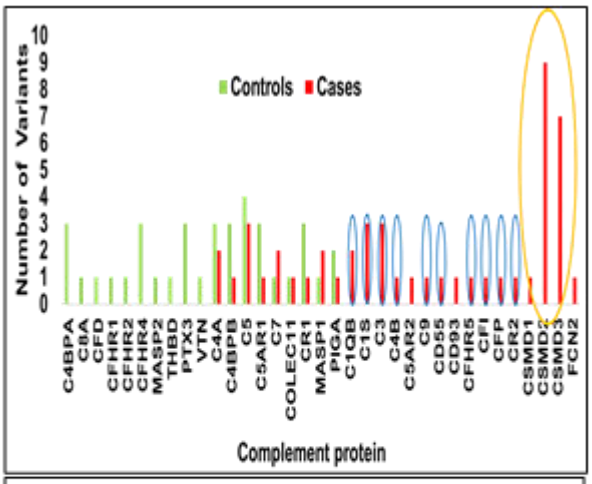

Fig 3: VUS identified in complement proteins in PE cases and controls. 16 VUS only in controls (green); 35 VUS in both cases and controls (green & red); 35 VUS in cases only (red). We will begin with analysis of the 15 VUS marked in blue in the cases. CSMD variants marked in orange.

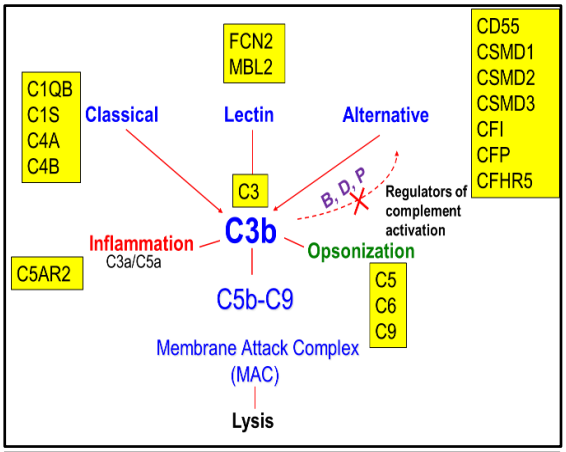

Fig 4: Variants identified in complement proteins in PE cases affect all three complement pathways (Classical, Lectin and Alternative) and the common terminal complex (MAC)

Table 3

Year 2	Planned
Aim 1	• Structure-Function analyses of 35 VUS and others identified in cases in Year 1.
Aim 2	• Obtain WES data from 154 PE patients from the Preeclampsia Registry™ and conduct analyses for CSMD variants. • Analyze differences in CSMD variant types and frequency between African Americans (WIHSC cohort) and Caucasians (Preeclampsia Registry cohort). • Determine the complement genes that are associated with PE, establish disease mechanisms, and define the clinical phenotype of high-risk PE patients who should undergo complement genetic sequencing in future cohorts and clinical practice.

Registry™ to obtain and analyze WES data for *CSMD* variants in an additional cohort of 154 PE patients that meet our inclusion criteria to validate the association of this regulator with PE (**Table 3**).

Research Design and Methods

Aim 1: **Define pathogenicity of genetic variants of uncertain significance (VUS) in complement proteins in PE.**

Approach. We have identified 121 distinct complement genetic variants (14 known pathogenic, 49 VUS and 58 benign) in complement proteins in our cohort of 65 PE patients enrolled within the first 6 months of the study. The 14 pathogenic variants are loss-of-function variants that disrupt protein expression (premature truncation, splice loss, etc) and do not require lab characterization since their ability to cause complement dysregulation is clear (**Fig 2**).

Functional assessment will be initially focused on the VUS, which include missense variants not previously characterized and in-frame insertion-deletion events. We will begin with the 15 VUS in 10 proteins which are well-known to be associated with disease and for which we have the most experience and established assays. These include C1QB, C1S, C3, C4B, C9, CD55, complement factor H-related 5 (CFHR5), complement factor I (CFI), properdin (CFP) and complement receptor 2 (CR2) (**Figs 3 and 4**).

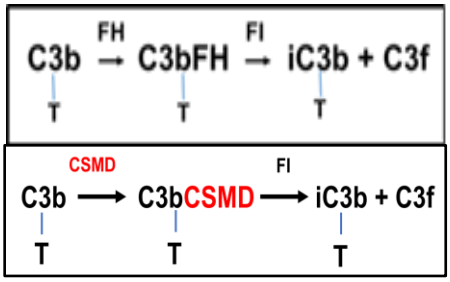

Fig 5. Cofactor activity. Inactivation of C3b by complement factor H (FH) or CSMD and factor I (FI) relative to its ability to amplify the complement pathway. T- target bound C3b.

Functional testing for the variants in remaining proteins will require modifications to our current assays which is well within our area of expertise. For example, it is known that CSMD regulates complement activation by acting as a cofactor for Factor I, similar to other known complement regulators such as Factor H, Membrane cofactor protein and Complement receptor 1 (**Fig 5**). The defect leads to a decrease in binding to complement component C3b which leads to a delay in cleavage of C3b, i.e. decreased complement regulatory activity known as cofactor activity.

Our standard approach (**Fig 6**) will be to: 1) prepare and express the variant protein in a mammalian system (always in parallel with the wild-type protein), 2) harvest the expressed and secreted protein from the supernatant, 3) quantify by ELISA and visualize by Western blot. Normal secretion of the variant would trigger steps 4 and 5 as described, 4) partially purify if necessary, 5) employ our set of functional assays for activity. Initially this consists of complement protein binding ELISAs, and assays for cleavage of complement fragments, C3b to iC3b. We have several examples of genetic variants where the cofactor activity is disrupted[12]. Western blots are performed as a cross-check for the ELISA and to confirm the expected protein sizes. Occasionally, the blot will show us an improper cleavage has taken place, or a product has an unusual size (loss of an N-linked sugar, for example).

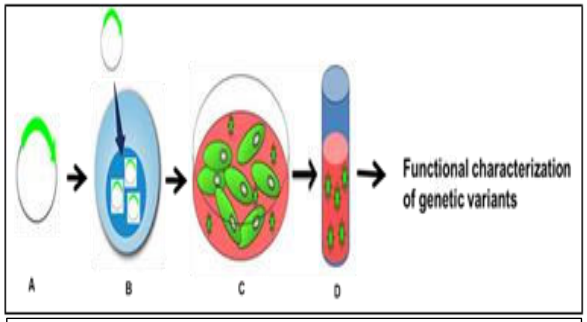

Fig 6. Experimental design for assessment of protein function. A. Site-directed mutagenesis of normal gene in plasmid DNA to create variant (green). B. In-vivo (bacterial) amplification and purification of DNA. C. Transfection of mutant into mammalian cells. D. Preparation of cell lysate and collection of supernatant for functional analysis.

Structural analyses of the variants will also be conducted in collaboration with N. Pozzi (St. Louis University) and S. Perkins (University College London, UK), both of whom are long time collaborators and co-authors on prior work[12-15] (letters of collaboration available). Pozzi is a structural biologist with expertise in serine proteases (such as Factor I) and Perkins is an authority on proteins with sushi domains (such as Factor H, CSMD, etc)

Pathogenicity determination. Variants will be considered pathogenic if they: 1) fail to express *in vitro* (as measured by Western blot and ELISA) or present as weak/abnormal bands in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Our prior

work has shown that some missense variants may affect protein stability/protein folding and thereby lead to decreased expression; and/or 2) at least one functional assay is abnormal. We have extensive experience with these methods[12-15].

Clinical correlations. Maternal history (BMI, preterm labor, proteinuria, end-organ dysfunction, infections, etc) and fetal complications (fetal loss, still birth, etc) are collected as part of the routine clinical evaluation protocols of WIHSC as well as Preeclampsia Registry™. After identifying any genes with a significant burden, we will include a secondary analysis using these clinical predictors in the regression model to establish the association between complement dysregulation and long-term clinical outcomes.

Aim 2: Determine the significance of *CSMD* mutations as a unique risk factor for PE

Approach. We have established a collaboration with the Preeclampsia Registry™ that enrolls patients through its online portal (www.preeclampsiaregistry.org) (see attached letter). The Registry is overseen by an Institutional Review Board and advised by a multi-disciplinary Scientific Advisory Council.

There are currently 154 PE patients in the Registry that meet **at least one inclusion criteria** for our study with some overlap in different categories (**Table 4**). 147 of the 154 PE patients are Caucasians, contrary to our current WIHSC cohort where >95% of the patients are AA. Participants have answered an enrollment questionnaire providing detail about their pregnancies and health history. Medical records, allowing for validation of patient reported information, DNA and subsequent WES data, have been collected for these patients. The WES data is available in ready-to-use format (as VCF and BAM files) and therefore variant analysis will be initiated without any delay.

Table 4. Number of patients from the Preeclampsia Registry™ that meet our inclusion criteria.

Cohort	With WES Data
Preterm PE <37 weeks	128
Recurrent PE	24
HELLP Syndrome	81
Acute Kidney Injury	27

We will obtain the WES data for the 154 patients and analyze it for the presence of variants in *CSMD* 1, 2, and 3 using American College of Medical Genetics (ACMG) guidelines[16]. The non-PE controls for these patients that have been matched for race, parity and gestational age will be obtained from those currently available at the WIHSC biobank. We will calculate the PE rare and common variant burden using logistic regression. Replication of *CSMD* variants in the Registry cohort will establish if this complement regulator is a general risk factor for high-risk PE or if it is a distinctive risk factor for AA. The Registry WES data is an exclusive resource that will also be used to identify variants in other proteins that may be specific to Caucasians. The VUS identified in this cohort will be functionally characterized using methods in Specific Aim 1 to establish pathogenicity.

Expected milestones, potential pitfalls and alternative approaches. Defining the functional consequences of complement genetic variants will facilitate better understanding of PE mechanisms and the role of novel complement receptors and regulators in PE. We will also ascertain PE risk variants unique to AA and Caucasians. This work will enable early identification of women at risk for developing early or recurrent PE and its associated systemic complications, particularly kidney disease and will further help to determine if complement system modulation is a potential therapeutic target. Identification of novel pharmaceutical targets may be additionally aided by knowing the genetic component of PE. The outcomes of this project could lead to lower maternal and fetal mortality, less dialysis dependency, and improved long-term maternal health.

We have the expertise in the molecular and structural biology of complement system to model the structure-function consequences of the novel variants and VUSs identified. Our extensive prior work on aHUS, C3 glomerulopathy, and AMD has demonstrated that this methodology leads to the proper classification of genetic variants as to their biologic significance[13-16]. We are poised to successfully perform this project because of our collaborations, a sufficiently-powered design, and our combination of critical skills. Furthermore, access to the WES data will allow us to identify and functionally analyze novel candidate targets in the future.

PLANS FOR OBTAINING ADDITIONAL EXTRAMURAL FUNDING ARISING FROM THE PROPOSED RESEARCH

Successful completion of this project will lay the foundation for conducting future studies that at this time are beyond the scope of the current proposal. We plan to investigate the role of *CSMD* variants identified in our cohort in the wildtype and heterozygous mice that have been developed and used by our collaborators (John Atkinson and Mike Holers) in prior studies[7]. These *CSMD1* knockout male mice models have demonstrated defects in fertility with profound anatomic and histopathologic derangement of the testes in association with increased complement C3 deposition. Moreover, maternal *CSMD1* genotype status has been associated with neonatal mortality. These data lay the foundation for our future studies that will focus on the effect of haploinsufficiency due to heterozygous *CSMD* variants and their role in pregnancy and PE.

We will also evaluate complement biomarkers in PE by systematically and sequentially measuring circulating levels of complement proteins and their activation fragments in cases and healthy controls to define early predictors of disease. Complement pathway activation and deposition will also be assessed by immunohistochemical staining of placental tissue to evaluate how histopathological features impact outcomes. Future plans also include establishing a larger multicenter referral network of patients with PE for genetic studies that will help to establish more robust cohorts for future grants. Identifying which complement pathway is activated in PE may be critical in understanding underlying risk factors and further correlating with and validating the genetic data.

In order to accomplish these studies, we plan to apply to the Preeclampsia Foundation, March of Dimes, Veterans Affairs (VA) Merit Award and an NIH R01. The VA's Million Veteran Program (MVP) stores data and biospecimens (coded DNA, buffy coat, and plasma samples) from patients that is made available to VA-affiliated investigators for genomic research. Currently women make up ~10% of the 830,000 MVP enrollees. In March 2021, VA launched efforts to increase women veteran participation in the program to aid in genetic research focused on women health issues. This is an invaluable resource we aim to utilize for our future investigations.

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