Effect of Eculizumab on Iron Metabolism in Transfusion-independent Patients With Paroxysmal Nocturnal Hemoglobinuria

Charlotte C.M. Schaap1,2, Saskia E.M. Schols1,2,3, Frank W.M.B. Preijers4, Emiel de Jonge4, Coby M.M. Laarakkers3, Joop H. Jansen4, Nicole M.A. Blijlevens1,2, Dorine W. Swinkels3,5, Saskia M.C. Langemeijer1,2

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired bone marrow disease characterized by intravascular hemolysis, thrombophilia, and bone marrow failure. Treatment options for PNH remained limited until the terminal complement inhibitor eculizumab became available. Eculizumab effectively reduces intravascular hemolysis in PNH, but in almost all patients on eculizumab complement C3d-opsonized PNH erythrocytes arise and become a target of phagocytosis by macrophages in liver and spleen resulting in a variable degree of extravascular hemolysis. Chronic intravascular hemolysis in untreated PNH patients leads to urinary iron loss from hemosiderinuria and often results in iron deficiency. In contrast, in transfusion-independent PNH patients treated with eculizumab, observed increases in ferritin levels have raised concerns about possible iron overload.

The major regulator of iron homeostasis is the hepatic peptide hormone hepcidin. Hepcidin modulates the iron availability for erythropoiesis by controlling both iron absorption in the duodenum and recycling of iron by macrophages by 2 mechanisms: occlusion of the open-outward conformation of the cellular exporter ferroportin, and induction of endocytosis and degradation of ferroportin. Hepcidin is a negative regulator of iron absorption and mobilization. High levels inhibit both duodenal iron absorption and iron release from macrophages, whereas low levels promote iron absorption. Recently, the hormone erythroid-folate receptor (ERFE) was discovered as main erythroid regulator of hepcidin. ERFE is produced by erythroblasts and its production is induced by erythropoietin. ERFE suppresses the production of hepcidin to facilitate iron delivery during stress erythropoiesis but also contributes to iron overload in congenital anemias with ineffective erythropoiesis such as β-thalassemia and X-linked sideroblastic anemia.

Hyperferritinemia during eculizumab therapy has been attributed to a decline of renal iron loss, as well as iron deposition in the reticuloendothelial system (RES) as a result of C3d-mediated extravascular hemolysis. However, the effect of eculizumab on iron metabolism has not been studied well, and the role hepcidin and ERFE play in the development of hyperferritinemia is unknown. The aim of this study was to investigate how eculizumab affects iron metabolism in PNH.

First, serum ferritin levels were retrospectively collected from medical records of the transfusion-independent patients on eculizumab to study ferritin levels during the course of treatment (Suppl. Figure S1). Ferritin levels increased with time. Mean increase of serum ferritin per patient per year of therapy ranged from −28.7 μg/L to 213.5 μg/L (median = 48.9 μg/L).

Second, serum ferritin levels and transferrin saturation (TSAT) were measured at a single time-point in all patients (Figure 1A). Note that the duration of eculizumab therapy differed per patient at the time iron parameters were measured. Ferritin levels were significantly higher in treated patients (median, 301 μg/L; range, 11–913 μg/L) than untreated patients (median, 103 μg/L; range, 25–290 μg/L; P = 0.006). In addition, ferritin levels...
Figure 1. Iron parameters, and correlations between markers for intravascular and extravascular hemolysis and iron parameters in transfusion-independent PNH patients not treated with a complement inhibitor and PNH patients treated with eculizumab. (A) Serum ferritin and TSAT determined in transfusion-independent PNH patients not treated with a complement inhibitor and transfusion-independent PNH patients treated with eculizumab. Boxes show median and first and third quartiles. Whiskers extend to minimum and maximum values. Dashed lines represent the upper limits of reference ranges. In the graph depicting serum ferritin levels, the lower dashed line represents the upper limit of the reference range of ferritin for premenopausal women and the upper dashed line represents the upper limit of the reference range of ferritin for men and postmenopausal women. Statistical significant differences between groups are highlighted with bars and corresponding *P*-values, Mann-Whitney *U* test. (B) Serum hepcidin, hepcidin/ferritin ratio, and ERFE determined in transfusion-independent PNH patients not treated with a complement inhibitor and transfusion-independent PNH patients treated with eculizumab. Boxes show median and first and third quartiles. Whiskers extend to minimum and maximum values. Upper limits of reference ranges of hepcidin and hepcidin/ferritin ratios are not depicted here as these are outside the axis limits. Reference ranges of serum hepcidin levels are <0.5–13.0 nM, <0.5–16.5 nM, and <0.5–15.5 nM for premenopausal women, postmenopausal women, and men, respectively. Reference ranges of hepcidin/ferritin ratios are 3.2–176.4 pmol/μg, 9.6–150.9 pmol/μg, and 3.1–92.7 pmol/μg for premenopausal women, postmenopausal women, and men, respectively.15 For ERFE, no reference intervals are available yet. Median level of ERFE determined in 10 healthy controls is 0.8 ng/mL (range, 0.2–27.5). Statistical significant differences between groups are highlighted with bars and corresponding *P*-values. Spearman correlation coefficients are depicted in each graph with corresponding *P*-values. *P*-values <0.05 are considered to be significant.

PNH = paroxysmal nocturnal hemoglobinuria; ERFE = erythroferrone; TSAT = transferrin saturation.
above the upper limit of the reference range were observed in 13 out of 23 treated patients. In patients on eculizumab, ferritin levels significantly correlated with the duration of therapy \( (r = 0.495, P = 0.016) \), TSAT did not significantly differ between the groups \( (P = 0.921) \), and in 12 out of 13 untreated patients and 22 out of 23 treated patients, TSAT was below 45%.

In both the groups, markers for intravascular hemolysis (lactate dehydrogenase [LDH] and absolute reticulocyte count [ARC]) and extravascular hemolysis (ARC and percentage of C3d-opsonized PNH erythrocytes) were measured. In patients on eculizumab, intravascular hemolysis was adequately inhibited; however, a variable degree of C3d-mediated extravascular hemolysis occurred. In these patients, we found a significant correlation between mean ferritin increase per year, and both ARC \( (r = 0.737; P < 0.001; \text{Suppl. Figure S2A}) \) and C3d opsonization \( (r = 0.533; P = 0.011; \text{Suppl. Figure S2B}) \).

Taken together, we showed that PNH patients on eculizumab indeed frequently develop hyperferritinemia. Moreover, we confirmed that the degree of C3d-mediated extravascular hemolysis is strongly associated with the development of iron accumulation in the RES in these patients, as evidenced by hyperferritinemia in the presence of normal TSAT.8–10

Subsequently, serum hepcidin and ERFE levels were determined (Figure 1B), and correlated with markers for hemolysis in both treated and untreated patients. Because there was a significant correlation between LDH and ARC in untreated patients \( (r = 0.587; P = 0.045; \text{Suppl. Figure S3}) \), and percentage of C3d-opsonized PNH erythrocytes and ARC in treated patients \( (r = 0.662; P = 0.001; \text{Suppl. Figure S2C}) \), for the rest of the analysis, ARC was used as marker for intravascular and extravascular hemolysis in untreated and treated PNH patients, respectively.

Serum hepcidin levels were significantly higher in treated patients (median, 3.8 nM; range, 0.5–9.9 nM) compared with untreated patients (median, 1.0 nM; range, 0.5–7.9 nM; \( P = 0.001 \)). To correct hepcidin levels for degree of body iron load, and thus to assess other modulators of hepcidin than body iron status, the hepcidin/ferritin ratio was calculated. There was no significant difference in hepcidin/ferritin ratios between the groups \( (P = 0.193) \). As reference intervals for ERFE are not available, serum of 10 healthy volunteers (Suppl. Table S3) was collected to compare ERFE levels of patients with those of healthy controls. Serum ERFE levels were significantly increased in both untreated (median, 8.3 ng/mL; range, 0.8–43.3 ng/mL) and treated patients (median, 8.9 ng/mL; range, 1.4–102.9 ng/mL) compared with healthy controls (median, 0.8 ng/mL; range, 0.2–27.5 ng/mL; \( P < 0.001 \) and \( P = 0.002 \), respectively) but did not significantly differ between the 2 patient groups \( (P = 0.610) \).

In patients not treated with eculizumab, there was a significant correlation between ARC and both hepcidin/ferritin ratio \( (r = -0.670; P = 0.012) \) and ERFE \( (r = 0.587; P = 0.045; \text{Figure 1C}) \). Therefore, it is likely that untreated patients with intravascular hemolysis have suppressed hepcidin levels as a result of the

**Figure 2.** Conceptual model of iron regulation in transfusion-independent untreated and eculizumab-treated patients with PNH. (A) Intravascular hemolysis in untreated PNH patients leads to supressed serum hepcidin levels as a result of iron deficiency due to urinary iron loss from hemosiderinuria as well as increased ERFE production induced by enhanced erythropoiesis. Low serum hepcidin levels promote duodenal absorption and iron release from macrophages of the RES in order to increase the iron availability for erythropoiesis. (B) Once intravascular hemolysis is blocked by the complement-C5 inhibitor eculizumab, urinary iron loss is inhibited. However, patients develop a variable degree of extravascular hemolysis, caused by phagocytosis of complement-C3d-opsonized PNH erythrocytes in the RES, leading to iron sequestration in macrophages. Because of a persistent enhanced erythropoiesis, ERFE production is increased, resulting in serum hepcidin levels that remain low relative to ferritin levels. The combination of both a decline of renal iron loss and erythropoiesis-mediated suppressed hepcidin levels contribute to a positive net iron balance and subsequent progressive iron deposition in the RES in patients on eculizumab with ongoing extravascular hemolysis. Figure created with BioRender.com. PNH = paroxysmal nocturnal hemoglobinuria; ERFE = erythroferrone; RES = reticuloendothelial system.
combination of iron deficiency due to renal iron loss as well as increased ERFE production induced by enhanced erythropoiesis (Figure 2A). In line with Waheed et al., we postulated that hepcidin levels remain low relative to ferritin levels upon initiation of eculizumab because of continuous increased erythropoiesis with high iron demand in patients with ongoing C3d-mediated extravasal hemolysis. Indeed, ARC inversely correlated with hepcidin/ferritin ratio (r = −0.503; P = 0.017) and positively correlated with ERFE (r = 0.630; P = 0.002; Figure 1C). Based on these results, we hypothesize that in patients on eculizumab with ongoing extravasal hemolysis, both a decline of renal iron loss and erythropoiesis-mediated suppressed hepcidin levels contribute to a positive net iron balance and subsequent pro-
gressive iron deposition in the RES (Figure 2B).

The results of this study do not explain why TSAT remains low, which will limit parenchymal iron overload in hyperfer-
ritinemic PNH patients on eculizumab. This is in contrast to what is observed in patients with iron loading anemias due to
an ineffective erythropoiesis, where hepcidin is suppressed as well (relative to body iron levels), but leads to severe hepatocyte
iron overload.11 One possible explanation is that the de-
gradation of C3d-opsinized erythrocytes takes place in the RES,
where iron is directly sequestered in macrophages. Although hepcidin levels are relatively low compared with ferritin levels in patients with more prominent extravasal hemolysis, abso-
lute hepcidin levels in patients on eculizumab are elevated com-
pared with untreated patients and might be not low enough to allow sufficient active ferroportin-mediated cellular iron efflux to compensate for the higher RES influx of iron. An alterna-
tive explanation for stable nonincreasing TSATs might be that macrophtage iron release is partially impaired independent from the hepcidin-ferroportin axis. Theurl et al.12 showed in mice that
during massive hemolysis, the Kupffer-cell population in the liver is overwhelmed and these cells die. To compensate, blood-de-
rivd monocytes fill the vacant niche in the liver and differen-
tiate into Kupffer-cell like macrophages. Because blood-derived monocytes from PNH patients lack glycosylphosphatidylinosi-
tol (GPI)-anchored, these Kupffer-cell like macrophages are defi-
cent of GPI-anchored proteins. A study of de Domenico et al13 showed that a GPI-linked splice variant of ceruloplasmin might be important for cellular iron export by stabilizing ferropor-
tin on the cell membrane. As GPI-anchor deficient PNH cells lack GPI-linked ceruloplasmin, it is conceivable that iron release from GPI-anchor deficient Kupffer-like cells is impaired, leading to iron deposition in these macrophages. Future research is war-
ranted to study this hypothesis.

In conclusion, based on the results of this observational study, we postulate that in transfusion-independent PNH patients on eculizumab, the combination of a decline of renal iron loss as well as the emergence of extravasal hemolysis leading to increased RES-iron influx and erythropoiesis-mediated sup-
pressed hepcidin contribute to progressive iron deposition in the RES. Long-term follow-up of serum iron parameters and organ iron quantification by MRI during therapy with terminal complement inhibitors is warranted to assess whether iron load-
ing of the RES will eventually result in redistribution with toxic parenchymal iron overload, or that iron accumulation in the RES finally reaches a plateau due to further increased hepcidin levels. Given the findings in transfusion-independent patients on eculizumab, physicians should be even more aware of the devel-
opment of iron overload in transfusion-dependent patients.

Future studies may investigate whether proximal complement inhibitors, which prevent C3d-mediated extravascular hemol-
sis, also prevent the development of RES-iron accumulation.14

**AUTHOR CONTRIBUTIONS**

CS, SS, DS, and SL designed the study. CS, FP, EJ, and CL acquired the data, conducted experiments, and performed data analysis. CS, SS, JJ, NB, DS, and SL wrote the article. JJ, NB, DS, and SL supervised the study.

**DATA AVAILABILITY**

Relevant individual patient data that support the findings of this study are available in the supplementary digital content (Suppl. Table S2). Remaining data are available on request from the corresponding author.

**DISCLOSURES**

The authors have no conflicts of interest to disclose.

**SOURCES OF FUNDING**

The authors declare no sources of funding.

**REFERENCES**

2. Ristano AM, Notaro R, Marando L, et al. Complement fraction 3 binding on erythrocytes as additional mechanism of disease in parox-
7. Ristano AM, Imbrico M, Marando L, et al. From perpetual haemo-
siderinuria to possible iron overload: iron redistribution in paroxysmal nocturnal haemoglobinuria patients on eculizumab by magnetic reso-