

**Complement factor H variants I890 and L1007 while commonly associated with atypical haemolytic uremic syndrome are polymorphisms with no functional significance.**

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## SUMMARY

Mutations and polymorphisms in the gene encoding factor H (*CFH*) have been associated with atypical Hemolytic Uremic Syndrome (aHUS), Dense Deposit Disease (DDD) and Age-related Macular Degeneration (AMD). Experimental evidence supports that many of these *CFH* genetic variations disrupt the regulatory role of factor H (FH), sustaining the concept that dysregulation of complement is a unifying pathogenic feature of these disorders. Evidence of a causal relationship with the disease is, however, not available for all *CFH* genetic variations found in patients, which is a potential cause of misinterpretations with important consequences for the patients and their relatives. I890 and L1007 are two of such *CFH* genetic variations. They have repeatedly been reported associated with aHUS and are also found in DDD and AMD patients. Here we report an extensive genetic and functional analysis of these *CFH* variants. Our results indicate that I890 and L1007 segregate together as part of a distinct and relatively infrequent *CFH* haplotype in Caucasians. Extensive analysis of the purified S890/V1007 (control) and I890/L1007 (disease-associated) FH protein variants failed to provide evidence that these amino acid substitutions have functional implications. These data, the presence of the I890 and L1007 variants in healthy individuals and their high frequency in Sub-Saharan African and African American populations strongly suggest that I890 and L1007 are FH rare polymorphisms unrelated with disease.

## INTRODUCTION

Complement factor H (FH) is the main regulator of the alternative pathway (AP) of the complement system. FH exerts this regulatory activity in three different ways: it binds to C3b, competing with factor B (FB) in the assembly of the AP C3-proconvertase complex (C3bB); it accelerates the decay of the AP C3-convertase (C3bBb); and acts as cofactor of factor I (FI) in the proteolytic inactivation of C3b<sup>1-3</sup>. FH regulates complement activation both in fluid phase and on cellular surfaces<sup>4-6</sup>, preserving complement homeostasis and preventing uncontrolled C3b deposition and host tissue damage.

FH is a relative abundant plasma protein that is secreted as a single-chain glycoprotein of 155 KD composed of 20 homologous domains of 60 amino acids<sup>7</sup>, named short consensus repeats (SCR). FH concentration in plasma is highly variable, ranging from 116 to 562 µg/ml<sup>8</sup>. Different interaction sites for C3b and polyanions have been identified along the 20 SCR of FH. The SCR 1-4 region is the unique C3b binding site capable to act as cofactor for FI in the cleavage of C3b and to accelerate the decay of AP C3-convertase<sup>9</sup>. Similarly, the C3b and polyanion binding site at SCR 19-20 determines the ability of FH to bind C3b deposited on the cell surface, being this region of FH essential for self-pathogen discrimination<sup>10,11</sup>.

Mutations and polymorphisms in the *CFH* gene are associated with atypical hemolytic uremic syndrome (aHUS), dense deposit disease (DDD), and age-related macular degeneration (AMD) (reviewed in<sup>12</sup>). The available data support the hypothesis that AP dysregulation is a unifying pathogenic feature of these diverse conditions. They also illustrate a remarkable genotype-phenotype correlation in which distinct genetic variations at *CFH* specifically predispose to aHUS, AMD or DDD. In fact, the functional characterization of these disease-specific *CFH* genetic variations is

being instrumental to understand the molecular basis underlying each of these pathologies.

In aHUS, a thrombotic microangiopathy characterized by thrombocytopenia, haemolytic anemia and acute renal failure, and where endothelial cell injury appears to be the primary pathogenic event, the most prevalent genetic alterations in the *CFH* gene are missense mutations that alter the C3b- and polyanion-binding site at the C-terminus of FH. These mutations rarely result in hypocomplementemia or decreased FH plasma levels<sup>13-16</sup>. Functional studies have demonstrated that these aHUS-associated FH molecules present normal regulatory activity in plasma but a limited capacity to protect cells from complement lysis<sup>17-20</sup>. This functional alteration is clearly distinct from the lack of complement regulation in plasma, leading to complete C3 consumption and severe hypocomplementemia that characterizes DDD patients.

DDD is a very rare form of glomerulonephritis with isolated C3 deposits characterized by the presence of dense deposits within the glomerular basement membrane (GBM)<sup>21</sup>. DDD is associated with complement abnormalities that lead to persistent reduction of C3 serum levels and to intense deposition of degradation products of C3 in the GBM. Among the different factors associated with these complement abnormalities are mutations in the *CFH* gene. These *CFH* mutations result in truncations or amino acid substitutions that impair secretion of FH into circulation or that eliminate the complement regulatory activities located at the N-terminus of FH<sup>22-24</sup>. Thus, *CFH* mutations that decrease FH in plasma, or eliminate its complement regulatory activity, lead to unrestricted activation of complement in plasma, causing damage to glomerular cells and deposition of complement products in the GBM.

Age-related macular degeneration (AMD), the most common cause of blindness in the elderly in developed countries, is characterized by drusen, lipoproteinaceous

deposits localized between the retinal pigment epithelium (RPE) and Bruch's membrane, that lead to an extensive atrophy of the RPE and overlying photoreceptor cells (geographic atrophy, GA) or aberrant choroidal neovascularization (CNV) under the macular area. AMD and DDD share pathological similarities with accumulation of complement-containing debris within the eye and kidney respectively. Indeed, AMD-like pathology is well-recognized in patients with DDD<sup>25</sup>. The identification of *CFH* as a major susceptibility locus for AMD and the characterization of multiple genetic variants in the *CFH* genomic regions conferring risk or protection to AMD indicate that the complement system plays a significant role in AMD pathogenesis<sup>26-30</sup>. However, *CFH* association data showed no overlapping between *CFH* at-risk polymorphisms for aHUS and AMD<sup>20</sup>.

The peculiar genotype-phenotype correlation between specific *CFH* genetic variations and a particular disease contrast with the situation of the *CFH* I890 and L1007 variations (in SCR 15 and SCR 17, respectively) that have been repeatedly reported associated with aHUS and are also found in DDD and AMD patients<sup>29, 31-33</sup>. To characterize the functional consequences of these *CFH* genetic variations we have purified the different FH protein variants to homogeneity from the plasma of appropriate carriers and tested their capacity to bind to surface-bound C3b, analyzed their cofactor activity in the FI-mediated inactivation of fluid phase C3b and performed FH-dependent hemolytic assays. None of these assays showed functional alterations in the regulatory activity of the FH, which strongly suggest that they are rare FH polymorphisms without functional consequences.

## RESULTS

Sequencing analyses of the *CFH* gene in the aHUS (n=259) and DDD (n=19) Spanish cohorts identified four aHUS patients (H54, H97, H142 and H244) and one DDD patient (GN3) carrying two nucleotide changes (c.2669 G>T; S890I and c.3019 G>T; V1007L) in heterozygosis. The same c.2669 G>T and c.3019 G>T nucleotide changes were also detected in heterozygosis in two out of 173 controls, an occurrence that is not significantly different to that found in patients. These Complement profiles and clinical data of the aHUS and DDD patients carrying the S890I and V1007L amino acid changes, as well as additional genetic alterations in other complement genes found in these patients are summarized in Table 1.

Two aHUS pedigrees were available for segregation analysis. In both cases it was demonstrated that the patients inherited both nucleotide changes (c.2669 G>T and c.3019 G>T) from the same progenitor, illustrating that they were carried by the same *CFH* allele. Further analyses of several SNPs within the *CFH* gene demonstrated that in all carriers, these I890 and L1007 amino acid changes associated with the same *CFH* haplotype, suggesting a single evolutionary origin for the I890/L1007 *CFH* haplotypes identified in the aHUS, DDD and control individuals (Table 2). Interestingly, the I890/L1007 *CFH* haplotype carries the AMD and DDD risk polymorphism His402, which may have implications for its association with AMD and DDD. The I890/L1007 *CFH* haplotype is probably old. This is supported by the existence of I890/L1007 *CFH* “recombinant” haplotypes like that of H97, affecting the promoter region of *CFH* or the genomic rearrangement that resulted in the generation of the *CFH::CFHR1* hybrid gene in H142.

As indicated, the I890/L1007 *CFH* haplotype in H142 is remarkable because, in addition, it encodes a *CFH::CFHR1* hybrid gene that resulted from a non-homologous recombination between the *CFH* and *CFHR1* genes. Thus, the FH protein encoded by this *CFH* allele carries a total of four amino acid changes compared to a normal *CFH* allele: I890 and L1007 and another two (L1191 and A1197) characteristic of the exon 6 of *CFHR1* fused to the exon 23 of *CFH* in the *CFH::CFHR1* hybrid gene (Table 2)<sup>34</sup>.

To purify the I890/L1007 FH variant from non-mutated FH in heterozygote carriers we used affinity chromatography with the MBI-7 anti-human FH monoclonal antibody<sup>35</sup>. This antibody specifically recognizes the H402 variant of FH and was used to capture the FH allele carrying the I890 L1007 amino acid changes from the plasma of patients H54 and H142, who are FH Y402H heterozygotes. The eluted protein was further purified by gel filtration and concentrated free of contaminants. We followed the same protocol to purify the S890/V1007 FH allele from control FH Y402H heterozygote donors (Figure 1). After quantification by ELISA, the purified proteins, S890/V1007, I890/L1007 and I890/L1007/L1191/A1197, were tested functionally in a number of different assays.

To test whether the I890/L1007 amino acid changes affect the cofactor activity for the FI-mediated proteolysis of C3b, S890/V1007 and I890/L1007 FH variants were mixed with C3b and incubated in the presence of FI at 37°C. After densitometric analysis of coomassie stained gels, the ratio between  $\alpha'$  chain/ $\beta$  chain of C3b was used to calculate the percentage of C3b cleavage. As illustrated, no differences in the cofactor activities between FH variants S890/V1007 and I890/L1007 (Figure 2A,B) or between S890/V1007 and I890/L1007/L1191/A1197 (Figure 2A,B,C) were appreciated, indicating that these amino acid substitutions do not have a significant effect on the cofactor activity of FH. As a control that our assays have appropriate sensitivity to

detect small functional alterations we have included in these experiments the V62 and I62 FH polymorphic variants previously, which shown to present slightly differences in their FI cofactor activities <sup>36</sup> (Figure 2D)

To explore the effect of the I890/L1007 amino acid changes in the interaction with C3b we performed a C3b binding plate assay. Purified C3b was immobilized on microtiter plates and identical quantities of the I890/L1007 and S890/V1007 FH variants were added and allowed to interact for two hours at 37°C. FH bound to C3b was detected using the Ox24 monoclonal antibody. FH was quantified in parallel in the same ELISA experiment. Our results show that the binding to C3b of the purified FH protein I890/L1007 was undistinguishable from that of the control FH S890/V1007 (Figure 3A). These results indicate that the amino acid changes in the SCR 15 and 17 do not alter the capacity of FH to bind C3b. We also used in these experiments the V62 and I62 FH polymorphic variants as it has also been shown previously that they present slight differences in their C3b-binding capacity <sup>36</sup> (Figure 3B).

To investigate the potential effect of the I890 and L1007 amino acid changes in the regulatory activity of FH on cell surfaces, the S890/V1007 and I890/L1007 FH variants were tested in a FH-dependent hemolytic assay developed in our laboratory. In these assays a human serum carrying a well-characterized *CFH* mutation that alters the C-terminus of FH <sup>18</sup>, was reconstituted with identical amounts of the S890/V1007, I890/L1007 or I890/L1007/L1191/A1197 FH variants and incubated with sheep erythrocytes in the presence of 7 mM Mg<sup>2+</sup> and EGTA. Lysis of erythrocytes in this assay inversely correlates with the capacity of FH to regulate the alternative pathway on the cellular surface. Our results indicate that the I890/L1007/L1191/A1197 FH variant presents decreased inhibition of the erythrocyte lysis compared with the native non-mutated variant of FH (figure 4B). This reduced capacity to regulate the alternative



pathway on the cellular surface was expected because this FH variant is also the product of a *CFH::CFHRI* hybrid gene<sup>34</sup>. In contrast, the FH variant from H54 showed no difference with the FH control and demonstrated to act efficiently in the protection against erythrocyte lysis. These data, again, indicates that the S890I and V1007L amino acid substitutions are not altering the capacity of FH to regulate the complement AP on the cellular surface.

## DISCUSSION

Mutation screening of complement genes in aHUS, DDD and AMD has become a laboratory routine. Identification of mutations helps diagnosis and provides useful information to anticipate the evolution of the disease in the patients and their response to treatments, conditioning clinical decisions. For example, among aHUS patients, those carrying *CFH* mutations have the worse prognosis and poorest renal transplantation outcomes, although they associate with good responses to plasma treatment. In addition, identification of mutations and polymorphisms associated with increased risk to these pathologies also influence the genetic counselling provided to patients and their relatives. It is therefore critical to obtain evidence supporting that the disease-associated mutations identified in these screenings have a causal relationship with the pathology. Here we have studied two FH amino acid substitutions, I890 and L1007, lacking these functional information that have repeatedly been found among Spanish aHUS and DDD patients and that have also been reported associated with aHUS in other Caucasian cohorts <sup>31-33</sup>. In addition, S890I and V1007L were described as rare polymorphisms associated with AMD <sup>29</sup>.

The peculiar concurrence of both S890I and V1007L amino acid substitutions in all these cases is explained by the segregation analysis performed here in two aHUS pedigrees that revealed that these two amino acid changes segregate together with a unique *CFH* haplotype characterized by a specific combination of SNPs (Table 2). The high allelic frequency of the S890I and V1007L polymorphisms in Sub-Saharan African (0,267; 0,317) and African American (0,455; 0,591) populations (rs515299 and rs534399, respectively), suggest that this *CFH* haplotype has an African origin and was

introduced in Caucasians some time ago. In support of this conclusion, one of the patients in our cohort carrying this haplotype (H244) has a Sub-Saharan African origin.

Carriers of the I890/L1007 *CFH* haplotype present normal FH levels in plasma with a contribution of the I890/L1007 FH allele of approximately 50% (Table 1). These data illustrate that these amino acid substitutions do not influence the expression/secretion of FH. To characterize the potential consequences of the S890I and V1007L substitutions in the functional activities of FH we performed three different functional assays using purified FH proteins. We tested the capacity of the I890/L1007 FH variant to bind to surface-bound C3b, analyzed its cofactor activity in the FI-mediated inactivation of fluid phase C3b and performed a FH-dependent hemolytic assay to determine its capacity to regulate the alternative pathway on cellular surfaces. None of these assays showed functional alterations in the regulatory activity of FH. This failure to detect functional alterations caused by the S890I and V1007L substitutions is not a consequence of a lack of sensitivity of our assays. They clearly revealed the subtle functional differences caused by the FH Val62Ile polymorphism or by the modification of the C-terminal region of FH that occurs in the *CFH::CFHRI* hybrid gene<sup>34, 36</sup>. We, therefore, concluded that the FH S890I and V1007L variants are most likely *CFH* polymorphisms without functional consequences. Furthermore, recent structural data have shown that SCR15 and SCR17, including these variations, are not implicated in the interaction between FH and C3b (Morgan et al. 2011).

Carriers of the I890/L1007 *CFH* haplotype in the Spanish aHUS cohort present other well-characterized mutations and/or polymorphisms in complement genes that may help to explain the development of the disease in these individuals. Thus, patient H54 carries in homozygosis the aHUS-conferring risk allele MCPggaac<sup>37</sup>; Patient H97

also carries in homozygosis the MCPggaac allele and, in addition, the mutation C86Y in the FI gene which produces a partial deficiency of FI in plasma (Table 1); and in patient H142, the I890/L1007 *CFH* haplotype also carries a *CFH::CFHR1* hybrid gene, which produces a FH protein with reduced capacity to regulate the alternative pathway on cellular surfaces (Figure 4B) <sup>34</sup>. Similarly, patient GN3 is positive for C3 nephritic factor (C3Nef). Lastly, an increased frequency of the I890/L1007 *CFH* haplotype in DDD and AMD should be expected because this *CFH* haplotype also carries the *CFH* H402 allele (rs1061170), a very strong risk factor for both AMD and DDD <sup>20,26</sup>.

In conclusion, we failed to provide evidence supporting a causal relationship of I890/L1007 with aHUS. The lack of functional consequences of the *CFH* S890I and V1007L amino acid substitutions, their presence in healthy individuals and their very high frequency in Sub-Saharan African and African American populations strongly suggest that S890I and V1007L are rare FH polymorphisms unrelated with the disease.

## MATERIALS AND METHODS

### *Complement analysis, mutation screening and genotyping.*

C3 and C4 concentrations were determined by nephelometry (Image®, Beckman Coulter) and FH plasma concentration was quantified by a specific sandwich ELISA method using polyclonal and monoclonal antibodies developed in house that does not cross react with the CFHRs proteins. No C3Nef was measured in serum plasma by standard procedures<sup>38</sup>. Patients and healthy volunteers were screened for mutations and polymorphisms in the *CFH*, *MCP*, *CFI*, *CFB*, *C3* and *THBD* genes by automatic DNA sequencing of PCR amplified fragments. Genomic DNA was prepared from peripheral blood cells according to standard procedures. Each exon was amplified from genomic DNA by using specific primers derived from the 5' and 3' intronic sequences as described<sup>15, 39-41</sup>. Automatic sequencing was performed in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Copy number variations in the *CFHR1-R3* genes were analyzed by MLPA as described<sup>42</sup>. The studies described herein received IRB approval (Comision de Bioetica, Consejo Superior de Investigaciones Cientificas and CEIC, Hospital Universitario La Paz, Madrid, Spain). Patients and their relatives gave their informed consent.

### *Proteins*

FH allele carrying the mutations I890 and L1007 was isolated from fresh plasma of aHUS patient H54 and a relative of the aHUS patient H142. We used a CNBr-activated sepharose 4B (GE healthcare) column coupled with the monoclonal antibody MBI-7 that exclusively recognizes the FH H402 protein variant<sup>35</sup>. Fractions containing

FH were collected, concentrated and applied to a gel-filtration column (Superose 6, GE Healthcare). Fractions containing the FH were pooled and purity of final preparations was confirmed in SDS-PAGE coomassie stained gels (Figure 1). C3 was purified by affinity chromatography and gel filtration as described previously<sup>43</sup>. C3b was generated by limited digestion with trypsin and re-purified by gel filtration as described above. C3b was obtained without any detectable contaminants or aggregates. Factor I was purchased from Comptech (Tyler, TX). Concentration of sample proteins was assessed using absorbance at 280 nm and molarities were calculated using an extinction coefficient for CFH of 1.95<sup>35</sup> and for C3 of 0.98 (Protean Software, DNASTar).

#### *Cofactor activity for FI-mediated proteolysis of fluid phase C3b*

The fluid-phase cofactor activity of FH was determined in a C3b proteolysis assay using purified proteins. In brief, C3b, FH and FI were mixed in 10 mM Hepes pH 7.5, 150 mM NaCl, 0.02% Tween 20 at final concentrations of 50 µg/ml, 4 µg/ml and 2.5 µg/ml, respectively. Mixtures were incubated at 37°C during 10 minutes. In another set of assays, samples were collected at 2.5, 5, 7.5 and 10 minutes of incubation. The reactions were stopped by the addition of 3µl of SDS sample buffer (2% SDS, 62.5 mM Tris, 10% Glycerol, 0.75% Bromophenol Blue). Samples were analyzed in 10% SDS-PAGE under reducing conditions. Gels were stained with Coomassie brilliant blue R-250 (BioRad) and proteolysis of C3b determined by measuring the cleavage of the  $\alpha'$  chain using a GS-800 calibrated densitometer (BioRad) and the MultiGauge software package (Fujifilm). The C3b  $\beta$  chain was used as an internal control to normalize the amount of protein added between samples. Percentage of cleavage was determined by

the ratio between  $\alpha'$  chain/ $\beta$  chain of C3b and setting as 0% of proteolysis by using a control FH in the absence of FI.

#### *ELISA C3b-binding assay*

The binding of FH variants to surface-bound C3b was determined by ELISA method. 96-well polystyrene microtiter plates were coated with C3b (2.5  $\mu$ g/ml) in coupling buffer (0.1 M NaHCO<sub>3</sub> pH 9.5) overnight at 4°C. The plate was blocked with washing buffer (20 mM Tris, 150 mM NaCl and 0.1% Tween 20) with 1% Bovine Serum Albumin for 1 hour at room temperature (RT). After washing, serial dilutions of FH variants (starting dilution was 2.5  $\mu$ g/ml) were added and incubated with surface-bound C3b for 2 hours at 37°C. After washing, the plate was incubated with anti-FH monoclonal antibody Ox24 for 1 hour at RT, and then with a secondary antibody coupled with horseradish peroxidase (DAKO). Colour reaction was developed with o-phenylene-diamine (DAKO) and absorbance measured at 492 nm. FH preparations used in the ligand assay were quantified in duplicate in the same ELISA plate using immobilised polyclonal anti-FH antibody to capture FH followed by the Ox24 and secondary antibodies to measure the amount of FH. Concentrations of FH were calculated from curves obtained using purified standard samples.

#### *Factor H-dependent hemolytic assay*

The capacity of FH to regulate the activity of the AP on cellular surfaces was assessed with a hemolytic assay using sheep erythrocytes and a serum carrying a well-

characterized *CFH* mutation, FH-W1183L<sup>18</sup>. In brief,  $1 \times 10^7$  sheep erythrocytes in AP buffer: VBS (2.5 mM barbital, 1.5 mM sodium barbital, 144 mM NaCl, pH 7.4) with 7 mM  $MgCl_2$  and 10 mM EGTA, were incubated with 10% FH-W1183L serum in AP buffer and increasing amounts of the different FH variants for 30 minutes at 37 °C. The reaction was stopped by adding VBS 20 mM EDTA. After centrifugation, supernatants were read at 414 nm. FH variants I890/L1007 and I890/L1007/L1191/A1197 were compared to the same control FH variant, S890/V1007, in two independent assays.  $\Delta$ FH without added FH was taken as 100% of lysis and serum diluted in AP buffer plus 20 mM EDTA were used as blank for spontaneous lysis.

## **DISCLOSURE**

Authors declare that they have no financial conflicts of interest.



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**TABLE 1.** Clinical and complement data of patients carrying the *CFH* I890 and L1007 genetic variants.

	<b>aHUS</b>				<b>DDD</b>
	<b>H54<sup>(1)</sup></b>	<b>H97</b>	<b>H142<sup>(2)</sup></b>	<b>H244<sup>(3)</sup></b>	<b>GN3<sup>(4)</sup></b>
<b>Age of onset</b> (years)	51	41	3.5	1	5.5
<b>C3</b> (700-1400 µg/ml)	59	123	94,3	135	135
<b>C4</b> (140-470 µg/ml)	7	24,5	24,2	31,5	31,5
<b>Total FH</b> (116-562 µg/ml)	127,4	256,8	150	316	161,8
<b>% FH<sub>I890-L1007</sub>*</b>	46%	47%	56%	-	-
<b>FI</b> (75-115%)**	99%	52%	104%	80%	103%
<b>Additional mutations</b>	none	CFI C86Y	<i>CFH::CFHR1</i> hybrid gene	none	none
<b>Del CFHR1-CFHR3</b>	none	none	het	none	none
<b>Autoantibodies</b>	none	none	none	none	C3Nef
<b>Renal status</b> (outcome)***	Deceased	ESRD	ESRD	MRI	MRI
<b>Transplantation</b> (recurrences)	none	Yes (no)	Yes (no)	none	none

- (1) Male patient who was diagnosed of post-transplant HUS (microangiopathic hemolytic anemia with schistocytes, and renal failure) associated with Tacrolimus and Cyclosporin threatment. He died one month later from pneumonia caused by Acynetobacter and Aspergillus.
- (2) After almost 4 years of haemodialysis, this male patient received a cadaver kidney transplant on September 2010. He was treated with eculizumab (Soliris®) before transplantation and every 15 days afterwards, following the protocol recommended by Alexion Pharmaceuticals. He is actually in good condition.
- (3) This is male child from Nigeria. He developed HUS after diarrhoea and a respiratory infection. He was under peritoneal dialysis and recovered partial renal function. No recurrences. Actually present moderate renal insufficiency
- (4) Diagnosis of DDD in this patient was established on the basis of renal biopsy (light microscopy, immunofluorescence and electron microscopy) performed at presentation of the disease. It was observed moderate mesangial hipercellularity and increased mesangial matrix without double-contour in the capillary walls. Immunofluorescence showed intense isolated granular C3 staining in the capillary walls and in the mesangium as nodular ring forms, negative for immunoglobulins or other complement proteins. EM revealed abundant electrodense ribbon-like deposits within the GBM and local electrodense deposits in the mesangium. The presentation was a nephritic syndrome with microhematuria and without proteinuria. He showed persistence hypocomplementemia C3 (8 mg/dl) and four months after was C3NeF positive. This situation was maintained for 12 years. In 1992, at age 20 years old, he presented proteinuria 5.75 g/day; Serum Creatinine: 1,0 ; Creatinine Clearance: 147ml/min and was remained C3Nef positive. He was treated with Prednisone. By 2007, after 33 years of evolution, the C3Nef titters were only detected as traces, with normalization of C3 levels and with an almost complete remission of the proteinuria after combined ACEI and ARB therapy. He presented ocular manifestations of AMD (drusen) at

age 33. The patient actually presents a moderate renal insufficiency (Creatinine: 1,9 ; Creatinine Clearance: 65 ml/min ) and proteinuria 1,2 g/day.

- \* In FH His402Tyr heterozygotes, levels of expression of the FH<sub>I890-L1007</sub> allele were determined by ELISA using anti FH 402His specific antibodies<sup>35</sup>.
- \*\* Factor I levels are referred to a reference pool of sera.
- \*\*\* ESRD, end stage renal disease; MRI, moderate renal insufficiency.

	<b>Promoter</b> -232 C>T	<b>V62I</b> c.184 G>A	<b>Y402H</b> c.1204 T>C	<b>Q672Q</b> c.2016 A>G	<b>E936D</b> c.2808 G>T	<b>S890I</b> c.2669 G>T	<b>V1007L</b> c.3019 G>T	<b>S1191</b> c.3645 C	<b>V1197</b> c.3663 T
<b>N1</b>	C	G	C	A	G	T	T	C	T
<b>N2</b>	C	G	C	A	G	T	T	C	T
<b>H54</b>	C	G	C	A	G	T	T	C	T
<b>H244</b>	C	G	C	A	G	T	T	C	T
<b>GN3</b>	C	G	C	A	G	T	T	C	T
<b>H97</b>	C	A	C	A	G	T	T	C	T
<b>H142</b>	C	G	C	A	G	T	T	<b>C</b>	<b>T</b>

**TABLE 2.** *CFH* haplotypes carrying the FH I890 and L1007 amino acid substitutions.

## FIGURE LEGENDS

### **Figure 1. SDS-PAGE coomassie-stained gel of the purified FH variants.**

The S890/V1007, I890/L1007 or I890/L1007/L1191/A1197 FH variants were purified from the plasma of a healthy individual and the aHUS patients H54 and H142, as described in Materials and Methods.

### **Figure 2. Cofactor activity of FH variants in the proteolysis of fluid phase C3b.**

C3b and FI were incubated with equal amounts of either S890/V1007, I890/L1007 or I890/L1007/L1191/A1197 FH variants for 10 minutes at 37°C and the reaction was stopped by the addition of SDS sample buffer. Samples were analyzed by SDS-PAGE under reducing conditions and gels were Coomassie-stained (a). A densitometric analysis of C3b proteolysis from triplicates of these experiments is shown in (b). A time-course analysis of C3b proteolysis is shown in (c). Fluid phase cofactor activity was measured by examining C3b cleavage at 2.5, 5, 7.5 and 10 minutes reaction for both control S890/V1007 (filled triangles) and I890/L1007/L1191/A1197 (open triangles) FH variants. Percentage of cofactor activity was determined by the ratio of C3b cleaved,  $\alpha'$ chain/ $\beta$ chain, and normalized to 0% proteolysis of control samples. Inset panel shows the double reciprocal plot of the S890/V1007 (open circles) and I890/L1007/L1191/A1197 (filled circles) of the cofactor activity curves. Multiple linear regression analysis showed no significant differences between the slopes for S890/V1007 and I890/L1007/L1191/A1197 cofactor activities. The sensitivity of our assay was demonstrated by including in the experiment a S890/V1007 FH carrying the I62 polymorphism (open circles).

### **Figure 3. Capacity of the FH variants to bind to C3b.**

The interaction between serial dilutions of purified FH with C3b deposited in 96-well plates is expressed as percentage of the amount of S890/V1007 FH variant bound to C3b at a concentration of 2.5 µg/ml. Means ± S.D. of three independent experiments are shown for I890/L1007 (open triangles), two samples of the S890/V1007 FH variant (filled triangles and filled circles) and for a S890/V1007 FH carrying the I62 polymorphism (open circles). This last one sample illustrate the sensitivity of our assay.

### **Figure 4. Inhibition of the lysis of sheep erythrocytes by the FH variants.**

A volume of serum from a control patient carrying a well-characterized mutation in *CFH* giving 100% lysis when added to sheep erythrocytes was mixed with different amounts of the purified FH variants. The lysis observed is shown as percentage of the lysis in the absence of added FH and was plotted against added FH. Means ± S.D. of three independent experiments are shown for S890/V1007 (open circles) and I890/L1007 (filled circles) (a) and for S890/V1007 (open circles) and I890/L1007/L1191/A1197 (filled circles) (b). Statistical differences as follows: \*:  $p < 0,05$ ; \*\*:  $p < 0,01$ ; \*\*\*:  $p < 0,001$ .









