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ORIGINAL ARTICLE

Elevated thrombin generation in patients with congenital disorder of glycosylation and combined coagulation factor deficiencies

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Abstract

Background: Congenital disorders of glycosylation are rare inherited diseases affecting many different proteins. The lack of glycosylation notably affects the hemostatic system and leads to deficiencies of both procoagulant and anticoagulant factors.

Objective: To assess the hemostatic balance in patients with multiple coagulation disorders by using a thrombin generation assay.

Method: We performed conventional coagulation assays and a thrombin generation assay on samples from patients with congenital disorder of glycosylation. The thrombin generation assay was performed before and after activation of the protein C system by the addition of soluble thrombomodulin.

Results: A total of 35 patients were included: 71% and 57% had low antithrombin and factor XI levels, respectively. Protein C and protein S levels were abnormally low in 29% and 26% of the patients, respectively, whereas only 11% displayed low factor IX levels. Under baseline conditions, the thrombin generation assay revealed a significantly higher endogenous thrombin potential and thrombin peak in patients, relative

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to controls. After spiking with thrombomodulin, we observed impaired involvement of the protein C system. Hence, 54% of patients displayed a hypercoagulant phenotype in vitro. All the patients with a history of stroke-like episodes or thrombosis displayed this hypercoagulant phenotype.

Conclusion: A thrombin generation assay revealed a hypercoagulant in vitro phenotype under baseline condition; this was accentuated by impaired involvement of the protein C system. This procoagulant phenotype may thus reflect the risk of severe vascular complications. Further research will have to determine whether the thrombin generation assay is predictive of vascular events.

KEYWORDS

coagulation disorder, congenital disorder of glycosylation, thrombin generation assay

1 | INTRODUCTION

Hemostasis is a physiological process that maintains blood flow and prevents blood loss (through clot formation) following vascular injury. Many coagulation proteins are involved in clot formation, and a deficiency in any one of them may lead to bleeding. Extension of the clot is regulated by physiological inhibitors, which may cause thrombosis if they are lacking or altered. The final result is a tightly regulated balance between procoagulant and anticoagulant factors.[1] The impairment of a single coagulation protein—usually resulting from mutation of the coding gene—may disrupt the hemostatic balance and thus lead to bleeding or thrombosis. On one hand, hemophilia is characterized by a lack in procoagulant factors VIII (FVIII) or IX (FIX), and results in severe bleeding.[2] On the other, a deficiency in antithrombin (AT), a key endogenous coagulation inhibitor, and the first thrombophilic risk factor to be discovered in humans, leads to thrombosis. Since the discovery of AT, procoagulant states resulting from impairment of the protein C (PC) system have been identified.[3] Some contexts (such as cirrhosis, the neonatal period, and disseminated intravascular coagulation) produce a combination of protein deficiencies that affect both procoagulant and anticoagulant factors.[4,5] A lesser known etiology for combined impairments of coagulation proteins is ascribed to congenital disorders of glycosylation (CDG), a large group of rare inherited diseases. To date, around 100 different CDG have been identified. Because of the importance of the glycosylation process for the function of many proteins, impaired glycosylation results in broad multisystem clinical manifestations. The most frequent CDG is phosphomannomutase deficiency (*PMM2*-CDG).[6] The clinical symptoms of *PMM2*-CDG may include failure to thrive, developmental delay, cerebellar ataxia, strabismus, skeletal findings, and cardiac or renal involvement.[7] Coagulation factors, most of which are glycoproteins, are also frequently affected in CDG. Interestingly, patients with CDG may have a coagulation pattern that does not correspond to hepatocellular failure, factor consumption, or vitamin K deficiency. Indeed, low levels of both clotting factors (FIX and FXI) and coagulation inhibitors (especially AT, PC and protein S [PS]) are usually reported in the literature.[7–10] Primary hemostasis might also be affected in patients with CDG; there is evidence of thrombocytopenia related to SLC35A1-CDG (a form affecting a specific sialic acid transporter [11]) or the enhancement of platelet aggregation in PMM2-CDG[12]even though the platelet N-glycoproteins appear to be normal.[13] In childhood, *PMM2*-CDG is marked by acute neurologic impairments mimicking stroke; these are commonly referred to as stroke-like episodes. The episodes occur mainly during periods of fever or after head trauma, and are often revealed by confusional state, focal neurological deficit with mono- or hemiparesis, and sometimes, epileptic seizures.[7,14] The hemostatic system's role in the pathophysiology of stroke-like episodes has not yet been characterized. During the first 72 hours of a stroke-like episode, magnetic resonance imaging may reveal vasogenic edema that is not restricted to arterial territories[14–16]; in contrast, ischemic occlusion is rarely observed.[17] Stroke-like episodes may affect up to 50% of patients with *PMM2*-CDG.[7] Thrombosis and hemorrhages are also reported in CDG, but are less frequent than stroke-like episodes.[7,16,18,19] Because of multiple coagulation abnormalities affecting both procoagulant and anticoagulant factors, the hemostatic balance is difficult to assess in vivo. Hemostatic balance is usually investigated by overall coagulation tests, such as the prothrombin time and the activated partial thromboplastin time; however, these tests have limitations. First, they explore only 5% of the total thrombin

Essentials

- Factor XI and antithrombin are mainly affected in congenital disorders of glycosylation.
- We assessed the hemostatic balance using thrombin generation assay.
- We evidenced a hypercoagulant phenotype in vitro in most patients.
- Patients with a history of stroke-like episodes or thrombosis displayed the hypercoagulant state.

potential (i.e., just enough to clot the plasma).[20] Furthermore, the overall coagulation tests are not sensitive to coagulation inhibitors in general and the PC system in particular (which cannot be activated in the absence of soluble thrombomodulin [sTM]). The thrombin generation assay (TGA) probes the whole thrombin formation process—from generation to inhibition—and thus takes account of both clotting factors and clotting inhibitors.[20,21] By combining different analytical conditions, all the anticoagulant systems can be investigated (i.e., the AT system under baseline conditions and the PC system in the presence of sTM).[22] Although the TGA has been used to study the coagulation balance in patients with cirrhosis,[23] in neonates,[24,25] and in patients with sepsis,[26] it has not previously been applied (in the presence and absence of sTM) to studies of patients with CDG.

2 | METHOD

2.1 | Study populations

Thirty-five patients with confirmed CDG were enrolled in this retrospective, multicenter study between 2010 and 2017. Patients being treated with anticoagulants were excluded from the study. Samples from 35 control subjects were also collected during consultations before minor surgery. Control subjects with known coagulation abnormalities or taking anticoagulants were excluded. The study was approved by the local investigational review board (Necker Children's Hospital, Paris, France; reference: 2018-TP-4). Patients were provided with study information and gave their consent to participation.

2.2 | Standard coagulation assays

Samples were collected in vacuum tubes containing 0.109 mol/L sodium citrate. Platelet-poor plasma was obtained after two cycles of centrifugation at 20°C for 15 minutes at 2000 × *g* (to achieve a platelet count <10 × 10^9/L), and then stored at −80°C. Standard coagulation tests were performed using the ACL TOP analyzer (Instrumentation Laboratory). Levels of FIX and FXI activity were measured in a clotting-based assay using lyophilized, deficient plasma from Siemens (Siemens Healthcare Diagnostics SAS) or Werfen (Instrumentation Laboratory). AT activity was determined using a chromogenic assay (Stachrom® ATIII, Diagnostica Stago SAS). PC and PS anticoagulant activities were determined in specific clotting-based assays (Staclot® PC and Staclot® PS, respectively; Diagnostica Stago SAS). The results were interpreted according to the age-appropriate reference interval for each assay.[5]

2.3 | The thrombin generation assay

To standardize the results as much as possible, the TGA was solely performed in the central laboratory at Necker Children's Hospital, using the calibrated, automated thrombogram method.[27] Samples were mixed with PPP reagent® (tissue factor: 5 pmol/L; phospholipids: 4 μmol/L) (Diagnostica Stago) in a 96-well plate (Immulon, 2HB clear U-bottom; Thermo Fisher Scientific). All samples were run in duplicate. Coagulation was triggered with calcium chloride in buffer containing the fluorogenic substrate (FluCa-kit reagent®, Diagnostica Stago). For each individual plasma sample, we used a thrombin calibrator (Diagnostica Stago) to correct for differences in sample color, inner filter fluorescence, and substrate consumption. Fluorescence was recorded for 60 minutes in a Fluoroskan Ascent microplate fluorimeter (Thermolab Systems), and the data were then analyzed using Thrombinoscope™ software (version 5.0.0.742; Diagnostica Stago). The endogenous thrombin potential (ETP), the thrombin peak, the time to the thrombin peak, and the lag time were recorded for each assay. To assess the impact of the PC system on the TGA result, experiments were also conducted in the presence of recombinant sTM (American Diagnostica) at a final concentration of 7.5 nmol/L. As described by Perrin et al,[26] an index "R" was calculated for the TGA parameters (i.e., the value in the presence of sTM divided by the value in the absence of sTM); the closer the ratio is to 1, the weaker the response to the PC system.

2.4 | Statistical analysis

All quantitative variables were expressed as the median (interquartile range). Statistical analysis was performed with Prism software (GraphPad Software Inc.). Intergroup comparisons were performed using the Mann-Whitney *U* test. Correlations were determined by calculating Spearman's coefficient. The threshold for statistical significance was set to $P < 0.05$.

3 | RESULTS

3.1 | Characteristics of the study populations

Thirty-five patients with confirmed CDG (15 males and 20 females; median age: 10 [4; 14] years) were enrolled in the study (Table 1). The control population comprised 35 volunteers (20 males and 15 females; median age: 8 [2; 14] years). The patient and control group did not differ significantly with regard to age ($P = .3115$). During a retrospective review of the participants' medical records, we found that 10 patients with CDG had a history of stroke-like episodes (n = 7), a thrombotic event (n = 2) or both (n = 1). No major bleeding (defined according to the International Society on Thrombosis and Hemostasis criteria) was reported, although bleeding related to esophageal varices occurred in two patients with mannose phosphate isomerase CDG. None of the blood samples were collected during an acute event.

3.2 | Combined coagulation protein deficiencies in patients with CDG

Given that FIX, FXI, AT, PC, and PS are primarily affected in CDG,[7–10] we chose to assay these coagulation factors. Indeed, we found that CDG syndrome affected the activity of clotting factors and plasma

TABLE 1 Clinical characteristics of patients with CDG, and levels of coagulation proteins

Patient (#)	Age at blood collection (y)	Sex	CDG	PT (%)	aPTT (ratio)	Fibrinogen (g/L)	Factor IX (%)	Factor XI (%)	Antithrombin (%)	Protein C (%)	Protein S (%)	Event
1	10	M	PMM2	98	1.07	2.7	78	95	92	111	89	
2	11	M	PMM2	95	1.02	–	100	98	96	113	123	
3	17	M	PMM2	75	**1.31**	3.6	97	**24**	**32**	**27**	70	SL/femoral artery thrombosis
4	5	F	PMM2	86	**1.26**	5.1	91	**21**	**38**	68	78	SL
5	10	F	PMM2	92	**1.23**	2.3	81	**31**	**35**	63	**38**	
6	12	F	PMM2	**47**	1.11	2.1	71	**34**	**45**	**24**	72	SL
7	13	F	PMM2	98	1.13	2.7	73	**48**	**51**	70	86	
8	12	F	PMM2	74	1.14	–	102	94	102	94	101	
9	13	F	PMM2	100	1.04	3.24	92	103	109	85	118	
10	16	F	PMM2	93	**1.37**	–	68	**27**	**34**	**41**	59	SL
11	8	M	PMM2	108	**1.31**	3.39	85	**32**	**48**	76	**42**	SL
12	14	M	ALG6	85	**1.41**	2.6	80	**19**	**43**	65	61	
13	12	M	ALG6	87	1.17	–	86	**20**	**43**	70	**53**	
14	18	F	MPI	76	**1.25**	2.8	73	**51**	**36**	**56**	62	
15	15	F	MPI	103	0.88	2.9	105	140	111	125	101	
16	5	M	PMM2	107	1.09	2.8	74	96	92	116	75	
17	2 m	M	MPI	**55**	**2.19**	**0.8**	**19**	**20**	**20**	**28**	**36**	
18	10	M	MPI	72	**1.43**	1.6	**47**	**48**	87	**48**	60	Jugular vein thrombosis/bleeding from esophageal varices
19	8	F	PMM2	96	**1.41**	2.02	68	89	**75**	100	64	
20	1	M	PMM2	103	0.97	4.0	71	**27**	**17**	51	–	SL
21	4	F	PMM2	71	**1.30**	2.7	81	**54**	**53**	70	66	
22	6	F	PMM2	110	1.05	2.1	84	90	100	111	62	
23	1	F	PMM2	90	1.06	3.6	**25**	**25**	**9**	**16**	**46**	SL
24	3	M	PMM2	67	0.97	3.3	143	140	**40**	68	**46**	
25	21	F	ALG12	–	–	2.6	96	**59**	**50**	141	69	NA
26	1	F	PMM2	**46**	–	**1.1**	**46**	**38**	**30**	**29**	112	
27	10	M	PMM2	82	1.08	2.2	103	119	113	73	59	
28	10	M	PMM2	92	1.13	2.1	108	71	**49**	61	76	Cerebral thrombophlebitis

(Continues)

TABLE 1 (Continued)

Patient (#)	Age at blood collection (y)	Sex	CDG	PT (%)	aPTT (ratio)	Fibrinogen (g/L)	Factor IX (%)	Factor XI (%)	Antithrombin (%)	Protein C (%)	Protein S (%)	Event
29	3	F	PMM2	87	1.05	3.8	100	**55**	**68**	**44**	**42**	
30	4	F	PMM2	97	1.15	3.7	64	**34**	**37**	**29**	**39**	SL
31	12	F	MPI	77	**1.21**	2.2	62	62	**77**	56	59	Bleeding from esophageal varices
32	2	M	DPM1	106	1.01	3.1	95	92	**63**	99	91	
33	18	F	DPAGT1	95	–	4.1	103	124	101	69	70	
34	25	F	PMM2	88	1.09	3.2	86	75	**77**	79	82	
35	19	M	CCDC115	97	1.14	–	82	**52**	**74**	108	**40**	

Coagulation protein levels are expressed as a percentage of the normative value for pooled plasma (set to 100%). Results in bold type correspond to values below the age-adjusted normative reference intervals.

Reference values—prothrombin time (PT): >70%; activated partial thromboplastin time (aPTT): ratio <1.2; fibrinogen: 1.5-3.5 g/L; factor IX (age up to 3 mo: >20%; 3-6 mo: >40%; 6 mo-15 y: >50%; >15 y: >60%); factor XI (age up to 3 mo: >25%; 3-6 mo: >50%; >6 mo: >60%); antithrombin (age 1-3 mo: >50%; >3 mo: >80%); protein C (age 1-3 mo: >40%; up to 15 y: >50%; >15 y: >70%); protein S (age 1-6 mo: >50%; up to 15 y: >60%; up to 50 y: >50%) [26].

Abbreviations: ALG12, mannosyltransferase 8; ALG6, alpha-1,3-glucosyltransferase; CCDC115, coiled-coil domain containing 115; DPAGT1, dolichol-phosphate N-acetylglucosamine-1-phosphotransferase; DPM1, dolichol-phosphate-mannose synthase; MPI, phosphomannose isomerase; NA, not available; PMM2, phosphomannomutase 2; SL, stroke-like episode.

inhibitors. Relative to the age-appropriate reference interval,[5] we found that 20 patients (57%) had low FXI levels, whereas only 4 (11%) displayed low FIX levels. Levels of AT, PC, and PS were also low in 25 (71%), 10 (29%), and 9 (26%) patients, respectively (Table 1). Only five patients in our series had a single factor deficiency (AT) (Figure 1), suggesting that AT is more likely to be affected in CDG syndromes. Interestingly, FXI levels were low in 19/25 (76%) patients with an AT deficiency, whereas PC and/or PS deficiency was associated with AT deficiency (14/15 patients), FXI deficiency (14/15 patients), or both (13/15 patients) (Figure 1). Moreover, levels of FXI correlated well with levels of AT (Spearman's r = .8443; P < .0001; Figure 1).

3.3 | The thrombin generation assay

In the patients with CDG, the baseline TGA revealed an abnormal thrombin generation profile relative to controls (Figure 2B). We observed a greater median ETP (1435 [1160-2163] nmol/L/min in patients with CDG vs 956 [893-1157] nmol/L/min in controls, P < .0001) and a greater thrombin peak (230 [193-284] vs 157 [110-207] nmol/L, respectively; P < .0001; Figure 2B). The lag time and the time to peak were shorter in patients with CDG than in controls (2.50 [2.00-3.00] vs 2.84 [2.67-3.50] minutes for the lag time, P = .001, and 5.23 [4.34-6.50] vs 7.00 [5.84-8.33] minutes for the time to peak, respectively, P = .0008; Figure 2B). Under baseline conditions, these changes in the thrombin generation profile evidenced a hypercoagulant state in the patients. Similar results were obtained when the TGA was performed at a low TF concentration (1 pmol/L) in 25 patients with CDG, relative to controls (Figure S1).

To evaluate the response to the PC system, the TGA was then performed in the presence of 7.5 nmol/L of sTM. Spiking with sTM is expected to decrease the ETP and the peak (relative to the baseline condition, as illustrated by the control thrombogram shown in Figure 2A). When the PC system is not activated, the relative decreases in the ETP and peak are not observed (see patient 6 in Figure 2A). The assay's sensitivity to the PC system is reflected by the R^{ETP} and R^{Peak} ratios. The response to PC system was altered in patients with CDG (relative to controls) as illustrated by the greater values of R^{ETP} (0.37 [0.26-0.62] vs 0.23 [0.14-0.32], respectively; P = .0005) and R^{Peak} (0.48 [0.33-0.74] vs 0.35 [0.28-0.49], respectively; P = .0104; Figure 2C). The impaired PC involvement in patients with CDG accentuates the hypercoagulant state observed in vitro under baseline conditions.

Last, the overall hemostatic balance for each patient was assessed with regard to the baseline ETP (in the absence of sTM, corresponding to the ability to generate thrombin) and R^{ETP} (reflecting impairment of the PC system; Figure 3). Baseline thrombin generation (reflected by the ETP) is influenced by the deficiencies in procoagulant factors (FIX and FXI) and AT, whereas R^{ETP} is influenced by the PC system. Nineteen of the 35 patients (54%) expressed a hypercoagulant phenotype in vitro (relative to controls), with elevated baseline thrombin generation (n = 9), impaired involvement of the PC system (n = 5), or both (n = 5). Moreover, all 10 patients with

CDG and a documented history of stroke-like episodes (n = 7) or thrombotic events (n = 2) or both (n = 1) displayed a hypercoagulant phenotype in the TGA. The small number of patients prevented us from performing a 2 × 2 contingency table analysis.

4 | DISCUSSION

In CDG syndrome, the lack of glycosylation affects many plasma glycoproteins, including hemostatic proteins, which generally contain several glycosylation sites.[28] As previously described in the literature on patients with CDG,[7–10] we observed in our series a characteristic coagulation pattern, with a decrease in both procoagulant and anticoagulant factors. AT and FXI were the main coagulation factors affected, with low levels in, respectively, 71% and 57% of the patients with CDG; this was probably because the lack of glycosylation is known to have a strong impact on the folding, secretion, and/or stability of these proteins.[29,30] Clearance might also be affected by the glycosylation defect; indeed, sialidase treatment of AT is associated with a significantly shorter in vivo half-life in rabbits.[31] The lack of glycosylation might also affect the coagulation proteins' activity as well as their levels. Indeed, the absence of AT's N-glycan chain at Asn 135 results in a greater affinity for heparin.[32,33] The PC and PS levels were also low in 29% and 26% of the patients, respectively, whereas only 11% of the patients displayed a FIX deficiency. PC also has four glycosylation sites, which are reportedly involved in the protein's secretion, its activation by the thrombin-TM complex or its anticoagulant activity.[34]

In the present study, we used an approach to assess the impact of combined deficiencies (affecting both procoagulant and anticoagulant factors) on the hemostatic balance in patients with CDG. The TGA enabled us to assess the entire thrombin formation process (from generation to inhibition), and thus to take account of both clotting factors and coagulation inhibitors. In patients with CDG, application of the TGA revealed higher levels of thrombin generation (i.e., a significant increase in ETP and the peak) and a shorter lag time and time to peak in patients with CDG, relative to controls. These results attest to a hypercoagulant state in vitro under baseline conditions. In order to assess the response to the PC system, we also performed the TGA after spiking with sTM[22]; we observed a significant impaired involvement of the PC system. This impairment was probably the result of the protein C deficiency evidenced in 29% of the patients. The lower sensitivity to the PC system in patients accentuated the hypercoagulant phenotype observed at baseline in vitro. This approach to characterizing hemostasis (i.e., applying the TGA under baseline conditions and then after activation of the PC system) has already been used to study the coagulation balance in patients with combined coagulation factor deficiencies. Indeed, in clinical settings affecting both procoagulant and anticoagulant factors such as cirrhosis[23,35] and the neonatal period,[25] the results of the TGA suggest that the overall hemostatic balance is not altered: a decrease in thrombin generation is counterbalanced by a decrease in sensitivity to the PC system. This probably explains the low bleeding tendency of patients with cirrhosis other than gastrointestinal bleeding, the pathophysiology of which is mainly related to portal hypertension and therefore leads to esophageal varices, rather than coagulation impairment.[36] Similarly, two patients in our series experienced bleeding related to esophageal varices and portal hypertension: a hypercoagulant TGA profile was seen for patient 18, whereas patient 31 had a normal profile. A relatively normal coagulation balance (according to the TGA) has been reported in neonates, despite markedly low levels of vitamin K-dependent coagulation factors.[25] In the context of septic shock, and despite a clear impairment of thrombin generation under baseline conditions (probably reflecting consumption coagulopathy), patients are highly resistant to the PC system—leading to an overall procoagulant phenotype in vitro.[26] Interestingly, the patients with CDG in our series who had experienced stroke-like episodes or thrombosis all displayed a hypercoagulant phenotype in vitro. This observation suggests that patients with a disrupted hemostatic balance are more exposed to the occurrence of stroke-like episodes or thrombosis. Furthermore, none of the patients with a normocoagulable phenotype had a history of thrombosis or stroke-like episodes. Despite this hypercoagulant in vitro phenotype in patients with CDG, the incidence of thrombosis in our series was low (three cases). We hypothesize that in vivo the prothrombotic effect of AT deficiency is counterbalanced by the low level of FXI—as suggested by the strong correlation between AT and FXI levels in our study population. Furthermore, it has been reported that patients with FXI deficiency have a low incidence of thrombotic events.[37,38] In our series, the patients were more likely to have a history of stroke-like episodes than a history of thrombotic events. In children, the occurrence of stroke-like episodes is not limited to PMM2-CDG syndrome; many disorders (such as migraine, epilepsy, and even tumors) can mimic stroke.[39] In patients with PMM2-CDG, the pathophysiology of stroke-like episodes has yet to be characterized. Recently, their similarity with acute events in channelopathy related to familial hemiplegic migraine has offered

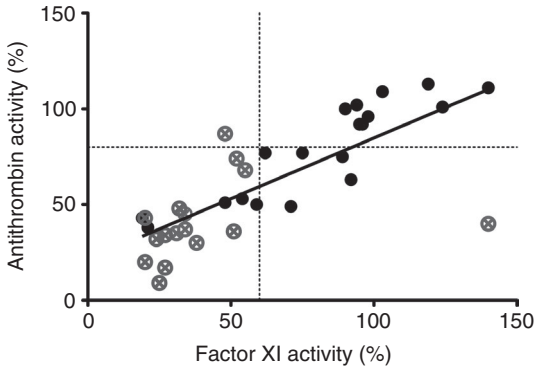

FIGURE 1 Correlation between factor XI (FXI) and antithrombin levels in patients with congenital disorders of glycosylation (CDG). Dashed lines represent the reference interval in children over the age of 6 months (for CDG 17, aged 2 months, the reference interval is >50% for antithrombin and >25% for FXI). Patients who also displayed protein C and/or PS deficiencies are represented by gray crossed circles. The correlation between FXI and antithrombin levels was determined using Spearman's rank test

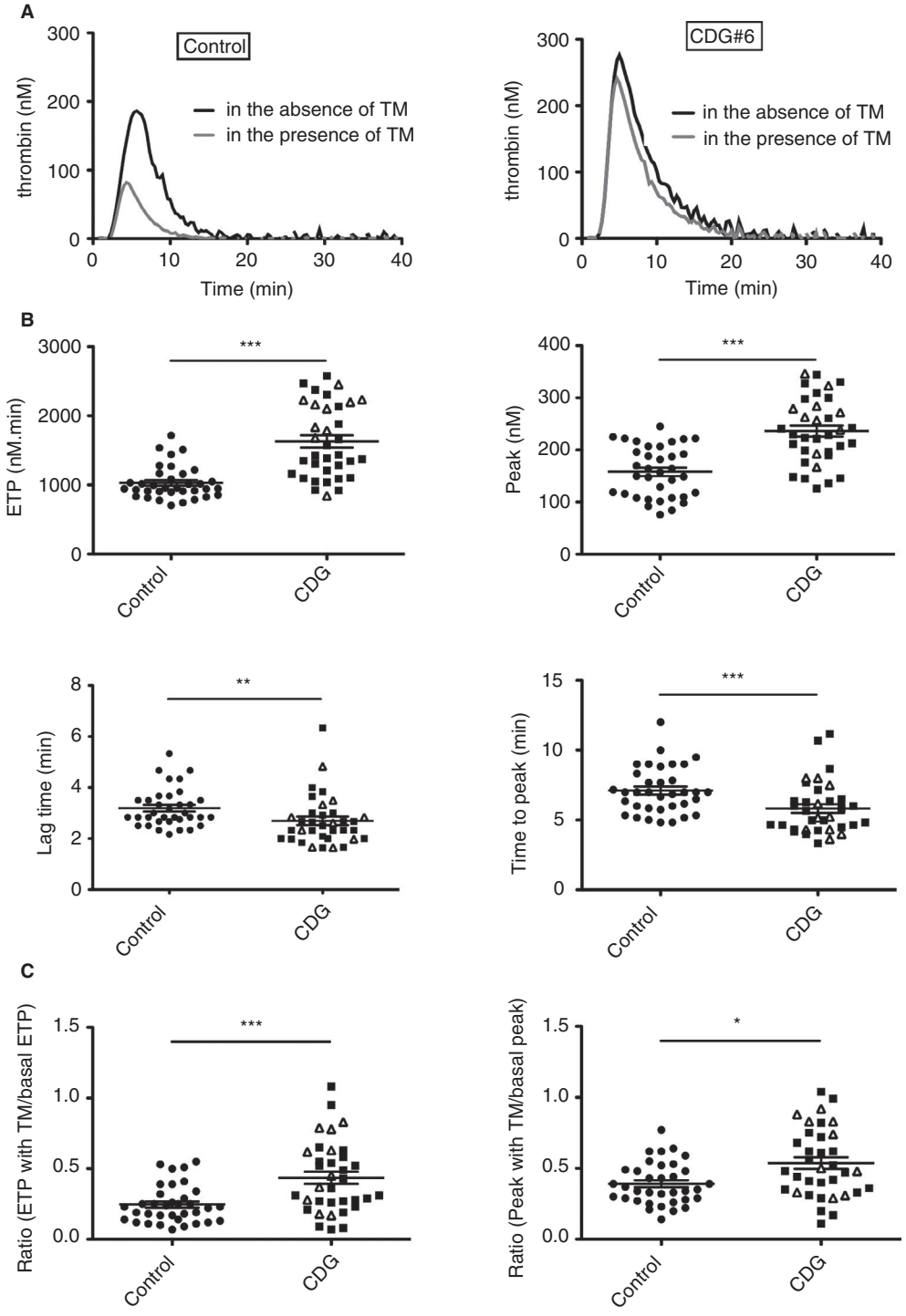

FIGURE 2 Results of the thrombin generation assay. Thrombin generation was performed with PPP reagent® (final concentrations: tissue factor, 5 pmol/L; phospholipids, 4 µmol/L). The endogenous thrombin potential (ETP, nmol/L/min), thrombin peak (peak, nmol/L), lag time (min), and time to peak (min) were recorded. A, Typical thrombograms. Thrombogram obtained in the absence of thrombomodulin (TM; black line) using plasma from controls (left panel). The addition of thrombomodulin (7.5 nmol/L) resulted in a decrease in thrombin generation (gray line) via the activation of protein C. Baseline thrombin generation in patient 6 (right panel) revealed a hypercoagulant profile, relative to controls. After the addition of TM, a hypocoagulant effect was no longer observed (gray line) because of an impairment of protein C. The data are representative of two independent experiments. B, Baseline thrombin generation. Each point corresponds to a study participant, and the mean ± SEM is also shown. Patients with a history of a stroke-like episode or thrombosis are represented by triangles. C, Response to thrombomodulin addition. Results are expressed as the RETP ratio (ETP in the presence of TM/ETP in the absence of TM) and the RPeak ratio (peak in the presence of TM/peak in the absence of TM). Data are presented as the mean ± SEM. *$P < .05$; **$P < .01$; ***$P < .001$

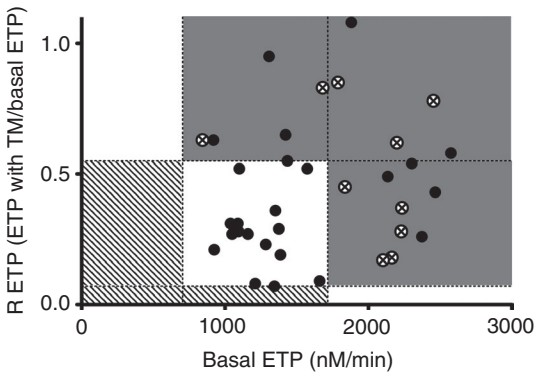

FIGURE 3 Distribution of patients according to their in vitro phenotype in the TGA. The quadrants are defined according to the highest values obtained in controls, delimiting three areas. The white area corresponds to a normal ETP and R index, or both an altered ETP and R index, or a high ETP with low R index (defined as normal coagulation phenotypes). The gray area corresponds to a high R index with a normal or high ETP, or a high ETP with a normal R index (defined as hypercoagulable phenotypes). The hatched area corresponds to an altered ETP with a normal or low R index, or a normal ETP with an altered R index (defined as hypocoagulable phenotypes). Patients with CDG who had a history of stroke-like episodes or thrombosis are illustrated as crossed circles

a research approach.[14] Even though there is no evidence of ischemic occlusion during stroke-like episodes in PMM2-CDG,[14–16] the hypercoagulability state may still contribute to their development. Indeed, as hypothesized in migraine with aura, the occurrence of a transient ischemic attack cannot be ruled out.[40,41]

Stroke-like episodes have several reported triggering factors. In particular, hyperthermia may reduce the activity of PMM2 and thus accentuate the glycosylation defects affecting coagulation factors. Indeed, temperature instability of several PMM2 variants has been reported in the literature.[42] Furthermore, for some AT variants, hyperthermia reportedly induced a conformational change in the protein and transient AT deficiency, leading to an increased risk of thrombosis.[43,44] Patients with *PMM2*-CDG and pre-existing coagulation abnormalities appear to be more sensitive to disturbing factors or trauma and thus disseminated intravascular coagulation, as already described during stroke-like episodes or in inflammatory disease.[17,45] Another case report indicated that surgery is also a critical situation because administration of FXI triggered a serious coagulation imbalance and disseminated intravascular coagulation.[46] These observations suggest that coagulation protein deficiencies in patients with CDG should be considered as a whole and not in isolation.

Our study had some limitations. In contrast to the purified sTM used in the assay, the patients' abnormally glycosylated TM may be less well secreted or less active. Indeed, the deletion of EGF-like domains 4 and 5 (which contain N-glycan chains) reduces thrombin's binding affinity and its cofactor activity.[47] Thus, mobilization of the PC system might have been more impaired in vivo in patients with CDG than in vitro. Although the plasma thrombomodulin concentration was higher in patients with CDG than in controls (2.95 [2.24-4.33] vs 2.06 [1.79-2.38] ng/mL, $P = .0009$; Figure S2), it was probably not high enough to counterbalance the procoagulant phenotype observed in the TGA. Indeed, the TM concentration used in the assay is 100 times higher than those measured in plasma. Moreover, antithrombotic effects of human recombinant soluble thrombomodulin was observed at plasma levels ranging from 100 to 1000 ng/mL.[48,49]

We did not explore the role of the second physiological thrombin inhibitor (α2-macroglobulin), levels of which are higher in children than in adults. Indeed, levels of α2-macroglobulin are significantly elevated up to the age of 10 years.[50] Alpha2-macroglobulin is also highly N-glycosylated, and abnormal isoforms were evidenced in one patient with CDG[51] and thus are possibly involved in the modulation of coagulation in patients with CDG. Furthermore, the TGA was performed on platelet-poor plasma that lacked the other blood cells involved in hemostasis (such as platelets). Last, the TGA is reportedly suitable for detecting an AT deficiency and seems less influenced by FXI. TGA is more sensitive to FXI in the presence of platelets and when the tissue factor concentration is low.[52–54] In our conditions, when TGA is assessed in platelet-poor plasma with high tissue factor concentration (5 pmol/L), the thrombin generation profile is primarily driven by antithrombin deficiency. However, with low tissue factor concentration (1 pmol/L), an impact of FXI deficiency cannot be ruled out.[55] In a subset of our patients and age-matched controls, TGA was also performed with low tissue factor concentration (1 pmol/L), and we observed significantly greater in the ETP and the thrombin peak in patients with CDG (Figure S1).

A prospective study will be necessary to determine whether the TGA phenotype predicts the occurrence of vascular events. Even though no stroke-like episodes and/or thrombosis were reported in study participants with no history of coagulation abnormalities, one cannot rule out the occurrence of a coagulation imbalance during a febrile episode or in another situation favoring the accentuation the N-glycosylation defect.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

All authors contributed to the preparation of the article and met the required conditions for authorship. D. Borgel and J. Corral designed the study. M. Serrano, M. Kossorotoff, and P. De Lonlay

visited patients and collected data. D. Lasne, N. Boddaert, A. Bruneel, N. Seta, and V. Vicente collected data. T. Pascreau performed the assays. T. Pascreau, M. E. de la Morena-Barrio, and E. Bianchini collected, analyzed, and interpreted the data. T. Pascreau and D. Borgel wrote the article. All the authors revised the article for critical content.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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