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Study of Anticoagulant, Procoagulant, and Fibrinolytic Pathways in Mexican Patients With Paroxysmal Nocturnal Hemoglobinuria

Aldo A. Scherling-Ocampo, Ángel G. Vargas-Ruíz and Xavier López-Karpovitch*

Department of Surgery Hematology and Oncology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico

ABSTRACT

Background: In Mexico, the frequency of thromboembolic events associated to paroxysmal nocturnal hemoglobinuria is 3%; a clone size > 50% in granulocytes has been associated with a higher risk of thromboembolic events. **Methods:** Between 2001 and 2012, 40 patients with paroxysmal nocturnal hemoglobinuria were studied. In 12 cases anticoagulant, procoagulant, and fibrinolytic pathways were analyzed. **Results:** Only two of 40 patients (5%) developed a thromboembolic event over a 25.5-year follow-up period. From 12 patients, 91.7% had a paroxysmal nocturnal hemoglobinuria clone > 50% in granulocytes and 83.3% a clone > 50 % in monocytes. Five of 12 cases had elevated FV levels and four showed increased FVIII, von Willebrand factor antigen, von Willebrand factor ristocetin cofactor activity and FX. Protein S and protein C were decreased in nine and three patients, respectively. Only antithrombin correlated positively with paroxysmal nocturnal hemoglobinuria clone size in monocytes ($p = 0.0442$), whereas von Willebrand factor ristocetin cofactor correlated negatively with lactic dehydrogenase levels ($p = 0.0186$). No statistically significant associations were recorded with all other factors. **Conclusion:** The low frequency of thromboembolic events in Mexican patients could partly be explained by the associations between anticoagulant system (antithrombin) with paroxysmal nocturnal hemoglobinuria monocyte clone size, and procoagulant system (von Willebrand factor ristocetin cofactor) with lactic dehydrogenase levels. (REV INVES CLIN. 2015;67:350-6)

Key words: Paroxysmal nocturnal hemoglobinuria. Clone size. Hemolysis. Coagulation. Thrombosis.

Corresponding author:
*Xavier López-Karpovitch
Department of Surgery Hematology and Oncology
Instituto Nacional de Ciencias Médicas y Nutrición
Salvador Zubirán
Vasco de Quiroga, 15
Col. Sección XVI, Del. Tlalpan, C.P. 14000
Ciudad de México, México
E-mail: xlopezk@gmail.com

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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired somatic abnormality of the phosphatidylinositol glycan class A gene (*PIG-A*), localized in the X chromosome, and involved in the synthesis of glycosylphosphatidylinositol (GPI) on hematopoietic cell membranes[1].

Thromboembolic events (TEE) are the main cause of death associated to PNH[2,3]. Possible involved mechanisms include: a suppressed fibrinolytic system, increased leukocyte-derived tissue factor[4], complement-mediated platelet and endothelial injury, platelet activation[5,6], endothelium or platelet-derived microparticles[7,8], free hemoglobin, bacteria and food antigens in the splanchnic circulation, decreased tissue factor pathway inhibitor (TFPI), and the size of the PNH clone[3].

The risk of TEE has been shown to be increased in cases bearing large (> 50%) granulocyte PNH clones, with a TEE 10-year incidence of 44% compared to 5.8% in patients with smaller clones[3].

In Mexico, patients do not have a high TEE rate compared to that reported in the international literature (up to 44%)[9]. Only 3% of Mexican PNH patients develop this complication[10]. Although there are studies that have analyzed different coagulation factors in PNH patients[11-13], to date there is no study in Mexico evaluating them. Thus, in the current study the clinical characteristics as well as the anticoagulant, procoagulant, and fibrinolytic profiles in a Mexican population were analyzed and correlated with PNH clone size in granulocytes and monocytes.

PATIENTS AND METHODS

Patients

Patients with PNH registered at our institute between January 2001 and December 2012 were included in the study. The diagnosis was established by flow cytometry in accordance with the guidelines for the diagnosis and monitoring of PNH (Borowitz, et al.)[14]. Patients were classified as having classic PNH, PNH in the setting of another specified bone marrow disorder, or subclinical (PNH-sc)[1]. Patients with comorbidities requiring anticoagulation as a result of a disease other than PNH (i.e., atrial fibrillation, primary thrombophilia) were excluded. No patient with PNH in whom coagulation studies were performed was under anticoagulation therapy.

Hemolysis evaluation

The presence of hemolysis was evaluated according to lactic dehydrogenase (LDH) values and was established with values ≥ 1.5-fold the upper limit of normal (ULN).

Flow cytometry

The diagnosis and evaluation of the granulocyte and monocyte PNH clone was conducted in samples obtained in ethylenediamine tetraacetic acid as per the guidelines for the diagnosis and monitoring of PNH[14]. A Becton Dickinson FACSCanto™ II eight color flow cytometer was used. At least 5,000 events were analyzed to obtain a sensitivity of at least 1% in the detection of PNH clones.

Procoagulant, anticoagulant, and fibrinolytic assays

Patient procoagulant profiles were evaluated by measuring the levels of fibrinogen (Fib), FV, FVIII, von Willebrand factor antigen (vWF:Ag), von Willebrand factor ristocetin cofactor activity (vWF:RCo), FIX, FX, and FXI. The anticoagulant factors measured were functional protein C (PC) activity, functional protein S (PS) activity, and antithrombin (AT). The fibrinolytic profile included D-dimer levels (DD), euglobulin lysis time (EL), plasminogen (Pg), plasminogen activator inhibitor-1 (PAI-1), and alpha 2-antiplasmin (AP).

Fibrinogen was quantified by using the modified Clauss method (Multifibren® U, Siemens), while the activity of factors V, VIII, IX, X, and XI was evaluated with a coagulometric method (Coagulation Deficient Plasma for each evaluated factor, Siemens) in a BCS® XP coagulometer (Siemens).

The vWF:Ag was quantified with an immunoturbidimetric method (vWF Ag, Siemens) and vWF:RCo, by turbidimetry (BC von Willebrand Reagent, Siemens). Anticoagulant pathway evaluation included measuring PC and AT functional activity using chromogenic substrates (Berichrom® Protein C and Berichrom® Antithrombin III [AT], respectively, both by Siemens). Functional PS was measured by coagulometry (Protein S Ac, Siemens).

Levels of Plg, PAI-1, and AP were determined with a chromogenic method (Berichrom Plasminogen, Berichrom PAI, and Berichrom alpha$_2$-Antiplasmin, respectively, all by Siemens). Levels of DD were measured by immunoturbidimetry (INNOVANCE® D-Dimer, Siemens) and EL was determined manually.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured with the coagulometric method (Dade® Innovin® and Dade® Actin® FSL Activated PTT Reagent, respectively, by Siemens).

Statistical analysis

This was an observational, analytic, retrospective, cohort study. Spearman's correlation coefficient was used to determine the correlation between procoagulant, anticoagulant, and fibrinolytic profiles with the PNH clone size in granulocytes and monocytes. The same test was used to analyze the correlation between LDH levels with the PNH clone size and the coagulation profiles. Statistical significance was reached with a type I error < 0.05. The STATA® v11.0 program was used.

Table 1. Demographic characteristics and laboratory data at diagnosis and follow-up in the 2001 to 2012 cohort

Variable	N
Gender, female/male	16/24
Age in years, median (range)	30.5 (10-76)
Follow-up in months	85 (0-306)
PNH type	
– Classic	5
– In the setting of another specified bone marrow disorder	9
– Subclinical	7
– Unclassifiable	19
Reticulocyte index (%)	3.3 (0.02-14.7)
– Clinical	5.1 (0.02-14.7)
– Subclinical	1.8 (0.63-3.25)
Hemoglobin g/dl	7.8 (2.4-17.2)
Neutrophils 10^9/l	1.6 (0.36-13.6)
Platelets 10^9/l	86 (2-271)
Lactic dehydrogenase u/l	1,371 (174-3,698)
Patients with granulocyte-deficient PNH clone > 50%	25/38
Patients with monocyte-deficient PNH clone > 50%	29-40
Patients with thrombosis	2
Deaths	2

PNH: paroxysmal nocturnal hemoglobinuria.

RESULTS

During the study period, the PNH registry included 50 patients. Ten patients were excluded since information on clone size was lacking. Of the remaining 40 cases, 16 (40%) were women, and the median age was 30.5 years. Regarding the presentation, 14 (35%) had a classical presentation or in the setting of another specified bone marrow disorder and seven patients had PNH-sc (17.5%). Nineteen cases (47.5%) were not classified since data concerning bone marrow aspirate, bone biopsy, or cytogenetic analysis were not available. Table 1 shows the demographic, clinical, and laboratory characteristics of the 40 patients.

Follow-up

Only 18 of 40 patients were in follow-up at the time of data analysis. In 12 of them (66.67%), assays to establish procoagulant, anticoagulant, and fibrinolytic profiles were performed (Table 2). None of the 12 patients developed a TEE during follow-up. Eleven patients (91.7%) had a PNH granulocyte clone size > 50%. In monocytes, clone size was > 50% in 10 cases (83.3%). In relation to the procoagulant profile, FV and FX were increased in five patients (41.7%) and FVIII, vWF:Ag, and vWF:RCo were also high in four patients (33.3%). Increment of all these parameters, except for FX, was recorded in only one patient (No. 5). In the remaining study population, a maximum of three parameters were simultaneously increased in any given patient. Analysis of the anticoagulant profile revealed that PS and PC levels were decreased in nine (75%) and three (25%) patients, respectively. The AT was slightly elevated in only two patients (Nos. 2 and 6). Regarding the tests evaluating fibrinolysis, DD and AP were increased in two and in one patient, respectively.

As shown in table 3, no association was established between the size of the clone in granulocytes and results of procoagulant, anticoagulant, and fibrinolytic assays. Also, there was no correlation between the size of the clone in monocytes and the procoagulant and fibrinolytic profiles. However, in the anticoagulant profile, a significant positive correlation was established between AT and the clone size in monocytes (Spearman rho = 0.6147; p = 0.0442) (Table 4).

Table 2. Procoagulant, anticoagulant, and fibrinolytic profiles in a selected cohort of patients

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12
Variable												
Sex/age	F/73	M/16	M/39	F/56	M/36	F/33	F/24	F/54	M/21	M/10	F/21	F/24
G clone size (> 50 %)	37	**99**	**85**	**93**	**98**	**99**	**93**	**86**	**99**	**99**	**69**	**96**
M clone size (> 50 %)	**59**	**100**	**87**	**97**	49	**100**	**93**	**89**	**100**	40	**96**	**74**
LDH (109-197 IU/l)	**242**	**3240**	**2124**	**2281**	**1397**	**1183**	**5077**	**3463**	**3845**	**539**	192	**2579**
Ret (0.5-2.5%)	2.03	**10**	**4.09**	**6.1**	**4.42**	1.82	**24.5**	2.17	0.02	**5.01**	**5.08**	NA
Plat (150-450 10^9/l)	**11**	**42**	**7**	157	**2**	**5**	150	**86**	**112**	207	**32**	154
PT (9.8-11.1 sec)	11.3	10.2	11.5	11.1	10.8	17.6	11.0	10.7	11.5	13.1	11.4	10.4
PTT (24.5-30.8 sec)	34.4	20.9	28.5	23.5	27.1	29.1	30.4	28.5	25.5	27.4	21.1	22.2
Fib (238-508 mg/dl)	456	483	282	**199**	245	494	**213**	321	**200**	**235**	319	323
FV (70-120%)	**121**	**138**	112	108	**128**	89	92	117	111	78	**125**	**132**
FVIII (60-150%)	148	**228**	126	**159**	**187**	**314**	101	135	118	146	130	147
VWF: Ag (50-160%)	**287**	160	**180**	134	**304**	**384**	92	110	97	109	115	107
VWF: RCo (50-150 %)	**290**	141	**216**	122	**249**	**266**	79	94	90	108	137	98
FIX 60-150%)	106	**185**	102	118	117	107	94	126	108	101	107	142
FX (70-120%)	91	**147**	97	**131**	111	**41**	95	109	103	94	**127**	**136**
FXI (60-140%)	81	**149**	88	96	81	**143**	74	108	93	74	106	110
PC (70-140%)	**64**	136	**63**	119	94	**64**	113	105	109	80	79	125
AT (75-125%)	109	**135**	114	109	87	**130**	85	104	114	108	86	109
PS (70-123%)	91	**66**	**64**	**30**	83	**50**	**35**	**37**	**61**	**62**	89	**38**
DD (50-334 mg/l)	269	204	**49**	305	**370**	298	172	258	256	250	**480**	236
EL (neg clot lysis > 2 hours)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Plg (75-140%)	125	133	93	118	107	123	100	115	103	99	112	117
PAI-1 (2.0-7.0 IU/ml)	4.9	3.9	4.8	3.4	5.2	5.0	4.1	4.1	3.9	3.8	3.9	4.1
AP (80-120%)	93	106	105	118	100	111	108	104	110	**124**	111	120

Numbers in parenthesis indicate reference values. **Bold** type indicates abnormal results. G: granulocytes; M: monocytes; LDH: lactic dehydrogenase: Ret: reticulocytes corrected by hematocrit; Plat: platelets; PT: prothrombin time; PTT: partial thromboplastin time; Fib: fibrinogen; vWF:Ag: von Willebrand factor antigen; vWF:RCo: ristocetin cofactor activity; PC: protein C; AT: antithrombin; PS: protein S; DD: D-dimer; EL: euglobulin lysis; Plg: plasminogen; PAI-1: plasminogen activator inhibitor-1; AP: alpha 2-antiplasmin; NA: not available.

Lactic dehydrogenase

Lactic dehydrogenase levels were correlated with all coagulation parameters, revealing a significant inverse association only with vWF:RCo (Spearman rho = –0.7212; p = 0.0186) (Table 5). In the 40 patients, LDH values also correlated directly and significantly with the clone size in granulocytes (Spearman rho = 0.3906; p = 0.0204) and in monocytes (correlation 0.4618; p = 0.0052) (data not shown).

Thromboembolic events and related manifestations

In the 40-patient cohort (Table 1), two men (5%) developed TEE. In both cases, PNH could not be classified although both had clinical and laboratory data of hemolysis. One of them, aged 49 years, had an ischemic stroke 73 months after the diagnosis of PNH. The second patient was 25 years old when he developed his first TEE, also manifested as an ischemic stroke (initial manifestation of the disease). Three years later this patient presented Budd-Chiari syndrome. Coagulation or fibrinolysis profiles were not available in either patient.

Cytogenetics

The karyotype was obtained in only six of the 12 patients and it was normal in all cases.

Mortality

In relation to both deaths, one was a result of community-acquired pneumonia in an 81-year-old man after a follow-up period of 247 months, and the other was due to a subarachnoid hemorrhage in a 76-year-old

Table 3. Correlation between paroxysmal nocturnal hemoglobinuria clone size in granulocytes and coagulation data in a selected cohort of patients (n = 12)

Variable	Spearman's rho	p value
Fib	–0.0280	0.93
FV	–0.2937	0.35
FVIII	0.3566	0.25
vWF:Ag	–0.0280	0.93
vWF:RCo	–0.1189	0.71
FIX	0.2308	0.23
FX	–0.0490	0.87
FXI	0.1818	0.57
PC	0.2448	0.44
AT	0.4266	0.16
PS	–0.2517	0.42
DD	–0.0490	0.87
Plg	0.0280	0.93
PAI-1	–0.1135	0.72
AP	0.2172	0.49
PT	0.2172	0.49
INR	0.2586	0.25
PTT	–0.2067	0.51

rho: rank-order correlation; Fib: fibrinogen; vWF:Ag: von Willebrand factor antigen; VWF:RCo: ristocetin cofactor activity; PC: protein C; AT: antithrombin; PS: protein S; DD: D-dimer; Plg: plasminogen; PAI-1: plasminogen activator inhibitor-1; AP: alpha 2-antiplasmin; PT: prothrombin time; INR: international normalized ratio; PTT: partial thromboplastin time.

Table 4. Correlation between paroxysmal nocturnal hemoglobinuria clone size in monocytes and coagulation data in a selected cohort of patients (n = 12)

Variable	Spearman's rho	p value
Fib	0.2294	0.4975
DD	–0.4220	0.1960
FV	–0.3119	0.3504
FVIII	0.4587	0.1558
vWF:Ag	0.0734	0.8302
vWF:RCo	0.0000	1.0000
FIX	0.3028	0.3655
FX	0.0092	0.9786
FXI	0.3762	0.2542
PC	0.2569	0.4457
AT	**0.6147**	**0.0442**
PS	–0.3945	0.2299
Plg	0.2661	0.4291
PAI-1	–0.3365	0.3117
AP	0.2253	0.5054
TP	0.0550	0.8723
INR	0.1439	0.6730
TTPa	–0.1193	0.7269

rho: rank-order correlation. **Bold** type indicates a statistically significant value. Fib: fibrinogen; vWF:Ag: von Willebrand factor antigen; vWF:RCo: ristocetin cofactor activity; PC: protein C; AT: antithrombin; PS: protein S; DD: D-dimer; Plg: plasminogen; PAI-1: plasminogen activator inhibitor-1; AP: alpha 2-antiplasmin; PT: prothrombin time; I NR: international normalized ratio; PTT: partial thromboplastin time.

man, after a six-month follow-up. In the first patient, PNH had been classified as subclinical disease, while the other was a classic PNH (Table 1).

DISCUSSION

To our knowledge, this is the first study on PNH that simultaneously measures clonal size and patient procoagulant, anticoagulant, and fibrinolytic factors in a Mexican population.

In our series, only 5% developed TEE throughout the course of their disease. This proportion is similar to that reported by Góngora-Bianchi, et al.[10] in a Mexican population, with an incidence of 3%. These data differ from publications on the frequency of TEE in PNH patients, which ranged between 15.5% and up to 44% in the USA, Brazil, and Europe[15,16].

Furthermore, mortality associated to TEE varies between 40 and 67%[17,18], differing from that reported in this study in which thrombosis-associated death was nil. Only one case of TEE in our study recurred (the first episode was an ischemic stroke and the second one was manifested as Budd-Chiari syndrome). This patient is currently under treatment with eculizimab.

Also, the size of the clone in granulocytes did not affect the risk of TEE in our patient, unlike the data reported by Hall[3]. In our study, of the 25 patients with a granulocyte clonal size > 50%, only two (8%) developed TEE throughout the course of the disease after a median follow-up of 85 months. The size of the clone in monocytes did not influence the risk of TEE since these two patients only represented 6.8% of the 29 patients with a PNH clone > 50%.

In the current study, no correlation was found between PNH clone size in granulocytes and any of the procoagulant, anticoagulant, or fibrinolytic profiles. However, PNH clone size in monocytes did correlate positively with AT levels. Of the three profiles, the one most frequently compromised was PS activity, since

Table 5. Correlation between lactic dehydrogenase levels and coagulation data in a selected cohort of patients (n = 12)

Variable	Spearman's rho	p value
Fib	–0.2970	0.4047
DD	–0.6121	0.0600
FV	–0.2485	0.4888
FVIII	–0.2848	0.4250
vWF:Ag	–0.6121	0.0600
vWF:RCo	**–0.7212**	**0.0186**
FIX	0.2848	0.4250
FX	0.1515	0.6761
FXI	–0.0545	0.8810
PC	0.5273	0.1173
AT	0.0303	0.9338
PS	–0.4909	0.1497
Plg	–0.4061	0.2443
PAI-1	–0.4000	0.2520
AP	–0.0851	0.8152
TP	–0.2364	0.5109
INR	–0.0671	0.8539
TTPa	–0.1515	0.6761

rho: rank-order correlation. **Bold** type indicates a statistically significant value. Fib: fibrinogen; vWF:Ag: von Willebrand factor antigen; vWF:RCo: ristocetin cofactor activity; PC: protein C; AT: antithrombin; PS: protein S; DD: D-dimer; Plg: plasminogen; PAI-1: plasminogen activator inhibitor-1; AP: alpha 2-antiplasmin; PT: prothrombin time; INR: international normalized ratio; PTT: partial thromboplastin time.

it was decreased in nine of the 12 patients (75%). Protein C was decreased in three of our patients (25%). When compared with the study by Grünewald, et al.[13], the only similar data were increases in FV, FVIII, FIX, FX, FXI, and AT, as well as a decrease in PS values and none, except for AT, correlated with PNH clonal size in our patients. Interestingly, Grünewald's study did not report any decrease in PC[13].

Unlike the increase in fibrinolysis referred by Grünewald, et al., Plg and PAI-1 levels were normal in our study and did not correlate with clonal size. Only DD was increased in two of our patients and AP was increased in one, but did not correlate with the size of the PNH clones in granulocytes or monocytes.

Griscelli-Bennaceur, et al. reported 12 patients with PNH mimicking aplastic anemia and PNH granulocyte clones > 50%[12]. None of these patients had abnormal levels of PC, PS, or AT, although three developed TEE. These findings differ from the coagulation abnormalities reported by Grünewald, et al.[13] and reproduced in our study.

Gralnick, et al. studied 11 patients with PNH, two of which had previously developed TEE. No abnormalities in the fibrinolytic system were detected (AP, PAI-1, tPA, DD, plasmin-antiplasmin complexes). In the procoagulant and anticoagulant profiles, only one patient showed a decrease in PS (activity only) and PC (activity and antigenic), attributed to concomitant oral anticoagulation. Antigenic PS was normal[11]. Hence, the results published by Gralnick, et al. do not coincide with our data in terms of elevated procoagulant factors and decreased PS.

Lee, et al. reported that a 1.5-fold increase in the LDH ULN levels was an independent risk factor for TEE development (OR: 7.0; p = 0.013). This complication was strongly associated to increased mortality in PNH patients (probability ratio: 6.85; p < 0.001). These authors found no evidence relating the clone size in granulocytes and the risk of TEE (p = 0.843)[19]. In our population, only 29 of 40 patients (72.5%) had a recent LDH determination, and 82.7% of them had a 1.5-fold increase above the ULN. Among these cases with significant LDH elevations, only two (8.3%) developed TEE during follow-up. Therefore, LDH values did not appear to influence the development of TEE in our patients, or the granulocyte PNH clone size, which correlated positively with LDH levels. Of interest was our finding that LDH values negatively correlated with vWF:RCo.

In summary, the current study reveals that Mexican patients with PNH have a low incidence of TEE and this complication is not associated with the clone size in granulocytes and monocytes or with LDH values. Furthermore, the abnormalities detected in the procoagulant, anticoagulant, and fibrinolytic profiles, including the correlation between AT and the clone size in monocytes, as well as that of LDH with the size of the clone in granulocytes and monocytes, are in line with data reported by other authors[11-13]. It is possible that the low frequency of TEE in Mexican patients could partly be explained by the positive association between AT and PNH monocyte clone size, and the negative association of vWF:RCo with LDH levels.

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