Paroxysmal Nocturnal Hemoglobinuria Clones in Children with Acquired Aplastic Anemia: A Multicentre Study

Fabio Timeus1,2*, Nicoletta Crescenzio2, Daniela Longoni3, Alessandra Doria2, Luiselda Foglia2, Sara Pagliano2, Stefano Vallero1, Valentina Decimi3, Johanna Svahn4, Giuseppe Palumbo5, Antonio Ruggiero6, Baldassarre Martire7, Marta Pillon8, Nicoletta Marra9, Carlo Dufour4, Ugo Ramenghi2, Paola Saracco2

1 Pediatric Onco-Hematology, Regina Margherita Children’s Hospital, Turin, Italy, 2 Pediatric Hematology, University of Turin, Turin, Italy, 3 Pediatric Department MBBM Foundation S. Gerardo Hospital, Monza, Italy, 4 Hematology Unit, G. Gaslini Children’s Hospital, Genoa, Italy, 5 Pediatric Onco-Hematology Department, Bambin Gesù Children’s Hospital, Rome, Italy, 6 Pediatric Oncology, Policlinico Gemelli, Rome, Italy, 7 Department of Pediatrics, University of Bari, Bari, Italy, 8 Pediatric Onco-Hematology Unit, University Hospital of Padua, Padua, Italy, 9 Department of Pediatric Haemato-Oncology, Santobono-Pausilipon Hospital, Naples, Italy

Abstract

A multicentre study evaluating the presence of glycosyl phosphatidyl-inositol (GPI)-negative populations was performed in 85 children with acquired aplastic anemia (AA). A GPI-negative population was observed in 41% of patients at diagnosis, 48% during immune-suppressive therapy (IST), and 45% in patients off-therapy. No association was found between the presence of a GPI-negative population at diagnosis and the response to IST. In addition, the response rate to IST did not differ between the patients who were GPI-positive at diagnosis and later developed GPI-negative populations and the 11 patients who remained GPI-positive. Two patients with a GPI-negative population >10%, and laboratory signs of hemolysis without hemoglobinuria were considered affected by paroxysmal nocturnal hemoglobinuria (PNH) secondary to AA; no thrombotic event was reported. Excluding the 2 patients with a GPI-negative population greater than 10%, we did not observe a significant correlation between LDH levels and GPI-negative population size. In this study monitoring for laboratory signs of hemolysis was sufficient to diagnose PNH in AA patients. The presence of minor GPI-negative populations at diagnosis did not influence the therapeutic response. As occasionally the appearance of a GPI-negative population was observed at cyclosporine (CSA) tapering or AA relapse, a possible role of GPI-negative population monitoring during IST modulation may need further investigation.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell (HSC) disorder characterized by the clonal expansion of a PIG-A mutated stem cell and consequent defective synthesis of glycosyl phosphatidyl-inositol-anchored proteins, complement-mediated hemolysis, increased incidence of thrombosis, and bone marrow failure. PNH and acquired aplastic anemia (AA) are closely related. Flow cytometry analysis (FCA) has revealed a high incidence of minor GPI-negative populations in pediatric AA patients. Yoshida et al [11] demonstrated the presence of GPI-negative cells in 21.4% (population size 0.04–0.81%) of 103 children with AA. Scheinberg et al [12] in a series of 152 adults and 45 children with AA observed the presence of GPI-negative populations in 40% of patients. Timeus et al [13] demonstrated the presence of minor GPI-negative

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* Email: fabio.timeus@unito.it
populations in 53% (population size 0.2–2.2%) of 17 AA patients studied at diagnosis. Sutton et al [14] observed a GPI-negative population in 9/23 severe AA and in 1/3 moderate AA. No correlation was found between GPI-negative populations and response to IST.

**Materials and Methods**

The present multicentre longitudinal study started in 2008 and was performed on 85 children with AA diagnosed in 8 AIEOP (Italian Association of Pediatric Hematology-Oncology) Centres (age at diagnosis 1–17 years, median = 10.7, 46 severe AA, 30 very severe AA, 9 non severe AA, see Table 1). Written informed consent to perform diagnostic and follow up examinations required in the treatment protocol of acquired aplastic anemia (approved by the single center Ethical Committees) was obtained by patient’s parents at time of diagnosis. A specific informed consent form for this study was not required by Ethical Committees as the PNH analysis was a diagnostic test performed on peripheral blood during routine follow up of patients and was not a genetic test. This statement was confirmed by Comitato Etico Interaziendale OIRM-S.ANNA-ORDINE MAURIZIANO (prot n. 24316/C28.1; website: www.cittadellasalute.to.it).

Among 30 patients from one single centre, 6 were newly enrolled, whereas for the other 24 (enrolled before 2008) we present an update of previously published data [13]. Thirty-nine were studied from diagnosis, 25 during IST, 20 off therapy and one selected case was analyzed for the first time at relapse after first line HSC transplantation (HSCT). Among the patients followed since diagnosis, 8 received an HLA matched sibling donor HSCT as first line therapy, one patient had spontaneous remission without therapy, 28 patients were treated with IST according to EBMTR protocols (cyclosporine A (CSA), anti-thymocyte globulin ± granulocyte colony stimulating factor). Horse anti-thymocyte globulin (hATG) was utilized in 14 patients, rabbit ATG (rATG) was utilized in 14 patients (all diagnosed after 2008, when hATG was not available in Italy). Two patients with NSAA were treated with CSA alone.

Samples in EDTA were centralized in the Turin laboratory and analyzed within 24 hours. Peripheral blood GPI-negative cells were detected by lack of CD59 expression on granulocytes by two-colour FCA for CD45/33/66b or CD45/33/66b/FLAER.

**Results**

A GPI-negative population was observed in 16 of 39 (41%) patients at diagnosis (population size 0.17–10.4%; absolute count 0.05–437.2 GPI-negative cells/μl), in 12 out of 25 patients (48%) studied during IST (population size 0.16–65.5%; 1.4–1735 GPI-negative cells/μl) and in 9 out of 20 patients (45%) studied when off-therapy (population size 0.16–4.0%; 8.5–155 GPI-negative cells/μl).

In 33 patients (16 followed since diagnosis, 9 in IST, 8 off-therapy), the GPI-negative population was sporadic or intermit- tents, whereas in 13 patients (9 followed since diagnosis, 3 in IST, 1 off-therapy) it persisted for more than 3 sequential controls. The GPI-negative population size was significantly smaller in the persistent group than in the sporadic (median 0, 31%, range 0.16–3.0% versus 0.74%, range 0.16–6.0%, p < 0.05). In 8 out of the 23 GPI-positive patients at diagnosis, a GPI-negative population (size 0.16–1.7%; 0.1–26.4 GPI-negative cells/μl) appeared later during IST.

Table 2 summarizes the evaluation of GPI-negative populations, neutrophil counts and the response to therapy in the 30 patients followed since diagnosis and treated with IST.

We did not observe a significant difference in the response to IST between patients GPI-negative and GPI-positive at diagnosis (2-tailed Fisher test = 1). No significant difference in the response to IST was also observed between the patients GPI-positive and the subgroup of patients with persistent GPI-negative populations.

**Table 1.** AIEOP centres participating to the study.

| AIEOP CENTRES                  | N. PATIENTS AT DIAGNOSIS | N. PATIENTS IN IST | N. PATIENTS OFF THERAPY |
|--------------------------------|--------------------------|-------------------|-------------------------|
| TURIN                          | 20                       | 5*                | 4                       |
| MONZA                          | 8                        | 6                 | 9                       |
| GENOA                          | 5                        | 11                | 4                       |
| NAPLES                         | 3                        | 1                 | 1                       |
| ROME BAMBINO GESU’ CHILDREN HOSPITAL | 2                | 0                 | 0                       |
| ROME POLICLINICO GEMELLI       | 0                        | 0                 | 2                       |
| BARI                           | 0                        | 2                 | 0                       |
| PADUA                          | 1                        | 0                 | 0                       |
| TOT                            | 39                       | 25                | 20                      |

Eighty-four AA patients described in the multicentre study. Thirty-nine were studied from diagnosis, 25 during IST, 20 off therapy. One more selected case (from Monza, not shown in this table) was analysed at relapse after HSC transplantation (HSCT) and is shown in Fig. 1B.

*The follow-up of one out of these five patients is shown in Fig. 1A.

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A GPI-negative population was observed at relapse after HSC transplantation (HSCT) and is shown in Fig. 1B.
| PATIENTS AT DIAGNOSIS | First line treatment | T0, pre-IST | T180, post IST | Response to therapy |
|----------------------|---------------------|-------------|----------------|-------------------|
|                      |                     | Nx10 E9/L   | % GPI-negative cells | GPI-negative cells/mL | Nx10 E9/L | % GPI-negative cells | GPI-negative cells/mL |               |
| D1                   | hATG+CSA            | 0.40        | 0.1700          | 0.68              | 2.10       | 0.1900              | 3.99              | NR             |
| D2                   | hATG+CSA            | 0.30        | 0.4000          | 1.20              | 0.58       | 0.0000              | 0.00              | NR             |
| D3                   | hATG+CSA            | 0.60        | 0.3000          | 1.80              | 1.40       | 0.2000              | 2.80              | NR             |
| D4                   | hATG+CSA            | 0.07        | 0.7364          | 0.51              | 2.11       | 0.0050              | 0.10              | PR             |
| D5                   | hATG+CSA            | 1.00        | 0.3000          | 3.00              | 1.43       | 1.1000              | 15.73             | PR             |
| D6                   | hATG+CSA            | 0.01        | 0.5000          | 0.05              | 4.64       | 0.0000              | 0.00              | PR             |
| D7                   | hATG+CSA            | 0.40        | 0.0000          | 0.00              | 1.90       | 0.0000              | 0.00              | NR             |
| D8                   | hATG+CSA            | 1.10        | 0.0000          | 0.00              | 5.90       | 0.2000              | 11.80             | NR             |
| D9                   | hATG+CSA            | 0.05        | 0.1000          | 0.05              | 1.48       | 0.1600              | 2.36              | PR             |
| D10                  | hATG+CSA            | 0.50        | 0.0600          | 0.30              | 2.04       | 0.3120              | 6.36              | PR             |
| D11                  | rATG+CSA            | 0.67        | 2.2000          | 14.74             | 1.05       | 0.2550              | 2.67              | NR             |
| D12                  | rATG+CSA            | 0.80        | 0.4800          | 3.84              | 1.30       | 0.2800              | 3.64              | NR             |
| D13                  | rATG+CSA            | 0.36        | 0.0055          | 0.01              | 0.06       | 0.1840              | 0.11              | NR             |
| D14                  | rATG+CSA            | 0.35        | 0.0000          | 0.00              | 0.42       | 0.4984              | 2.09              | NR             |
| D15                  | rATG+CSA            | 0.98        | 0.0070          | 0.06              | 1.00       | 0.2000              | 2.00              | PR             |
| D16                  | rATG+CSA            | 0.40        | 0.0370          | 0.14              | 3.83       | 0.1562              | 5.98              | CR             |
| D17                  | hATG+CSA            | 0.80        | 0.5500          | 4.40              | 0.60       | 0.0000              | 0.00              | CR             |
| D18                  | hATG+CSA            | 0.40        | 0.0000          | 0.00              | 0.40       | 0.0700              | 0.28              | PR             |
| D19                  | hATG+CSA            | 0.40        | 0.0000          | 0.00              | 2.80       | 0.0060              | 0.16              | CR             |
| D20                  | rATG+CSA            | 0.40        | 0.0850          | 0.34              | 0.06       | 0.1100              | 0.06              | NR             |
| D21                  | rATG+CSA            | 0.10        | 0.0020          | 0.02              | 2.30       | 0.7985              | 18.36             | NR             |
| D22                  | rATG+CSA            | 0.40        | 0.0269          | 0.10              | 0.40       | 0.0000              | 0.00              | NR             |
| D23                  | CSA                 | 0.40        | 0.0328          | 0.13              | 0.60       | 0.0000              | 0.00              | NR             |
| D24                  | CSA                 | 0.10        | 0.0000          | 0.00              | 0.80       | 0.0000              | 0.00              | CR             |
| D25                  | rATG+CSA            | 1.70        | 2.7640          | 46.98             | 0.60       | 1.1457              | 6.87              | PR             |
| D26                  | rATG+CSA            | 2.20        | 0.0000          | 0.00              | 2.50       | 0.0000              | 0.00              | NR             |
| D27                  | rATG+CSA            | 0.40        | 0.0450          | 0.18              | 0.40       | 0.0000              | 0.00              | NR             |
| D28                  | rATG+CSA            | 0.01        | 0.6500          | 0.06              | 0.01       | 0.6600              | 0.06              | NR             |
| D29                  | rATG+CSA            | 0.08        | 0.0000          | 0.00              | 2.27       | 0.0000              | 0.00              | NR             |
| D30                  | hATG+CSA            | 0.50        | 0.0326          | 0.16              | 1.40       | 0.0000              | 0.00              | PR             |

hATG = horse ATG; rATG = rabbit ATG; CSA = cyclosporin. In patients treated with hATG evaluation at 180 days showed 2 complete responders (CR, 14%), 7 partial-responders (PR, 50%) and 5 non-responders (NR, 36%), whereas in patients treated with rATG evaluation at 180 days showed 1 CR (7%), 2 PR (14%), 11 NR (79%) (2-tailed Fisher test p = 0.0542). Among the 11 patients GPI negative at diagnosis, CR and PR were respectively 1 (9%) and 4 (36%), whereas NR were 6 (55%). Among the 19 patients GPI positive at diagnosis CR and PR were respectively 3 (16%) and 5 (26%), whereas NR were 11 (58%) (2-tailed Fisher test p = 1). In all the 4 GPI-negative patients who were treated with rATG the clone persisted at day +180, whereas it disappeared in 4/7 patients receiving hATG up-front. doi:10.1371/journal.pone.0101948.t002
GPI-negative population: \( R = 0.15; \) \( \Delta \) neutrophils versus \( \Delta \) absolute count of GPI-negative population: \( R = 0.44 \). The response rate to IST did not differ also between the 7 patients GPI-negative at diagnosis who remained GPI-negative at day +180 (2 PR) and the 4 patients GPI-negative at diagnosis in whom the GPI-negative population disappeared at day +180 (1 CR, 2 PR) (2-tailed Fisher test \( p = 0.24 \)). In patients GPI-negative at diagnosis who remained GPI-negative at day +180 we observed a trend towards a reduction of the clone size.

Among the 25 patients studied during IST, two had a GPI-negative population that appeared during the tapering of CSA. One of these patients is described in Figure 1A. The other, a GPI-positive patient from the Monza Centre (see Table 1) had a GPI-negative population of 2.8% that appeared during CSA tapering.

In 2 patients previously GPI-positive, a GPI-negative population appeared at time of relapse when off therapy (update of previously published data). In the selected patient treated with first line HSCT, a GPI-negative population was present after graft failure (autologous reconstitution 100% recipient) at the time of AA relapse and disappeared after starting IST with CSA (Figure 1B).

Excluding the 2 patients with a GPI-negative population >10%, we did not observe a significant correlation between LDH levels and GPI-negative population size \( (R = 0.019) \). In the 2 patients (patients AAa and AAb) with a GPI-negative population >10%, mild to moderate hemolysis was observed. Patient AAa who developed a frank PNH without hemoglobinuria is described in details in Figure 1A. Patient AAb showed at diagnosis: Hb = 73 g/L, reticulocytes = 123.0 x 10^9/L, LDH = 2448 U/L, without hemoglobinuria. He was treated with HSCT from a sibling matched donor as first-line therapy, with disappearance of the GPI-negative clone. He has been considered as AA/PNH. No thrombotic event was reported in the whole cohort of AA patients.

**Discussion**

In the present study conducted on a large number of pediatric AA patients we observed a significant rate of minor GPI-negative populations at diagnosis similar to observations in adults [2,9,10,12]. Noteworthy, 2 patients (one at diagnosis and one during follow up) showed major (>10%) GPI-negative populations...
with moderate hemolysis and without hemoglobinuria, and were considered as AA/PNH.

In agreement with other reports [11,12,14] and with our previous study [13], we did not find a positive correlation between the presence of pre-treatment GPI-negative populations and favorable response to IST. The appearance of a GPI-negative population in patients previously GPI-positive at diagnosis, reported as uncommon by others [15], was seen in a high proportion (42%) of our IST-treated patients. The observation of fluctuation of GPI-negative populations during IST or the appearance of GPI-negative populations in previously GPI-positive patients during IST or even off therapy, suggests complex interactions between stem cell immune-mediated damage and immune-suppression, such as transient or persistent reactivation of the immune attack. Moreover, without a mutation analysis is impossible to state if the GPI-negative cells observed in the periodical analysis of the same patient belong to the same or different clones.

The patients with a persistent GPI-negative population are an interesting subgroup that might be biologically different from the “sporadic” [16,17]. Unexpectedly, the size of GPI-negative population was smaller in the “persistent” compared to the “sporadic” patients, while there was no difference in the response to IST.

Although the number of patients receiving IST upfront does not enable definitive conclusions, we observed that in hATG-treated subjects GPI-negative populations disappeared more frequently than in those who were given rATG. Occasionally, the appearance of a GPI-negative populations was observed during CSA tapering or at AA relapse. One such example is the patient shown in Figure 1A who before developing a frank hemoglobinuria during follow up, showed an increased GPI-negative population at the first CSA tapering. This GPI-negative population disappeared for 12 months after the CSA dose was increased. Published data about changes in GPI-negative populations size during IST are limited and this finding may be important clinically. Thrombosis is the most severe complication of PNH. We did not observe thrombosis in the two AA/PNH patients neither in the patients with minor GPI-negative populations.

As PNH is extremely rare in children, a considerable delay in diagnosis has been previously described in literature [16,17], supporting the utility of a careful monitoring with flow cytometry of patients at risk for PNH, as AA patients [4,18]. In our study, positive laboratory signs of hemolysis were observed in patients who had GPI-negative neutrophils greater than 10%, whereas LDH levels were not informative about the presence of minor GPI-negative populations. Therefore, our experience suggests that a careful monitoring for laboratory signs of hemolysis may be sufficient for the diagnosis of symptomatic PNH in AA patients, whereas a systematic flow cytometry follow up represents a more complex and expensive approach. However, the possibility, even if very rare, of a frank PNH without signs of hemolysis has been reported [19,20] and in severely aplastic patients laboratory markers of hemolysis might not be altered in presence of PNH clones large as percentage but small as absolute number.

Finally, the biological role of minor GPI-negative populations in AA and their relationship with immune-suppression may need further investigation.

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Author Contributions

Conceived and designed the experiments: FT PS. Performed the experiments: NC AD LF SP. Analyzed the data: FT PS NC AD LF SP SV. Contributed reagents/materials/analysis tools: DL CD UR VD JS GP AR BM MP NM. Wrote the paper: FT PS. Provided clinical data about the patients and critical advice on the manuscript writing: DL CD UR. Provided clinical data about the patients: VD JS GP AR BM MP NM.

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