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Thrombophilia screening in patients with autoimmune hemolytic anemia: a single-center analysis

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Running head: thrombophilia screening in AIHA

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To the Editor

Thrombotic complications have been reported in approximately 15–20% of patients with autoimmune hemolytic anemia (AIHA) [1-6]. Contributing factors likely include nitric oxide scavenging by circulating free hemoglobin, the release of erythrocyte-derived microparticles [7-9], and a multifaceted interplay among autoimmunity, autoinflammation, complement activation, and the coagulation system, as well as coexisting conditions. A recent systematic review estimated a pooled risk ratio of 2.63 (95% CI, 1.37–5.05) for thrombotic events in AIHA [7]. Retrospective series have identified several clinical risk factors for thrombosis, including severe anemia at onset (hemoglobin, Hb <6 g/dL), intravascular hemolysis (lactate dehydrogenase, LDH >1.5× the upper limit of normal), prior splenectomy, and the presence of Evans syndrome [5,10-13]. However, the role of routine thrombophilia screening in AIHA has not been established yet. The primary objective of this study was to assess the prevalence of inherited and acquired thrombophilia in AIHA and its association with incident thrombotic events. Secondary objectives included estimating incidence rates of thrombosis and evaluating the impact of antithrombotic prophylaxis.

We systematically enrolled a cohort of 92 patients with a confirmed AIHA according to current guidelines [1]. Patients underwent a thorough thrombophilia screening including the collection of an accurate personal and family history of thrombosis (venous/arterial thrombotic events, VTE/ATE), and of anamnestic risk factors, followed by sampling of peripheral blood for inherited and acquired forms. Thrombotic complications were classified as occurring prior or after AIHA onset and as venous or arterial. Additionally, anticoagulant or anti-platelet prophylaxis during thrombotic episodes was registered, allowing the evaluation of the incidence of thrombosis according to the ongoing prophylaxis. The study was approved by the ethical committee as a substudy of the CYTOPAN trial [NCT05931718].

The median age at first AIHA episode was 60 years (45-72) and 59% were females (Table 1). Most patients had a warm AIHA (57%), with a direct antiglobulin test (DAT) positive for IgG+C in one third, followed by cold agglutinin disease (CAD, 31%), and the rarer mixed and atypical forms (12%). During the retrospective follow-up, the patients experienced a median of 2 (1-4) hemolytic episodes.

Thrombophilia abnormalities were detected in 20/92 patients (22%), including deficiencies in antithrombin, protein C, or protein S, observed in 6/92 (7%), factor V Leiden or prothrombin *G20210A* mutations in 1 patient each; finally, anti-phospholipid antibodies were detected in 10/92 (11%), 5 triple positivity, 2 double positivity, 2 lupus anticoagulant only, and 1 isolated anti-cardiolipin positivity. Other acquired risk factors of thrombophilia evaluated included combined oral contraceptive reported in 26/54 (48%) of women, previous cancer in 14 (15%), recent surgery or trauma in 50 (54%), cardiovascular disease (CVD) risk factors in 40 (43%), and autoimmune comorbidities in 21 (23%). Personal history of VTE/ATE was documented in 4 patients (4.3%), while a positive family history was noted in 50 cases (54.3%). Concerning conditions associated with AIHA that may increase the thrombotic risk, 8 had Evans syndrome, 1 patient had a classic hemolytic paroxysmal nocturnal hemoglobinuria (PNH) with high disease activity (LDH 1.5 x ULN and anemia), and 1 essential thrombocythemia (ET) with 900x10^9/L platelets on hydroxyurea. Finally, a total of 3 pregnancies occurred after AIHA diagnosis among 54 women: one was in a woman with prior intrauterine growth restriction (IUGR), who delivered successfully without complications; a second woman, with an uncomplicated pregnancy history before AIHA, experienced a IUGR during her post-AIHA pregnancy but then completed it successfully; the third woman had a hydatidiform mole after AIHA diagnosis and interrupted the pregnancy. Notably, thrombophilia

screening was performed after a median of 4 years (IQR 0-39 years) post AIHA diagnosis, thus limiting the reliability of the acquired risk factors in this analysis.

Among the 92 patients, 20 (22%) experienced a total of 29 thrombotic events: 11 at AIHA diagnosis, including 8 VTE and 3 ATE. Among those with VTE at AIHA onset, 3 patients had concurrent Evans syndrome, and 2 had a prior history of VTE. An additional 18 thrombotic events occurred after AIHA diagnosis, consisting of 14 VTEs and 4 ATEs. Of the 14 VTEs, 12 occurred during AIHA relapses while 2 during AIHA remission.
Table 2 compares the frequency of thrombophilia risk factors between patients who experienced thrombosis at/after AIHA diagnosis and those who did not. The distribution of prothrombotic risk factors was broadly similar. This was clear for inherited thrombophilia abnormalities detected in a greater proportion among patients without thrombosis (17/72, 24% versus 3/20 15%). On the other hand, it should be noted that all 7 patients with double or triple anti-phospholipid antibodies positivity experienced multiple thrombotic episodes, as well as thrombosis while on anticoagulants. Concerning conditions associated with AIHA, the patient with PNH experienced a massive pulmonary embolism during the diagnostic phase and without heparin treatment, while those with TE and myelodysplastic neoplasm had no thrombosis. No associations between pregnancy complications and thrombophilia during AIHA were noted.
Figure 1 shows thrombosis-free survival for the whole cohort. Over a cumulative observation period of 183,245 days (approximately 501.7 patient-years) the incidence of thrombotic events was 3.6 (95%CI 2.2-5.5) per 100 pt-yr. The incidence was 10.6 (2.6-18.6) per patient-years in patients without thrombophilia, and 6.7 (0– 19.2) per patient-years in those with thrombophilia.

Supplementary table 1 shows the analysis of the incidence of thrombotic events based on antithrombotic prophylaxis exposure. Considering patients receiving prophylaxis (N=20), those receiving anticoagulation with vitamin K antagonists (VKA; n=13) accounted for 25.3 patient-years, during which 2 VTEs and 1 ATE occurred, yielding incidence rates of 7.9 VTEs and 3.9 ATEs per 100 patient-years. Among those on antiplatelet therapy alone (APT; n=5; 16.2 patient-years), 1 VTE was recorded (no ATEs), with an incidence rate of 6.2 VTEs per 100 patient-years. No thrombotic events were observed in the 2 patients who received combined VKA and APT (2 patient-years). Regarding thrombophilia screening, 5/20 were positive, of whom 2 had a thrombotic event (1 VTE and 1 ATE); 3 had a prior history of thrombosis (one had PNH).
Concerning patients not-on prophylaxis (N=72), 11 VTE and 3 ATE occurred, corresponding to incidence rates of 2.4 VTEs and 0.7 ATEs per 100 patient-years. Notably, this group had a longer observation time of 458.1 patient-years compared with those receiving prophylaxis. 15/72 had a positive thrombophilia screening of whom only 3 had a thrombotic event (2 VTE and 1 ATE).
All in all, while thrombotic events were more frequent in the no-prophylaxis group in absolute terms, incidence rates appeared higher in patients receiving antithrombotic therapies, possibly due to the shorter observation period and a higher baseline thrombotic risk in the latter (Incidence Rate Ratio 3.0 (0.99-9.14).
In this study, we describe the presence of inherited or acquired thrombophilia in about 20% of patients with AIHA, the former accounting for about 7% and the latter being much more frequent. Our findings do not support a clear correlation between thrombotic events and positive thrombophilia screening, particularly for congenital risk factors (factors V andII mutations, natural anticoagulants deficiency). The relationship with acquired risk factors is less clear-cut as it may be biased by their intrinsic time-dependent variability as well as by the time from the screening to the thrombotic event. There is great debate on the utility of thrombophilia screening in different settings, as recently outlined by the American society of Hematology 2023 guidelines

[14]. The latter mainly issued strong recommendations against screening, although with several exceptions, but did not consider hemolytic or autoimmune conditions [14].
As a matter of fact, our findings support a possible effect of dual and triple anti-phospholipid antibodies positivity on the cumulative incidence of thrombosis. Consistently, anti-phospholipid positivity was shown to be associated with thrombotic risk in large retrospective AIHA series and was present in 2/3 patients experiencing thrombosis while on prophylaxis in this study [5,10]. Furthermore, the autoimmune nature of the disease further corroborates to test anti-phospholipid antibodies in the clinical practice, as also suggested by the recent recommendations for Evans syndrome [15]. Altogether our findings support the decision to avoid the screening for inherited thrombophilia in AIHA patients, while testing for acquired risk factors, particularly anti-phospholipid antibodies, is advised. We would suggest to screen all patients at diagnosis, and then to re-evaluate those positive as per current guidelines (i.e. after 12 weeks).
Notably, AIHA remains the main trigger for thrombosis, with a incidence rate of 3.6 (2.2-5.5) per 100 patient-years, and a strong association with hemolytic activity. In fact, of the 29 thrombotic events, 11 occurred during the first AIHA episode, and 12 during subsequent hemolytic relapses. Conversely, AIHA type and primary versus secondary form did not significantly influence thrombotic risk; among diseases associated with AIHA, PNH was the only associated with thrombosis, in line with the vicious thrombophilia observed in this disease.

Regarding anti-thrombotic prophylaxis, we observed a higher cumulative incidence of thrombosis in patients already on prophylaxis as compared to those without. On the contrary, in a 2-year prospective cohort of 174 AIHA patients, no thrombotic events occurred in those who received low molecular weight heparin prophylaxis, while 5 events were observed in the non-prophylaxed group [10]. All in all, while prophylactic anticoagulation is recommended during hemolytic flares, the prolonged anticoagulation during AIHA remission should be evaluated on a case-by-case basis, mainly accounting for acquired risk factors. In this setting, age, cardiovascular risk factors, thrombophilic comorbidities, autoimmune diseases, and the positivity of anti-phospholipid antibodies may suggest long-term prophylaxis and inform a different AIHA therapeutic approach (i.e. avoiding splenectomy). The great uncertainty on the duration of anticoagulant prophylaxis is also outlined in the recent ASH 2023 guidelines [14].
The main limitation of this study is its retrospective design, the shorter follow up while on anticoagulant prophylaxis, and the limited number of thrombotic events. Nonetheless, a significant strength lies in the comprehensive and systematic evaluation of thrombotic risk factors and outcomes in a large cohort of AIHA patients managed in a referral center.

In conclusion, routine congenital thrombophilia screening is not warranted in AIHA, while the evaluation of acquired and modifiable risk factors, particularly testing for anti-phospholipid antibodies, is advisable. AIHA remains the major risk factor for thrombosis, warranting thromboprophylaxis during haemolytic events.

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Table 1. Baseline characteristics of the study population at the first autoimmune hemolytic anemia (AIHA) episode. Values are given as N(%) unless otherwise specified.

	All patients **N=92**
Male/Female, n (%)	38(41) / 54(59)
Age at first AIHA episode, years **Median (IQR)**	60 (45-72)
ABO group, 0 /non-0, n	34/37
Haemolytic events number, median (IQR)	2 (1-4)
AIHA thermal classification	
Warm	53 (58)
IgG	37 (69)
IgG+C	16 (31)
Cold	29 (11)
Mixed/atypical	10 (11)
AIHA type	
Idiopatic	65 (71)
Autoimmunity	17 (18)
LPD	7 (8)
Other	1 LR-MDS, 1 PNH, 1 MPN
Evans syndrome	8* (9)
Lines of treatment, median (range)	2 (1-6)
Steroids	89 (97)
Rituximab	72 (78)
Splenectomy	0 (0)
Cytotoxic immunosuppressors	5 (5)
Others	14** (15)

*** 7 warm, 1 mixed/atypical.**

****nipocalimab, isatuximab, obexelimab, sutimlimab, parsaclisib, fostamatinib**

AIHA was classified according to the positivity of direct anti-globulin test (DAT) into warm (IgG or IgG+C3d), cold (C3d and agglutinin titer >64), mixed (IgG+C3d and cold agglutinin titer >64), and atypical cases (IgA positive or DAT negative ones). AIHA was idiopathic in 71%, and secondary to lymphoproliferative disorders, autoimmune diseases, myelodysplastic syndrome (MDS), paroxysmal nocturnal hemoglobinuria (PNH) and myeloproliferative neoplasm (MPN) in the remaining; 8 patients had Evans syndrome. Patients received a median of 2 treatment lines (range: 1–6), with corticosteroids (n=89) and rituximab (n=72) being the most commonly administered therapies. No patients underwent splenectomy, while cytotoxic immunosuppressants (n=5) and other treatments (n=14) were used less frequently.

Table 2. Thrombotic risk factors and thrombophilia abnormalities among patients with autoimmune hemolytic anemia (AIHA). Screening included evaluation of inherited (antithrombin, AT, protein C, PC, and protein S, PS, levels, factor V Leiden, factor II G20210A mutation) and acquired forms (anti-phospholipid antibodies, namely positivity for the lupus anticoagulant LAC, anti-cardiolipin antibodies IgG/IgM, and anti-beta2 glycoprotein I antibodies IgG/IgM). Numbers are expressed as numbers (%) unless otherwise specified.

Prothrombotic risk factors before AIHA	**Thrombosis* at the first AIHA N=20**	**No thrombosis N=72**	**Total N=92**
Oc use/pregnancy	4 (20)	22(30)	26 (28)
Previous Cancer	2 (10)	12 (17)	14 (15)
Surgery/trauma	4 (20)	46 (64)	50 (54)
CVD risk factor**	5 (25)	35 (49)	40 (43)
Autoimmune diseases	2 (10)	19(26)	21 (23)
Personal history of VTE/ATE before AIHA	4 (20)	4 (5)	10[§] (11)
Family history if VTE/ATE before AIHA	4 (20)	46 (64)	50[§] (54)
Thrombophilia abnormalities, n (%)	3 (15)	17 (24)	20 (22)
AT, PC, PS	1 (5)	5 (7)	6 (7)
Factor V Leiden	0	1 (1.4)	1 (1)
Prothrombin G20210A mutation	0	1 (1.4)	1 (1)
Lupus anticoagulant anticardiolipin and/or anti B2 antibodies	2 (10)	8 (11)	10 (11)[†]
Multiple defects	0	2 (3)	2 (2) [‡]

*VTE or ATE

**at least one comorbidity among ipertension, dislipedemia and diabetes.

†5 triple positive, 2 double positive, 2 lupus anticoagulant, 1 single ACA positivity

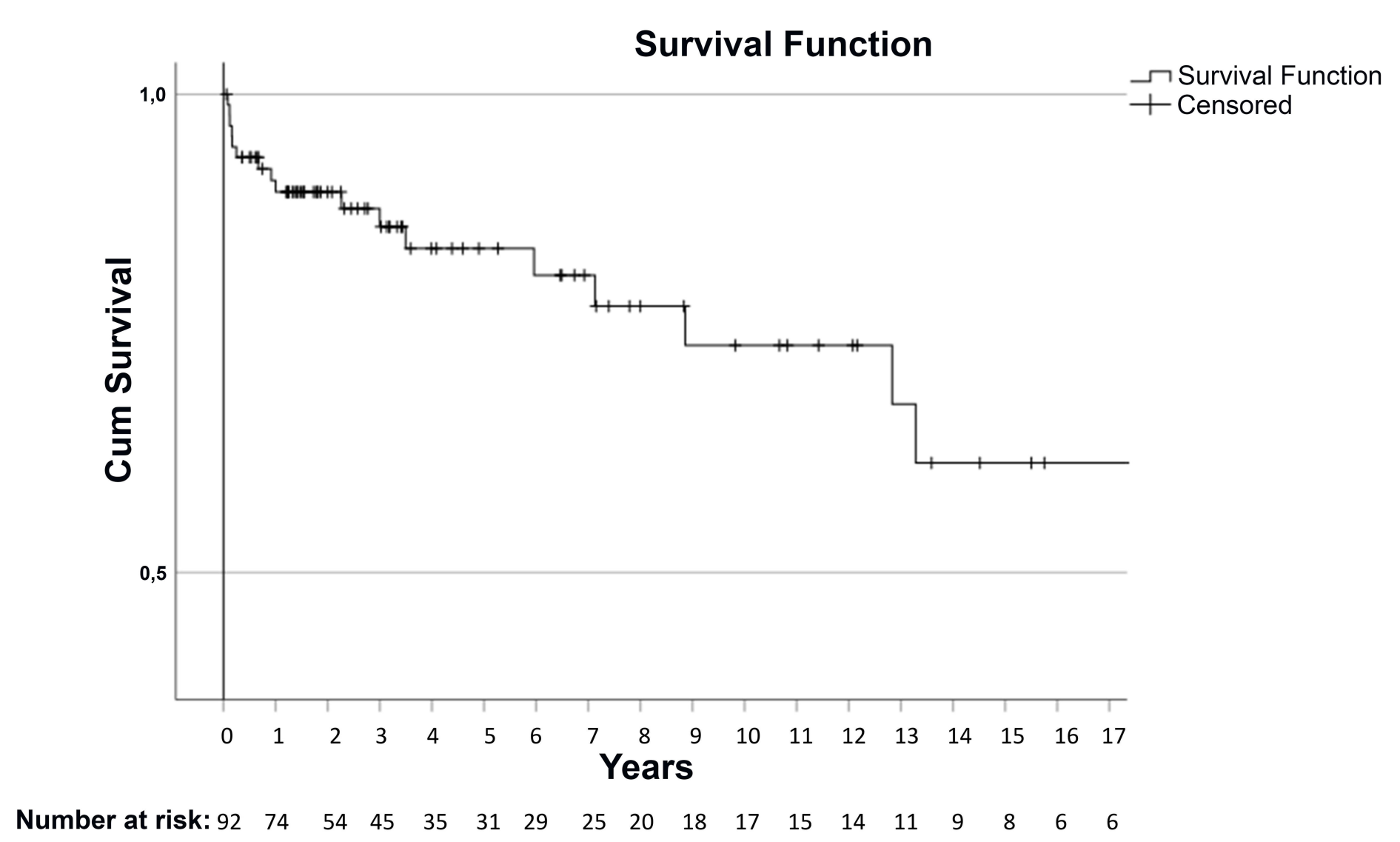
‡ 1 PC+ AT, 1 FVL + PS

§ 6 patient had both personal and family history of VTE/ATE before the 1 AIHA event

AIHA was classified according to the positivity of direct anti-globulin test (DAT) into warm (IgG or IgG+C3d), cold (C3d and agglutinin titer >64), mixed (IgG+C3d and cold agglutinin titer >64), and atypical cases (IgA positive or DAT negative ones). Venous/arterial thrombotic events, VTE/ATE; CVD cardiovascular disease; Oc oral contraceptive.

Figure legends

Figure 1. Thrombosis free survival in patients with autoimmune hemolytic anemia.
The cumulative incidence of thrombosis was analyzed by Kaplan-Meier method. The exposure time was calculated as the time between the first AIHA episode to the venous (VTE)/arterial thromboembolism (ATE) or to the last follow up for patients not experiencing thrombosis. In those patients receiving thromboprophylaxis the exposure time was calculated as the time from the start of prophylaxis to the thrombotic event/end of follow up.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Years
Number at risk: 92 74 54 45 35 31 29 25 20 18 17 15 14 11 9 8 6 6

Supplementary Table 1. Incidence rates of thrombotic events (arterial/venous, ATE/VTE) in autoimmune hemolytic anemia AIHA)

	Total observation period (92 patients)	Period without prophylaxis (72 patients)	Period on oral anticoagulants (OAC) (13 patients)	Period on anti-platelet agents (APT) (5 patients)	Period on both OAC and APT (2 patients)
Exposure days→years	183245 →501,7	167326 → 458.1	9249 →25.3	5935 →16.2	735 →2
VTE, n	14	11	2	1	0
ATE, n	4	3	1	0	0
Incidence rate IR *100 pt-yr (95%CI)	**Total**	**No prophylaxis**		**Prophylaxis ***	
VTE	2.8 (1.6-4.6)	2.4 (1.3-4.2)		6.9 (1.9-18.4)	
ATE	0.8 (0.3-1.9)	0.7 (0.2-1.7)		2.3 (0.2- 10.7)	
Total	3.6 (2.2-5.5)	3.1 (1.8-5.0)		9.2 (3.1-21.8)	
Total Cumulative Incidence at 1 year		9.8 (2.9-16.7)		15.4 (-4.2-35)	

* Among the 4 patients with events occurring while on prophylaxis, 3 patients had a prior thrombotic episode.