Supplemental information

Study participants

Atypical HUS was diagnosed in patients included in this study based on microangiopathic hemolytic anemia and thrombocytopenia defined on the bases of hematocrit less than 30%, hemoglobin level less than 100g/L, serum lactate dehydrogenase level higher than 460U/L, undetectable haptoglobin level, fragmented erythrocytes in peripheral blood smear, and platelet count less than $150x10^9$/L associated with acute renal failure. Familial aHUS was diagnosed when two or more members of the same family were affected by the disease at least 6 months apart and exposure to a common trigger infectious agent was excluded. Sporadic aHUS was diagnosed when one or more episodes of the disease manifested in a subject with no familial history of the disease. One hundred fifty-four consecutive patients of the International Registry of HUS/TTP analyzed in *CFH*, *MCP*, factor I (*CFI*), *C3*, factor B (*CFB*), and thrombomodulin (*THBD*) genes were screened for genomic rearrangements affecting *CFH*, *CFHR1*, *CFHR2*, *CFHR3* and *CFHR5*. The study was approved by the Ethics Committee of the Azienda Sanitaria Locale, Bergamo (Italy), and informed consent was obtained in accordance with the Declaration of Helsinki.

Complement profile assessment.

Serum concentrations of C3 and C4 were evaluated by kinetic nephelometry. FH, CH50 serum levels and SC5b-9 plasma levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA). Screening for FH autoantibodies was undertaken using an ELISA assay (Dragon Durey et al, *J Am Soc Nephrol*, Feb 2005;16(2):555-563). The normal ranges were set as mean ±2 standard deviation of the values recorded in healthy subjects. Value up the

higher limit of the normal ranges were considered as high, whereas values below the lower limit of the normal ranges were considered as low.

Mutation screening and genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. PCR products of the coding sequence and the intronic flaking regions of *CFH,* a *MCP, CFI,* C3*, CFB,* and *THBD* were analyzed by direct fluorescent sequencing. Genotype of single nucleotide polymorphisms (SNPs) rs3753394, rs800292, rs1061170, rs3753396, rs1065489, and rs7144 were undertaken using direct fluorescent sequencing on the 3730 DNA Analyzer (Applied Biosystem).

Screening for genomic disorders

Screening for genomic disorders affecting *CFH*, *CFHR1*, *CFHR2*, *CFHR3*, and *CFHR5* was undertaken using multiplex ligation-dependent probe amplification (MLPA). Details of the probes used are given in Table 1. These probes include those in a kit from MRC Holland (SALSA MLPA kit P236-A3 ARMD) and homemade probes for *CFH* exon 19, intron 20, intron 21, intron 22 and exon 23 (in bold in Table 1), which were analyzed in a separate assay. The control DNA was obtained from local blood donors. For the MLPA reaction 100 ng genomic DNA and 2-fmol probe were used. Incubations and PCR amplification were performed on a 2720 thermal cycler (Applied Biosystem); 1 ul of product and 0.3 ul di LIZ500 internal size standard were made up to 10 ul using Hi-Di Formamide and samples were run on the 3130xl Genetic Analyzer (Applied Biosystem). Peak areas for each sample were determined using Gene Mapper software (version 3.7). A probe ratio between 0.7 and 1.3 was taken to be indicative of two copies of the DNA. A probe ratio higher than 1.3 and lower than 1.7 suggested a heterozygous duplication.

CGH arrays.

Copy number variations and genomic rearrangements in the *CFH-CFHRs* region were assessed by a custom-designed high-density (median resolution of 110 bp) CGH 15k microarrays (Agilent Technologies, Palo Alto, CA, USA) spanning the RCA gene cluster region in chromosome 1q32. DNA samples were labelled, mixed with DNA from a normal control fully characterized at the RCA gene cluster region and hybridized to the microarray according to the manufacturer's protocols. Microarray data were extracted and visualized using the Feature Extraction Software v8.1 and CGH Analytics v3.2.25 (Agilent Technologies, Valencia, Spain). Data analysis was performed using the ADM-1 algorithm, as supplied within "Genomic Workbench Standard Edition 7.0" (Agilent Corp, Santa Clara).

Identification of the breakpoint

The breakpoint of the duplication resulting in the *CFHR1/CFH* hybrid gene was mapped by a long-range PCR amplification across *CFHR1* intron 4 (forward primer 5'-ACCATCATCAATTTCAAAACCCGT) and *CFH* intron 23 (reverse primer 5'-CAATTTCATTTTTTATGTATTGTTTTAC). The PCR was performed with 50 ng of gDNA from peripheral blood leukocytes using AccuPrime Taq DNA polymerase High Fidelity kit (Life Technologies) in presence of 3.375 mM $MgSO_2$ and 0.75 nM of each primer. The amplification was performed using the following thermocycler program (2720 Applied Biosystem): 95°C 10 min, 35 cycles of 95°C 45 sec, 60°C 2 min, 72°C 3 min and elongation at 72°C for 20 min. The PCR product was sequenced in both directions by direct fluorescent sequencing on the 3730 DNA Analyzer (Applied Biosystem) with the same primers of the PCR step and plus internal primers. The list of primers is shown in Supplemental table 1. To confirm the *CFHR1/CFH* rearrangement we performed a multiplex PCR on genomic DNA using a common forward primer in *CFHR1* intron 5 (5'-CCTAATTCTCATACATTAAACATAA), a reverse primer in *CFHR1* exon 6 (5'-

TCCCCGTTTACACACAAATTCAG), and a reverse primer in *CFH* intron 23 (5'-GTAAAACAATACATAAAAAATGAAATTG) in the same reaction.

FH and FHRs western blotting

Sera (diluted 1:1000) from the proband, his wife and controls with varying copy numbers of *CFHR1* were run on SDS-PAGE 8% gels under reducing conditions, blotted onto nitrocellulose, and blocked with 5% dried milk/TBS 1X/Tween 0.1%. Blot was incubated overnight at 4°C with goat anti-human FH polyclonal antibody (1:1000, Merck chemicals) or MBC125, a mouse IgG2a mAb raised against a recombinant protein containing the first three SCRs of FHR1, in TBS 1X/BSA 1%/ Tween 0.1%. After three washing in TBS 1X/Tween 0.1% the blot was incubated with secondary HRP-conjugated antibodies in TBS 1X/BSA 1%/Tween 0.1%. After one hour and half at room temperature, the blot was washed three times with TBS 1X/Tween 0.1% and then twice with TBS 1X. Blot was then developed using Pierce ECL Western blotting substrate (Thermo Scientific).

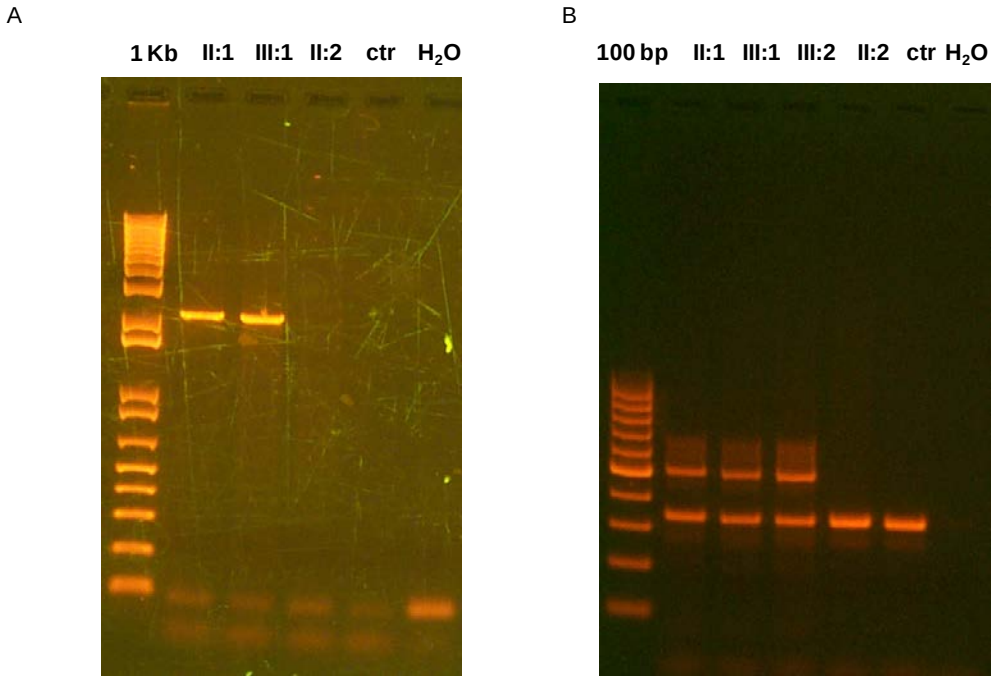
C5b-9 deposition on endothelial cells

The human microvascular endothelial cell line of dermal origin (HMEC-1, a kind gift of Dr. Edwin Ades and Francisco J. Candal of CDC and Dr. Thomas Lawley of Emory University, Atlanta, GA) was cultured as described (Ruiz-Torres et al, Thromb Haemost 2005;93:443-452). For these experiments, HMEC-1 were plated on glass slides and used when confluent. Cells were activated with ADP 10 µM (Sigma-Aldrich) for 10 minutes, thereafter cells were incubated for 4 hours with serum from the proband, his daughter, his son, his wife or from a control diluted 1:2 with test medium (HBSS with 0.5% BSA). In additional experiments serum from the proband's wife who does not carry the genomic duplication and the hybrid *CFHR1/CFH* gene was added with increasing amounts (17, 25 and 50 µg/ml in diluted serum) of wild-type "purified FHR1" from control sera. Considering the published

concentration range (70-100 µg/ml) of FHR1 in normal human serum (Heinen S et al, Blood 2009; 114: 2439-2447), addition of 17/25 µg/ml FHR1 in diluted serum (corresponding to 34/50 µg/ml in undiluted serum) would mimic the effect of an extra copy of *CFHR1*, while addition of 50 µg/ml FHR1 mimics the effect of 2 extra *CFHR1* copies.

At the end of the incubation step HMEC-1 were fixed in 3% paraformaldehyde and stained with rabbit anti-human complement C5b-9 (Calbiochem) followed by FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Soluble Complement Receptor 1 (sCR1, TP-10, 150 µg/ml, a gift from CellDex), an inhibitor of the classical, alternative, and lectin pathways of complement activation, was added to duplicate samples of each serum. A confocal inverted laser microscope (LSM 510 Meta; Zeiss) was used for acquisition of the fluorescent staining on endothelial cell surface. Fifteen fields, systematically digitized along the surface, were acquired using a computer-based image analysis system. The area occupied by the fluorescent staining was evaluated by automatic edge detection using built-in specific functions of the software Image J (NIH, Bethesda, MD), and expressed as pixel^2 per field analyzed. For each sample the mean of 15 fields (excluding the lowest and the highest values) was calculated. Results are expressed as mean ± SE. Data were analyzed by ANOVA. P values of less than 0.05 were considered to be statistically significant.

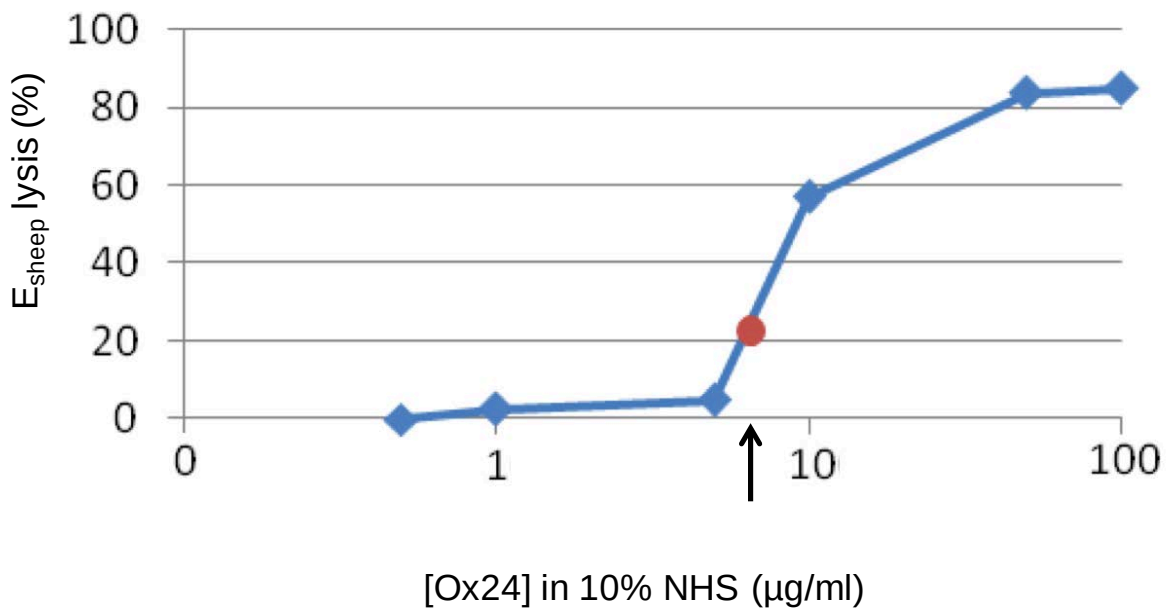
Supplemental Figure 1



Panel A. Agarose gel of the long-PCR showing the product of the PCR with the primer pair annealing to *CFHR1* intron 4 and *CFH* intron 23 from genomic DNA of the proband (II:1) and the affected daughter (III:1). Genomic DNA from the healthy proband's wife (II:2) and an healthy control (ctr) gave no band.

Panel B. Results of multiplex PCR on genomic DNA utilizing a common forward primer in *CFHR1* intron 5, a reverse primer in *CFHR1* exon 6, and a reverse primer in *CFH* intron 23. The reaction resulted in both a 334-bp wild-type *CFHR1* fragment and a 500-bp hybrid fragment in the proband (II:1), his affected daughter (III:1) and his unaffected son (III:2) carrying the duplication and the *CFHR1/CFH* hybrid; while only the 334-bp wild-type *CFHR1* fragment was obtained in the proband's wife (II:2), without the duplication and in an healthy control (ctr).

Supplemental Figure 2



Titration curve to diminish the amount of functional FH in normal human serum for competition assay between FH and FHR1 in the sheep erythrocyte assay. We have added an anti N-terminal FH antibody (OX24) to a 10% normal human serum dilution and tested the effect on sheep red cell hemolysis. Lysis started (black arrow in the figure) with 6.5 µg/ml OX24 in 10% normal human serum, when about 50% of FH was inactivated.

Supplemental Table 1

Primer Sequence	
Forward	ACCATCATCAATTTCAAAACCCGT
Reverse	**CAATTTCATTTTTTATGTATTGTTTTAC**
Forward	**TTGACCAATATCTTAATCAAACAAA**
Forward	**CCTAATTCTCATACATTAAACATAA**
Forward	**CATCGATTACCATTTTAAGTTTATTT**
Forward	TATTATATAAAGTGCTGTGTTTGTA
Forward	TATTTTGCTGTTGGTAACAAAATAA
Reverse	GAACTTATCATTGCTGCTTTTGT

Primer sequences used for bidirectional sequencing of the 1955 bp specific amplicon. In bold are shown primers used for identification of the *CFHR1/CFH* hybrid gene breakpoint region.