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Original Article

Diagnostic Screening of Paroxysmal Nocturnal Hemoglobinuria: Prospective Multicentric Evaluation of the Current Medical Indications

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Background: Although consensus guidelines have been proposed in 2010 for the diagnostic screening of paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry (FCM), so far no study has investigated the efficiency of such medical indications in multicentric vs. reference laboratory settings.

Methods: Here we evaluate the efficiency of consensus medical indications for PNH testing in 3,938 peripheral blood samples submitted to FCM testing in 24 laboratories in Spain and one reference center in Brazil.

Results: Overall, diagnostic screening based on consensus medical indications was highly efficient (14% of PNH^+ samples) both in the multicenter setting in Spain (10%) and the reference laboratory in Brazil (16%). The highest frequency of PNH^+ cases was observed among patients screened because of bone marrow (BM) failure syndrome (33%), particularly among those with aplastic anemia (AA; 45%) and to a less extent also a myelodysplastic syndrome (MDS; 10%). Among the other individuals studied, the most efficient medical indications for PNH screening included: hemolytic anemia (19%), hemoglobinuria (48%) and unexplained cytopenias (9%). In contrast, only a minor fraction of the patients who had been submitted for PNH testing because of unexplained thrombosis in the absence of cytopenia, were positive (0.4%).

Conclusions: In summary, our results demonstrate that the current medical indications for PNH screening by FCM are highly efficient, although improved screening algorithms are needed for patients presenting with thrombosis and normal blood cell counts. © 2016 International Clinical Cytometry Society

Key terms: fluorescence cytometry; hematology; standardization; flow cytometry; myelodysplastic syndrome

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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal hematopoietic stem cell disorder characterized by an acquired somatic mutation of the *PIG-A* gene which results in complete absence and/or lower expression of proteins which are anchored to the cell surface membrane through glycophosphatidylinositol (GPI) (1–5). In the last decades, multiparameter flow cytometry (FCM) has proven to be a robust method for the detection of GPI-deficient cells and the diagnostic screening of PNH (6–8). Despite this, diagnosis of PNH still remains a challenge due to the rarity of the disease (annual incidence rate of 0.5-1.3 cases/million individuals) (9,10), its heterogeneous clinical presentation (1–5) and the relatively limited awareness about its broad clinical spectrum which ranges from non-immune hemolytic anemia associated with hemoglobinuria and unexplained thrombosis, to smaller PNH clones in the context of a bone marrow (BM) failure syndrome -e.g. aplastic anemia (AA) or myelodysplastic syndrome (MDS)-, or even subclinical (i.e. asymptomatic) forms of presentation, in the absence of laboratory evidence of hemolysis (1–5).

Since delayed diagnosis has proven to increase morbidity, and even shorten the life expectancy of PNH patients (3–5), consensus recommendations have been made in recent years aiming at a more efficient diagnostic screening of the disease (7,8,11,12). Thus, general agreement exists on that flow cytometry testing for PNH should be requested in patients with: (1) nonimmune (e.g., Coombs negative) hemolytic anemia; (2) intravascular hemolysis associated with hemoglobinuria or unexplained hemolysis, in association with iron deficiency, abdominal pain, thrombosis, and/or cytopenia (e.g., neutropenia and/or thrombocytopenia); (3) unexplained thrombosis particularly at unusual sites (e.g., Budd-Chiari syndrome), and; (4) BM failure syndrome, including AA, low-grade MDS and idiopathic cytopenias of undetermined significance (ICUS) (7). Despite such consensus recommendations have been widely adopted and used, to the best of our knowledge no study has been reported so far in which the efficiency of PNH testing for each of these medical indications had been prospectively evaluated in parallel in multicenter vs. reference center settings.

In the present study, we evaluated the efficiency of diagnostic screening for PNH by FCM in two different

laboratory settings: (1) in a multicenter setting from an European country where specific guidelines have been proposed, updated and adopted (Spain) (11,12), and; (2) in a large reference laboratory in Sao Paulo (Brazil) where samples can be referred at no cost for the patient/medical doctor.

PATIENTS AND METHODS

Patients and Samples

Overall, information about 3,938 peripheral blood (PB) samples from an identical number of individuals prospectively submitted between January 2011 and December 2014 for diagnostic screening of PNH by flow cytometry was collected at 24 flow cytometry laboratories of the Iberian Society of Cytometry (SIC) in Spain (1,718 samples) plus one reference laboratory in Sao Paulo, Brazil (2,220 samples). Cases with a previously established diagnosis of PNH who were submitted for disease monitoring/re-evaluation were specifically excluded from this study. Referred samples were grouped according to consensus medical indications (7,8,11,12) based on which they were submitted for PNH testing, including both clinical and biological signs/symptoms of classical PNH in the absence of a previous hematological disorder (3,032/3,938 samples; 77%), and cases who had a previous diagnosis of an hematological disorder associated with a BM failure syndrome (906/3,938; 23%).

Flow Cytometry Testing for PNH

Consensus flow cytometry diagnostic screening methods (7,8,12–14) were used at each individual laboratory for the detection of GPI-deficient cells among mature PB neutrophils and monocytes following either the 2010 International guidelines (7) or the 2010 guidelines of the Spanish Society of Hematology (11,12) (Supporting Information Tables s1 and s2). The following GPI-associated markers were analyzed: FLAER (87% of the cases), CD14 (98%), CD16 (37%), CD24 (93%), and/or CD157 (5%) (Supporting Information Tables s1 and s2). In those cases with GPI-deficient mature neutrophils and monocytes, expression of CD59 (100% of cases) was also analyzed on red blood cells (Supporting Information Table s2). A case was defined to be PNH positive (PNH^+) when GPI-deficient cells were found in $\geq$2 different cell lineages (e.g. monocytes and neutrophils) at frequencies >0.01% of all leukocytes (minimum sensitivity across all participating laboratories).

External Quality Assurance

The vast majority of the samples from the Spanish multicenter setting (1,536 samples) were collected by laboratories ($n = 19/24$) participating in the external quality assessment (EQA) scheme for PNH screening by flow cytometry of the SIC. Overall, the rate of PNH^+ samples detected was higher among these groups vs. those not participating in the EQA scheme (10.7% vs. 4.4%, respectively; $P = 0.003$), the overall number of samples analyzed by laboratories not participating in the EQA of the SIC being too small for accurate separate statistical analysis of the rate of PNH^+ samples obtained according to distinct medical indications.

Statistical Methods

Mean values and their standard deviation, as well as median and range, were calculated for all continuous variables analyzed; for categorical variables frequencies were used. In order to determine the statistical significance of differences observed between groups, either the χ^2 test or the Fisher's exact test were used for categorical variables; for continuous parameters the Student's *t* test was employed. Correlation studies were performed by the Pearson test. *P*-values <0.05 were considered to be associated with statistical significance. For all statistical analyses, the SPSS software package (SPSS 20 Inc., Chicago, IL) was used.

RESULTS

Overall Frequency of PNH^+ Cases

Overall, at the 25 laboratories contributing to this study, GPI-deficient cells were detected in 563/3,938 samples (14%) (Table 1). Based on the general population covered by the participating laboratories from Spain (≈17.6 million people), the estimated annual incidence of new PNH cases would be of ≈2.5 cases/million individuals per year, ranging between 2.3 and 2.8 cases per million in 2011 and 2014, respectively. When patients with previously diagnosed hematological associated disorders (mostly AA and MDS) were excluded from the analysis, the annual incidence of PNH was of 0.6 cases per million individuals per year (range: 0.5 to 0.8 cases/million individuals, in 2012 and 2013, respectively). Similar data for the Brazilian population covered by the diagnostic screening reference laboratory could not be assessed.

Prevalence of PNH^+ Cases per Consensus Medical Indications

Globally, GPI-deficient cells were more frequently detected among patients who had prior diagnosis of a hematological disorder such as AA and MDS (270/906 PNH^+/screened cases; 30%), vs. those presenting with clinical and laboratory signs/symptoms of PNH, in the absence of a prior hematological disease (263/3,032; 8.7%) (Table 1).

Within the BM failure syndrome patients' group (Table 1), GPI-deficient cells were more frequently detected among AA patients (243/541; 45%) and to a lesser extent also, among MDS cases (26/266; 9.8%); in contrast, they were less commonly observed among patients with chronic myeloproliferative neoplasms (MPN), among whom a single PNH^+ case was detected (a $JAK2^+$ essential thrombocythemia) out of 21 cases tested (4.8%) (Table 1). As expected, no GPI-deficient cells were detected among patients with prior diagnosis of acute leukemia, lymphoproliferative disorders,

Table 1
Frequency of Cases Showing GPI-Deficient Cells in Peripheral Blood at Diagnosis According to the Medical Indications That Triggered for PNH Screening by Flow Cytometry

Medical indications for PNH screening	Frequency of PNH+ cases
Individuals with clinical and biological signs/symptoms of PNH in the absence of a previous hematological disorder (*n* = 3,032)	**8.7%**
Hemoglobinuria (*n* = 73)	47.9%
Hemolytic anemia (*n* = 382)	18.6%
Subtotal hemolysis (n = 455)	*23.3%*
Unexplained cytopenias including anemia (*n* = 393)	22.4%
Unexplained cytopenia without anemia (*n* = 772)	5.1%
Anemia, not otherwise specified (*n* = 468)	3.6%
Subtotal cytopenia (n = 1,633)	*8.8%*
Thrombosis with nonhemolytic anemia and/or other cytopenias (*n* = 73)	13.7%
Thrombosis without anemia and/or other cytopenia (*n* = 800)	0.4%
Subtotal thrombosis (n = 873)	*1.5%*
Iron deficiency (*n* = 57)	0%
Other (*n* = 14)	0%
Subtotal others (n = 71)	*0%*
Patients with hematological disorders (*n* = 906)	**29.8%**
Aplastic/hypoplastic anemia (*n* = 541)	44.9%
Myelodysplastic syndrome (*n* = 261)	9.8%
Subtotal BM failure (n = 802)	*33.3%*
Chronic myeloproliferative neoplasm (*n* = 21)	4.8%
Other hematological and/or immunological disorders (*n* = 78)	0%
Subtotal other non-BM failure disorders (n = 99)	*1.0%*
Total (*n* = 3,938)	**14.3%**

A case was defined as being PNH^+ once GPI-deficient cells were detected at frequencies ≥0.01% of total leukocytes, in ≥2 cell lineages.

monoclonal gammopathies, autoimmune diseases and solid tumors (0/78) in whom diagnostic screening for PNH was requested (Table 1).

In turn, among those individuals who were submitted because of presenting with PNH-associated signs/symptoms, in the absence of a prior diagnosis of an hematological disorder (Table 1), the frequency of PNH^+ cases was clearly higher among cases presenting with hemolysis (106/455; 23%), followed by patients who had unexplained cytopenias (144/1,633; 8.8%) and to a much less extent, patients who presented with unexplained thrombosis (13/873; 1.5%). In more detail, the most reliable signs for PNH screening among this group of individuals, included: hemoglobinuria (35/73; 48%), unexplained cytopenias including anemia (88/393; 22%), non-immune hemolytic anemia (71/382; 19%), and thrombosis associated to (non-hemolytic) anemia and/or another cytopenia (10/73; 14%). A much lower frequency of PNH^+ samples was observed among cases tested because of unexplained cytopenias in the absence of anemia (39/772; 5.1%) or unspecified anemia (17/468; 3.6%). Meanwhile, the frequency of cases with GPI-deficient cells was rare among those patients who presented with unexplained thrombosis in the absence of anemia and other cytopenias (3/800; 0.4%). Of note, no GPI-deficient cells were detected among samples tested because of anemia associated with iron deficiency (0/57) and/or other rare triggering factors not considered in the above mentioned consensus medical indications (0/14).

Upon comparing the frequency of PNH^+ cases between the two different laboratory settings similar trends were observed for the results observed in the 24 Spanish centers vs. the reference Brazilian laboratory (Table 2). Despite this, a significantly higher frequency of cases carrying GPI-deficient cells was observed in the Brazilian reference center vs. the multicenter setting in Spain, particularly as regards cases presenting with hemoglobinuria (57% vs. 25% PNH^+ cases; $p < 0.02$), hemolytic anemia (30% vs.7.8%; $p < 0.001$), unexplained cytopenias including anemia (42% vs.5.6%; $p < 0.001$) and AA (58% vs.35%; $p < 0.001$). In contrast, no statistically significant differences were observed between both laboratory settings for the other medical indications analyzed (unexplained cytopenias in the absence of anemia, unspecified anemia, and unexplained thrombosis with or without anemia) (Table 2).

Different panels of GPI-associated markers were used by distinct laboratories in Spain (Supporting Information Table s1). Of note, the frequency of PNH^+ cases detected was higher when FLAER vs. non-FLAER based panels (as allowed by the 2010 guidelines of the Spanish Society of Hematology) were used for PNH screening (10.8% vs. 7.4%; $P = 0.02$; Supporting Information Table s3). Usage of FLAER was associated with detection of significantly more PNH^+ cases among samples screened because of AA (40.3% vs. 25.5%; $P = 0.006$) and cytopenia (4.9% vs.1.1%; $P = 0.02$), while no statistically significant differences were found for other medical indications. Interestingly, the SIC EQA for PNH screening program run in parallel with this study also showed that FLAER was associated with a significantly higher reproducibility of the results of PNH screening (Supporting

Table 2
Frequency of Cases Showing GPI-Deficient Cells in Peripheral Blood at Diagnosis According to the Medical Indications That Triggered for PNH Screening by Flow Cytometry at Different Centers/Laboratory Settings

Medical indications for PNH screening	Frequency of PNH+ cases: Spanish laboratories ($n = 1,718$)	Frequency of PNH+ cases: Brazilian reference laboratory ($n = 2,220$)
Individuals with clinical & biological signs/symptoms of PNH in the absence of a previous hematological disorder	**4.0%**	**11.4%***
Hemoglobinuria	25.0%	56.6%*
Hemolytic anemia	7.8%	29.6%*
Subtotal hemolysis	*9.4%*	*35.5%**
Unexplained cytopenias including anemia	5.6%	42.2%*
Unexplained cytopenias without anemia	3.2%	5.7%
Anemia, not otherwise specified	1.4%	4.7%
Subtotal cytopenia	*3.6%*	*11.4%**
Thrombosis with non-hemolytic anemia and/or other cytopenias	7.3%	21.9%
Thrombosis without anemia and/or other cytopenia	0.8%	0.2%
Subtotal thrombosis	*1.7%*	*1.4%*
Iron deficiency	0%	0%
Other	0%	0%
Subtotal others	*0%*	*0%*
Patients with hematological disorders	**21.7%**	**45.1%***
Aplastic/hypoplastic anemia	35.2%	57.7%*
Myelodysplastic syndrome	9.7%	10.0%
Subtotal BM failure	*25.2%*	*46.7%**
Chronic myeloproliferative neoplasm	5.3%	0%
Other hematological and/or immunological disorders	0%	0%
Subtotal other non-BM failure disorders	*1.1%*	*0%*
Total	**10.1%**	**16.4%***

A case was defined as being PNH^+ once GPI-deficient cells were detected at frequencies $\geq$0.01% of total leukocytes, in $\geq$2 cell lineages. *$P < 0.05$ vs. Spanish laboratories.

Information Table s4). Usage of other GPI-associated markers (Supplementary Table s3) did not show a significant impact on the detection of PNH^+ cases (CD16, CD24), or were tested only in a limited number of samples (CD157).

Demographics of PNH^+ vs. PNH^- Patients

All children ($\leq$14 years) that tested PNH^+ presented with AA (Table 3), whereas none of those screened because other signs/symptoms of PNH different from AA did; however, the number of cases analyzed in the

Table 3
Frequency of Cases Showing GPI-Deficient Cells in Peripheral Blood at Diagnosis According to Age and the Medical Indications that Triggered for PNH Screening by Flow Cytometry

Medical indications for PNH screening	Frequency of PNH^+ cases: $\leq$14y ($n = 159$)	15y-39y ($n = 1,282$)	40y-59y ($n = 1,087$)	$\geq$60y ($n = 1,199$)
Hemoglobinuria	0%#	57.6%	58.8%	28.6%
Hemolytic anemia	0%	27.9%*	23.8%*	7.9%
Subtotal hemolysis	*0%*	*34.6%**	*28.9%**	*10%*
Unexplained cytopenias including anemia	0%#	32.4%*	29.9%*	15.3%
Unexplained cytopenias without anemia	0%	9.3%**	4.1%	3.0%
Anemia, not otherwise specified	0%	5.3%	4.5%	1.9%
Subtotal cytopenia	*0%*	*13.1%**	*10.0%**	*6.1%*
Thrombosis with non-hemolytic anemia and/or other cytopenias	0%#	22.2%	12.5%	7.7%
Thrombosis without anemia and/or other cytopenia	0%	0.3%	0.3%	0.8%
Subtotal thrombosis	*0%*	*1.4%*	*1.2%*	*1.9%*
Aplastic/hypoplastic anemia	35.8%	56.7%**	45.0%	34.2%
Myelodysplastic syndrome	0%#	25.0%	7.8%	9.2%
Chronic myeloproliferative neoplasm	0%#	0%	0%	11.1%
Subtotal hematological disorders	*31.7%**	*54.3%***	*28.3%**	*16.7%*
Total	**11.9%**	**19.4%****	**12.5%***	**8.9%**

A case was defined as being PNH^+ once GPI-deficient cells were detected at frequencies $\geq$0.01% of total leukocytes, in $\geq$2 cell lineages. #less than 10 cases analyzed in this groups, all PNH^-. AA: aplastic/hypoplastic anemia. MDS: myelodysplastic syndrome. *$P < 0.05$ vs. $\geq$60 years age group. **$P < 0.05$ vs. all the other age groups.

hemoglobinuria and MDS screening-based subgroups was very limited (3 and 2 cases, respectively) to draw any definitive conclusions.

Among adults, a significantly higher frequency of PNH^+ cases was observed among screened PB samples from 15–39 years-old individuals vs. the 40–59 years and ≥60 years age groups (19.4% vs.12.5% and 8.9%; $P < 0.001$) (Table 3). According to the distribution of PNH^+ cases per medical indication, individuals between 16–39 years showed a significantly greater frequency of PNH^+ results for samples screened because of AA (56.7% vs.45.0% and 34.2%; $P = 0.002$ and $P < 0.001$, respectively) and unexplained cytopenia without anemia (9.3% vs. 4.1% and 3.0%; $P = 0.02$ and $P = 0.003$, respectively). Interestingly, older individuals (≥60 years) showed a significantly lower frequency of PNH^+ cases quite similar to younger adults (16–39 years-old and 40–59 years-old age groups) among cases screened because of hemolytic anemia (7.9% vs. 27.9% and 23.8%; $P < 0.001$ and $P = 0.001$, respectively) and unexplained cytopenias including anemia (15.3% vs. 32.4% and 29.9%; $P = 0.001$ and $P = 0.005$, respectively). No association with age was observed for the frequency of PNH+ cases among individuals showing anemia without other cytopenias, or thrombosis, as well as MDS ($P > 0.05$) (Table 3). Similarly, no statistically significant differences were observed as regards the frequency of PNH^+ cases in males (14%) vs. females (13%).

Distribution of GPI-Deficient and GPI-Normal Cells per Medical Indication

Overall, PNH^+ cases presenting with hemolysis were those who showed the highest percentage of GPI-deficient red blood cells (RBC), neutrophils and monocytes at diagnosis (37%±27%, 72%±30% and 76%±28%, respectively); differences reached statistical significance ($P < 0.001$) vs. cases who presented with unexplained cytopenias (18%±27%, 35%±41% and 39%±41%, respectively), MDS (9%±11%, 27%±36% and 31%±38%, respectively) and AA (9%±18%, 19%±29% and 21%±30%, respectively). Of note, the significantly increased percentage of GPI-deficient cells observed among individuals presenting with hemolysis (vs. those who had unexplained cytopenias, MDS and AA, respectively) was due, not only to significantly higher absolute counts of GPI-deficient RBC (1,087 ± 776 vs. 506 ± 826, 157 ± 312, and 203 ± 528 cells $\times 10^3/\mu L$, respectively; $P < 0.001$) neutrophils (1,986 ± 1,791 vs. 825 ± 1,688, 886 ± 1,437, and 242 ± 583 cells/μL, respectively; $P < 0.01$) and monocytes (324 ± 245 vs. 136 ± 182, 91 ± 148 and 51 ± 102 cells/μL, respectively; $P < 0.001$) observed among these cases, but also because of significantly decreased counts of cells expressing normal GPI levels, including RBC (1,953 ± 1,001 vs. 2,419 ± 1,001, 2,764 ± 544, and 2,584 ± 854 cells × $10^3/\mu L$, respectively; $p \leq 0.01$), neutrophils (593 ± 768 vs. 1,012 ± 914, 1,688 ± 1,847, and 1,031 ± 905 cells/μL, respectively; $P \leq 0.002$) and monocytes (91 ± 120 vs. 191 ± 177, 158 ± 113, and 221 ± 369 cells/μL, respectively; $P \leq 0.03$). In addition, PNH^+ cases presenting with hemolysis also showed increased relative and absolute counts of GPI-deficient neutrophils and monocytes vs. PNH^+ cases who had been screened because of unexplained thrombosis (72%±30% vs. 43%±43% and 76%±28% vs. 57%±42%; 1,986 ± 1,791 vs. 620 ± 1,043 and 324 ± 245 vs. 147 ± 133 cells/μL; $P \leq 0.04$), together with decreased counts of neutrophils and monocytes expressing normal GPI levels (593 ± 768 vs. 2,938 ± 3,417 and 91 ± 120 vs. 219 ± 240 cells/μL; $P < 0.01$); in contrast, no significantly differences were observed between both patient groups as regards total, PNH^+ and PNH^- RBC numbers ($P > 0.05$).

Regarding PNH^+ cases who had AA at diagnosis, significantly ($P < 0.001$) lower percentages and absolute counts of GPI-deficient RBC (9%±18% and 203 ± 528 cells $\times 10^3/\mu L$), neutrophils (19%±29% and 242 ± 583 cells/μL) and monocytes (21%±30% and 51 ± 102 cells/μL) were detected vs. those observed for individuals presenting with hemolysis (37%±27% and 1,087 ± 776 cells $\times 10^3/\mu L$; 72%±30% and 1,986 ± 1,791 cells/μL; 76%±28%, and 324 ± 245 cells/μL; respectively) and unexplained cytopenias (18%±27% and 506 ± 826 cells $\times 10^3/\mu L$; 35%±41% and 825 ± 1,688 cells/μL; 39%±41%, and 136 ± 182 cells/μL; respectively). In addition, AA cases who were PNH^+ also showed significantly lower percentages and absolute counts of GPI-deficient RBC (9%±18% and 203 ± 528 cells $\times 10^3/\mu L$) ($P < 0.001$) than PNH^+ cases who were screened because of unexplained thrombosis (36%±26% and 1,315 ± 713 cells $\times 10^3/\mu L$); however similar numbers of GPI-deficient neutrophils and monocytes ($P > 0.05$) were found in these two patient groups. No statistically significant differences were found as regards normal GPI-expressing cell counts ($P > 0.05$) among patients with unexplained cytopenias, thrombosis, MDS, and AA.

DISCUSSION

Consensus recommendations for PNH screening have been redefined in 2010 (7,11). Since then, such recommendations have been widely accepted and adopted. Despite this, information about the actual efficiency of such medical indications remains very limited (15) in both reference laboratories and in smaller multicenter settings. Here we evaluated for the first time the efficiency of current medical indications for the diagnostic screening of PNH by FCM in a large multicenter setting vs. a single reference laboratory. As expected for an ultra-rare disease, the overall incidence of PNH^+ cases within the multicenter setting in Spain was very low, in line with the expected incidence of the disease (9,10). In addition our results indicate that the current medical indications for PNH testing (7,8,11,12) are rather efficient with an overall rate of positive cases of around 14%, or (even) higher in young adults (≈20%). Despite this, the highest frequency of PNH^+ cases was found among individuals diagnosed with aplastic anemia/hypoplastic BM and those who had hemoglobinuria followed, to a less extent, by cases presenting with unexplained cytopenia including anemia, non-hemolytic anemia, thrombosis with non-hemolytic anemia/cytopenias, and

(hypoplastic) MDS. A lower but still significant rate of PNH^+ cases was also found among cases with unexplained cytopenias in the absence of anemia, or those who had unexplained anemia (not otherwise specified). In contrast, PNH testing for cases presenting with unexplained thrombosis in the absence of anemia or another cytopenia, as well as for patients diagnosed with other hematological/immunological disorders in the absence of a BM failure syndrome, emerged as worthless, with extremely low rates of PNH positivity. Overall, these results are in line with previous observations reported in the literature mostly in smaller patient series and specific diagnostic groups (15–34).

Several technical questions about the precise methods and reagents to be used for PNH screening remained open in the consensus guidelines published by the Spanish Society of Hematology (12) and the International PNH consensus panel (7) in 2010–2011, including which GPI-associated proteins might be better for the identification of GPI-deficient granulocytes and monocytes (7,12), and whether FLAER must be a mandatory marker in all combinations (12). Overall, the data here presented would support that FLAER should be mandatory in all combinations used for PNH screening, particularly for the evaluation of samples presenting with lower percentages of GPI-deficient cells such as those submitted because of an unexplained cytopenia and AA, as it emerged as the only single marker with a significant impact in the rate of detection of PNH^+ cases among samples submitted for PNH testing because of both medical indications. In contrast, the two GPI-associated granulocyte proteins most commonly used (CD16 and CD24) seem to be equally efficient for detecting GPI-deficient cells, while other markers (i.e.CD157) that can adequately identify GPI-deficient cells among both granulocytes and monocytes need to be further investigated, as they were used in a minority of samples here analyzed. Of note, FLAER would not only be associated with a greater frequency of detection of PNH^+ cases but it also showed a higher reproducibility of the results, based on the data extracted from the EQA for PNH screening organized by the SIC. Interestingly, we also observed a lower rate of PNH^+ cases among those laboratories that did not participate in the SIC EQA program, suggesting that the ability to detect GPI-deficient cells might be improved with targeted training. This supports previous studies showing that the risk of misdiagnosing PNH by flow cytometry is higher among less experienced laboratories, particularly for those samples presenting with low (i.e., <1%) numbers of GPI-deficient cells (35).

Interestingly, significant differences were observed in our study as regards the frequency of PNH^+ cases between the 24 Spanish centers and the reference laboratory in Brazil, but with overall similar trends. Altogether, these results confirm the relatively high overall efficiency of PNH testing in both settings. Of note, the percentage of PNH^+ cases was significantly higher in both settings than previously reported in a single reference laboratory in the US (15), despite samples referred for PNH monitoring were also included in this later series, while these cases were specifically excluded from our study.

From the above three large patients series, PNH positivity was almost always higher in the reference center in Brazil, particularly for cases presenting with AA, hemoglobinuria, Non-Hemolytic anemia and unexplained cytopenias. Although this might be partially explained because all cases in Brazil were analyzed with FLAER, the frequency of samples carrying detectable GPI-deficient cells among those centers using FLAER in Spain remains lower than that of the reference Brazilian laboratory. This might be also due to the fact that cases that had prior diagnosis of PNH by other methods had been submitted for confirmation/testing by FCM at the reference laboratory in Brazil when free-of-charge testing started in this center in 2010, at the moment of starting this study. Alternatively, the increase frequency of cases testing positive in the reference center in Brazil could be due to the usage of more stringent criteria for PNH testing, associated with a higher diagnostic probability. However, it should be note that no significant differences were observed among the Spanish vs. Brazilian laboratories for several other medical indications (unexplained cytopenias without anemia and anemia not otherwise specified) in line also with the low frequencies (<5%) reported for these later cases in the literature (15).

Most importantly, patient conditions different from consensus medical indications (7,8,11) (e.g., clinical and laboratory pictures presenting in the absence of hemolysis and cytopenias) were associated with very low rates of positivity (<1.5%) in both the multicenter and reference laboratory settings, in line with previous observations (15). Altogether, these results reinforce the recommendation for PNH testing in individuals presenting with hemoglobinuria, hemolytic anemia and unexplained cytopenias including anemia (7,8,11), at the same time, they point out the need for refined screening algorithms to increase the efficiency of PNH testing in other cases. As an example, we showed that despite the overall frequency of PNH^+ cases among samples tested because of unexplained thrombosis was comparable to most previously reported series/setting (15–19), the combination of unexplained thrombosis with anemia/cytopenia was associated with a significantly higher rate of PNH positivity of 14% vs.1.5% for other thrombosis patients.

Thrombosis probably represents the most severe and life-threatening complication of PNH, its frequency being relatively low at disease onset, particularly among patients without hemolysis (1–5,36). Because of this and the high prevalence of thrombosis in the general population, the overall frequency of PNH^+ cases among newly screened thrombotic patients is usually low (<0.3%–1.4%) (15–18). However, a more in depth review of PNH^+ cases reported in the literature among patients presenting with unexplained thrombotic events

(16,18,19) shows that these cases frequently display also cytopenia(s) or even signs of a BM failure syndrome (16,19), except for a few patients who showed minor (<0.5%) PNH^+ clones (18). These results are fully in line with the clear association observed here between the presence of PNH^+ cells and cytopenias among cases screened because of unexplained thrombosis, independently of their age; in contrast, patients with thrombosis who had normal blood cell counts, typically tested negative for PNH. These findings suggest that among cases presenting with unexplained thrombosis, PNH testing should focus on those having cytopenias, independently of age. Further investigations are required to define more efficient screening algorithms for PNH testing in cases that present with unexplained thrombosis and normal cell counts in PB.

The efficiency of PNH testing among patients with BM failure syndrome has been more extensively investigated in the literature (20–34) due to the well-known association between the presence of GPI-deficient cells among AA patients and response to immunosuppressive therapy (23–31), as well as progression to symptomatic PNH (32). Overall, a BM failure syndrome associated with prior diagnosis of a hematological malignancy was the medical indication most frequently associated with PNH positivity in our series, both in adults and in children or adolescents. These results emphasize the need for PNH testing in AA patients of any age, including also younger AA cases (30–32). In line with our observations, previous studies have shown that GPI-deficient cells are frequently found in AA (20–30) and MDS (20,22,24,26,33,34) patients, while they are detected only in few MPN cases (37). Despite the exact frequency of PNH+ AA cases varies substantially in the literature (range 22% to 89%) (20,21,23,26), it is currently estimated to be ≈40% of cases (22,25,30,31), as also found in our series with slight differences according to the age of the patients. An even higher variability has been reported in the literature for the frequency of PNH+ cases among MDS patients (range 1.8% to 41% of cases), depending on patient selection criteria (20,22,24,26,33,34). Thus, GPI-deficient cells are more commonly found among low-grade MDS patients who show features of an hypoplastic BM than in other MDS subtypes (22,33,34). An overall rate of ≈10% PNH+ cases was found in MDS patients from the two laboratory settings investigated in this study. Also, one MPN sample out of a few cases tested PNH+ in our series. Despites PNH testing for MPN patients is currently not recommended, several cases have been recently described in which *JAK-2*, and *CALR* mutations, as well as *BCR-ABL* gene rearrangements have been associated with PNH-positivity (37–40), the precise frequency of PNH+ cases among MPN patients deserving further investigations. In contrast to what is described above for AA and MDS cases, presence of hemolysis or cytopenias does not appear to be sufficient indication for PNH testing in patients diagnosed of hematological and/or immunological disorder other than AA, MDS, and MPN, such as leukemia/lymphoma or autoimmune diseases (4,7,41).

Previous reports have shown that patients who present with hemolysis have significantly larger percentages of GPI-deficient RBC, monocytes and neutrophils than cases who emerged with BM failure (1,5,42), as also found here. Interestingly however, in our series, PNH patients presenting with mild (hemolytic) symptoms (e.g., unexplained anemia and/or other cytopenias) or unexplained thrombosis, had intermediate percentages of GPI-deficient cells between those observed among PNH patients presenting with hemolytic and aplastic disease. Of note, the size of the PNH clone is typically defined on the basis of the proportion of GPI-deficient cell, such percentage typically correlating well with the corresponding absolute counts of GPI-deficient cells in PB, as found among our cases. In contrast, GPI-normal counts in PB per se, are regarded as being of limited relevance. Interestingly, here we found a significant decreased number of GPI-normal cell in PB of all different patient groups, including cases who did not show a BM failure syndrome; unexpectedly, the number of GPI-normal cells varied significantly depending on the medical indications that triggered PNH testing, depletion of "normal" hematopoietic cells being significantly more pronounced among PNH^+ cases who presented with hemolysis vs. all other patient groups. Of note, the percentage of PNH^+ patients that fulfilled the diagnostic criteria for BM aplasia (neutrophils <1,500 cells/uL) and severe BM aplasia (neutrophils <500 cells/uL) (43) based on PB counts of GPI-normal neutrophils, was >90% and 60% among PNH^+ cases with hemolytic disease; even more, >50% and 25% of PNH^+ patients who showed unexplained cytopenia/anemia and/or thrombosis had GPI-normal neutrophil count in PB compatible with BM aplasia and severe BM aplasia, respectively, such rates being similar to those observed among AA and MDS patients. These results support the notion that an underlying BM defect exists in virtually all PNH^+ patient subgroups, although it might be masked by the parallel expansion of GPI-deficient cells, particularly in patients presenting with classical PNH (2–5,10,44). In addition, our findings also suggest that in those PNH patients in whom the GPI-defective hematopoiesis is not able to overcome the underlying BM defect, diagnosis of AA will most likely precede the detection of GPI-deficient cells, whereas in the remaining PNH^+ cases, symptoms of hemolysis would predominate. Since hemolytic symptoms and BM failure require different treatment approaches, quantification of GPI-deficient as well as GPI-normal cells in PNH patients, might be more informative than just the percentage of GPI-deficient cells, for more accurate evaluation of the effects of therapy, for monitoring the extent of GPI-deficiency and for evaluating the degree of suppression vs. recovery of residual GPI-normal hematopoietic cells.

In summary, the results here reported indicate that current medical indications for PNH testing are highly

efficient, particularly among cases with previous diagnosis of aplastic anemia/hypoplastic BM and MDS, as well as in individuals presenting with hemoglobinuria and/or unexplained hemolytic anemia, cytopenias associated or not with thrombotic events. Those results are in line with current standard of care. In contrast, new diagnostic algorithms are required for a more efficient screening of patients presenting with unexplained thrombosis in the absence of cytopenias. Further studies in which the utility of introducing sensitive parameters for hemolysis (e.g., haptoglobin levels (45–47)) in the diagnostic algorithm are investigated might contribute to better select for cases presenting with unexplained thrombosis in the absence of cytopenias, that should be screened for PNH. Most interestingly, our results also show that a depressed "normal" residual hematopoiesis coexists with the PNH clone in all patient groups, particularly among PNH^+ cases presenting with hemolysis, pointing out the potential utility of the evaluation of the PB counts of both GPI-deficient and GPI-normal cells for more efficient monitoring of PNH patients.

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