CLINICAL TRIALS AND OBSERVATIONS

Rare germline *complement factor H* variants in patients with paroxysmal nocturnal hemoglobinuria

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> **KEY POINTS**
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> - Rare germline *CFH* variants are over-represented among patients with PNH.
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> - Patients with PNH with rare germline *CFH* variants were more likely to be transfusion-dependent under eculizumab.

Patients with paroxysmal nocturnal hemoglobinuria (PNH) are susceptible to complement-mediated intravascular hemolysis and thrombosis. Factor H (FH) is the main regulator of the complement alternative pathway, which protects cells from unwanted complement-mediated damage. Although FH is not a glycosylphosphatidylinositol-linked molecule, it may play a role in PNH. We sought to determine if rare germline variants in *complement factor H* (*CFH*) affect the PNH course, screening 84 patients with PNH treated with eculizumab for rare variants in *CFH*, *CFI*, and *C3* genes. We compared the allelic frequencies with populational data and a geographically-matched control group, looking for an association between presence of the variants and treatment response (transfusion independence by 6 months). Sixteen patients presented rare variants, 9 in *CFH* (10.7%). Germline *CFH* variants were more frequent among patients with PNH than among controls ($P = .02$) or public data ($P < .001$) and were more likely to be transfusion-dependent at 6 months after eculizumab initiation ($P = .015$). With a median follow-up of 5.8 years, 8 of 9 patients with the *CFH* variant received transfusions, and 2 developed thromboses. None of the patients with the *CFH* variant had severe aplastic anemia from eculizumab initiation until 6 months. We demonstrated for the first time that rare *CFH* variants are over-represented among patients with PNH and that germline genetic background may affect the response to eculizumab.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disorder, that manifests as intravascular hemolysis, bone marrow failure and thrombosis. It is caused by somatic mutations in the *PIGA* gene, resulting in impaired glycosylphosphatidylinositol (GPI) biosynthesis.[1] GPI anchors cell–surface proteins, including the decay-accelerating factor (CD55) and the membrane inhibitor of reactive lysis (CD59), thus modulating the alternative and terminal complement pathways. Impaired GPI synthesis leads to complement-mediated hemolysis.[2] The most widely available PNH treatment, eculizumab, binds to C5, halting intravascular hemolysis and reducing thrombosis occurrence.[3-5] Clinical responses vary from complete to none.[6,7]

Some patients may need higher doses of eculizumab to avoid residual intravascular hemolysis.[8,9] C5 inhibition enhances the accumulation of C3 fragments on PNH red blood cells (RBCs),

triggering extravascular hemolysis.[10] Specific *C5* variants are resistant to eculizumab.[11] In addition, a common *CR1* variant predisposes to suboptimal responses to eculizumab.[12]

We hypothesized that rare germline variants in regulatory genes implicated in the complement alternative pathway may influence disease presentation or response to eculizumab, notably transfusion dependence, 6 months after treatment initiation.

Study design

Data collection

We studied patients diagnosed with PNH, who were treated with eculizumab for at least 6 months and were enrolled in the French Registry of Marrow Failure Syndromes, with available samples for molecular profiling with next-generation sequencing (NGS). All participants signed informed consent. This research was carried out per the Declaration of Helsinki.

The Comité de protection des personnes Ouest VI approved the research, registry, and biobank.

Gene sequencing

Genomic DNA was extracted from blood, buccal swabs, and fibroblast samples. All coding and flanking intronic sequences of *CFH, CFI, MCP,* and *C3* genes were analyzed by NGS.[13,14] Rare variants were defined as minor allelic frequency (MAF) <0.1% in the general population. Those variants in which the genetic change affected the protein function were considered pathogenic.[15] Other variants were classified as being of uncertain signifcance (VUS).[15,16] We confirmed the constitutional nature of the variants by sequencing DNA extracted from buccal swabs or cultured skin fibroblasts.

Statistical analyses

Qualitative and quantitative variables are presented as numbers and percentages or medians and interquartile ranges, respectively. Our cohort of patients with rare variants was compared with a control group of 80 healthy adults from France and from public data (1000 Genomes).

Transfusion dependence was the primary end point, defined as >2 RBC transfusion episodes during 6 months. The secondary outcomes were failure-free survival (FFS), defined as the first RBC transfusion after 28 days from eculizumab initiation, an increase in eculizumab dose, thrombosis, or death and event-free survival (EFS), defined as FFS also including hematopoietic cell transplantation or evolution to a WHO (2016)–defined myeloid malignancy. Symptoms of anemia or a hemoglobin level below 8 g/dL, triggered blood transfusions.

EFS and FFS were analyzed using the Kaplan-Meier estimator. The impact of variables on outcomes was tested using Fisher or Wilcoxon and log-rank tests. We present EFS and FFS with 95% confidence intervals (CI). We used the R software for statistical analyses.

Results and discussion

We screened 84 patients for genetic variants and evaluated 83 patients for primary and secondary objectives (supplemental Figure 1; available on the *Blood* website).

To the best of our knowledge, it is for the first time that we report the frequency of rare variants in *CFH* (9/84, 10.7%), *CFI* (3/84, 3.6%), and *C3* (4/84, 4.8%) genes in patients with PNH (Table 1). We confirmed the germline nature of *CFH* variants in 10 patients using DNA from buccal swabs (n = 2) or fibroblasts (n = 8).

We found that *CFH* variants were significantly over-represented among patients with PNH when compared with healthy controls (*P* = .02) or to public data (*P*<.001, supplemental Table 1). In contrast to *CFH*, the frequency of *CFI* or *C3* variants was not increased, highlighting FH's role in RBC protection.

FH, a central alternative pathway regulator, prevents the formation of the C3 convertase and regulates C3 convertase decay and the degradation of C3b on cells' surfaces, acting as a cofactor to factor I.[17] In PNH cells, only CR1 and FH protect RBCs against complement. FH protection is crucial for PNH cells' survival.[18] Enhanced surface levels of FH on the RBCs of CD55 or CD59–deficient mice and patients with PNH protected the RBCs against complement-mediated hemolysis.[19]

Based on available data, 3 out of 8 rare *CFH* variants were pathogenic, causing alternative pathway deregulation. These 3 unstudied variants may affect FH-cell binding. In the absence of functional studies, the relevance of identified variants in the pathophysiology of PNH remains unclear. However, the over-representation of rare variants in disease-specific populations indicates the relevance of the gene to disease pathogenesis.[15,20,21]

The duration of PNH diagnoses ranged from 1978 to 2019; the first and last eculizumab initiations took place in 2005 and 2019, respectively. The transfusion recommendations were stable during these 14 years (supplemental Table 1). Two patients started eculizumab, having relapsed for PNH after hematopoietic stem cell transplantation for aplastic anemia with autologous reconstitution. Twenty-seven patients received 1200 mg (instead of 900 mg) of eculizumab owing to suboptimal responses, of which 3 had a rare *CFH* variant.

Compared with wild-type, patients bearing a *CFH* variant were older at PNH diagnosis; other baseline characteristics were not different. During follow-up, the results of the diagnoses of 7 patients evolved to a myeloid malignancy. None of these patients had a rare variant in the studied genes.

The *CFH* variants or transfusion requirements before eculizumab initiation were associated with transfusion dependence at 6 months after eculizumab initiation in univariate analysis (75.7% vs 33.3% for *CFH* variant and wild-type, respectively, *P* = .015, supplemental Table 3); no other baseline characteristics affected transfusion dependence. All 3 patients with pathogenic *CFH* variants and 1 with pathogenic *CFI* were transfusion-dependent for 6 months (supplemental Table 5).

Median follow-up was 5.8 years (95% CI, 5.2-7.3). Six-year FFS and EFS were identical because transfusions preceded other events as 39.3% (95% CI, 28.1-50.3) and 0% for wild-type and *CFH*-variant groups, respectively (Figure 1; supplemental Figure 3; supplemental Table 4). All but 1 patient with *CFH* variant received transfusions after eculizumab loading dose (PNH037 had a VUS and remained transfusion-independent [supplemental Table 5]; eculizumab started after a thrombotic event). Two patients with *CFH* variant had a first thrombosis under eculizumab therapy. None of the patients with *CFH* variant fulfilled the criteria for severe aplastic anemia after eculizumab initiation. Overall, patients younger in age at PNH diagnosis, without a *CFH* variant, and transfusion independent before eculizumab treatment had a better 6-year EFS (supplemental Table 4).

We did not find any association among common variants in *CR1* (associated with lower protein expression) or in *CFH* p.His402-Tyr (implicated in other complement-mediated diseases)[22] with disease presentation or eculizumab response (supplemental Table 3; supplemental Table 6; supplemental Table 7; supplemental Figure 4).

Table 1. Rare germline CFH variants among patients with PNH

UPN	Gene	Variant	Transcript	Status	MAF (%)	CADD score	In vitro functional characterization[23]	Summary of the effect	Classification in this report
PNH016	CFH	c.472G>A	p.Val158Ile	Hz	0.018	0.001	yes	Any identified functional defect[24]	VUS
PNH028	CFH	c.2077G>A	p.Asp693Asn	Hz	0.016	20.8	no	Low FH plasma level	Pathogenic
PNH030	CFI	c.672T>A	p.Asp224Glu	Hz	novel	0.038	yes	Any identified functional defect[25]	VUS
PNH032	C3	c.664T>A	p.Phe222Ile	Hz	0.005	27.3	no		VUS
PNH032	C3	c.4100T>C	p.Ile1367Thr	Hz	0.081	17.41	no		VUS
PNH032	CFI	c.1246A>C	p.Ile416Leu*	Hz	0.02	18.64	yes	Significantly reduced expression compared with WT[26]	Pathogenic
PNH033	CFH	c.3004G>C	p.Gly1002Arg	Hz	0.078	17.45	no	Low FH plasma level with Alternative pathway C3 consumption	Pathogenic
PNH037	CFH	c.2651C>A	p.Ser884Tyr	Hz	0.032	17.24	yes	Any identified functional defect[24]	VUS
PNH038	CFI	c.1534G>A	p.Gly512Ser	Hz	0.0017	33	yes	Any identified functional defect[25]	VUS
PNH046	C3	c.4100T>C	p.Ile1367Thr	Hz	0.081	17.41	no		VUS
PNH060	C3	c.749G>T	p.Gly250Val	Hz	novel	19.09	no		VUS
PNH073	CFH	c.1366G>A	p.Glu456Lys	Hz	0.0008	2.658	no	Normal C3 level in plasma	VUS
PNH078	C3	c.1119+1G>A	IVS10+1	Hz	novel	35	no	Splice site variant	Pathogenic
PNH087	CFH	c.2295A>T	p.Leu765Phe	Hz	0.0036	0.035	no	Normal C3 level in plasma	VUS
PNH097	C3	c.3083T>G	p.Leu1028Arg	Hz	novel	26.7	no		VUS
PNH097	CFH	c.472G>A	p.Val158Ile	Hz	0.018	0.001	yes	Any identified functional defect[25]	VUS
PNH102	CFH	c.2867C>T	p.Thr956Met	Hz	0.1	14.99	yes	Any identified functional defect[25]	VUS
PNH103	CFH	c.388G>A	p.Asp130Asn	Hz	0.014	22.6	yes	Normal C3 and FH levels in plasma, minor defects in complement regulation[23]	Pathogenic
PNH108	C3	c.4100T>C	p.Ile1367Thr	Hz	0.081	17.41	no		VUS

Allelic frequency has been taken from the Genome Aggregation Database (gnomAD). PNH032 carried a pathogenic CFI variant (p.Ile416Leu) and 2 C3 variants of undetermined significance (p.Ile1367Thr and p.Phe222Ile); PNH097 carried 2 VUS variants in C3 (p.Leu1028Arg) and CFH (p.Val158Ile).

CADD, combined annotation dependent depletion; Hz, heterozygous; VUS, variants of undetermined significance.

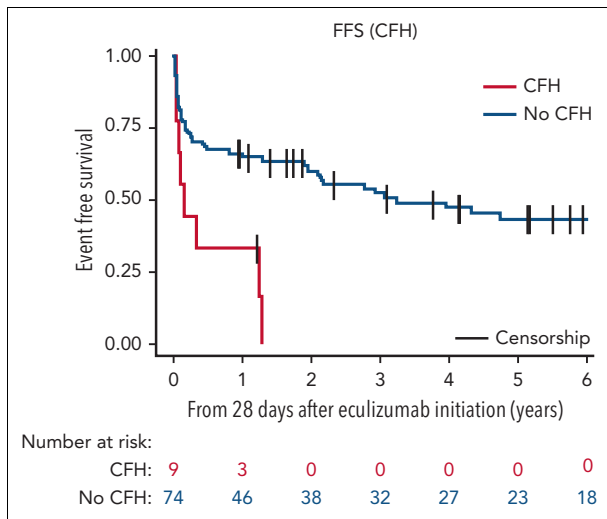*The allelic frequency for p.Ile416Leu in the African population is 1.2%.

Figure 1. FFS of patients with PNH bearing a rare *CFH* variant (red) vs without a *CFH* variant (blue). The 6-year FFS for the no-CFH group was 39.3 months (95% CI, 28.1-50.3). Events included, first red blood cell transfusion after the eculizumab loading dose, thrombosis, eculizumab dose increase to 1200 mg, or occurrence of death.

Our data support that FH protection is necessary for the survival of PNH cells, even during C5 cleavage inhibition. Decreased FH regulation on RBCs under C5 inhibition would further amplify C3 fragment–deposition on PNH cell surfaces, enhancing extravascular hemolysis and contributing to poor eculizumab responses. We did not observe striking differences in hemoglobin or lactate dehydrogenase between patients with or without a rare *CFH* variant (supplemental Figure 2) from day 28 to 6 months. These observations do not favor intravascular hemolysis as the cause for the suboptimal response to anti-C5 treatment, but suggest a more indolent evolution.

The limitations of this study were the retrospective design, the possibility of referral bias (national reference center), and the lack of subgroup or multivariate analyses owing to the disease rarity. Our results require an independent validation.

Patients bearing these *CFH* variants may respond differently to proximal complement inhibitors. Screening for *CFH* variants may help clinicians understand the suboptimal response to terminal inhibition and possibly adapt treatment with new proximal complement inhibitors.

Authorship

Contribution: P.H.P. helped in designing the project, participated in patient recruitment, collected, analyzed, interpreted clinical and molecular data, and wrote the manuscript; J.-E.G. performed statistical analysis on clinical data; F.S.d.F., R.B., and Y.B. participated in patient recruitment and collected samples; P.-E.D. participated in patient recruitment and collected clinical data; P.V.M, A.D., and S.R. performed molecular analyses; A.-C.L. collected clinical data; L.L. and J.S. prepared and biobanked samples and gave helpful intellectual insights; G.S. participated in patient recruitment, gave helpful intellectual insights, and edited the manuscript; V.F.-B. performed molecular analyses, interpreted molecular data, helped elaborate and sponsor the project, and edited the manuscript; R.P.d.L. helped in designing and sponsoring the project, participated in patient recruitment, analyzed, interpreted clinical and molecular data, and edited the manuscript; and all authors read and approved the final version of the manuscript and are accountable for all aspects of the work.

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