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Research Article

Detection of Subclinical/Minor PNH Clone(S) in Patients with Thrombotic Events

Ioanna Anastasopoulou[1*], Paraskevi Kotsis[2], Stilliani Kokori[3], George Lallas[1], Sofia Gkotsi[1], Olga Katsarou[1], Konstantinos Konstantopoulos[4]

[1]Blood Unit and National Reference Center for Congenital and Bleeding Disorders, Laikon General Hospital, Athens, Greece; [2]Transfusion Medicine Department Clinical and Laboratory Research, School of Health Sciences, University of Thessaly, Thessaly, Greece;[3]Laboratory of Hematology and Blood Transfusion Department, University General Hospital Attikon, Athens, Greece; [4]Professor Hematology, University Medical School of Athens, Athens, Greece

ABSTRACT

Background: Paroxysmal nocturnal hemoglobinuria is a rare acquired disorder of a pluripotent hematopoietic stem cell, caused by a *PIG-A* gene somatic mutation. Thrombosis occurs in 40% of PNH patients and consists a main cause of morbidity. More than one-third of thrombotic episodes are located in hepatic veins, the inferior vena cava and cerebral veins.

Study objective: To evaluate the presence and role of PNH subclinical/minor clone(s) in idiopathic VTE patients, with no known congenital or acquired thrombophilia.

Study design: 181 patients having experienced idiopathic thrombosis and 100 healthy controls were screened for PNH clone(s) presence by flow cytometry. Testing was performed on white blood cells using FLAER and on red blood cells using CD59. The study was focused on the presence of minor clone(s), according to cut-off values raised from the healthy population.

Results: Ten out of 181 patients were revealed with a small PNH clone in both cell lines (WBCs and RBCs). The presence of a minor clone was not found to be associated with thrombosis prognosis or recurrence. Conclusions: PNH screening should be performed (especially) in young patients with unexplained thrombosis or thrombosis in unusual sites. According to our data, it is not concluded that the parameters tested are associated with disease prognosis or VTE recurrence. In certain populations, a higher percentage of minor clone(s) was observed; re-examination and systematic follow-up might be useful for specific individuals cases. Further studies with a larger number of individuals may be needed.

Conclusion: Paroxysmal nocturnal hemoglobinuria is a disorder in patients more than one-third of thrombotic episodes is located in hepatic veins, the inferior vena cava and cerebral veins. Mainly it is focussed under the PNH clones in thrombotic patients. Majorly the young individuals are experienced arterial thrombosis and splanchnic vein thrombosis.

Keywords: Paroxysmal nocturnal hemoglobinuria; Minor PNH clones; Unexplained thrombosis; Flow-cytometry; Flaer

Abbreviations: PNH: Paroxysmal Nocturnal Hemoglobinuria; VT: Venous Thromboembolism; *PIG-A* gene: Phosphatidylinositol Glycan Class-A gene; GPI: Glycosyl-phophatidylinositol; DAF: Decay Accelerating Factor; MIRL: Membrane Inhibitor of Reactive Lysis; SVT: Splanchnic Vein Thrombosis; FLAER: Fluorescent Labeled Inactive toxin Aerolysin; ICCS: International Clinical Cytometry Society

INTRODUCTION

Physicians worldwide treat patients suffering from venous thromboembolism (~1/1000 individuals annually). Thromboembolism is a multifactorial disease, the pathogenesis of which involves both circumstantial and genetic factors [1]. Beyond hereditary thrombophilia, other factors that increase thrombosis risk

Correspondence to: Ioanna Anastasopoulou, Blood Unit and National Reference Center for Congenital and Bleeding Disorders, Laikon General Hospital, Athens, Greece, E-mail: gianastasopoulou@gmail.com

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include ageing, immobilization, surgery, pregnancy, oral contraceptive intake, hormone replacement therapy, and several other clinical conditions (Inflammation, cancer, antiphospholipid syndrome, myeloproliferative diseases, PNH, etc.). The risk of thrombosis is lifelong, thrombotic episodes usually occur when one or more circumstantial risk factors come into play. Hereditary thrombophilia occurs in a high percentage of the patients presenting with thrombosis, the commonest causes of which (~15%-20%) are associated with genetic inherited factors such as Factor V Leiden or FIIG20210A mutation) [2]. Acquired thrombophilia can occur under several clinical circumstances mentioned above [3,4]. PNH is a rare acquired disorder of a pluripotent hematopoietic stem cell, characterized by complement-mediated hemolytic anemia, hemoglobinuria, cytopenia, and predisposition to thrombosis. It is caused by a somatic mutation in the X-linked *PIG-A* gene, whose product is involved in the synthesis of Glycosyl-Phosphatidylinositol (GPI), a glycolipid structure that serves as an anchoring mechanism for cell membrane-bound proteins. Mutation results in complete or partial silencing of the GPI anchor production; thus, a clone of GPI-anchored surface proteins deficient cells arises. Clinically important proteins absent or partially deficient on PNH cells, are the complement regulatory proteins CD55 (DAF) and CD59 (MIRL) as well as other GPI-linked proteins such as CD14, CD16, CD24, etc. Especially the CD59 absence (or deficiency) from erythrocytes surface leads to intravascular hemolysis, hemoglobinuria and anemia [5-9]. Venous thrombosis in PNH is a common disease manifestation (occurring in up to 40% of PNH patients) and a main cause of morbidity [9-13]. Budd-Chiari syndrome is considered a common thrombotic complication and may lead to hepatic failure with a high risk of mortality, while portal vein thrombosis, cerebral vein and sinus thrombosis are also common [11,14]. Arterial thrombotic events occur in a minority of PNH patients but they are not of less mortality risk [15-17]. More than 20% of PNH cases experience recurrence or have been found to present with multiple site thrombosis [18,19]. The high incidence of thrombosis in PNH patients is not completely understood; however several postulations have been proposed to explain this association, such as intravascular hemolysis, nitric oxide depletion, platelet activation, impaired fibrinolysis, etc [12,13,15,20]. Although the clone size is associated with thrombosis risk (large clones increase thrombotic risk) it is not yet established that clone size is the only determinator for thrombosis manifestation and recurrence. The presence of minor PNH clone(s) might indicate a possible risk factor, as thrombotic episodes have been noticed in patients with smaller clones, as well [11,18,21-23]. Investigators suggest that patients with intravascular hemolysis findings and/or cytopenia, those with unexplained thrombotic events especially in unusual sites or those experienced thrombotic episodes while on anticoagulation, should be screened for PNH [15,17,19,24,25]. Flow cytometry is considered the gold standard for PNH investigation, as it can evaluate the GPI-bound proteins' lack, with high sensitivity and specificity [24-26].

As VT is a multifactorial disease, our study's primary objective was to estimate the presence and possible implication of subclinical/minor PNH clone(s) in thrombosis occurrence, in young patients with unexplained thrombotic events, after the exclusion of congenital or acquired thrombophilia.

METHODOLOGY

Patients

Our study's cohort included: a) 181 patients with thrombosis, negative for known thrombophilia screening. Each patient underwent a physical examination, and demographics and medical history were taken. Laboratory investigation included routine blood tests, liver and kidney function tests, thrombophilia screening and JAK2V617F mutation detection for the SVT cases (data not shown). Thrombophilia screening included Antithrombin III, Protein C and S, APC-R, FV Leiden, FII G20210A investigation and APS investigation (LA, anti-cardiolipin and anti-b2GPI IgG/IgM Abs). Patients' cohort consisted of the following sub-populations: Pulmonary Embolism (PE) (n=65), Splanchnic Vein Thrombosis (SVT) (n=31), Vein Thrombosis (Other VT) in classical or in unusual sites (n=49), arterial thrombosis any kind (Arterial) (n=36), and b) 100 healthy controls.

Patients' inclusion criteria

Patients 18-55 years old who attended the Laikon Hospital Thrombosis outpatient Clinique for a 36 months period (11/2016–11/2019), who experienced the first thromboembolic episode at age <40 years old and did not prove to have congenital or acquired thrombophilia.

Patients' exclusion criteria

Patients <18 and >55 years old pregnant women. Women on oral contraceptives or hormone replacement therapy. Cases with a systemic disease known to predispose to acquired thrombosis (cancer, anti-phospholipid syndrome, myeloproliferative disorder, collagen diseases, sickle cell disease, thrombotic thrombocytopenic purpura, etc.). Cases with an already known congenital or acquired thrombophilia and subjects already diagnosed as PNH cases.

Included individuals were tested for the presence of PNH clone(s) by Flow Cytometry (FC). Testing was performed on WBCs (monocytes and neutrophils) using monoclonal antibodies against GPI-linked proteins and Flaer (named as Mono Flaer and Neutro Flaer, respectively), and on RBCs using CD59 and Glycophorin-A (named as Red CD59). Our goal was to study the involvement of minor PNH clones in the occurrence of thrombotic event(s), according to the clinical cut-off values determined by our normal cohort. Four patients with known PNH were also tested as positive controls and for gating purposes (their results were not included in the statistical analysis).

The study was carried out in accordance with the Helsinki Declaration and was approved by the Ethics Committee of our hospital. All included individuals signed informed consent.

Materials

Whole EDTA anticoagulated blood (stored up to 24 hours at 4°C) was used. After staining with monoclonal antibodies, samples were analyzed on a 6-parameter SPACE flow-cytometer by sysmex. Analysis was performed using Flow-max 2.8 software and results were stored in flow-cytometer files.

Ready-to-use monoclonal antibodies labeled with the corresponding fluorochromes were used. More specifically:

Flaer analysis on WBCs

For monocytes analysis: Anti-human CD45-APC (clone H130, Biolegend), anti-human CD14-PE (clone 63D3, Biolegend), anti-human CD33-PeCy5 (clone WM53, Biolegend), Flaer-ALEXA Fluor 488 pro-aerolysin reagent (FL2S-C 50 μg, Cedarlane).

For neutrophils analysis: Anti-human CD45-APC (clone H130, Biolegend), anti-human CD24-PE (clone ML5, Biolegend), anti-

human CD15-PeCy5 (clone W6D3, Biolegend), Flaer-ALEXA Fluor 488 pro-aerolysin reagent (FL2S-C 50 μg, Cedarlane).

CD59 expression on RBCs

Anti-human (glycophorin-A) CD235ab-FITC (clone HIR2, isotype: mouse IgG2b, Biolegend), anti-human CD59-PE (clone p282-H19, Biolegend).

Sample preparation

Monocyte/Neutrophils analysis with Flaer (WBCs analysis): Staining in 100 μl whole blood, 5 μl CD45-APC, 5 μl CD14-PE, 5 μl CD33-PeCy5 and 5 μl Flaer-ALEXA for monocytes (Tube 1) or 5 μl CD45-APC, 5 μl CD24-PE, 5 μl CD15-PeCy5 and 5 μl Flaer-ALEXA for neutrophils (Tube 2), were added. Tubes were incubated 15 min at RT in the dark and red cell lyses (using Quicklysis by Cytognos) followed. After incubation 10 min in the dark at RT, analysis on the flow cytometer was performed.

CD59 expression analysis in erythrocytes (RBCs analysis): Dilution of the original sample 200x in sodium chloride solution. Preparation of the CD235ab-FITC working solution 1:40 in NaCl 0.9%. Staining 20 μl diluted sample plus 2 μl of the CD235ab-FITC working solution plus 10 μl CD59-PE (Tube 3). Samples were incubated 15 min at RT in the dark and then washed (twice), in order to eliminate excess fluorochrome and nonspecific binding. After the addition of 1ml sodium chloride solution, analysis on the flow cytometer was performed.

Negative controls: Negative controls were prepared in order to verify compensation settings and delineate the analysis populations in each series of analyzes. More specifically, for WBCs assay: preparation of two tubes in which the monocytes/neutrophils protocol was followed without the addition of Flaer, and for RBCs: preparation of a tube in which the CD59 expression protocol was followed, without the addition of CD59-PE.

Gating

WBCs analysis: Analysis of 100,000 cells. Monocyte delimitation as CD33-PeCy5 positive/intermediate SSC and neutrophils delimitation as CD15-PeCy5 positive/high SSC. Quantification of monocytes negative for CD14-PE/Flaer-ALEXA (PNH clone) and neutrophils negative for CD24-PE/Flaer-ALEXA (PNH clone) (Figure 1).

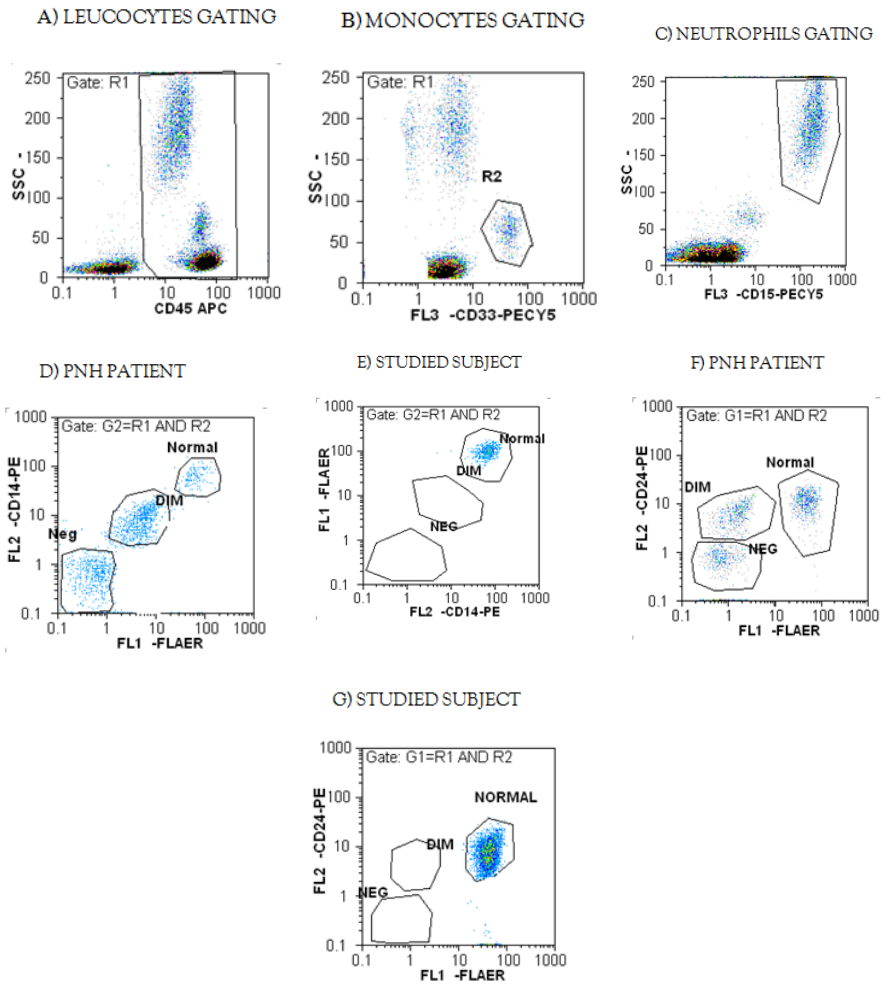

Figure 1: Flow-cytometry analysis on WBCs. (A) Leucocytes gating based on CD45. (B) Monocytes gating based on CD33 and scattering characteristics of the target cells. (C) Neutrophils gating based on CD15 and scattering characteristics of the target cells. (D) Monocytes clone analysis in a PNH patient. (E) Monocytes clone analysis in a studied subject. (F) Neutrophils clone analysis in a PNH patient. (G) Neutrophils clone analysis in a studied subject.

RBCs analysis: Analysis of 100,000 cells in the gating of red cells (CD235ab-FITC positive cells). Quantification of erythrocytes as CD59-PE negative (PNH type-III), CD59-PE dim (PNH type-II) and CD59-PE positive (normal/type-I) (Figure 2). The developed methodology concerning analysis and delimitation strategy was based on ICCS guidelines [25]. Possible subclinical PNH clones in neutrophils and monocytes were identified with side scatter (CD15 for neutrophils and CD33 for monocytes), as well as the non-expression of a leukocyte index in combination with Flaer (CD24- for neutrophils and CD14- for monocytes).

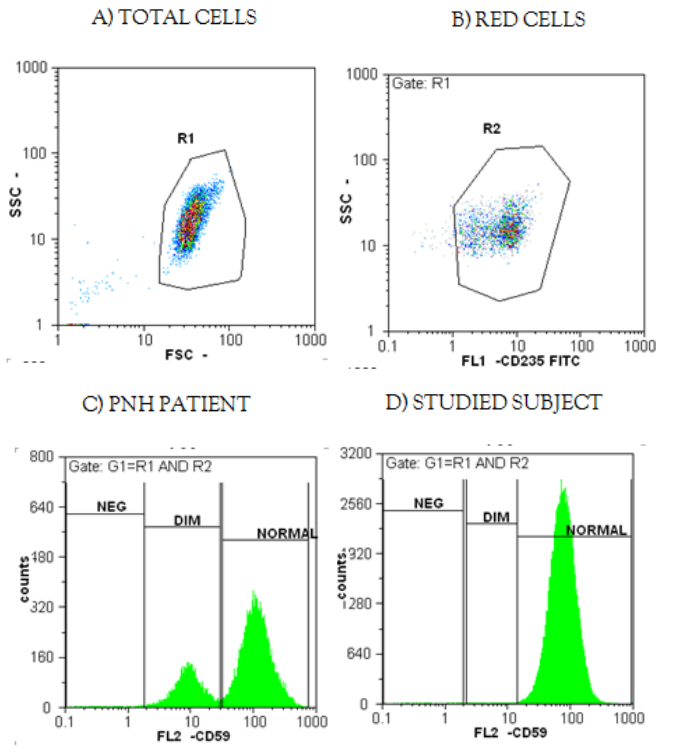

Figure 2: Flow-cytometry analysis on RBCs. (A) Total cells gated. (B) Red cells analyzed. (C) PNH clone analysis on red cells (CD59) in a PNH patient. (D) PNH clone analysis on red cells (CD59) in a studied subject.

Statistical analysis

Statistical analysis was focused on the presence of subclinical PNH clone(s) in both WBCs and RBCs, as mentioned above. Evaluation of minor PNH clones and their size was done according to clinical cut-off values (mean+2SD), determined by the normal individuals' values (N=100), tested in parallel with patients' samples. Continuous variables are presented with mean and standard deviation or median Interquartile Range (IQR), while qualitative variables are presented with absolute and relative frequencies. For the comparisons of proportions, chi-square and Fisher's exact tests were used. Normal distributed variables were compared between the study groups using Student's t-test, while Mann-Whitney test was used for the comparison of non-normal variables between different patient groups. Logistic regression analysis was used in order to find independent factors associated with recurrence in the group of patients with thrombosis. Adjusted Odds Ratios (OR) with 95% Confidence Intervals 95% (CI) was computed from the results of the logistic regression analyses. All p values reported are two-tailed. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 22.0).

RESULTS

In order to study correlations between the populations tested, the participants' cohort was divided into two groups:

a) Group-1 (2 populations): patients in total vs. normal controls,

b) Group-2 (5 populations): patients' subpopulations separated by thrombosis event type (PE, SVT, Other VT, and Arterial) vs. normal controls.

Although it is known by literature that typical PNH flow cut-offs are set at 0.01% for red cells and neutrophils, our cut-off values did not reach such low levels [12,25,27-29]. For monocytes, our cut-off values were in agreement with the literature, in which it is reported that, due to the low number of events that can be acquired, cut-off values range around 0.1% [27,28]. Thus, cut-off values determined by our normal cohort were evaluated as follows; for WBCs, monocytes=0.099%, (mean=0.029, SD=0.035), neutrophils=0.073% (mean=0.025, SD=0.024); and for RBCs, CD59=0.095%, (mean=0.037, SD=0.029). None of the samples tested showed a population with clear PNH clone in WBCs or RBCs (as expected), except the positive controls whose results were not included in the statistical analysis.

Participants' demographics and clinical characteristics are presented in Table 1. Patients' mean age was 41.0 yrs (SD=10.4) and controls' 41.2 yrs (SD=10.1), while the majority of the participants were men, shows the comparison of flow-cytometry indices values in group-1 (patients in total vs. controls) (Table 2). Statistically significant lower Mono Flaer, Neutro Flaer and Red CD59 values were found in normal subjects as compared to patients with thrombosis (p=0.015; p=0.008 and p=0.002, respectively).

Table 1: Demographics and clinical characteristics of the studied cohorts.

	Patients (N=181)	Controls (N=100)	
	N (%)	N (%)	P
Gender			
Men	103 (56.9)	66 (66.0)	0.136+
Women	78 (43.1)	34 (34.0)	-
Age at check time, mean (SD)	41.0 (10.4)	41.2 (10.1)	0.876++
Thrombosis event			
PE	65 (35.9)	-	-
SVT	31 (17.1)	-	-
Other VT	49 (27.1)	-	-
Arterial	36 (19.9)	-	-
Age at 1st event mean (SD)	37 (9.9)	-	-
Multiple site thrombosis	58 (32.0)	-	-
Recurrence	91 (50.3)	-	-
Family history	56 (31.3)	-	-
Co-morbidities			
Obesity	5 (2.8)	-	-
Dyslipidemia	6 (3.3)	-	-
Diabetes	2 (1.1)	-	-
Hypertension	10 (5.5)	-	-
Varicose veins	8 (4.4)	-	-
Lower limbs valves deficiency	2 (1.1)	-	-
Sjögren syndrome	3 (1.7)	-	-
Rheumatoid arthritis	1 (0.6)	-	-
Vasculitis	1 (0.6)	-	-
Thrombotic event types per subpopulation			
PE (N=65)	65/65 (100)	-	-

SVT (N=31)	Mesenteric vein	8/31	(25.8)	-	-
	Portal vein	15/31	(48.4)		
	S. Budd-Chiari	2/31	(6.5)		
	Splenic vein	6/31	(19.4)		
	Lower extremities	30/49	(61.2)		
Other VT (N=49)	Upper extremities	3/49	(6.1)	-	-
	Retinal vein occlusion	4/49	(8.2)		
	Iliofemoral vein	7/49	(14.3)		
	Inferior vena cava	3/49	(6.1)		
	Jugular vein	1/49	(2.0)		
	Subclavian vein	1/49	(2.0)		
Arterial (N=36)	Acute limb ischemia	3/36	(8.3)	-	-
	Coronary heart disease/MI	12/36	(33.3)		
	Abdominal aorta	1/36	(2.8)		
	Renal artery	2/36	(5.6)		
	Vertebral artery	2/36	(5.6)		
	Ophthalmic artery	4/36	(11.1)		
	Stroke	12/36	(33.3)		

Note: +Pearson's chi-square test; ++Student's t-test

Table 2: Comparison of flow cytometry indices values between the two studied populations.

	Group-1	Mean (SD)	Median (Range)	P+
Mono Flaer	Patients in total	0.08 (0.14)	0 (0-0.09)	0.015
	Controls	0.03 (0.04)	0 (0-0.06)	
Neutro Flaer	Patients in total	0.04 (0.06)	0.03 (0.01-0.06)	0.008
	Controls	0.02 (0.02)	0.02 (0.01-0.04)	
Red CD59	Patients in total	0.11 (0.21)	0.04 (0.02-0.13)	0.002
	Controls	0.04 (0.03)	0.03 (0.02-0.05)	

Note: +Pearson's chi-square test.

Appearance and allocation of results values above cut-off levels per subpopulation and patients in total, for all three flow cytometry indices, are shown in Table 3. Overall, 24.9% of the patients were found with Mono Flaer values >0.099, 13.8% with Neutro Flaer >0.073, and 28.7% presented with Red CD59 >0.095. Higher values at Mono Flaer were noticed in SVT cases (38.7%), while in the Arterial sub-cohort higher values were noticed at both Mono Flaer and Red CD59 (30.6% and 33.3%, respectively). In the Other VT sub-cohort a higher percentage was found at Red CD59 (36.7%).

Table 3: Results above cut-off levels for different subpopulations and patients in total.

	Mono Flaer		Neutro Flaer		Red CD59	
	N	%	N	%	N	%
PE (N=65)	13	20	8	12.3	15	23.1
SVT (N=31)	12	38.7	4	12.9	7	22.6
Other VT (N=49)	9	18.4	5	10.2	18	36.7
Arterial (N=36)	11	30.6	8	22.2	12	33.3
Patients in total (N=181)	45	24.9	25	13.8	52	28.7

In both groups (patients in total vs. controls and sub-cohorts vs. controls), patients' percentages with Mono Flaer >0.099 and Neutro Flaer >0.073 were significantly higher compared to the correspondent percentages of the controls values (Table 4). Similarly, percentages of patients with Red CD59 >0.095 were significantly higher compared to the correspondent percentages of the controls (p=0.001) in both groups (Table 5).

In general, in the patients' cohort, 10 subjects out of 181 (5.5%) were revealed with minor PNH clone in both cell lines (WBCs and RBCs). The majority of the cases revealed with a minor PNH clone at monocytes and CD59 (5 cases, 2.8%), while the relevant percentages for minor PNH clones at neutrophils and CD59 or at all three indices (monocytes, neutrophils and CD59), were 1.1% (2 cases) and 1.7% (3 cases), respectively. More specifically for PE sub-cohort there was one case (1.5%) with a minor PNH clone (WBC/monocytes and RBC/CD59). For the SVT sub-cohort three cases (9.7%) were found (2 at WBC/monocytes and RBC/CD59, 6.5% and 1 at WBC/monocytes-neutrophils and RBC/CD59, 3.2%). For Other VTs there were two cases (4.1%, 1 in WBC/monocytes and RBC/CD59 and 1 at WBC/monocytes-neutrophils and RBC/CD59), while the Arterial subgroup revealed with four samples with minor clones (11.1%, 1 for WBC/monocytes and RBC/CD59, 2 for WBC/neutrophils and RBC/CD59 and 1 for WBC/monocytes-neutrophils and RBC/CD59).

In order to identify independent risk factors related to recurrence in the patients' cohort, a logistic regression analysis was performed and Odds Ratios (95% CI) were obtained. Logistic regression analysis for patients' recurrence was based on their demographics and personal history, as well as on their initial thrombotic event type. Recurrence rates varied significantly depending on thrombosis type and positive family history. According to our data, thrombotic event type and positive family history were independently related to the presence of recurrence. Tested flow cytometry indices and the presence of minor PNH clone(s) were not predictive of recurrence (Table 6).

Table 4: Participants' values in WBCs for each group, according to cut-off levels.

		WBCs									
		Mono Flaer					Neutro Flaer				
		<0.099		>0.099		P Pearson's × 2	<0.073		>0.073		P Pearson's × 2
		N	%	N	%		N	%	N	%	
Group -1	Patients in total	136	75.1	45	24.9	<0.001	156	86.2	25	13.8	0.01
	Controls	95	95	5	5		96	96	4	4	
Group -2	PE	52	80	13	20	<0.001	57	87.7	8	12.3	0.026+
	SVT	19	61.3	12	38.7		27	87.1	4	12.9	
	Other VT	40	81.6	9	18.4		44	89.8	5	10.2	
	Arterial	25	69.4	11	30.6		28	77.8	8	22.2	
	Controls	95	95	5	5		96	96	4	4	

Note: + Fisher's exact test.

Table 5: Participants' values in RBCs for each group, according to cut-off levels.

		Red CD59				P Pearson's × 2 test
		<0.095		>0.095		
		N	%	N	%	
Group- 1	Patients in total	129	71.3	52	28.7	<0.001
	Controls	99	99	1	1	
Group-2	PE	50	76.9	15	23.1	<0.001
	SVT	24	77.4	7	22.6	
	Other VT	31	63.3	18	36.7	
	Arterial	24	66.7	12	33.3	
	Controls	99	99	1	1	

Table 6: Multiple logistic regression analysis in patients' cohort (dependent variable: recurrence).

		OR (95% CI)+	P
Thrombosis Event	SVT (reference)		
	PE	2.24 (0.56-8.98)	0.255
	Other VT	7.27 (1.98-26.74)	0.003
	Arterial	3.11 (0.75-12.88)	0.117
Gender	Male (reference)		
	Female	0.94 (0.41-2.14)	0.876
Age at 1st event		1 (0.96-1.04)	0.989
Family history	No (reference)		
	Yes	2.49 (1.02-6.05)	0.044
Presence of rare cells with PNH phenotype	No (reference)		
	Yes	1.38 (0.5-3.81)	0.531
Flow cytometry indices	Mono Flaer	1.12 (0.05-25.41)	0.941
	Neutro Flaer	14.55 (0.02-9018.02)	0.414
	Red CD59	0.72 (0.07-7.08)	0.778

Note: +Odds Ratio (95%Confidence Interval).

DISCUSSION

The existence of PNH clone(s) represents a certain risk factor for thrombosis. In many PNH patients, venous thrombosis (especially in abdominal or intracranial veins) is an important complication with mortality of about 30%. The prothrombotic state in PNH is extremely complex, with many different factors involved (platelet and endothelial cell activation, intravascular lysis, etc.); patients present with an activated coagulation system even in the absence of thrombosis. Several studies have shown that the probability of a thromboembolic event in patients with PNH disease is directly related to PNH clone size [11,21,22]; it has been reported that a 10% increase in PNH clones corresponds to a 1.64 times higher risk for thromboembolic event[23]. However, some patients with small clone size were found to have developed thrombosis, which suggests that clone size is not the only risk factor for thrombosis, but it may be involved in the onset of an episode.

To our knowledge, for individuals with a confirmed thromboembolic event, a study by Lazo-Langer et al. in 2015 has been published, concerning a large number of patients (n=388, mean age=57 yrs) with at least one episode of demonstrated DVT or PE and including patients with risk factors for VTE such as thrombophilia, oral contraceptive intake and hormone replacement therapy [30]. The presence of minor PNH clones was evaluated in neutrophils and red cells, using the typical modern flow cut-off values of 0.01%. Our population consisted of 181 younger individuals (mean age: 40 yrs), with unexplained thrombosis events and no evidence of congenital or acquired thrombophilia or other risk factors for VTE. Patients' cohort was separated according to thrombosis event type, including PE cases, splanchnic vein thrombosis events, VT cases in classical and unusual sites and arterial thrombotic events. We evaluated the presence of minor PNH clones in neutrophils, monocytes and red cells. The purpose of our study was to investigate whether the presence of these clones might be critical for an episode occurrence and if so, in which of the above four thrombotic types mentioned, these minor clones appear more often.

According to ICCS guidelines, PNH clones are characterized by size values >1%; in our cohort, no type-III/II clones were identified, however, given the hypothesis of the possible involvement of even smaller clones in the occurrence of thromboembolic events, an

attempt to evaluate their cut-off values was made [25,27]. The percentage of individuals with Mono Flaer ≥ 0.099% differed significantly between the group-1 populations (p=0.001), with higher values being in the patients' cohort. A significant difference was also noticed between group-2 subpopulations (p=0.001), with a higher percentage being noticed in SVT and Arterial patients. Similarly, in neutrophils a statistical significance was noticed in both groups, with the patients' cohort presenting with higher values compared to healthy controls (p=0.010 for group-1 and p=0.026 for group-2). The Arterial sub-cohort revealed with the higher values.

It has been reported that a variation in the size of PNH clones is observed between monocytes and neutrophils in some cases [27,28]. Clone size in neutrophils has been traditionally considered a better index, Borowitz et.al state that monocytes evaluation provides confirmatory information; however, following references claim that both neutrophils and monocytes lineages should be checked, as in some cases monocytes reflect clone size more accurately than neutrophils [25,27-29]. The majority of the cases present with larger PNH clones in monocytes, data not completely understood on a pathophysiology base (probably due to stimulation, lifespan, etc.) [27]. It is known that the results that should be taken into account are those appearing with a clone in both cell lines (WBCs and RBCs) [25,27-29]. In our study, 10/181 patients presented with a small clone in both WBCs and RBCs, with the majority including small clone(s) at monocytes and RBCs, which might be an explanation for the high difference noticed between our results and Lazo-Langer's study results, in which only one case was found with a minor clone in both WBCs and RBCs (testing was performed on neutrophils and RBCs) [30].

Although individuals with higher values than the cut-off in only one index are not considered to carry a PNH clone, we have noticed that in the SVT sub-cohort there was a high percentage of such values at monocytes (38.7%) and in the Other VT and Arterial cases at RBC/CD59 (36.7% and 33.3%, respectively). The Arterial subgroup showed a high percentage of higher values at monocytes (30.6%), as well. The question to be answered was, whether these results could be used as an 'alert' index for a possible future thromboembolic event. The hypothesis should be assessed by screening a larger number of individuals and a follow-up study. Re-assessment of participants might prove useful for a possible appearance of a new minor clone in the future. It is known that as clone size changes over time, in patients with absence of clinical manifestations and small PNH clones, monitoring over time is required, while in patients with larger clones size and clinical symptoms, testing should be performed twice a year [17,28]. On the other hand, clone size might decrease or become undetectable, so in cases of clinical manifestations' deterioration (i.e. hemolysis or thromboembolic event), immediate testing is recommended [12,17,25]. According to Manivannan et al., "the monitoring is essential because patients with very small clone size are followed with observation alone" [12]. Taking into consideration that minor PNH clones are more common in bone marrow failure (AA/MDS) with no predominant hemolysis [20,27,31,32]. A follow-up at defined intervals should be carried out in 'suspicious cases', in order to rule out an abnormality due to clonal expansion [15,25]. Shen et al., in their attempt to clarify the clonal expansion mechanism and the distinct behavior of PNH clones, assumed that additional somatic mutations of an ancestral or a facilitating nature may be involved [33]. No mutational analysis was performed in our samples; however, additional somatic mutations could be an explanation for the small clones' existence in our cohort, if their persistence will be confirmed by follow-up. Furthermore, as it has been shown that *PIG-A* mutations were detected in healthy individuals at a frequency of approximately 0.002% (mutations that appear to arise from colony-forming cells rather than from stem cells), the question that arises is if our results reflect the prevalence of transient minor PNH clones in the general population and if these clones have an impact on thrombotic risk. Repeating PNH flow cytometry testing might help to ascertain the persistence of rare populations of PNH cells [16,33,34].

The occurrence of thromboembolic events consists a major problem for the general population, while the absolute risk of venous thrombosis ranges at 1%-2% per year and increases with age. The risk of recurrence after the first thromboembolic event is about 5%-7%; patients who have already experienced thromboembolism appear with a higher risk of developing a new one, regardless of the time passed since the first onset, although the recurrence risk (especially for anticoagulated patients) decreases over time [3,35]. In our patients' cohort, results from multiple logistic regression analysis, with recurrence as the dependent variable, indicated that thrombotic event type and positive family history were found to be independently related to recurrence. The proportion of patients with recurrent episodes varied significantly depending on the type of the initial thrombotic event. According to our data, the Other VT cases were found 7.27 times more likely to recurrent than the SVT ones (p=0.003), while cases with positive family history were more likely (2.49 times, p=0.044) to experience recurrence than those with no family history. Concerning the cases noticed with minor clone(s) at both cell lines in our study, the issue raised was whether these individuals were more likely to experience VTE recurrence than the rest ones, although the majority of our patients are on lifelong anticoagulation. No thrombosis recurrence has been reported in these patients since the beginning of the study. Tested flow cytometry indices and the presence of minor PNH clone(s) were not predictive of recurrence.

Study limitations

This is a retrospective study. Patients' follow-up is ongoing. A limited number of individuals were checked in this study. In particular, SVT and Arterial cohorts consisted of small patient numbers. A larger number of tested subjects are needed for more reliable results to be obtained.

Concerning the monoclonal antibodies used, the literature extensively mentions the use of CD64 for better monocyte gating, however, in the present study monocytes' delineation was based upon the use of CD33 and scattering characteristics of the target cells.

In the populations checked, complement pathway proteins, in order to clarify possible complement activation even if no clear PNH clone was observed, were not tested.

CONCLUSION

To our opinion, screening for PNH clones presence should be performed in patients with suspected PNH disease, as well as, in young individuals with unexplained thrombosis or thrombosis at unusual sites, although from our study it is not concluded that the parameters tested are associated with disease prediction or the appearance of a future thrombosis.

None of the parameters tested nor the presence of subclinical clone(s) appears to have any effect on the likelihood of recurrence.

An unresolved issue is whether in patients with no PNH clone presence or those with a minor clone appearance in the first check-up, a follow-up should be carried out, especially in recurrent thrombotic cases.

According to our data, the above question has mainly to do with patients who have experienced arterial thrombosis and splanchnic vein thrombosis, as higher percentages of small PNH clones in both cell lines were noticed in these two sub-cohorts.

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