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General Article

GENERAL ARTICLE

Effect of rare coding variants in the *CFI* gene on Factor I expression levels

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Abstract

Factor I (FI) is one of the main inhibitors of complement activity, and numerous rare coding variants have been reported in patients with age-related macular degeneration, atypical hemolytic uremic syndrome and C3 glomerulopathy. Since many of these variants are of unknown clinical significance, this study aimed to determine the effect of rare coding variants in the complement factor I (*CFI*) gene on FI expression. We measured FI levels in plasma samples of carriers of rare coding variants and *in vitro* in the supernatants of epithelial cells expressing recombinant FI. FI levels were measured in 177 plasma samples of 155 individuals, carrying 24 different rare coding variants in *CFI*. In carriers of the variants p.Gly119Arg, p.Leu131Arg, p.Gly188Ala and c.772G>A (r.685_773del), significantly reduced FI plasma levels were detected. Furthermore, recombinant FI expression levels were determined for 126 rare coding variants. Of these variants 68 (54%) resulted in significantly reduced FI expression in supernatant compared to wildtype (WT). The recombinant protein expression levels correlated significantly with the FI level in plasma of carriers of *CFI* variants. In this study, we performed the most comprehensive FI expression level analysis of rare coding variants in *CFI* to date. More than half of *CFI* variants lead to reduced FI expression, which might impair complement regulation *in vivo*. Our study will aid the interpretation of rare coding *CFI* variants identified in clinical practice, which is in particular important in light of patient inclusion in ongoing clinical trials for *CFI* gene supplementation in AMD.

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Introduction

The complement system is an ancient part of the immune system, bridging innate and adaptive immunity. Activation can be triggered via three different pathways: the classical, lectin and alternative pathways. The alternative pathway is continuously activated at a low level by spontaneous tick-over of complement component C3, making tight regulation of complement essential to prevent excessive activation. Two central inhibitors that are required for maintaining this balance are Factor H (FH) and Factor I (FI) (1).

Due to this delicate balance, it is not surprising that complement dysregulation plays a role in a broad spectrum of diseases. Examples of diseases where genetic variation causes excess complement activation are age-related macular degeneration (AMD), atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy (C3G) (2,3). AMD is a major cause of blindness among elderly in the Western world, and with aging of the population, a further increase of its prevalence is expected during the next 20 years (4). AMD is hallmarked by accumulation of extracellular deposits called drusen between the retinal pigment epithelium (RPE) and Bruch's membrane. In late stages of AMD, drusen can cause atrophy of the RPE, leading to photoreceptor loss (dry AMD), or trigger the invasion of newly formed blood vessels, which tend to leak and cause rapid vision loss (wet AMD) (5). aHUS and C3G, on the other side, are rare kidney disorders affecting approximately one to two out of a million, respectively (6,7). In aHUS characteristic features are microangiopathic thrombocytopenia, hemolytic anemia and renal failure. C3G is characterized by glomerular C3 deposits leading to renal failure (8). In a few cases, retinal drusen were reported in C3G and aHUS patients (9), but the most striking feature is the shared association with complement dysregulation in AMD, aHUS and C3G (3,10). Rare coding variants in the complement factor I (*CFI*) gene are associated with AMD (3), and 4–8.4% of aHUS patients carry variants in *CFI* (11,12). In C3G the association with *CFI* is less striking, but pathogenic variants are found at a low frequency (13). Interestingly, 26% of rare variants reported in *CFI* have been detected in both AMD and aHUS ((14,15) and https://www.complement-db.org/home.php, 09.01.2020), including the p.Gly119Arg (rs141853578) variant, which confers a high risk for AMD (16,17).

FI consists of five domains, FI membrane attack complex (FIMAC) domain, CD5 domain, two low-density lipoprotein receptor type A (LDLRA) domains and a serine proteinase (SP) domain, containing the catalytic site of FI. A recent study noted clustering of *CFI* variants in the SP domain of FI in aHUS and in the CD5 domain in AMD, but a substantial proportion of variants identified in both AMD and aHUS patients are spread across the whole protein (14).

Among AMD cases, a younger age at onset was observed in carriers of rare variants in complement components, including the p.Gly119Arg variant in *CFI*, compared to non-carrier AMD cases (18). Several rare coding variants in the *CFI* gene have been reported to confer a high risk of AMD with odds ratios as high as 20 (16), and rare variants in the *CFI* gene have been reported to occur in families with AMD (19,20). A phase 1/2 clinical trial for subretinal *CFI* gene supplementation in AMD is currently ongoing (https://www.clinicaltrialsregister.eu/ctr-search/trial/2017-003712-39/GB).

Furthermore, aHUS patients with end-stage renal failure are at high risk of recurrence after kidney transplantation in presence of pathogenic *CFI* variants (21). Rare variants in the *CFI* gene therefore have a high clinical relevance for both AMD and for aHUS.

However, interpreting the pathogenicity of rare coding variants in the *CFI* gene remains challenging. In AMD most of the rare *CFI* variants have been identified in only a small number of individuals, and due to lack of power, it is therefore difficult to prove their involvement with statistical associations (16). For some variants, such as the p.Gly119Arg, reduced expression of FI has been reported in recombinantly expressed protein and in plasma of heterozygous carriers (17,20). For other variants plasma FI levels in patients have been reported, but often only in one or a small number of carriers. For the majority of *CFI* variants reported in literature, however, expression levels have not been reported (16). Therefore, the aim of this study was to perform a comprehensive analysis of the effect of 126 rare coding variants in the *CFI* gene on FI expression levels (summarized in Fig. 1 and Supplementary Material, Table S1). We measured FI levels in plasma samples of carriers of rare heterozygous missense variants and *in vitro* in the supernatants of epithelial cells expressing recombinant FI. In addition, we determined how well these measurements correlated with each other and with *in silico* predictions.

Results

Plasma FI levels in carriers of rare CFI variants

To determine the effect of rare coding variants on FI expression, plasma samples of carriers were selected from the EUGENDA cohort (for AMD patients and controls) and from the Radboudumc aHUS/C3G cohort. In total plasma samples of 155 individuals were analyzed, of which 106 (95 EUGENDA and 11 aHUS/C3G) individuals carried a single heterozygous rare coding variant in *CFI* and two individuals carried two heterozygous rare coding *CFI* variants. For one of these two individuals, the family pedigree indicates compound heterozygosity (Table 1). For 13 patients plasma samples from follow-up visits were available (Supplementary Material, Table S2). Of the plasma samples of individuals collected from the EUGENDA cohort, 72 rare variant carriers were affected by AMD, 2 could not be graded, and 23 were graded as no AMD (of these 23, 3 were below the age of 60). The mean age was 72.31 years (Table 1). Additionally, plasma samples of 10 aHUS patients and 1 C3G patient were included in this study (Table 2). The mean age of aHUS/C3G patients (30.91 years) was lower than the mean age of controls (73.33 years), non-carrier AMD cases (73.17 years) and the mean age of the combined carrier groups from the EUGENDA cohort (71.89 years) (one-way ANOVA, Dunnett's *post hoc* test; $P < 0.0001$ for all three groups).

For each plasma sample, FI levels were determined with enzyme-linked immunosorbent assay (ELISA) in triplicate, and mean values of the triplicate measurements for each individual sample are shown in Figure 2. The normal range was determined as mean FI $\pm$ 2 SD using of (36.4 $\pm$ 16.7 µg/ml) in plasma samples of non-carrier control individuals. Significantly reduced FI levels were observed for carriers of the heterozygous c.355G>A (p.Gly119Arg), c.392T>G (p.Leu131Arg) and c.563G>C (p.Gly188Ala) variants compared to both non-carrier controls and AMD cases. Carriers of the c.772G>A (r.658_773del) variant showed significantly reduced levels compared to non-carrier AMD cases. Furthermore, the individuals carrying the heterozygous variants c.191C>T (p.Pro64Leu) and c.1420C>T (p.Arg474*) showed levels below the lower limit of the normal range at 19.7 µg/ml. The carrier of the two heterozygous c.1217G>A (p.Arg406His) and c.602dupG (p.Arg202Lysfs*10) variants showed FI levels below the normal range, while the carrier of the compound heterozygous c.563G>C (p.Gly188Ala) and c.781G>A

Table 1. Summary of individuals included from EUGENDA cohort

cDNA change[a]	Protein change[b]	N	AMD	No AMD < 60 years	Mean age in years (SD)	No. of females
N/A—non-carrier controls		24	0	–	73.33 (1.97)	14
N/A—non-carrier AMD case		23	23	–	73.17 (2.29)	14
37T>G	Phe13Val	2	1 (1CG[c])	–	73.14 (10.78)	2
148C>G	Pro50Ala	1	1	–	78.04 (–)	1
191C>T	Pro64Leu	1	1	–	73.52 (–)	0
215C>G	Thr72Ser	2	1 (1CG[c])	–	67.53 (5.22)	2
355G>A	Gly119Arg	18	14	1	66.51 (10.82)	10
392T>G	Leu131Arg	7	5	1	71.26 (10.209)	4
454G>A	Val152Met	1	0	–	70.62 (–)	1
563G>C	Gly188Ala	4	3	1	51.15 (15.18)	1
772G>A	Ala258Thr; r.658_773del[d]	3	2	–	71.8 (2.25)	2
781G>A	Gly261Ser	1	0	–	69.46 (–)	1
782G>A	Gly261Asp	13	9	–	75.41 (6.12)	9
1025G>A	Gly342Glu	1	1	–	78.41 (–)	1
1217G>A	Arg406His	5	4	–	69.44 (24.02)	3
1322A>G	Lys441Arg	10	6	–	73.19 (6.4)	4
1342C>T	Arg448Cys	5	5	–	75.47 (11.55)	4
1420C>T	Arg474*	2	2	–	85.86 (9.84)	1
1421G>A	Arg474Gln	1	0	–	81.12 (–)	1
1637G>A	Trp546*	1	1	–	83.15 (–)	1
1657C>T	Pro553Ser	15	12	–	75.72 (6.35)	9
1709G>C	Ser570Thr	2	2	–	76.58 (2.89)	2
563G>C, 782G>A[e]	Gly188Ala, Gly261Asp[e]	1	1	–	66.17 (–)	1
1217G>A, 602dupG	Arg406His, Arg202Lysfs*10	1	1	–	77.8 (–)	1
Total rare variant carrier		97	72	3	71.89 (10.91)	61
Total EUGENDA		144	95	3	72.31 (9.04)	89

[a]Based on NM_000204.4, on GRCh37.
[b]Numbering including the signal peptide.
[c]Images of these patients could not be graded.
[d]The c.772G>A variant was previously shown to lead to skipping of exon 5 (22, 23).
[e]Based on segregation analysis in family members, these variants were demonstrated to be compound heterozygous.

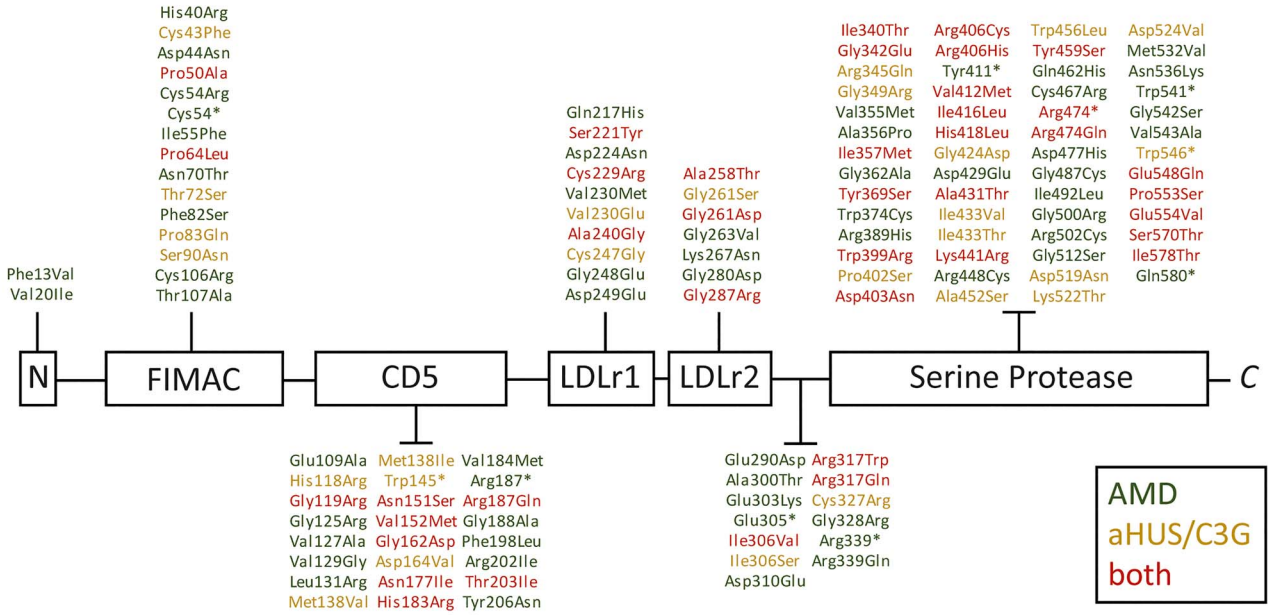

Figure 1. Distribution of rare missense or truncating variants in CFI included in this study. The listed variants were found in AMD (green), aHUS/C3G (yellow) or both in AMD and aHUS/C3G (red). Variant selection was based on (14, 16). Details of the shown variants are summarized in Supplementary Material, Table S1.

Table 2. Summary of aHUS/C3G patients carrying rare missense variants in *CFI*

cDNA change[a]	Protein change[b]	N	Mean age in years (SD)	No. of females
355G>A	Gly119Arg	1	40 (–)	1
454G>A	Val152Met	1	45 (–)	1
685T>C	Cys229Arg	1	21 (–)	1
1025G>A	Gly342Glu	1	46 (–)	0
1071T>G	Ile357Met	2	14 (2.83)	0
1217G>A	Arg406His	1	8 (–)	0
1322A>G	Lys441Arg	1	5 (–)	0
1420C>T	Arg474*	1	57 (–)	1
1642G>C	Glu548Gln	1	51 (–)	0
1657C>T	Pro553Ser	1	39 (–)	1
Total aHUS/C3G cohort		11	30.91 (18.80)	5

[a]Based on NM_000204.4, on GRCh37.
[b]Numbering including the signal peptide.

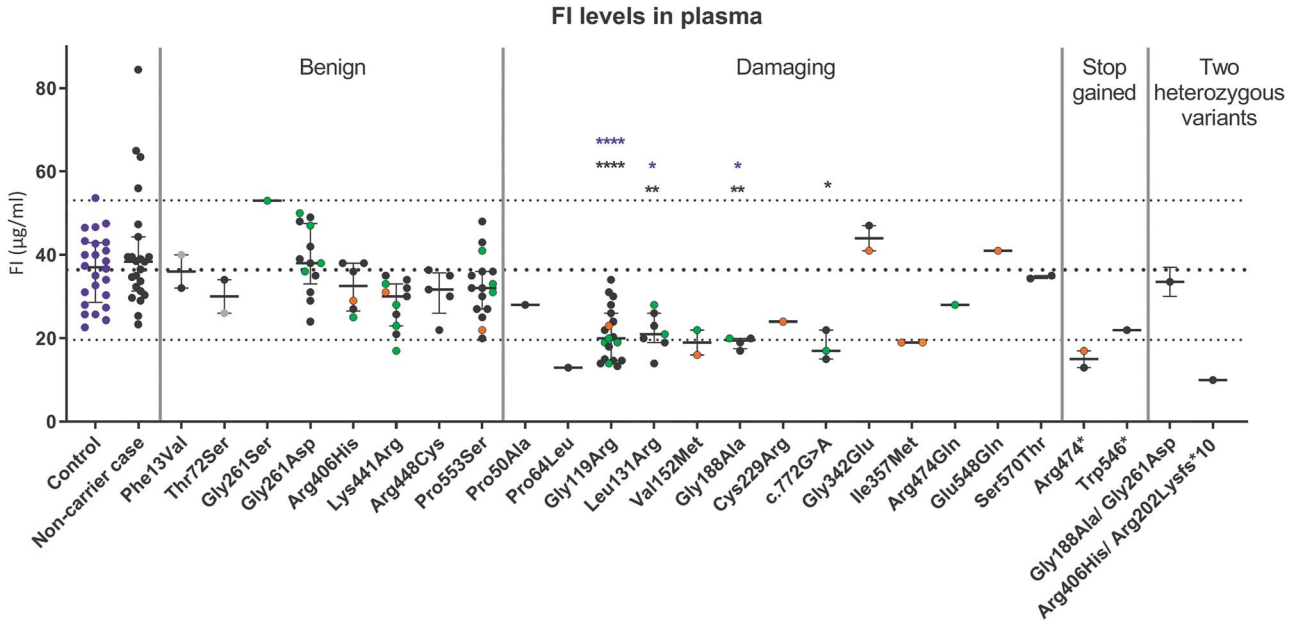

Figure 2. FI levels in plasma. Levels were determined with ELISA. AMD cases are indicated in black, no-AMD rare variant carriers are indicated in green and aHUS/C3G cases are indicated in orange. Individuals that could not be graded for AMD are indicated in gray. Dotted lines indicate the normal range [control mean ± 2 SD (36.4 µg/ml ± 16.7 µg/ml)]. For each group the median FI level is indicated by lines and interquartile ranges by error bars. Variants are categorized as benign with a CADD score <20 and damaging with a CADD score >20. Rare variant carriers were compared to non-carrier controls and non-carrier AMD cases using Kruskal–Wallis test with Dunn's *post hoc* test. Variants for which only a single carrier was measured were excluded from the statistical analysis. ****$P < 0.0001$, **$P < 0.005$, *$P < 0.05$.

(p.Gly261Asp) variants had normal FI levels. Plasma FI levels of carriers of the heterozygous variants c.454G>A (p.Val152Met) and c.1071T>G (p.Ile357Met) clustered around the lower limit of the normal range.

For 13 carriers plasma samples collected at follow-up visits were available (Supplementary Material, Table S2). To determine the variability of FI levels at different time points, FI levels were also determined in these follow-up samples (Fig. 3). The time interval between the different sampling time points was between 1 and 9 years. Three out of six carriers of the c.355G>A (p.Gly119Arg) variant showed persistently low FI over the period of several years (individuals AMD 5, AMD 8 and AMD 12). Some natural variation of plasma FI levels was observed, resulting for two carriers (individuals AMD 9 and AMD 3) in that one out of three and one out of two plasma FI levels, respectively, are above the lower limit of the normal range at 19.7 µg/ml.

Effect of rare CFI variants on expression of recombinant FI

In light of the natural variation in plasma FI levels *in vivo*, and since plasma samples were not available for carriers of all rare variants reported in literature (14, 16), 126 rare coding variants were selected for recombinant expression analysis *in vitro*. Expression levels of mutant and wild-type (WT) recombinant FI proteins were determined with ELISA in supernatants and lysates of transfected HEK293T cells (Fig. 4). For 68 of the 126 (54%) rare *CFI* variants, significantly reduced expression compared to recombinant WT FI expression level was observed in supernatants. Of these 68 variants, 46 (37% of 126) showed an expression level of the mutant FI recombinant protein that was less than 50% compared to the WT FI expression level. For 32 (25% of 126) variants, the expression levels of the mutant FI protein was less than 25% compared to the WT expression level.

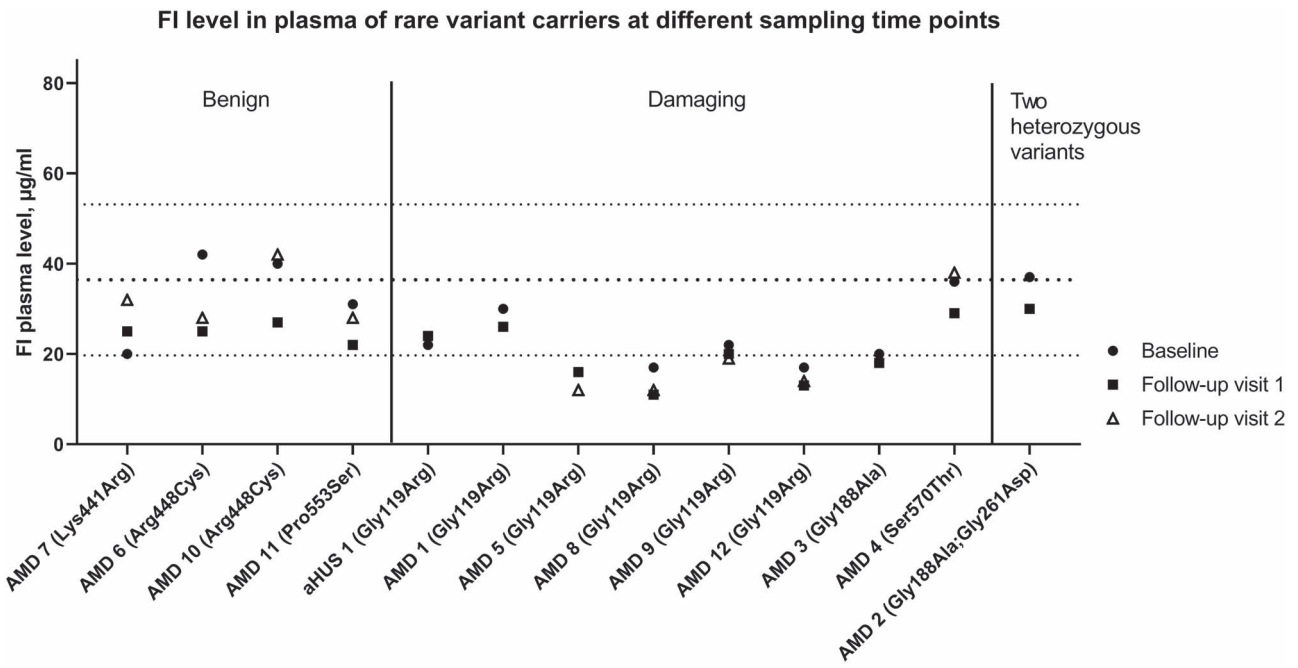

Figure 3. Follow-up plasma FI levels are plotted per carrier. Each sample was measured in triplicate with ELISA. Follow-up data were collected over the period of 1–9 years. Dotted lines indicate the normal range [control mean ± 2 SD (36.4 µg/ml ± 16.7 µg/ml)]. Variants are categorized as benign with a CADD score <20 and damaging with a CADD score >20.

To ensure that reduced recombinant protein expression levels were not an artefact caused by reduced binding of the monoclonal anti-FI antibody used in the ELISA, the FI expression levels of all mutant proteins that showed levels <30% of WT expression by ELISA were also analyzed on western blot using a polyclonal-FI antiserum. In total 36 variants with expression <30% and 8 additional variants with expression >30% compared to WT were analyzed on western blot. For all 44 analyzed mutant proteins, expression in the ELISA was in agreement with expression on western blot (Supplementary Material, Fig. S1). For three truncating variants c.913G>T (p.Glu305*), c.1015C>T (p.Arg339*) and c.1738C>T (Gln580*), remaining expression of 69%, 32% and 89% of WT expression was observed in the ELISA (Fig. 4). After precipitation of the supernatants, the presence of truncated FI was also confirmed at the expected approximate sizes of 33 kDa for c.913G>T (p.Glu305*) and 37 kDa for c.1015C>T (p.Arg339*). The variant c.1738C>T (Gln580*) causes truncation of four amino acids and is detected at the same height as WT FI with 64 kDa (Supplementary Material, Fig. S2).

To determine whether the variants alter the secretion of recombinant FI, the ratio of FI level in supernatant to the FI level in lysate was calculated for all samples for which FI expression was observed above the mock background signal in either supernatants or lysates (Fig. 4C). The recombinant mutant proteins carrying the variants c.454G>A (p.Val152Met) and c.1376A>C (p.Tyr459Ser) stand out with a ratio >3, suggesting faster secretion. The variants c.913G>T (p.Glu305*), c.930C>A (p.Asp310Glu), c.269G>A (p.Ser90Asn) and c.560G>A (p.Arg187Gln) show a similar direction of effect with a ratio >2. The only variant with a significantly lower ratio compared to WT is c.412A>G (p.Met138Val) with a ratio of 0.69, suggesting retention in the cell and/or reduced secretion.

In order to evaluate whether variants clustering in a certain domain are more likely to affect FI expression, the median expression levels of FI mutant proteins were compared for each domain (Fig. 5A). No statistically significant differences were detected between expression levels of variants clustering in the different domains. However, tendencies for low expression are observed for variants clustering in the CD5 and LDLR1 domains, resulting in the lowest median expression with 52% and 24% of WT expression, respectively. In line with this observation, 78% (18 out of 23) and 75% (6 out of 8) of variants within the CD5 and LDLR1 domains are significantly lower expressed compared to WT. In the remaining domains, the percentage of significantly lower expressed variants ranges from 0% (in the signal peptide and C-terminus) to 57% (in the LDLR2 domain) (Table 3).

Table 3. Number of variants analyzed and recombinant protein expression for each domain of the FI protein

Domain	Total N	Reduced expression (%)
Signal peptide	1	0 (0)
Interdomains	16	6 (38)
FIMAC	14	6 (43)
CD5	23	18 (78)
LDLRA1	8	6 (75)
LDLRA2	7	4 (57)
Linker region	2	1 (50)
SP domain	52	27 (52)
Terminus	3	0 (0)

Additionally, the expression levels of recombinant protein were stratified by disease phenotype (Fig. 5B). The median expression for variants found in AMD or aHUS/C3G is 64% of WT expression for both diseases. For variants found in both AMD and aHUS/C3G, the median expression is 68.5% of WT expression. This is also underscored by the number of FI mutant proteins that are significantly lower expressed for each group: 54% (14 out of 26) of variants found in aHUS/C3G, 55% (33 out of 60) of variants found in AMD and 53% (21 out of 40) of variants found in AMD and aHUS/C3G.

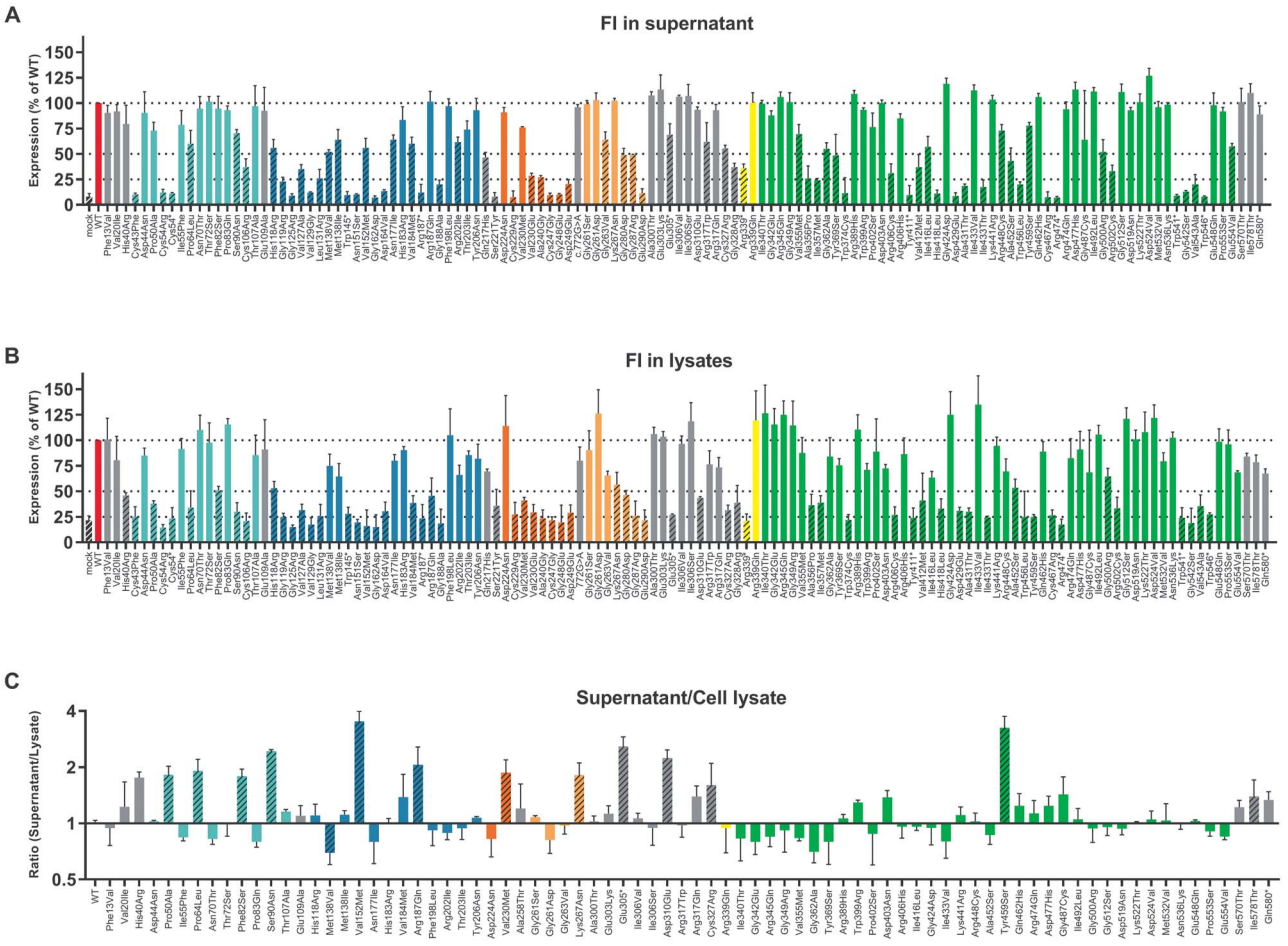

Figure 4. Recombinant expression of FI. Expression is presented as % of WT expression in supernatants (**A**), lysates (**B**) and the ratio of supernatant/lysate on a Log2 scale for variants with expression significantly higher than mock (**C**). Transfection of HEK293T cells was performed twice in two wells, and the median with the interquartile range is shown. The WT and mock sample are indicated by red and black, respectively, and domains are indicated by color according to the following: interdomains in gray, FIMAC in turquoise, CD5 in blue, LDLRA1 in dark orange, LDLRA2 in light orange, linker region in yellow and SP domain in green. Dotted lines indicate 100%, 50% and 25% of WT expression. Expression levels of the variants were compared to WT with Kruskal–Wallis test followed by uncorrected Dunn's *post hoc* test. Statistically significant differences ($P < 0.05$) are indicated by transverse stripes.

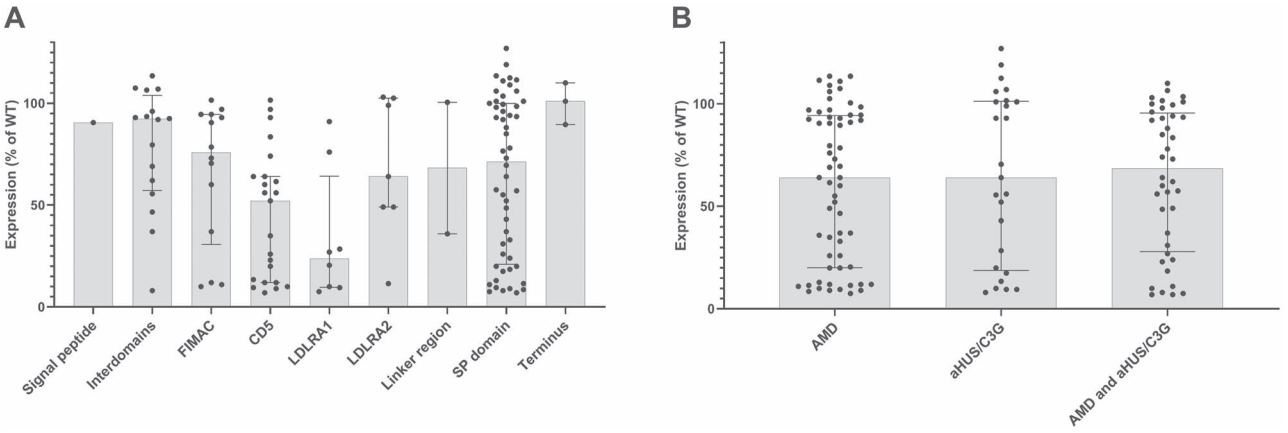

Figure 5. Recombinant FI expression of variants in each domain (**A**) or for each phenotype (**B**). The median expression (% of WT expression) is shown, and error bars indicate the interquartile range. Individual variants' expression is indicated with black dots (median expression as % of WT). All groups were compared to each other with Kruskal–Wallis test. For (**A**) the test was significant with $P = 0.034$, and Dunn's *post hoc* test was performed, resulting in $P > 0.05$ for all multiple comparisons. In (**B**) no overall significant difference was observed ($P = 0.805$), and therefore no *post hoc* test was performed.

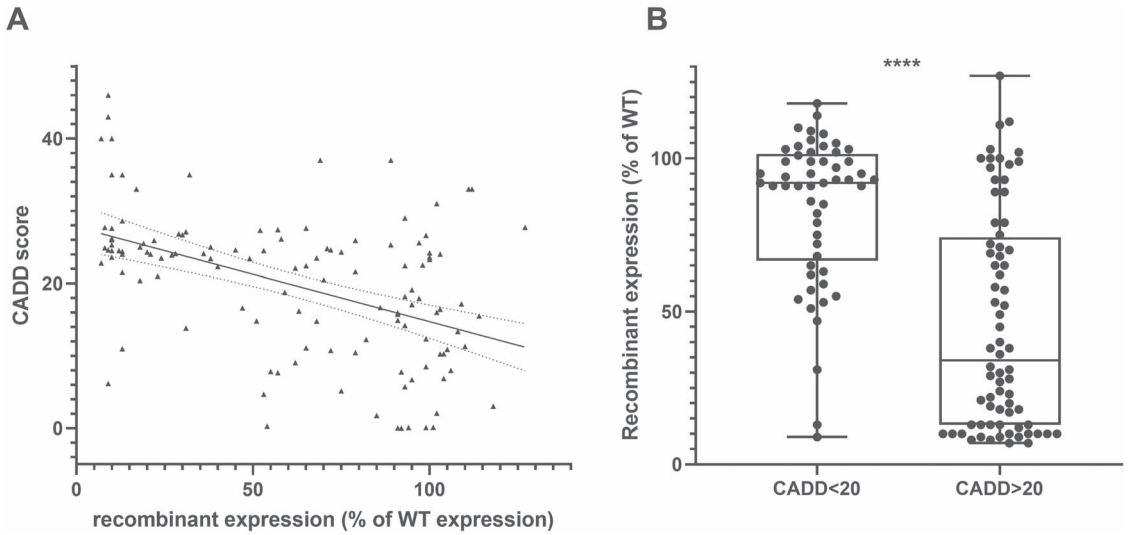

Figure 6. Comparison of recombinant FI expression (% of WT expression) and CADD scores. (**A**) Recombinant expression (% of WT expression) is plotted against the CADD Phred score. Spearman r correlation is significant with $P < 0.0001$, and $R^2 = 0.2192$ (slope and intercept of $Y = -0.1305^*X + 27.77$) was calculated. (**B**) Expression levels are compared using a cutoff CADD Phred = 20. Each data point is shown within the boxplot. The box includes the 25th and 75th percentile and the median. Whiskers indicate the minimum and maximum, and one sample Wilcoxon test was performed, indicating significant difference of expression between the groups at $P < 0.0001$.

We next determined whether the Combined Annotation Dependent Depletion (CADD) score, an algorithm often used in clinical practice to predict pathogenicity of coding variants, can be used as a predictor of the effect of rare *CFI* variants on FI protein expression. CADD scores were calculated with an in-house annotation pipeline, and the resulting CADD Phred values were plotted against the recombinant expression levels (Fig. 6A). A weak but significant correlation ($R^2 = 0.2192$, $P < 0.0001$) was observed between the CADD Phred score and the recombinant protein expression levels. Variants with a CADD Phred >20 showed significantly reduced recombinant protein expression levels compared to variants with a CADD Phred score <20 ($P < 0.0001$; Fig. 6B). Of 72 variants with a CADD Phred > 20, reduced protein expression of 50% of the WT was detected for 42 variants (and for 30 variants below 25% expression).

Recombinant protein expression of rare CFI variants correlates with FI plasma levels of carriers

In order to better compare recombinant FI expression with plasma FI levels, the percentage of plasma FI levels of carriers relative to the plasma FI levels in control individuals was calculated before running the correlation analysis (Fig. 7). The recombinant protein expression correlated significantly with the FI level in plasma of carriers of *CFI* variants ($R^2 = 0.5396$, $P < 0.0001$). Of note, the splice variant c.772G>A (r.658_773del) was excluded from the analysis, as this variant was reported to cause skipping of exon 5 (22,23). For this variant the recombinant expression (97% of WT expression) does not resemble the reduced levels in plasma (50% of control mean). For the plasma FI levels of nine variants clustering outside of the 95% confidence interval (CI) of the fitted line (c.685T>C (p.Cys229Arg), c.454G>A (p.Val152Met), c.191C>T (p.Pro64Leu), c.1421G>A (p.Arg474Gln), c.1322A>G (p.Lys441Arg), c.215C>G (p.Thr72Ser), c.1642G>C (p.Glu548Gln), c.1025G>A (p.Gly342Glu) and c.781G>A (p.Gly261Ser)), plasma FI levels of four variants (c. 685T>C (p.Cys229Arg), c.191C>T (p.Pro64Leu), c.1421G>A (p.Arg474Gln) and c.781G>A (p.Gly261Ser)) were only measured in a single carrier.

Discussion

In this study we performed a comprehensive analysis of the effect of 126 rare coding variants on FI expression levels. We measured FI in plasma samples of carriers of rare missense variants *in vivo* and demonstrated that 4 (17%) of 23 variants analyzed lead to significantly reduced FI levels in plasma. In addition, we measured FI levels *in vitro* in the supernatants of epithelial cells expressing mutant recombinant FI and demonstrated that 68 (54%) of 126 of rare *CFI* variants lead to significantly reduced levels of FI compared to recombinantly expressed WT FI protein.

FI plasma levels were determined in carriers of 23 different rare coding variants. For 16 variants FI levels were measured in more than one carrier, enabling statistical comparison with control groups (Fig. 2). Carriers of the heterozygous variants c.355G>A (p.Gly119Arg), c.392T>G (p.Leu131Arg) and c.563G>C (p.Gly188Ala) have significantly reduced levels compared to non-carrier cases and controls, which is in line with previous observations from our group and literature (17,20,24,25). Three carriers of the splice variant c.772G>A (r.658_773del) have significantly reduced levels compared to non-carrier AMD cases. This variant was previously reported to cause skipping of exon 5 and thereby leads to haploinsufficiency or even FI deficiency in homozygous carriers (22,24,26–29). Additionally, carriers of the heterozygous variants c.191C>T (p.Pro64Leu), c.1071T>G (p.Ile357Met), c.1420C>T (p.Arg474*) and c.1217G>A (p.Arg406His)(;)c.602dupG (p.Arg202Lysfs*10) have plasma FI levels below the normal range; however only one or two carriers are available for each group, limiting robust interpretation of the effects of these variants. Plasma samples of carriers of rare *CFI* variants are often not available or are available for only one or two carriers, and a natural variation in concentration was noted between carriers of the same variant and among samples of the same individual sampled at different time points (Figs 2 and 3). In addition,

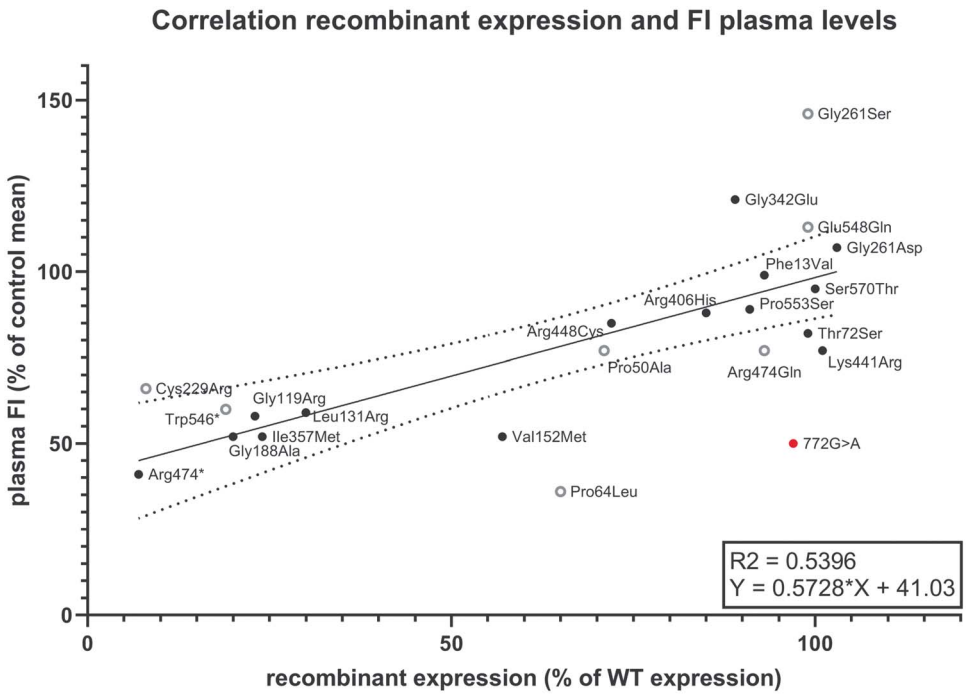

Figure 7. Correlation of recombinant FI expression (% of WT expression) and FI levels in plasma (% of mean control FI level). Variants for which only FI levels in plasma of a single carrier were measured are indicated with circles, and variants for which more than one carrier was measured are indicated with a dot. The splice variant c.772G>A (r.658_773del) and WT/control samples were excluded from the correlation analysis and straight-line fitting. Dotted lines indicate 95% CI of fitted line. Pearson correlation is significant with $P < 0.0001$.

heterozygous carriers of rare variants also carry a normal copy of the *CFI* gene, and WT protein expression from the normal allele might mask the effect of the mutant allele. These factors combined make it challenging to assess the effect of genetic variants on FI plasma levels.

In light of these observations, we decided to clarify the effects on expression of rare coding variants in *CFI* found in aHUS, AMD or C3G patients *in vitro*. In total 126 variants were selected based on Geerlings *et al.* (14,16) (Fig. 1). Of the 126 variants we included in this study, only for 27 variants recombinant expression levels were published previously (summarized in Supplementary Material, Table 1). Of these 27 variants, 14 were reported to cause reduced levels in supernatant (17,23,25,27,30). For two variants [c.148C>G (p.Pro50Ala) and c.949C>T (p.Arg317Trp)], published results on recombinant expression levels were conflicting (25,30,31). In our study, expression levels of mutant recombinant protein were determined for 126 rare coding variants, including the 27 for which levels were determined previously. For 68 (54%) of the 126 rare *CFI* variants, significantly reduced expression compared to recombinant WT FI expression level was observed in supernatants. All 14 variants previously reported to cause reduced recombinant expression in supernatants were also in our study significantly reduced compared to recombinant WT FI expression. For the variant c.414G>T (p.Met138Ile), normal expression was reported (31); however here we observe significant reduction of mutant protein expression with 65% of recombinant WT FI expression. Of all 126 variants, 46 (37%) variants showed recombinant protein expression levels less than 50% compared to WT recombinant protein, and 32 (25%) of these variants show even less than 25% expression compared to WT recombinant protein in supernatant (Fig. 4A).

To determine whether the variants cause retention in the cells or alter the secretion of recombinant FI protein, the ratio of supernatant/lysate was calculated for all variants for which expression above the background signal of mock in either supernatants or lysates was found (Fig. 4C). For the variant c.412A>G (p.Met138Val), a significantly reduced ratio (0.69) compared to WT was observed, indicating that the variant might lead to retainment in the cell lysates and/or reduced secretion. For the variants c.128G>T (p.Cys43Phe), c. 434G>A (p.Trp145*), c.452A>G (p.Asn151Ser), c.1071T>G (p.Ile357Met), c.1291G>A (p.Ala431Thr), c.1367G>T (p.Trp456Leu) and c.1420C>T (p.Arg474*), retainment in cell lysates was previously reported (25,30). However in our dataset, none of these variants were expressed significantly higher compared to mock in neither supernatants nor lysates and were therefore not included in this analysis.

On the other hand, some variants in our data have significantly higher levels in supernatants compared to lysates, with the variants c.454G>A (p.Val152Met) and c.1376A>C (p.Tyr459Ser) standing out with ratios > 3, followed by the variants c.913G>T (p.Glu305*), c.930C>A (p.Asp310Glu), c.269G>A (p.Ser90Asn) and c.560G>A (p.Arg187Gln) with ratios >2, potentially suggesting faster secretion. However, this effect might be due to degradation of intracellularly retained protein.

The 126 *CFI* variants that were analyzed for their effect on FI expression level are distributed throughout all domains of the FI protein (Fig. 1). A comparison to determine whether variants in certain domains are more likely to affect FI expression did not result in statistically significant differences; however variants in the CD5 and LDLRA1 domain tend to cause lower FI expression (Fig. 5A and Table 3). The LDLRA1 and LDLRA2 domains both bind

a calcium cation, which is predicted to have structural implications for the protein (32). In line with this prediction, the only variant [c.870A>C (p.Glu290Asp)], directly changing one of the six calcium-binding amino acids, causes reduced FI expression (12% of WT expression).

The lowest proportion of variants affecting FI expression was detected in the FIMAC domain: 43% of variants are expressed significantly lower compared to WT. This domain was reported to be essential for FI function likely by acting on the SP domain and playing a role in FH binding (32–34). This suggests that the normally expressed variants from this study potentially impair FI function.

The SP domain is highly disordered in the absence of a cofactor (32,35). In our study approximately half of the variants in this domain (52%) cause reduced expression compared to WT. Since this domain contains the catalytic site for C3b and C4b degradation, functional analysis is needed to clarify whether the normally expressed variants in this domain cause functional impairment. For six variants in this domain, functional analyses were previously reported using recombinant protein, but only the variants c.1019T>C (p.Ile340Thr) and c.1571A>T (p.Asp524Val) caused loss of function in C3b and C4b degradation assays (30,31,36).

The median expression levels of recombinantly expressed variants were also stratified by disease phenotype, but no differences in expression were observed between the groups (Fig. 5B). A potential explanation is that both AMD and aHUS are multifactorial diseases. Carrying a rare, pathogenic variant in *CFI* increases disease risk substantially; however additional genetic variants and (environmental) risk factors play an essential role in the disease outcome (10,37). AMD is caused by genetic variants in several disease pathways including the complement system, lipid metabolism and extracellular matrix remodeling, next to other risk factors such as aging and lifestyle (37). In aHUS mutations in complement components pre-dispose to disease, in addition to a second, environmental hits such as infections or pregnancy (10). From our data it can be deduced that FI levels alone do not define the clinical phenotype. Figure 2 shows that there is variability in FI levels, even among individuals carrying the same FI variant. Also, many patients have FI levels in the normal range. This further supports that a combination of factors determine the disease risk, rather than FI levels alone.

Prediction algorithms are often used in clinical practice to analyze whether a variant might be pathogenic or not. In order to determine how well the CADD Phred score correlates with recombinant expression levels, the calculated CADD Phred score was plotted against the recombinant expression in supernatants, resulting in $R^2 = 0.2192$ (Fig. 6A). While the overall correlation is not very strong, a significant decrease of expression is observed among variants with a CADD Phred >20 (Fig. 6B). Strikingly, of 72 variants with a CADD Phred > 20, reduced protein expression of 50% was detected for 42 (58%) variants, indicating that high CADD Phred scores (>20) are fairly good predictors for FI expression levels. Among the remaining 30 variants with a CADD score >20 is the splice variant c.772G>A (r.658_773del), for which the effect cannot properly be assessed in the *in vitro* FI expression assay. For four other variants with a CADD score>20 but normal FI levels [c.148C>G (p.Pro50Ala), c.1571A>T (p.Asp524Val), c.1019T>C (p.Ile340Thr) and c.1555G>A (p.Asp519Asn)], functional tests were previously published, and for all variants except for c.148C>G (p.Pro50Ala), a loss of function was reported (30,31). Therefore, it is likely that a large proportion of the remaining 25 rare *CFI* variants with a CADD Phred >20 affect FI function, which should be investigated in future studies.

In order to determine how well *in vitro* data from recombinant FI expression correspond to plasma FI levels of heterozygous carriers *in vivo*, a correlation analysis was performed (Fig. 7), resulting in an $R^2 = 0.5396$. The previously mentioned splice variant c.772G>A (r.658_773del) was excluded from the correlation, as splicing defects cannot be assessed in recombinant expression assays using cDNA constructs. For four variants [c.685T>C (p.Cys229Arg), c.191C>T (p.Pro64Leu), c.1421G>A (p.Arg474Gln) and c.781G>A (p.Gly261Ser)] that are clustering outside of the 95% CI of the fitted line, FI plasma levels could only be determined in a single carrier. Therefore, whether this is simply individual variation of FI levels *in vivo* or effects like alternative splicing cannot be determined with this dataset. For the c.191C>T (p.Pro64Leu) variant, only one further carrier was reported in literature with serum FI levels around 20 µg/ml (54% of control mean) (24), which is more in agreement with the recombinantly expressed protein in this study (60% of WT expression). Thus, although the correlation of plasma FI levels with recombinant FI expression is high, the natural spread of FI plasma levels hampers a robust analysis in individual carriers of rare variants. This indicates that measuring recombinant FI protein expression is a more sensitive method to determine the effects of genetic variants on expression levels.

In summary, we performed the most comprehensive expression level analysis of rare coding variants in *CFI* to date. More than half of *CFI* variants lead to reduced FI expression, which likely impairs complement regulation in *CFI* variant carriers *in vivo*. By providing recombinant expression data for 126 rare coding variants, our study will aid the interpretation of rare coding *CFI* variants identified in clinical practice, which is in particular important in light of patient inclusion in ongoing clinical trials for *CFI* gene supplementation in AMD (https://www.clinicaltrialsregister.eu/ctr-search/trial/2017-003712-39/GB).

Methods

Patients

Carriers of rare variants in *CFI* were selected from the European Genetic Database (EUGENDA) and from the aHUS/C3G cohort of the Radboudumc. All enrolled participants gave written informed consent and were pseudo-anonymized with a database identifier code.

From the EUGENDA cohort, 144 individuals were selected based on genotype and AMD grading. Of the selected individuals, 95 were diagnosed with AMD. In total 97 carriers of rare missense variants in *CFI* were included and 47 non-carrier controls or non-carrier AMD cases. Non-carrier controls and non-carrier AMD cases were above the age of 67 years at sampling time point. AMD diagnosis was obtained by evaluation of retinal images according to the Cologne Image Reading Center and Laboratory protocol. AMD was characterized as the presence of pigmentary changes together with at least 10 small drusen (<63 µm) or the presence of intermediate (63–124 µm) to large drusen (≥125 µm diameter) near the macula. Individuals showing neovascularization or geographic atrophy were also included as AMD cases. The study was approved by the local ethics committees on Research Involving Human Subjects and conducted according to the Declaration of Helsinki. From the aHUS/C3G cohort, 11 patients carrying a rare missense variant in *CFI* were selected. Ten patients were diagnosed with aHUS, defined as a presence of hemolytic anemia, thrombocytopenia and acute renal failure

that was not preceded with infection with Shiga toxin-producing *Escherichia coli* (8). One patient presented with C3G, defined as active glomerulonephritis combined with predominantly C3 and absent or marginal immunoglobulin deposition in renal biopsy (38).

EDTA plasma samples were collected and centrifuged according to standard protocols and frozen at −80°C within 1 h. Genomic DNA isolation from peripheral blood was performed according to standard procedures. Genetic analysis was described previously and was done by whole-exome sequencing (14,39) or exome chip analysis (3) or using single molecule molecular inversion probes and next generation sequencing for target selection and sequencing (de Breuk *et al*. manuscript in preparation).

Selection and cloning of rare missense variants

For recombinant expression studies, rare coding variants in *CFI* with a minor allele frequency (MAF) of <1.5% were selected based on Geerlings *et al*. (14,16). All 126 variants included are summarized in Supplementary Material, Table S1 and the distribution throughout FI is shown in Figure 1. MAF was obtained from the genome aggregation database (https://gnomad.broadinstitute.org/, on 18.10.2019). Full-length *CFI* cDNA (NM_000204.4, on GRCh37) was cloned into a pcDNA3 expression vector (Invitrogen, USA) as described (40). Variants were introduced using the Q5 site-directed mutagenesis kit (New England Biolabs, USA). Mutagenesis primers were designed using http://nebasechanger.neb.com/ or by designing overlapping primers including the desired nucleotide change. All mutagenesis primers are listed in Supplementary Material, Table S3. Sanger sequencing of the whole CFI insert, using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA), was performed to confirm presence of the desired variant and absence of additional unwanted changes introduced by PCR.

Expression of recombinant FI

HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (Sigma, USA), 1 mM sodium pyruvate (Sigma), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma). Cells at approximately 90% confluency were transfected with pcDNA3 constructs expressing WT or mutant FI using lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions. After recovery in supplemented DMEM, transfected cells were kept for 2 days in serum-free Opti MEM Glutamax (Gibco, USA) before conditioned media were collected. Cells were lysed with buffer containing 1% Triton-X100, 20 mM Tris–HCl, 150 mM NaCl and 5 mM EDTA, supplemented with Complete Mini Protease Inhibitor Cocktail (Merck, USA) immediately before use. Cell debris was removed from supernatants and lysates by centrifugation. Each *CFI* variant was transfected twice in duplicate.

FI ELISA

Concentrations of native FI in EDTA plasma and of recombinant FI in conditioned cell media and cell lysates were determined using sandwich enzyme-linked immunosorbent assay (ELISA). High binding microplates (Greiner) were coated with sheep anti-human FI antibody (LabNed, Netherlands) diluted 1000× in coating buffer (15 mM Na_2CO_3*10 H_2O, 35 mM $NaHCO_3$, pH 9.6). Plates were blocked first with SuperBlock (ThermoFisher Scientific, USA) and then with 1% bovine serum albumin (BSA, Sigma, USA) in PBS. The plates were washed four times with 200 µl PBS 0.02% Tween after blocking and between all the following incubation steps. Samples, standards and antibodies were diluted in PBS with 0.02% Tween and 0.2% BSA. Serum-purified FI was used as standard (CompTech, USA). After applying the samples, FI was detected with 1 µg/ml mouse anti-human FI (OX-21, ProSci, USA). As the secondary antibody 0.02 mmol/l goat anti-mouse horseradish peroxidase labeled Ig (Dako, Germany) was used, followed by detection with o-phenylenediamine dihydrochloride substrate (Dako, Germany), diluted according to manufacturer's instructions. All conditioned supernatants were measured in duplicate and all plasma samples in triplicate.

SDS-PAGE and western blot

For validation of reduced recombinant FI expression levels on western blot, conditioned supernatants were denatured immediately with NuPAGE™ lithium dodecyl sulfate (LDS) Sample Buffer (ThermoFisher Scientific, USA) under non-reducing or reducing conditions (50 mM Dithiothreitol). Some supernatants were first precipitated by incubating with trichloroacetic acid (TCA) for 10 min at 4°C and then washed twice with ice-cold acetone. The pellet was dried and resuspended in 1× NuPAGE™ LDS sample buffer (ThermoFisher Scientific) prior to further use. All samples were separated on SDS-PAGE (Invitrogen, USA) and transferred to a polyvinylidene difluoride membrane. After transfer, the membrane was blocked with superblock (ThermoFisher Scientific, USA) and then incubated with goat anti-human FI (Quidel, USA). Donkey anti-goat IRDye800 (Rocklands, USA) antibody was used as detection antibody. For imaging the Odyssey infrared system was used (Li-Cor, USA).

Statistical analysis

Statistical analysis and data visualization were performed with IBM SPSS Statistics 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 8.2.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. FI plasma levels were compared with non-carrier controls and non-carrier AMD cases using Kruskal–Wallis with Dunn's *post hoc* test. The age between the different groups (aHUS/C3G patients, non-carrier controls, non-carrier AMD cases and the combined *CFI* rare variant carrier groups from the EUGENDA cohort) was compared using one-way ANOVA with Dunnett's *post hoc* test. Recombinant FI expression of variants was compared with WT expression using Kruskal–Wallis test and Dunn's *post hoc* test. The presented *P*-values are not corrected for multiple testing, and the number of comparisons and individual *P*-values for each test is listed in Supplementary Material, Table S4.

For the correlation analysis of recombinant mutant FI protein expression levels with *in vivo* plasma levels of carriers of the matching variant, the percentage of the FI plasma concentration in carriers relative to mean FI plasma level in the control group was calculated. If several carriers with a variant were available, the mean FI plasma level of all carriers was used to determine the percentage relative to the mean FI plasma expression level in controls. The data were compared using Pearson correlation. The control group and recombinant WT sample was excluded from the correlation analysis as both were set to 100%.

Supplementary Material

Supplementary Material is available at *HMG* online.

Conflict of Interest statement: A.I.d.H. is a consultant for Ionis Pharmaceuticals, Gyroscope Therapeutics, Gemini Therapeutics and Roche.

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