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ORIGINAL ARTICLE

Features, reason for testing, and changes with time of 583 paroxysmal nocturnal hemoglobinuria clones from 529 patients: a multicenter Italian study

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Abstract

In this study, we aimed at disclosing the main features of paroxysmal nocturnal hemoglobinuria (PNH) clones, their association with presentation syndromes, and their changes during follow-up. A large-scale, cooperative collection (583 clones from 529 patients) of flow cytometric and clinical data was entered into a national repository. Reason for testing guidelines were provided to the 41 participating laboratories, which followed the 2010 technical recommendations for PNH testing by Borowitz. Subsequently, the 30 second-level laboratories adopted the 2012 guidelines for high-resolution PNH testing, both upon order by the local clinicians and as an independent laboratory initiative in selected cases. Type3 and Type2 PNH clones (total and partial absence of glycosyl-phosphatidyl-inositol-anchor, respectively) were simultaneously present in 54 patients. In these patients, Type3 component was sevenfold larger than Type2 ($p < 0.001$). Frequency distribution analysis of solitary Type3 clone size ($N = 442$) evidenced two discrete patterns: small (20% of peripheral neutrophils) and large (> 70%) clones. The first pattern was significantly associated with bone marrow failure and myelodysplastic syndromes, the second one with hemolysis, hemoglobinuria, and thrombosis. Pediatric patients ($N = 34$) showed significant preponderance of small clones and bone marrow failure. The majority of PNH clones involved neutrophils, monocytes, and erythrocytes. Nevertheless, we found clones made exclusively by white cells ($N = 13$) or erythrocytes ($N = 3$). Rare cases showed clonal white cells restricted only to monocytes (6 cases) or neutrophils (3 cases). Retesting over 1-year follow-up in 151 cases showed a marked clone size increase in 4 cases and a decrease in 13, demonstrating that early breaking-down of PNH clones is not a rare event (8.6% of cases). This collaborative nationwide study demonstrates a clear-cut difference in size between Type2 and Type3 clones, emphasizes the existence of just two classes of PNH presentations based on Type3 clone size, depicts an asymmetric cellular composition of PNH clones, and documents the possible occurrence of changes in clone size during the follow-up.

This study was conceived and coordinated by Luigi Del Vecchio, and the present manuscript is dedicated to his memory.

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Keywords Paroxysmal nocturnal hemoglobinuria · Flow cytometry · Myelodysplastic syndromes · Hemolytic anemia · Atypical thrombosis · Aplastic anemia

Abbreviations

AA	Aplastic anemia
BMF`	Bone marrow failure
CEINGE	Institute for Advanced Biotechnologies, Naples University
DAT	Direct anti-globulin test
FCM	Flow cytometry
FLAER	Fluorescent aerolysin
GPI	Glycosyl-phosphatidyl-inositol
HA	Hypoplastic anemia
ICD	International classification of the diseases
MDS	Myelodysplastic syndromes
PNH	Paroxysmal nocturnal hemoglobinuria
RFT	Reason for testing
SCAPE	Standardization of a cytometric approach to PNH clone evaluation

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disease, and although no clear-cut data are available, its incidence is around 1–2 per million, with a prevalence ranging from 10 to 20 per million [1]. PNH is an acquired form of hemolytic anemia, generated by the clonal expansion of a hematopoietic stem cell bringing a somatic mutation in a gene involved in an early step of glycosyl-phosphatidyl-inositol (GPI) anchor synthesis [2, 3]. Hemolysis, hemoglobinuria, increased risk for thrombosis, and variable grades of bone marrow failure (BMF) characterize PNH [4–7]. When this mutation occurs in a hematopoietic stem cell, a total or partial deficiency of the entire set of GPI-linked proteins occurs in the derived blood cells [7–10]. The absence or the reduction of GPI-linked complement regulatory proteins such as CD59 and CD55 causes complement-mediated intravascular hemolysis and platelet activation (via partially unknown mechanisms), which largely contribute to morbidity [11].

The close relationship between PNH and aplastic anemia (AA) or hypoplastic anemia (HA) suggested that auto-reactive T cells directed towards hematopoietic stem cells could be of relevance [12, 13]. The expansion of a PNH (GPI−) stem cell clone may be the consequence of an immunological attack against normal (GPI+) stem cells [14–16]. However, even in normal individuals, small PNH clones can be disclosed at a very low frequency [17, 18]. During the last 10 years, consistent evidence has been accumulated in favor of an autoimmune mechanism [19, 20].

The current golden standard for quantifying PNH clones is multicolor flow cytometry (FCM) [21–26]. FCM permits the characterization of PNH clones in population studies and makes it possible to correlate the affected cell phenotype to a variety of syndromes [21, 27].

The Italian national FCM archive (from now on ClonePNH—https://www.clonotecaepn.it) described in this paper represents the first large-scale unified collection of FCM data and clinical information regarding patients with PNH in Italy. Under the coordination of the CEINGE Institute at the University of Naples, it was built up over the time by the independent collaboration of more than 70 FCM laboratories (41 of which participated actively in this study), that contributed by entering data from all cases who had a reason for testing, with both positive and negative results.

In the present study, we aimed at exploring the frequency distribution of PNH clone size, type, and cell phenotype in adult and pediatric patients, as well as its association with the four main clinical syndromes accompanying the disease onset (i.e., bone marrow failures, myelodysplasia, hemolytic anemia/hemoglobinuria, atypical thrombosis). We also aimed at finding an answer to a basic question regarding PNH, such as the prevalence and relative size of Type2 clones (carrying a mutation with just a partial GPI defect) and Type3 clones (cases with a mutation causing a complete GPI defect). Lastly, we aimed at investigating the occurrence of spontaneous changes in PNH clones during the follow-up.

Materials and methods

Reason for testing (RFT) and ClonePNH Database Construction ClonePNH archive are online dedicated database, and participants in this project need to be previously trained, qualified, and registered.

The main non-overlapping syndromes considered as RFT, inducing physicians to order a PNH test were (a) aplastic anemia, (b) bone marrow failure, (c) hemolytic anemia, (d) myelodysplastic syndromes, (e) idiopathic cytopenias, (f) atypical thrombosis, (g) hemoglobinuria, (h) not declared. Overall, these syndromes represent the global tabular list of ICD-9-CM entries connected to PNH [28], and participating centers were asked to enter only the item with the major clinical impact. Since RFT represented the only clinically relevant information considered in this study, we recommended the participating centers to be accurate when entering data regarding the accompanying syndromes at disease onset. Seventy-eight laboratories distributed in 17 out of 20 Italian regions actively participated to data collection, and 41 to this study. We report here an analysis of the data collected between 2009 and 2014.

Flow cytometric analyses

The term "PNH clone" was used to identify the GPI-deficient cell population(s) defined by the FCM approach, namely neutrophils, monocytes, and erythrocytes, showing various proportions of reduced or absent GPI-linked markers. At first, participants were following the 2010 guidelines by M. Borowitz [29]. Over time, the guidelines for high-resolution PNH clone analysis were adopted by the 30 larger laboratories. CEINGE Institute acted as the provider of technical and scientific guidance by giving recommendations on the assay setup, on cell events to be collected (i.e., > 100,000 red cells and neutrophils, and at least 100 GPI-deficient monocytes) [26], and as the second-opinion reference center. If patients under study received transfusions or had hemolytic crises, it was recommended to perform the cytometric analysis of PNH clones after at least 20 days from overt hemolytic crises and/or transfusions.

In order to improve reproducibility and to ensure a uniform analytical strategy in studying PNH clones by FCM, participants in the ClonePNH project were trained by a national course named Standardization of a Cytometric Approach to PNH Clone Evaluation (SCAPE), lasting 4 months. To date, nine editions of the course have been run, structured with a conventional kick-off meeting, a series of 20 FCM exercises to be carried out as homework including the circulation of samples for comparison and a final meeting in which results were compared as in external quality assessment systems. Outlier results in comparison rounds were reviewed for possible analytical errors. Results with z-score of more than 10 were discarded to prevent unnecessary distortion. The vast majority of centers adhered to the PNH external quality assurance scheme by UKNEQAS.

The most commonly used markers were CD59 for red blood cells (RBC) [29], CD24, CD66b, Fluorescent Aerolysin (FLAER), and CD157 [24] for neutrophils, CD14, FLAER, and CD157 [24] for monocytes. The most common gating strategy was based upon Glycophorin-A (CD235a) and CD45 for detecting RBC, CD45, CD33, and CD15 for neutrophils, CD45, CD33, and CD64 for monocytes. Clone size was assessed by enumerating the percentage of GPI-negative or deficient cells, in selective capture gates including all the relevant events of a given lineage. Neutrophils were preferred to RBC and monocytes for screening since they represent the most frequent leukocyte population in the vast majority of cases and because they do not suffer from biases associated to hemolysis and transfusion support [5, 26]. The identification of Type2 and Type3 components was carried out by analyzing neutrophils, following the recommendations of the current literature [24–26]. A retrospective analysis of the used gating strategy and of the preferred antibodies demonstrated that almost all laboratories participating in the study adhered to the same technique, and that in the majority of cases, they used the same antibodies, clones, and fluorochromes.

A threshold of 0.01% for neutrophil PNH clones was agreed to consider a patient as PNH clone positive [24–27], irrespective of the clinical symptoms [5, 6]. The coordinating center (CEINGE Institute) acted both as a trainer and as data supervisor, and also performed confirmatory testing on request in doubtful cases (Supplementary Table 1) [29].

Statistical analyses were performed by using the SPSS package, IBM [New York, USA].

All studies in this paper were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008. Although this type of study did not require a formal clearance by ethical committees, all persons gave their informed consent prior to the inclusion of their data in the national repository.

Results

PNH clone detection and type

The ClonePNH database included a total of 3783 analyses. PNH clone data of treatment-naive patients at first presentation were entered in the national registry. A subgroup of entries was also related to subjects undergoing retesting over time, with a field indicating if any therapy was administered or not. Overall, PNH clones were 583, (Type2 alone, Type3 alone or the combination between Type2 and Type3) which were detected in 529 patients (13.9% of all entries) (Fig. 1).

Among positive cases, 447 patients (84.4%) showed an isolated Type3 clone, 28 (5.3%) an isolated Type2 clone, while 54 (10.2%) displayed the simultaneous presence of Type2 and Type3 clones (Table 1). PNH clones were always detected among neutrophils, with the exception of five cases in which only red cells were analyzed and neutrophil typing was missing. Cases with PNH clones < 1% and with the putative presence of a Type2 component were always referred to CEINGE for confirmation.

PNH clone size and association to disease

The difference in median values of Type3 and Type2 clone sizes was highly significant (62.15% and 8.45%, respectively; $p < 0.001$) (Table 2). Data extracted from quartiles showed that 95% CI for odds ratio measuring the probability of having concomitant Type2 and Type3 clones was 1.47–1.86, assuming a Type3 clone size > 50%. Conversely, in the presence of a small Type3 clone, the occurrence of an accompanying Type2 component was very rare.

Analysis of variance between the observed frequency distribution of 442 Type3 clone sizes and their normal theoretical distribution evidenced significant differences, with a striking

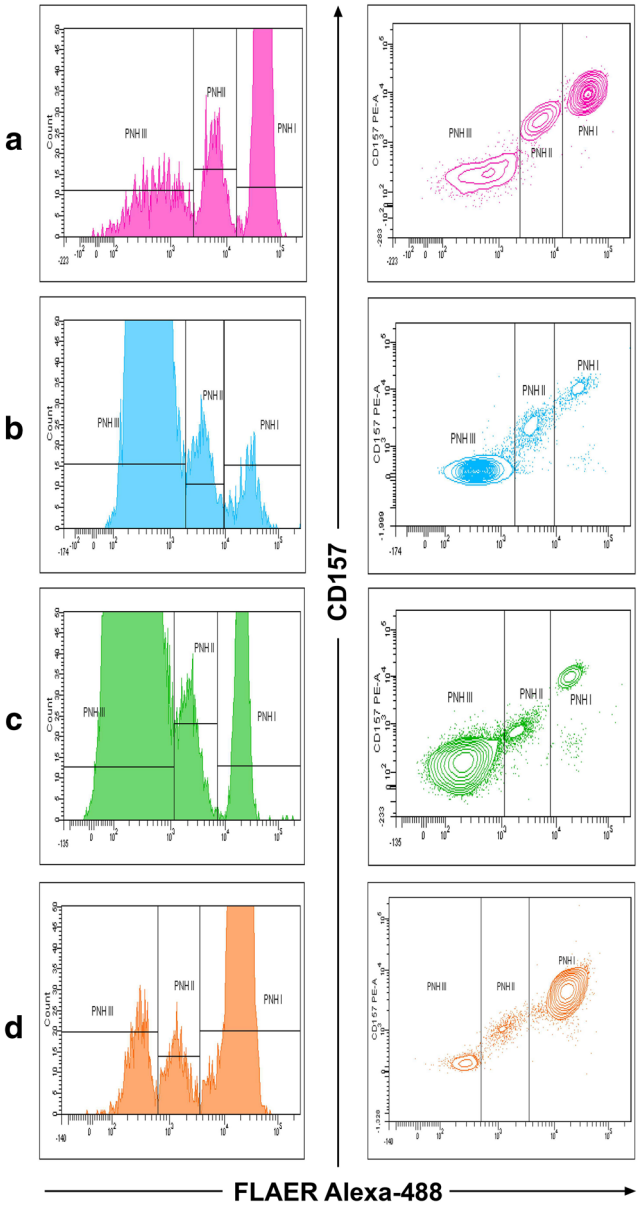

Fig. 1 Four representative cases [A–D] of flow cytometric discrimination of Type1, Type2, and Type3 components in neutrophils. FLAER, fluorescent aerolysin conjugated with Alexa-488. Contour plots show the bi-parametric analysis of FLAER conjugated to Alexa-488 and CD157 conjugated to phycoerythrin. Note the unequivocal discrimination of Type2 clones

Table 1 Distribution of PNH clone types among the 529 positive cases detected over 3783 screened patients

Type of clone	No. of samples	% of samples
Solitary Type2	28	5.3
Simultaneous Type2 + Type3	54	10.2
Solitary Type3	447	84.4
Total	529	100%

polarization of cases with small (≤ 20%) and large (> 70%) clone size ($p < 0.001$) (Fig. 2).

We analyzed the frequency distribution and size of Type3 clones according to RFT in a subset of 386 cases in which an RFT assignment was available. In order to simplify patients' stratification, we decided to gather aplastic anemia, bone marrow failure, and idiopathic cytopenias under a single "bone marrow failures" group. Similarly, hemolytic anemia and hemoglobinuria were also put in a single group. Myelodysplastic syndromes and atypical thromboses made the two other groups.

Overall, among the 529 patients with PNH clones, only 225 (42.53%) showed overt PNH clinical symptoms (30 in BMF group, 12 in MDS, 172 in HA, and 11 in thromboses).

A skewed frequency towards small clones in the bone marrow failures group was observed ($N = 144$, median of clone size from 1 to 10%, skewness 1.3, Fig. 3A) and in myelodysplastic syndromes ($N = 44$, median of clone size from 1 to 10%, skewness 1.16, Fig. 3B). By contrast, a skewing towards a larger clone size was observed in patients with hemolysis and/or hemoglobinuria as RFT ($N = 183$, median of clone size from 80 to 90%, skewness − 0.8, Fig. 3C) and atypical thrombosis ($N = 15$, median of clone size from 80 to 90%, skewness − 0.9, Fig. 3D). However, four cases with 0.1 to 10% clone size had thrombosis as RFT (Fig. 3D).

U-Mann Whitney test performed to compare the median clone size in bone marrow failures or myelodysplastic syndromes versus hemoglobinuria/hemolysis or versus atypical thrombosis showed significant differences ($p < 0.001$). By contrast, differences between bone marrow failures and myelodysplastic syndromes and between hemolysis and atypical thrombosis were not significant ($p = 0.5$; $p = 0.9$, respectively).

Pediatric patients showed a clear-cut skewing towards small clones, compared to adults (skewness 1.17 vs 0.2, respectively) (Fig. 4).

PNH clone size and cell type

Neutrophil and monocyte PNH clone size were compared, and the findings in the two white cell types were well correlated (Pearson correlation R value 0.943, $p < 0.0001$, Fig. 5). However, a few cases showed a mainly neutrophil (i.e., monocyte clone < 5% and neutrophil clone size > 20%, 3 cases) or a mainly monocyte (i.e., neutrophil clone < 5% and monocyte clone > 20%, 6 cases) pattern (Fig. 5A, rectangle a and b, respectively), as defined arbitrarily.

The clone size, as measured by RBC and neutrophils also showed a fair correlation, with a R value of 0.509, $p < 0.0001$ (Fig. 5B). It is noteworthy that 12 of such cases showed a mainly neutrophil pattern (rectangle "a"). The size of defective RBC clones at the moment of study was often lower as compared to neutrophils but three cases showed a mainly

Table 2 Size of PNH clones according to Type3 and Type2 phenotype

	Number	Mean	SD	Min	25th percentile	Median*	75th percentile	Max
Type3	54	53.34	38.59	0.04	8.30	62.15	91.00	100.00
Type2	54	18.01	22.71	0.13	2.00	8.45	22.40	85.90

Descriptive statistics of Type3 and Type2 components in cases with the simultaneous presence of both clone types [$N = 54$]

Note that the median size of Type3 clones was about sevenfold larger as compared to Type2. *$p < 0.001$

RBC pattern (Fig. 5B, rectangle "b"). The correlation between RBC and monocytes displayed a similar R value and similar proportion of a mainly RBC and a mainly white cell population (data not shown).

PNH clone changes over time

We assessed the PNH population changes over a 1-year follow-up, using therapy-naive cases that were analyzed more than once. No patients under study were submitted to bone marrow transplantation. In order to focus only on changes from small clones to middle-large clones and vice versa, patients with initially intermediate clone size between 20 and 30% were deliberately excluded from this analysis, to avoid the inclusion of small random changes. Sixty-four cases with a small clone size ≤ 20% and 87 cases with a large clone size (≥ 30%) were considered. Among the 64 small clone cases, 4 (6.3%) increased their size to > 30% (Fig. 6A). Among the 87 middle-large clone cases, 13 (14.9%) decreased their size to < 20% (Fig. 6B). Taken together, within this group of 151 cases, 134 (88.7%) remained stable during 1-year follow-up and 17 (11.3%) significantly changed their size.

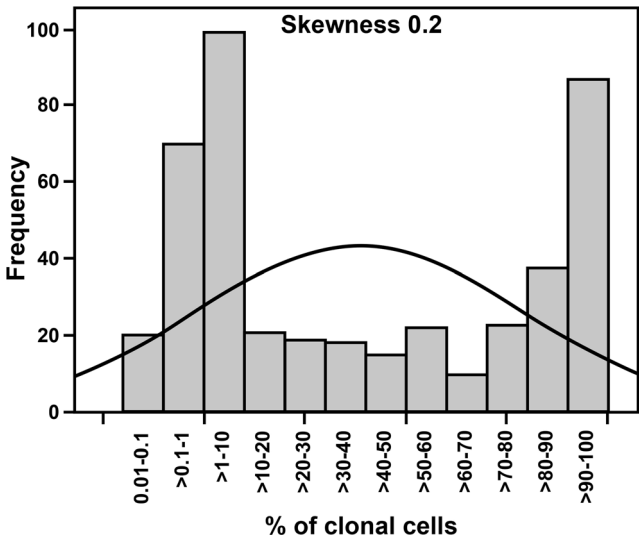

Fig. 2 Distribution of size classes of Type3 clones [$N = 442$]. The shape of clone size frequency distribution was evaluated by skewness assessment by the natural method of moments. In this figure, compared to the normal theoretical distribution [solid line], the actual distribution displayed two divergent peaks [one composed by small clones and another by large clones]

Discussion

Our study describes the features of a large series of newly discovered PNH clones (583 cases) in therapy-naive patients collected into a national data repository over a 6-year period and in some cases retested. This involved the coordinated effort of more than 70 Italian FCM laboratories expressly trained to screen and characterize PNH clones. A similar data collection performed in the USA was described [27] but in that case, samples were centrally examined by a single reference laboratory.

The usage of term "clone" in our study as in many other papers on PNH is just to identify the major GPI-deficient cell population(s) for sake of simplicity, since it is well known that PIG-A mutations can also be non-clonal [19].

The national database ClonePNH (https://www.clonotecaepn.it) is aimed at including all the data deriving from a reason for testing upon both a clinician's order and upon the likelihood of a possible underlying PNH conditions, as judged by the laboratory manager during the diagnostic workup of unexplained cytopenias, DAT-negative hemolysis, or thrombophilic states with unusual thromboses. This combined and coordinated approach has proved effective in detecting a number of hitherto unnoticed PNH clones, a significant fraction of which (65.3%) was associated with clinical symptoms [30].

Despite FCM screening criteria have evolved with time, in accordance with the sequential publication of technical guidelines [22, 25, 26, 29], during the last year of the study, the screening procedure has focused on neutrophils only, with the adoption of a validated, simplified 2-color method by 44 centers [30]. The acquired experience on PNH clone screening with time produced the evidence that it is appropriate to focus the FCM screening on neutrophils only, because such cells are easy to be collected in large numbers and are not modified by the hemolytic waves affecting red cells [26].

We compared the frequency of Type3 and Type2 clones. The presence of Type2 clones was a rarer phenomenon as compared to Type3. The expected occurrence of a Type2 component was higher in the presence of a large size of Type3 component. Interestingly, the size of Type2 clones was rather constantly about sevenfold smaller than Type3 clones (median value 8.45 vs 62.15%, respectively, $p < 0.001$). These findings are to a certain extent in line with the so-called "escape

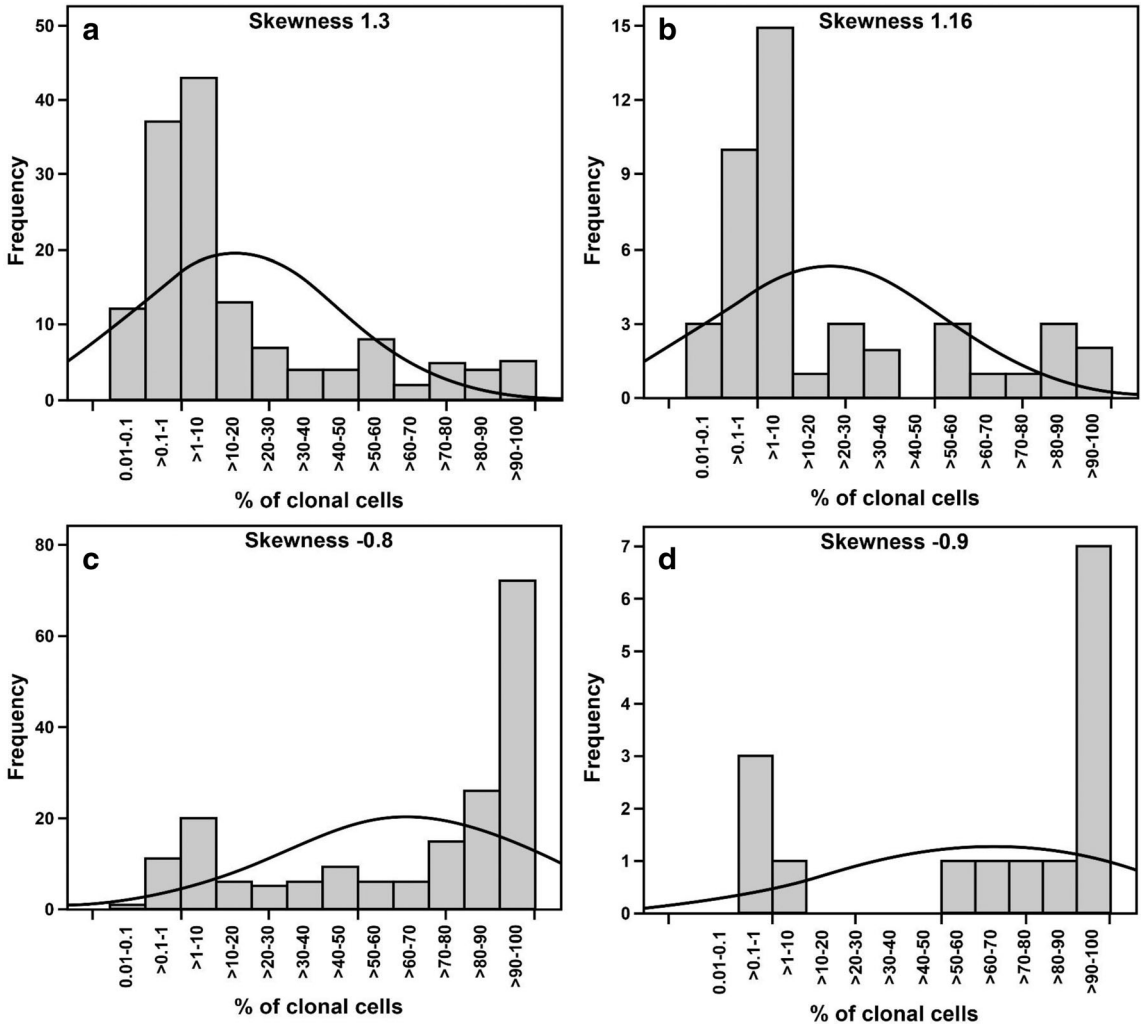

Fig. 3 Distribution of size classes of Type3 clones according to reason for testing. Panel A: bone marrow failures [$N = 144$]. Panel B: myelodysplastic syndromes [$N = 44$]. Panel C: hemolysis and/or hemoglobinuria [$N = 183$]. Panel D: atypical thrombosis [$N = 15$]. PNH clones distribution in A and B displayed a significant tendency to occupy the "small clone zone." PNH clones distribution in C and D displayed a significant tendency to occupy the "large clone zone"

theory" [1]: the Type2 clone, in competition with Type3 clone in the same bone marrow microenvironment and in the presence of an immune attack to normal hematopoietic stem cells, may display a less powerful "Darwinian fitness" as compared to Type3. Future research may be addressed at evaluating the presence of different types of PIG-A mutations in Type3 and Type2 clones.

Our analysis of frequency distribution of PNH clone size suggested the presence of a hitherto unknown preexisting condition driving a bimodal pattern made of small or large clone sizes. In a study by Nowak [31], 44 PNH patients were tested for HLA alleles and haplotype association. The haplotype A*2501 Cw*1203 B*1801 was associated with AA/PNH and small clones, while the haplotype DRB1*1501-DQB1*0602 was linked to florid PNH and large clones. Thus, PNH clone size seems to depend also from genetic factors potentially influencing immune response background. Of interest, another study never published on regular journals [27], in which FCM tests were carried out by a central laboratory, showed a frequency distribution quite similar to that of our study, although no quantitative comparisons were provided.

Stratifying PNH clones by RFT evidenced different frequency distributions with different kind and grade of skewness. BMF and MDS showed a prevalence of small clones with a "left shift" of size distribution, while hemoglobinuria cases showed a prevalence of large clones with a "right shift." Interestingly, cases with increased thrombotic risk showed a pattern similar to hemoglobinuria, with a majority of large clones and very few cases with small PNH clones. The latter are of particular interest. In our series, 4 cases with < 10% clone size presented with an episode of atypical thrombosis, so demonstrating that the presence of overt hemolysis and of a large clone is not a necessary prerequisite for an increased thrombotic risk (Fig. 3D).

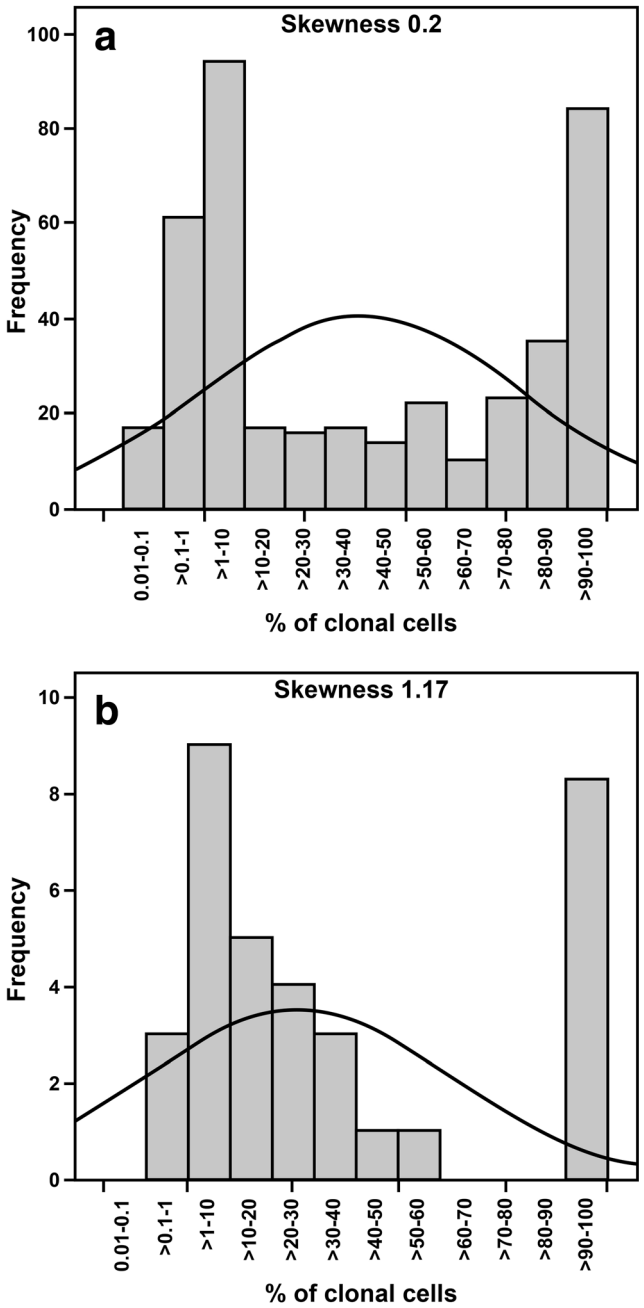

Fig. 4 Distribution of size classes of Type3 clones according to patients' age. Panel A, adult patients [$N = 408$]. Panel B, pediatric patients [$N = 34$]. Note the skewness of clone sizes towards the "small zone" in pediatric patients

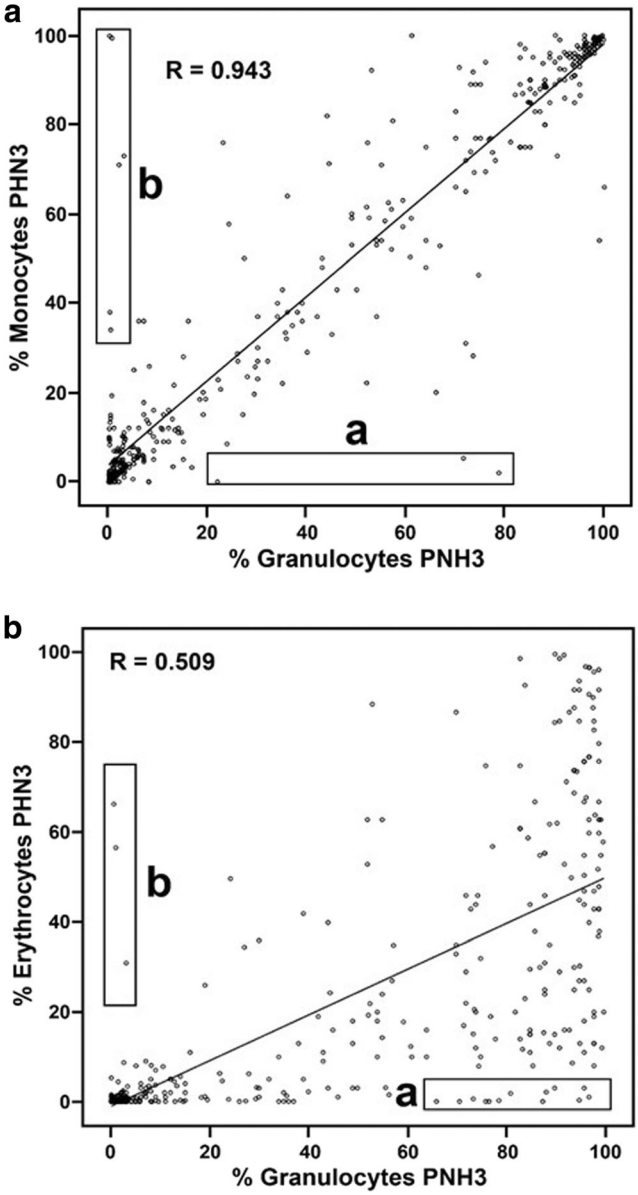

Fig. 5 Panel A. Analysis of correlation between Type3 clone sizes in neutrophils and monocytes [$N = 414$, $R = 0.943$, $p < 0.0001$]. Rectangle "a": mainly neutrophil clones [$N = 3$]. Rectangle "b": mainly monocyte clones [$N = 6$]. Panel B. Analysis of correlation between Type3 clone size in neutrophils and erythrocytes [$N = 414$, $R = 0.509$, $p < 0.0001$]. Rectangle "a": mainly neutrophil clones [$N = 12$]. Rectangle "b": mainly erythrocyte clones [$N = 3$]

Differently from Parker classification [32], that recognizes three distinct clinical classes of PNH patients, our findings are more consistent with the existence of just two different cellular patterns in PNH. The first one shows large clones and a clear-cut hemolytic/thrombotic tendency, while the second one presents small clones and a prevalent clinical picture of AA or MDS, with an increased thrombotic risk in some cases. Taken together, the frequency distribution, we have found that the "small clone" group includes the 25th percentile size class (1–10%), while the "large clone" group mostly includes the 75th percentile (size class 80–90%), consistently with the absence of an intermediate size population (Fig. 2).

In our study, pediatric cases showed a prevalence of small clones. Three previous studies investigated PNH clones in pediatric series, but in a less detailed manner. A retrospective analysis of florid PNH patients selected from a single excellence-center reported 12 PNH clones, six of which accompanied by documented atypical thrombosis [33]. The second multicenter study focused on PNH occurrence in AA,

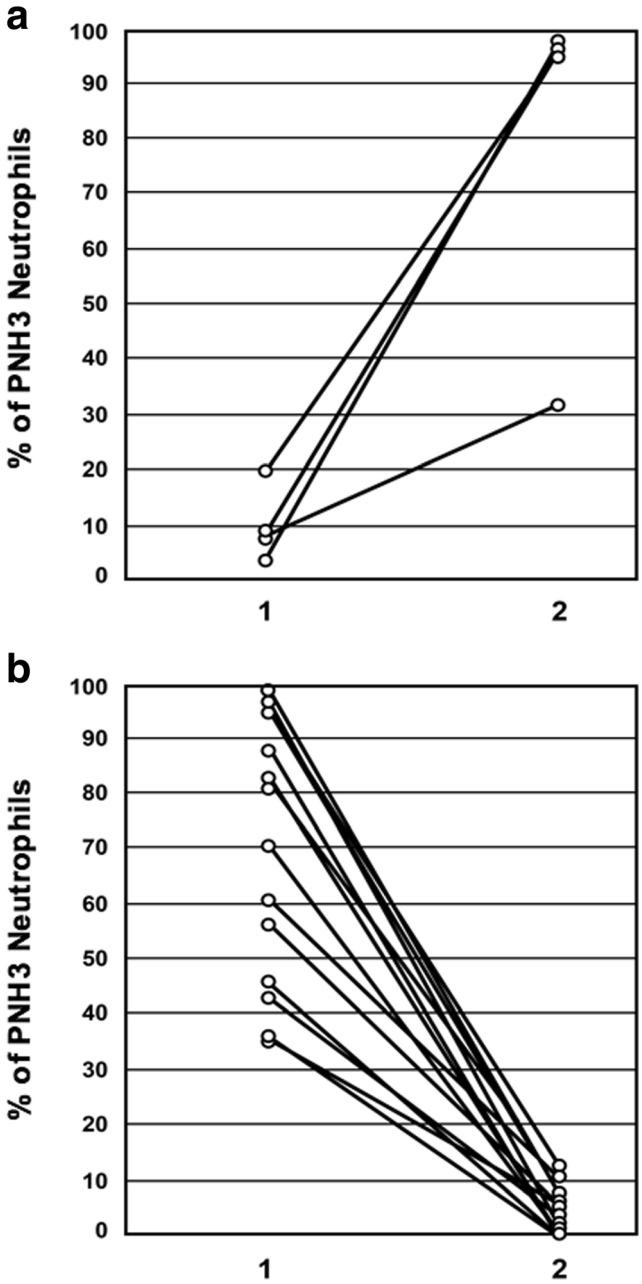

Fig. 6 Type3 clone size changes over 1-year observation in therapy-naive patients: increase or decrease from diagnosis to the last clone size assessment [12 months after]. Clones showing marked [< 20 to > 30%] increase from first to last clone size assessment are shown in panel A. Clones showing marked [> 30 to < 20%] decrease from first to last clone size assessment are shown in panel B

reporting 39 patients showing small clones and no evidence of thrombosis [34]. The third study compared demographic and clinical features of children and adults in the International PNH Registry. As compared to adults, children showed more frequent bone marrow failures, while thrombotic events seemed to be less prominent [35]. Taken together, the results of such studies are consistent with our series, in which pediatric cases included a broader spectrum of RFTs. Our study demonstrates that, despite most PNH clones in children are of small size, a florid PNH with a large clone can occur in 23.5% of cases (8/34, Fig. 4).

Our analysis of monocyte and neutrophil clones has demonstrated a consistent similarity between the two cell subsets (with the exception of rare "mainly neutrophil" or "mainly monocyte" cases). We are aware that the existence of "mainly monocyte" cases may pose a caveat on the use of neutrophils as the main target cell population when screening for PNH clones. Conversely, the lower monocyte representation in white blood cells may pose a serious technical limitation in PNH patients, sometimes hampering the collection of an adequate event number in routine screening and preventing high-resolution analysis. For the same reason, great attention should be paid on putative small Type2 clones in monocytes, since the analysis can be often inaccurate and prone to a number of technical artifacts [30]. The red cell PNH clone patterns were significantly divergent from the white cell findings, since RBC PNH clone sizes were often smaller. This could be due to the PNH RBC loss caused by intravascular hemolysis and PNH RBC dilution generated by transfusion support during follow-up. Our findings cannot however exclude also the involvement of a stem cell giving rise to larger amounts of white cells as compared to RBC. In other words, some PNH clones could develop essentially as "white cell" with only marginal involvement of RBC and without hemolytic manifestations. It is not yet clear whether "white cell" PNH clones can preferentially generate thrombotic manifestations. Twelve cases with a white cell PNH clone exclusively detectable on neutrophils and monocytes, but not RBC, are present in our archive. These cases are candidate for a nested cohort study in order to test the thrombotic risk of such a patient subset. In a recent review article, Brodsky [5] proposed to consider as florid PNH even cases in which the presence of a large clone was accompanied by thrombosis and not hemolysis, thus de facto implementing the Parker classification published in 2005 [32].

The frequency of cases showing an increase of PNH clone from small to large with time is low (6.3%), in accordance with a previous study [36]. The percentage of cases showing a decrease in clone size is higher, around 15%. Thus, the increase of clone size with time seems less frequent than the reduction. No patients among the 13 in whom a decrease in clone size took place underwent bone marrow transplantation or received immunosuppressive treatment, thus this phenomenon could be considered as a spontaneous change. To the best of our knowledge, a decrease from 35–100% to 0–15% within 1-year follow-up in non-transplanted subjects was not described before.

A limitation of our study is the restriction of the analysis to FCM data and their correlation with non-overlapping ICD-9-CM clinical categories: the previous configuration of the national database did not allow the inclusion of many clinical variables. Therefore, the present study did not specifically

address the relationship of FCM findings to clinical details and therapeutic data.

Follow-up studies of people with small, clinically silent PNH clones are warranted [36], and we concur with this recommendation. However, implementing a systematic screening of patients at risk in underexplored regions may be more effective in disclosing new PNH cases, thus providing therapeutic options and prophylactic benefits to patients with hitherto undiagnosed PNH. These results can be reached by stimulating the interaction between expert clinicians and good hematology and FCM laboratories, as our experience seems to demonstrate [30]. Our systematic screening activity, along with the recording of both negative and positive cases in a network of national contributors with a unified data repository, seems able to collect a larger number of incident and prevalent cases than expected by previous population studies [37, 38, 39].

Compliance with ethical standards

All studies in this paper were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008. Although this type of study did not require a formal clearance by ethical committees, all persons gave their informed consent prior to the inclusion of their data in the national repository.

Conflict of interest The authors declare that they have no conflict of interest.

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