**METHODS**

**Patients**

We have a current cohort of 65 patients who have been referred to us over the past four years with a possible diagnosis of aHUS. The diagnosis was made by their treating physician based on each patient’s clinical presentation of anemia, thrombocytopenia and acute kidney injury. In some cases, the referral has been to help determine a more definitive diagnosis and in others, we have been asked to assist with clinical decision-making in regard to need for and duration of therapy. These patients have undergone genetic testing at Genomic and Pathology Services (GPS) at Washington University School of Medicine. Eight Factor I variants have been identified in this cohort. Three of eight variants were known to be pathogenic. The remaining five were included in this study. The clinically validated aHUS/C3 Glomerulopathy next generation sequencing-based assay is derived from the Agilent Clinical Research Exome capture reagent (Agilent Technologies, Inc., Santa Clara, CA), with analysis of all exonic sequences for 13 genes (ADAMTS13, C3, CD46, CFB, CFH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CFI, DGKE, and THBD) having known associations with these diseases. CFHR3-CFHR1 copy number was assessed using multiplex ligation-dependent probe amplification (MLPA). Variants are annotated according to human genome assembly GRCh37/hg19 using Human Genome Variation Society nomenclature and classified into five categories based on the guidelines established by the ACMG, Level 1: pathogenic; Level 2: likely pathogenic; Level 3: VUS; Level 4; likely benign and Level 5: benign.

This study was approved by the Institutional Review Board (IRB) of Washington University School of Medicine. Residue numbering includes the 18 amino acids of the signal peptide.

**Serum FI levels**: Enzyme-linked immunosorbent assay (ELISA) was used to determine the antigenic levels of Factor I. For patients #1 and 3, the test on serum was conducted by the Blood Center of Wisconsin (<https://www.versiti.org/medical-professionals/products-services/diagnostic-labs>) and for patient # 4, it was conducted by the Molecular and Otolaryngology Laboratory at the University of Iowa (<https://morl.lab.uiowa.edu/>).

**Mutagenesis**

A 6x histidine carboxyl-terminal tagged CFI cDNA cloned in pcDNA3 was purchased from GenScript (Piscataway, NJ, USA). The variants were produced using the QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Stratagene, Santa Clara, CA). All cDNA clones were sequenced in their entirety to assure fidelity.

**Expression and purification of recombinant Factor I**

Human embryonic kidney 293T cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech, Herndon, VA) containing 10% heat-inactivated fetal calf serum (FCS, Harlan, Madison, WI), L-glutamine (2 mM, Sigma, St. Louis, MO), penicillin and streptomycin (10,000 U/ml and 100 mg/ml, Cellgro® Mediatech), non-essential amino acids and sodium pyruvate. Short-tandem repeat (STR) profiling of human cell lines has been used to authenticate the identity of these cells. The STR analysis at ATCC meets requirements for funding, publication and quality control. For transfection, the DMEM was replaced with OptiMEM® (Invitrogen, NY). Transient transfections were performed using X-tremeGENE HP DNA transfection reagent (Roche) and supernatants were collected after 48 h. The recombinant FI produced from mammalian cells was variably secreted as both a single polypeptide chain, i.e, pro-I(s), and as a mature FI composed of a disulfide-linked heavy chain and light chain. To overcome this issue and thereby increase the yield of mature FI, we co-transfected FI with furin cDNA cloned in pCMV 24.

**Cell lysates**

Cells (4 x 107 cells/ml) were lysed in 1% NP-40, 0.05% SDS in TBS with 2 mM phenylmethane sulfonyl fluoride (PMSF) at 4◦C for 30 min followed by centrifugation at 12,000 x gfor 10 min. Supernatants were collected and stored at a -80◦C.

**Quantification and Western blotting**

The quantity of each recombinant FI mutant protein was determined by ELISA. The capture antibody, OX21 (Serotec, Oxford, UK), was coated at 1 µg/ml overnight at 4◦C and then blocked for 1 h at 37◦C (1% BSA and 0.1% Tween 20 in PBS). Dilutions of wild-type (WT), variant FI samples and purified human FI (Complement Technologies, San Diego, CA) were incubated for 1 h and then washed with PBS containing 0.05% Tween- 20. Next, sheep anti-FI (Abcam, Cambridge, MA) was applied for 1 h at 37◦C. After washing, HRP-coupled rabbit anti-sheep IgG (Abcam, Cambridge, MA) was added and incubated for 1 h at 37◦C. After washing, 3, 3ʹ, 5, 5ʹ-Tetramethylbenzidine (TMB) substrate (Pierce, Rockford, IL) was added. Subsequently, reaction was stopped using 2N H2SO4 and absorbance at 450 nm was assessed.

Western blots of supernatants and purified proteins were electrophoresed under reducing and non-reducing conditions using 10% SDS-PAGE, transferred to nitrocellulose, and probed with 1:5,000 goat anti-FI Ab (Quidel, San Diego, CA) followed by a 1:10,000 HRP-conjugated rabbit anti-goat IgG (Abcam, Cambridge, MA). The polyclonal Ab to FI predominantly recognized epitopes on the heavy chain. Data obtained were confirmed by the use of positive (purified proteins) and negative controls via WB, flow cytometry and ELISA.

**C3b cleavage assays**

Assays to measure the breakdown of C3b (i..e., human C3b cofactor assays) were performed using the FI variant and its three cofactors (FH, CR1, MCP). Following incubation, breakdown of the α' chain of C3b into α1, α 41 and α 43 was compared to WT. For these assays, the complement proteins were diluted in physiologic salt (150 mM NaCl) or low salt (25 mM NaCl) buffer. C3b (10 ng; Complement Technologies Tyler TX) was incubated at 37◦C with WT or variant FI (10 ng in MCP, 20 ng in FH and 15 ng in CR1 assays) and appropriate cofactor (100 ng MCP, 200 ng FH or 150 ng CR1) in a total volume of 15 µl/reaction. Kinetic analyses of the WT and variants were performed at 0, 10, 20 and 30 min. At each time point, 15 µl of the reaction was mixed with 7 µl of 3 x Laemmli reducing sample buffer to stop the reaction and then heated at 95◦C for 5 min. The samples were electrophoresed on a 10% Tris-glycine gel and then transferred to nitrocellulose for WB analysis. Membranes were rinsed with TBS-T (0.05%Tween-20) for 5 min and blocked overnight with 5% nonfat dry milk in PBS. Blots were probed with a 1:5,000 dilution of goat anti-human C3 (Complement Technologies, Inc, Tyler, TX, USA) followed by HRP-conjugated rabbit anti-goat IgG and developed with SuperSignal substrate (Thermo Fisher Scientific, Waltham, MA, USA). The signal detected on radiographic films was scanned using a laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). All assays were performed at least three times on each FI sample.

**Structural analyses** The FI variants were mapped on crystal structures of C3b (**Figure 2**) bound to its regulators, using the Pymol software 7.

**Statistics**

To determine if a variant had a lower level of cofactor activity than WT, area under the curve (AUC) was computed for degradation of the αʹ chain of C3b (in %) and for the generation of the fragments α1, α41 or α43 (in %) between 0 min and 30 min. P-value was computed based on one-sided t-test for the difference of AUC between WT and variant FI. R programming language (CRAN, https://cran.r-project.org/) was used for statistical computations.