Received: 31 October 2022 | Accepted: 9 January 2023

DOI: 10.1111/bjh.18662

BJHaem
BRITISH JOURNAL OF HAEMATOLOGY

LETTER TO THE EDITOR

The long-acting anti-C5 ravulizumab results in C3 binding to PNH red cells similar to its parental molecule eculizumab

The extreme and peculiar susceptibility of paroxysmal nocturnal haemoglobinuria (PNH) red cells to activated complement is the main mechanism of intravascular haemolysis and anaemia in this acquired disease.[1] Thus, the introduction of complement 5 (C5) inhibition by a monoclonal antibody, eculizumab, has been a major advance in the clinical management of PNH.[2] In fact, eculizumab treatment abrogates intravascular haemolysis in almost all PNH patients and, eventually, reduces its clinical consequences: anaemia, risk of thrombosis, smooth-muscle dystonia.[3] However, in almost all PNH patients treated with eculizumab, a distinct population of PNH red cells binds fragments of complement C3 (C3) because the activation of C3 on cell surface is not blocked.[4,5] This is an important consequence of C5 inhibition because the PNH red cells opsonized with C3 fragments, mostly C3d,[6] become potential targets of the reticulo-endothelial system, likely *via* interaction with macrophage complement receptor 3,[7] with consequent variable degrees of extravascular haemolysis.[4] In the majority of patients this iatrogenic C3-mediated extravascular haemolysis (testified by the persistent reticulocytosis) is clinically unremarkable; however, it may reduce eculizumab clinical benefits in a portion of patients with few (18–31%) who remain transfusion-dependent.[3,4,8] Despite this limit, eculizumab has radically modified the natural history of PNH: in fact, it has proven to be safe, even during pregnancy,[9] and clinically effective resulting, eventually, in an improved quality of life and in an almost normal survival.[3]

These excellent clinical results have prompted the development of a variety of novel anti-C5 molecules with the aim to replicate its effectiveness and to address some issues as the C5 genetic variants, found almost only in Far East Asia patients, that prevent eculizumab binding and effectiveness[10] or the burdensome schedule that requires eculizumab intravenous dosing every 14 ± 2 days.[2] Among several anti-C5 molecules currently in clinical development (reviewed in[11]), the monoclonal antibody ravulizumab is an eculizumab derivative with a 4-fold longer half-life because 4 amino acid substitutions have increased endosomal C5 dissociation and antibody recycling *via* the neonatal Fc receptor.[12] Recently, ravulizumab has been licensed by FDA and EMA as two randomized controlled studies have proved that it is noninferior to eculizumab despite a longer dosing interval (56 ± 7 days).[13]

The clinical studies on the novel anti-C5 molecules, including those with ravulizumab, have not assessed C3d binding to PNH red cells. Since the discovery of the C3 binding, in our institutions the workout for the follow up of PNH patients treated with complement inhibitors includes the quantification of C3d binding.[4] Therefore, we have routinely performed this quantification in 14 patients initially enrolled in the ravulizumab phase 3 clinical studies and in 1 patient treated with ravulizumab on compassionate basis, a signed informed consent was obtained according to a protocol approved by the local Institutional Review Board.

We observed that in the patients who were naïve to anti-C5 therapy, the start of therapy with ravulizumab ($n = 2$) was associated with the prompt *de novo* appearance of a population of PNH red cells bound with C3d (Figure 1A), similar to what happens in those starting with eculizumab (Figure 1B).[4,6]

We have previously modelled *in vitro* the phenomenon of C3d binding by exposing PNH red cells to complement activation in the presence of eculizumab.[6,14] Thus, in order to verify whether the degree of C3d binding to PNH red cells was different between the two inhibitors, we have tested *in vitro* the C3d binding on red cells from PNH patients naïve to complement inhibitors ($n = 5$). Briefly, 2% red cell suspensions were incubated at 37°C in saline with 84% of pooled ABO-compatible sera from healthy donors, additional $MgCl_2$ 1.25 mM and 200 mg/mL of either eculizumab or ravulizumab; C3 binding was measured by flow cytometry after activation of complement alternative pathway by mild acidification (11.5 mM HCl: final pH between 7.0 and 6.8).[6] We found that 2 and 18 h after complement activation a similar fraction of PNH red cells become C3d bound irrespective of the drugs, eculizumab or ravulizumab, used for the C5-blockade (Figure 1C; $p > 0.999$). In keeping with this *in vitro* observations, we found that in 13 patients treated for at least 6 months with eculizumab neither the percentage of PHN red cells (Figure S1) nor that of PNH red cell population bound with C3d changed significantly with the switch and after at least 36 months on ravulizumab (15.5 *vs.* 19.7%; $p = 0.094$) (Figure 1D). This relative stability of the percentage of PNH red cells (Figure S2) and of PNH red cells bound with C3d at the switch from eculizumab to ravulizumab has been confirmed also in the subset of 11 patients who have been sequentially tested every 6 ± 1 months since the start of ravulizumab therapy (Figure 1E; $p = 0.192$).

This C3 binding, anticipated by previous *in vivo*[4] and *in vitro*[6] studies on eculizumab, does not detract from the

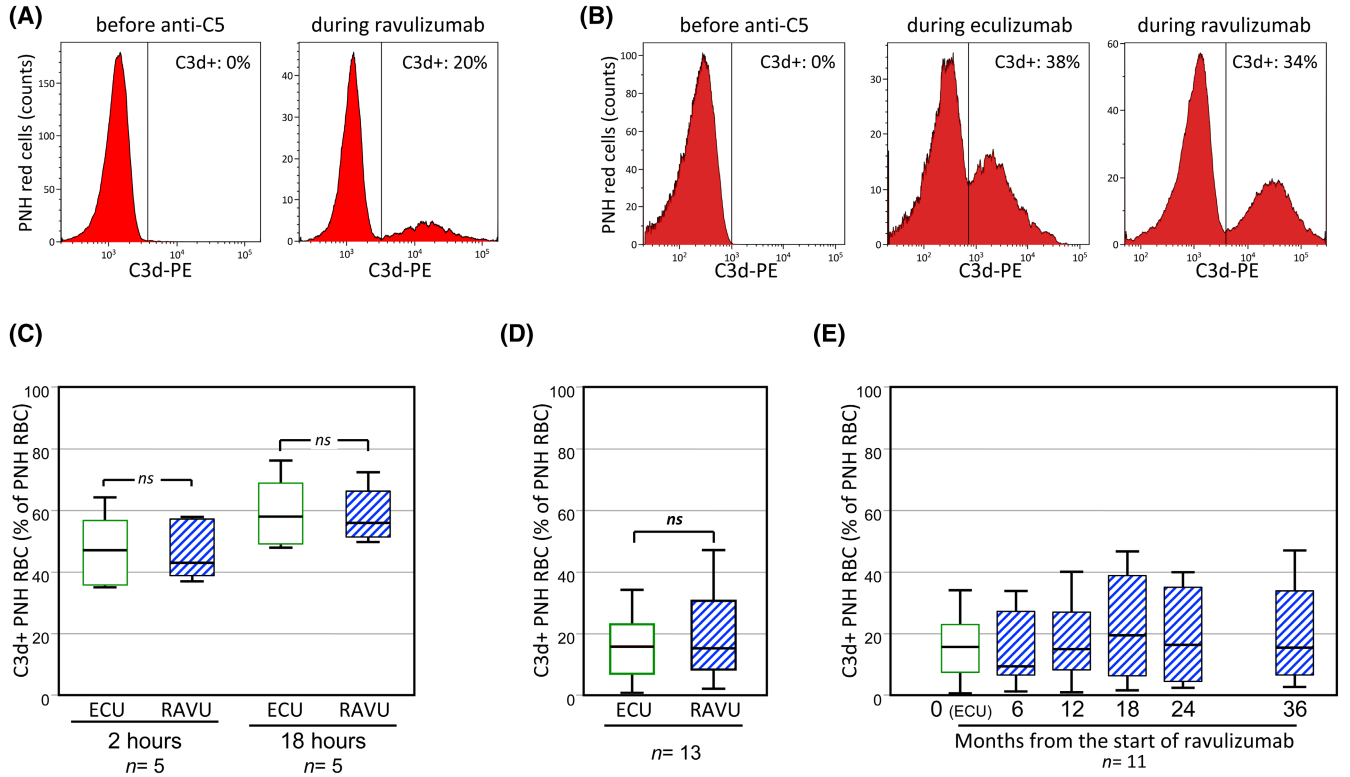

FIGURE 1 *In vivo* and *in vitro* C3d binding to PNH red cells of patients treated with ravulizumab. (A) C3d binding to PNH red cells in a PNH patient naïve to anti-C5 therapy who started ravulizumab. Before any treatment histogram plot shows no PNH red cell bound with C3d (left panel), during the therapy with ravulizumab the histogram plot shows 2 distinct populations of PNH red cells, one bound (C3d+) and one not bound (C3d–) with C3d fragments (right panel). C3 binding was assessed by flow cytometry with anti-C3d-neoantigen (A250, Quidel, USA) secondary stained with PE-conjugated polyclonal rabbit-anti-mouse antibodies (Dako Cytomation, Denmark). PNH red cells have been identified with an APC-conjugated monoclonal antibody anti-CD59 (Mem43, Serotec, UK). CD59-negative red cells (PNH red cells) have been gated and shown in the histogram plots that report on the X-axis the C3d-PE relative fluorescence and on the Y-axis the counts (number of events) of PNH red cell. Flow cytometry has been performed either with Accuri C6 (BD, USA) or Citoflex S (Beckman Coulter Life Science, USA), flow cytometry data were analysed and plotted by Kaluza Analysis Software v.2.1 (Beckman Coulter Life Science, USA). (B) C3d binding to PNH red cells in a PNH patient who switched from eculizumab to ravulizumab. C3d binding to PNH red cells in a PNH patient naïve to anti-C5 therapy (left panel), during eculizumab treatment (middle panel) and after the switch to ravulizumab (right panel). Flow cytometry histogram plots from a representative patient. (C) C3d binding to PNH red cells after complement activation *in vitro*. C3 binding to PNH red cells from 5 PNH patients C5-inhibitor-naïve has been studied *in vitro* in the presence of either eculizumab or ravulizumab. The box and whisker plot shows the proportion of PNH red cells bound with C3d (C3d PNH RBC) after 2 and 18 h since complement activation *in vitro* in the presence of 400 mg/ml of either eculizumab (empty boxes) or ravulizumab (striped boxes). (D) C3 binding to PNH red cells during eculizumab or ravulizumab therapy. Proportion of PNH red cells bound with C3d (C3d + PNH RBC) after at least 6 months of eculizumab (empty box) and after at least 36 months since switch to ravulizumab (striped box) in 13 PNH patients. (E) Kinetics of C3 binding to PNH red cells after the switch from eculizumab to ravulizumab. Proportion of PNH red cells bound with C3d (C3d + PNH RBC) assessed every 6 ± 1 months from the start of ravulizumab therapy (striped boxes) in 11 patients previously treated with eculizumab for at least 6 months. The percentage of C3d binding during eculizumab treatment is shown at time 0 (empty box). Data are shown by box and whisker plots: the bottom and top of the box show the 25th and 75th percentile, the horizontal line inside the box shows the median, and the ends of the whiskers represent the 5th and 95th percentile. Statistical significance ($p \leq 0.05$) was assessed by the non-parametric Wilcoxon's or Friedman tests for paired samples as appropriate using GraphPad Prism v.5.0 for Windows (GraphPad Software). ns, not statistically significant.

merits of ravulizumab that maintains the efficacy of eculizumab with a more consistent control of free C5 levels[15] and with a much lesser frequent dosing (26 *vs.* 6.5 per year). Thus, ravulizumab is expected to have a positive impact on the life of PNH patients because it alleviates the burden of treatment and, possibly, it may reduce the risk of pharmacokinetic breakthrough haemolysis.[16]

Nevertheless, these *in vitro* and *in vivo* observations confirm that C3d binding to PNH red cells is a phenomenon inherently associated with the inhibition of C5 and, likely, with the inactivation of any other component of the terminal complement pathway.[17] In keeping, C3 binding has been documented also when C5 is blocked *in vitro* by agents other than monoclonal antibodies as coversin.[18] Therefore, any C5 inhibitor, including ravulizumab, is not expected to prevent the progressive C3 binding to PNH red cells, eventually leading to the onset of C3-mediated extravascular haemolysis. Since this iatrogenic event is inseparable from anti-C5 therapy, it might be addressed only by the introduction, when clinically justified, of proximal complement inhibitors either in monotherapy or in combination with an anti-C5.[1]

AUTHOR CONTRIBUTIONS

Michela Sica and Caterina Nannelli performed the experiments and participated in designing the research, collecting and analysing data, and writing the paper. Federica Barone

performed the experiments and participated in designing the research, collecting and analysing data, writing the paper and providing clinical care. Patrizia Ricci participated in performing the research and in data collection. Luana Marano provided clinical care and collected data. Maria De Angioletti helped in performing the research and in analysing data. Eros Di Bona and Antonio M. Risitano helped in designing the research, provided clinical care and participated in analysing data and writing the paper. Rosario Notaro designed the research, analysed data, participated in the clinical management of the patients and wrote the paper. All the authors reviewed the paper and ratified the final version.

CONFLICTS OF INTEREST

E. Di Bona has received honoraria from Alexion Pharmaceuticals. A.M.R. has received honoraria from Apellis, Sobi, Novartis, Roche, Samsung, Pfizer and Alexion Pharmaceuticals. R.N. has received lecture fees from Alexion Pharmaceuticals, served as member of Investigator Board for BioCryst, Sobi Pharmaceuticals and Alexion Pharmaceuticals. The other Authors have no conflict of interest.

ACKOWLEDGEMENTS

We thank the patients, the families and the "Associazione Italiana Emoglobinuria Parossistica Notturna" for their continuous support.

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SUPPORTING INFORMATION

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