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The Potential Role of Neutrophils in Enhancement of Thrombotic Risk in Patients with Primary Antiphospholipid Syndrome

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ABSTRACT

Background: Antiphospholipid syndrome (APS) is an autoimmune condition characterized by high levels of antiphospholipid antibodies, which increase the risk of fetal loss, arterial and venous thrombosis**.** Neutrophils have long been observed in thrombi, but the specific mechanisms by which they contribute to thrombus development remain unclear.

Objective: To evaluate the role of neutrophils in enhancement of thrombotic risk in patients with primary antiphospholipid syndrome.

Material and Methods: 60 patients with primary APS participated in this study, along with 30 age- and sex-matched healthy volunteers serving as the control group. 30 patients in the patient group had prior thrombotic events, and 30 patients had no prior thrombotic events. The enzyme-linked immunosorbent assay was used to measure the lupus anticoagulant, anticardiolipin antibodies, anti-B2 glycoprotein, matrix metalloproteinase -2(MMP-2), monocyte chemoattractant protein-1(McP-1), and citrullinated histone (H3cit). While spectrophotometry was used to assess myeloperoxidase (MPO) activity.

Result: In groups I and II, the patients' mean ages were (42.93± 7.15) and (42.70± 8.30), respectively. The disease duration was (5.25 ±2.7 years) in group I and (5.54± 2.9 years) in group II. The recruited patients were distributed between the two groups, with 39 females and 21 males. Regarding MPO activity, MMP2, McP1, and H3cit, there was a significant difference between APS patients with prior thrombotic events and APS without such events, as well as between both groups and control.

Conclusion: Since neutrophil activation pathways are major regulators of both arterial and venous thrombosis, APS patients may benefit from medications that target these pathways to prevent thrombosis.

Keywords Antiphospholipid, Venous thrombosis, Arterial thrombosis, Neutrophils, NETosis.

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune condition with no known origin that is characterized by high levels of antiphospholipid antibodies (aPLs), which increase the risk of fetal loss, arterial and venous thrombosis [1]. There is no known cure for APS, and current treatments focus on suppressing coagulation rather than addressing the underlying pathophysiology. Despite being frequently associated with systemic lupus erythematosus (SLE), APS is now widely acknowledged to exist as a primary autoimmune syndrome with thrombosis and pregnancy loss as its cardinal manifestations [2].

Vascular beds of different sizes, including arterial and venous circuits, may be compromised by thrombosis brought on by APS. The most frequent locations for venous and arterial thrombosis, respectively, are the deep veins of the lower extremities and the cerebral circulation. Thrombosis may also occur in unusual sites including visceral arteries and veins and the cerebral venous sinuses [3].

As triggers of APS-related thrombosis, platelets, endothelial cells, and monocytes have previously received the greatest attention [4]. However, recent research has focused on the potential role of neutrophils in APS and their significance in pathologic coagulation, particularly venous thrombosis being the first cells present at the site of thrombosis, even before platelets [5-7]. Neutrophil extracellular traps (NETs), also produced by neutrophils, are chromatin-based extracellular structures that are primarily made of DNA and histones, which both originate from the nucleus and have been coated with substances derived from cytoplasm such as myeloperoxidase and neutrophil elastase granular proteins. Recent studies have revealed that NETs may also play a role in thrombosis by inactivating some anticoagulant factors and activating the intrinsic coagulation cascade, even though NETs were first identified for their function in host defense against pathogens. Moreover, the NET structure acts as a scaffolding for the formation of clots. Recent studies have analyzed the role of neutrophils in APS. These studies revealed that endothelial cells, neutrophils, monocytes, trophoblasts, and platelets are key players of APS progression. In this regard, evidence has shown that neutrophils are important leukocytes for the innate immune response, and they promote pyroptosis-mediated inflammation and thrombosis in APS patients [8,9].

Even though neutrophils have long been observed in thrombi, their specific roles in the process of thrombus formation are not yet fully grasped. Hence, the current study's objective was to evaluate the role of neutrophils in enhancement of thrombotic risk in patients with definite or probable primary

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antiphospholipid syndrome (APS), so that we can decide how to effectively use these data to develop focused interventions that reduce the pro-thrombotic environment without impairing patients' capacity to fight infections.

MATERIALS AND METHODS

Study populations and design:

This cross-sectional observational study was conducted at Department of Rheumatology, Rehabilitation and Physical Medicine, Faculty of Medicine, Tanta University over the period of 6 months from December 2022 to May 2023. The study was initiated on 60 patients with primary antiphospholipid syndrome (APS) including those with persistently positive lupus anticoagulant (LA) antibodies (backed up by two tests 12-week intervals). The patients' group involved 30 patients with history of thrombotic events (group I) and 30 patients without history of any thrombotic event, group II (Patients had only one laboratory criteria for APS and pregnancy morbidity with either thrombocytopenia, cardiac valve disease, livedo-reticularis, or cognitive dysfunction as non-criteria manifestations). The recruited patients fulfilled the laboratory requirements for APS in accordance with the most recent Sydney classification criteria [10]. Furthermore, 30 healthy volunteers of matched age and sex who were free of infection six weeks before to study inclusion and had no history of thrombosis were included in this study as a control group.

Exclusion criteria:

Patients with history of autoimmune systemic diseases, current infection and pregnant women were excluded.

Ethics-Related Matters:

The Tanta University Faculty of Medicine's Local Research Ethics Committee gave the study protocol their seal of approval (Approval code: 36155/12/22). The relevant committee on human experimentation's (institutional and national) ethical guidelines were followed throughout the entire process. All study participants gave their agreement after being made aware of its goals and the fact that the data would only be used for legitimate scientific purposes. When writing this publication, we adhered to the STROBE reporting requirements. . This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Methods:

All patients were subjected to the following:

1. **Full history taking, general examination, and local rheumatologic examination**.
2. **Laboratory evaluation:**
   - Lupus anticoagulant (LA), was assessed using dilute Russell's viper venom time (Drvvt)
   - Anti cardiolipin antibodies (aCL) and anti B2 glycoprotein I (b2GPI), IgG and IgM were determined by enzyme-linked immunosorbent assay (ELISA).
   - Myeloperoxidase (M po) activity was determined by spectrophotometry.
   - Matrix metalloproteinase 2 (MMP2), monocyte chemoattractant protein-1(McP1), and citrullinated histone H3 (H3 cit) were determined by enzyme-linked immunosorbent assay (ELISA). Chemicals and solvents used unless otherwise described were purchased from Sigma (Sigma, St Louis, USA).
3. **Adjusted Global Antiphospholipid Syndrome score (a GAPSS):**
   Was calculated, considering arterial hypertension (1 point), hyperlipidemia (3 points), and LA (4 points), aCL (5 points), b2GPI (4 points) results with maximum score of 17 points [11].

Blood sampling

Five mL of venous blood were collected with sterile disposable syringes, blood was collected in plain sterile tubes then centrifuged for 15 minutes at 1000 rpm, for serum separation by means of dry clean Pasteur pipette. The samples were divided and frozen at -80˚C. All groups were subjected to the blood measurement of the following:

1-Spectrophotometric assay of serum myeloperoxidase (MPO) enzyme activity:

Myeloperoxidase activity was assayed according to **Xia and Zweier,** 1997 using O-dianisidine and H_2O_2. Briefly, the presence of H_2O_2 as oxidizing agent, MPO catalyzes oxidation of O-dianisidine into oxidized O-dianisidine yielding a brown colored product. Enzyme activity was expressed as the change in the absorbance per minute at 412 nm wavelength. Results were represented as (U/L). Colorimetric assay was done using Biosystem spectrophotometer (BTS 350 semi-automatic analyzer, Spain) [12].

2-Immunoassay:

Enzyme-linked immunosorbent assay (ELISA) was used to detect serum matrix metalloproteinase-2 (MMP-2) and monocyte chemoattractant protein-1 (MCP-1/CCL2) using commercial ELISA kits supplied by RayBiotech, Inc. Norcross, USA, catalogue number: ELH-MMP2 and ELH-MCP1 respectively and citrullinated histone H3 (CitH3) using ELISA kit supplied by My BioSource California, USA, catalogue number: MBS7254090. All ELISA techniques were done according to the manufacturer's protocol and read on microplate reader (Stat Fax®2100, Fisher Bio block

Scientific, France), at 450 nm with correction wavelength set at 570 nm.

Statistical analysis

Utilizing SSPS software version 20, data analysis was carried out. Quantitative data were presented as mean, standard deviation, and range. Qualitative data were presented as number and percentage and were compared by chi square test. Spearman's rho was used to test for correlations The Kruskal-Wallis test was used to compare the participants' abnormally distributed data. Comparing quantitative data between the patient groups and controls was done using a one-way ANOVA test. When an analysis of variance test indicates a significant result, a Post Hoc test is used to identify specific mean differences between the three groups.0.05 was used as the cut-off point for statistical significance.

RESULT

Table 1 lists the characteristics of the APS patients and healthy controls. Along with sixty LA positive patients, thirty healthy volunteers were also included. Each APS patient in group I had a history of thrombosis, with 10 cases of both arterial and venous thrombosis, 5 cases of venous thrombosis, and 15 cases of both. Twenty patients in group II were tested positive for LA and anti-b2GPI, while ten patients were tested positive for LA alone. All patients in group I were triple positive for all aPL antibodies. In group I, every patient was taking an antithrombotic medication. 25 of them were given vitamin K antagonist, three were given low-dose aspirin, and two were given both. Twelve patients had hypertension, eight had hyperlipidemia, and six had concurrent diabetes mellitus **(Table 2)**.

Table 1: Features of study populations

			(Group I) (n=30)	(Group II) (n=30)	Control (n=30)	P
Age (Mean± SD)**,** years			42.93±7.15	42.70±8.30	41.50±9.94	0.006*
Duration of the disease (Mean± SD)**,** years			5.25 ± 2.7	5.54 ± 2.9	-	-
Sex	Female	N, %	19(63.3)	20(66.7)	17(56.7)	0.718
	Male	N, %	11(36.7)	10(33.3)	13(43.3)	
Comorbid diseases	Present	N, %	15(50)	6(20)	3(10)	0.002*
	Absent	N, %	15(50)	24(80)	27(90)	
Hb, mg/dl (Mean± SD)			9.97±1.03	10.53±2.03	11.13±0.86	0.008*
Platelets ^{X}1000			171.33±29.36	201.0±38.0	252.0 ±47.0	<0.001*
WBCs ^{X} 1000			6.60±1.73	7.27±1.82	7.35±1.37	0.162
Lupus anticoagulant antibodies	Positive		30(100)	30(100)	0(0)	<0.001*
	Negative		0(0)	0(0)	30(100)	
Anti cardiolipin, Ig M	Positive		20(66.7)	2(6.67)	0(0)	<0.001*
	Negative		10(33.3)	28(93.3)	0(0)	
Anti cardiolipin, Ig G	Positive		21(70)	3(10)	0(0)	<0.001*
	Negative		9(30)	27(90)	0(0)	
Anti b2 glycoprotein, Ig M	Positive		24(80)	0(0)	0(0)	<0.001*
	Negative		6(20)	30(100)	0(0)	
Anti b2 glycoprotein, Ig G	Positive		25(83.3)	0(0)	0(0)	<0.001*
	Negative		5(16.7)	30(100)	0(0)	

*APS: Anti phospholipid syndrome *: significant P, Group I: Aps with thrombotic events, Group II: APS devoid of thrombotic events. WBC: white blood cells, Hb: Hemoglobin.*

Table 2: Clinical features and thrombotic risks in the studied subjects

		(Group I) (n=30)	(Group II) (n=30)	Control (n=30)
APS clinical manifestations	**Thrombosis**	30(100)	0(0)	0(0)
	Pregnancy morbidity	23(76.7)	30(100)	0(0)
	Both	23(76.7)	0(0)	0(0)
Thrombosis	**Arterial**	5(10)	0(0)	0(0)
	Venous	15(50)	0(0)	0(0)
	Both	10(33.3)	0(0)	0(0)
Pregnancy morbidity	**Early**	14(46.7)	27(90)	0(0)
	Late	9(30)	3(10)	0(0)
	Premature delivery	0(0)	0(0)	0(0)
Thrombotic risk	**Triple positivity**	30(100)	0(0)	0(0)
	Arterial hypertension	12(40)	4(13.3)	3(10)
	hyperlipidemia	11(36.7)	2(6.7)	1(3.3)
	a GAPSS ≥17	12 (40)	0(0)	0(0)
Anticoagulation therapy during sampling		30(100)	16(53.3)	0(0)

Values of categorical variables are expressed as number and (percentage), APS: anti-phospholipid syndrome, a GAPSS: Adjusted Global antiphospholipid syndrome score.

Furthermore, there was a significant difference between both groups as regard MPO activity, matrix metalloproteinase 2, McP1, and H3cit and significant difference between both groups and controls (**Table 3**).

Table 3: Differences in the levels of (NETs) markers in APS patients

		(Group I) (n=30)	(Group II) (n=30)	Control (n=30)	P-value
MPO activity (µg/L)	Range	160.7 – 201.7	25.4 – 37.4	4.3 – 6.2	<0.001*
	Mean ± SD	182.10 ± 11.66	31.59 ± 3.88	5.28 ± 0.54	
MMP 2 (ng/ml)	Range	233.5 – 399.2	145.8 – 205.7	8.1 – 15.2	<0.001*
	Mean ± SD	301.99 ± 44.24	177.33 ± 19.18	11.58 ± 1.88	
McP 1 (pg/ml)	Range	287.9 – 329.5	190.6 – 233.3	9.7 – 23.8	<0.001*
	Mean ± SD	311.33 ± 11.61	210.21 ± 11.91	12.58 ± 2.47	
H3cit (ng/ml)	Range	250.4 – 325.4	185.2 – 250.9	2.6 – 7.5	<0.001*
	Mean ± SD	304.01 ± 21.43	210.63 ± 18.06	5.56 ± 1.19	

*APS: Antiphospholipid syndrome, LA: Lupus anticoagulant antibodies, MPO: Myeloperoxidase, MMP2: Matrix metalloproteinase 2, McP1: Monocyte chemoattractant protein-1, H3cit: Citrullinated histone H3, *: significant P*

In addition, lupus anticoagulant antibodies also correlated positively with MPO activity (**Figure 1 A, B, C**), matrix metalloproteinase 2 (**Figure 2 D, E, F**), McP1 (**Figure 3 G, H, I**), and H3cit (**Figure 4 J, K, L**) in APS patients in both groups and controls.

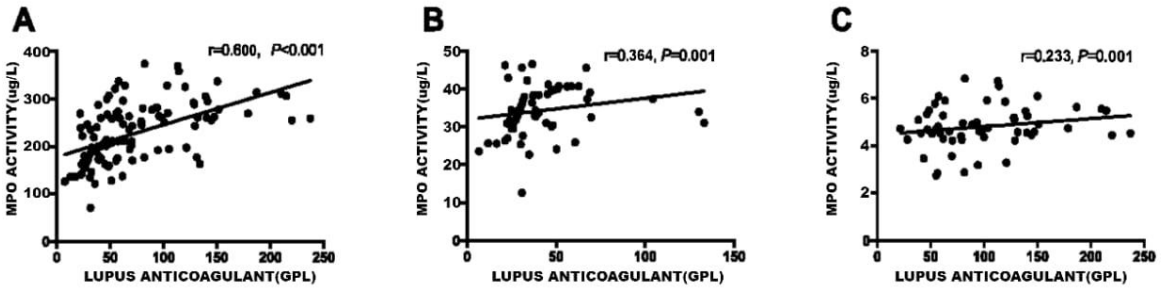

Figure (1): *Correlation between MPO activity and serum lupus anticoagulant of APS patients with thrombotic event (A), APS patients without thrombotic event (B), or control group(C).*

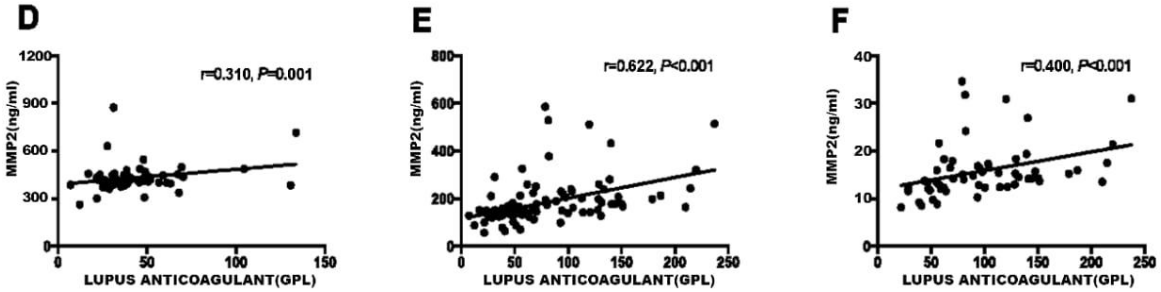

Figure (2): *Correlation between MMP2 and serum lupus anticoagulant of APS patients with thrombotic event (D), serum lupus anticoagulant of APS patients without thrombotic event (E) or control group(F)*

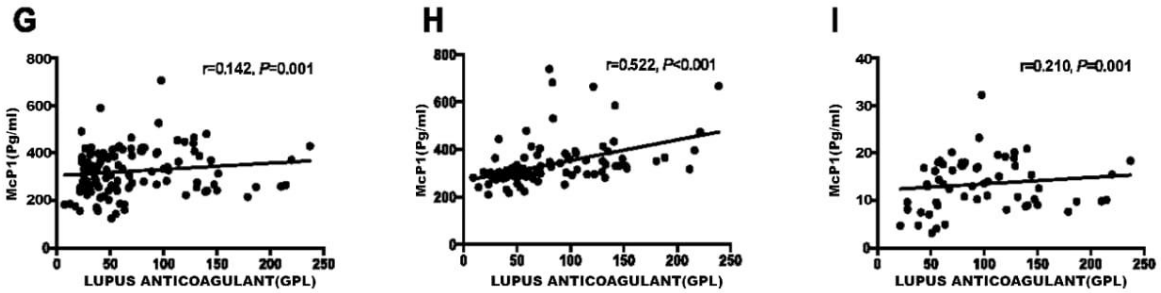

Figure (3): *Correlation between McP1 and serum lupus anticoagulant of APS patients with thrombotic event (G), APS patients without thrombotic event (H) and control group (I)*

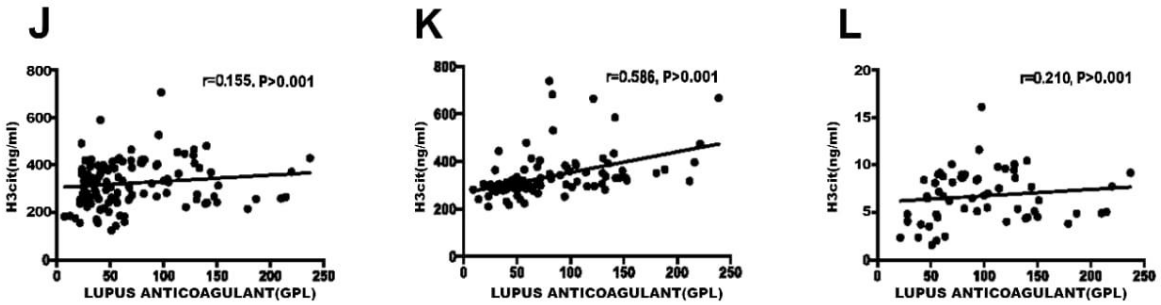

Figure (4): *Correlation between H3cit and serum lupus anticoagulant of APS patients with thrombotic event (J), APS patients without thrombotic event (K) and control group (L).*

DISCUSSION

Neutrophils play a crucial role in controlling venous and arterial thrombosis. Therefore, evaluation of their activity proposes a novel immunomodulatory strategy, and anti-neutrophil activation pathway medications may offer thrombosis protection in individuals with antiphospholipid syndrome. An intriguing issue that merits more research is one that was previously covered in primary APS [13]. The relationship between aPL and NET release was originally demonstrated in a study in 2015[14], and this result has since been confirmed by multiple research groups [15,16]. Large amounts of NETs released from neutrophils were seen in the blood of patients with APS more than healthy individuals [14]. Additionally, individuals with triple positive APL antibodies and high GAPSS had a tendency to have the most circulating NET residues and human monoclonal anti-2GPI antibodies also increased NET release leading to increased risk of thrombotic recurrence [14,17].

This was noticed in 40% of our APS patients in group I who had high GAPSS. Furthermore, the NADPH oxidase, TLR4 signaling, and Mac-1-mediated adhesion all played a part in the mechanism by which aPL increased NET release [16]. APL-mediated thrombosis in mice was studied in vivo using a few limitation models of venous thrombosis. APS IgG-treated mice had sizable thrombi that were enriched in NETs. Deoxyribonuclease treatment and neutrophil depletion both brought the level of thrombosis in APS mice to that of control mice [18]. Neutrophils play a crucial part in thrombosis, although despite compelling evidence to the contrary, there is still debate on how neutrophils affect the thrombotic process. Numerous research from the past ten years confirms that neutrophils do, in fact, play a significant role in pathologic venous and arterial thrombosis. Therefore, it is important to determine how neutrophils affect individuals with confirmed or likely primary antiphospholipid syndrome in terms of increased thrombotic risk. Hence, we assessed the activity of myeloperoxidase, matrix metalloproteinase 2, McP1, and H3cit in those patients in our study. The fact that H3cit positive NETs were demonstrated to be implicated in numerous diseases [19] as well as thrombus formation [20] corroborated this. One explanation for APS's elevated risk of thrombosis is that the antiphospholipid (aPL) antibody exposure primes neutrophils, causing them to produce more NETs. Innate immune cells are activated by aPL antibodies, particularly anti-2GPI antibodies, as has already been demonstrated [21]. By contrasting neutrophils taken from patients with APS with healthy controls, **Yalavarthi *et al.*,** [14] demonstrated that there were higher levels of cell-free DNA in plasma from APS patients and that after two hours of incubation in serum-free medium, their neutrophils create more spontaneous NETs. Additionally, administration of APS serum to regulate neutrophils led to an increase in NET formation. Curiously, **van der Linden *et al.***'s ***studies*** [22] did not reveal a noticeably higher NET production.

In our study, we also noticed a significant difference between antiphospholipid syndrome patients with thrombotic events and those without previous thrombotic events as regard MPO activity, matrix metalloproteinase 2, McP1, and H3cit and significant difference between both group and control. Moreover, these NETs markers were significantly correlated with serum lupus anticoagulant level. This comes in agreement with **Yalavarthi *et al.*,** [14] who found NETs circulate at elevated levels in patients with APS. They also demonstrated that anti-β2 glycoprotein I antibody and other IgG fractions from individuals with APS promoted NET release when β2GPI was present on the surface of neutrophils. Another study by **Leffler *et al.*,** [23] provides another reason for the higher occurrence of NETs in these individuals by showing that NETs are not degraded as well in APS patients. Likewise, **Meng *et al.*,** [18] proved that APS thrombi were enriched in NETs using in vivo mouse models. Thus, NETosis was the result of APS IgG stimulating mouse neutrophils. Also demonstrated by this team of researchers was the ability of DNase treatment and neutrophil depletion to prevent thrombosis in APS mice.

Although, **Reshetnyak *et al.*,** in their study revealed that the elevated levels of the MPO-DNA complex were considered a promising biomarker for lupus nephritis, disease activity, and immunological disorders in SLE patients and were not associated with thrombotic events. The MPO-DNA complex is a specific marker of NETosis that was found to increase in the serum of SLE patients but not APS [9]. Neutrophils release chromatin webs into the extracellular environment, as described by **Brinkmann and colleagues** [24]. These microbicidal proteins are known as neutrophil extracellular traps (NETs), and they are produced by neutrophils whose granules and cytoplasm have become entangled in tangles of histones and decondensed extracellular DNA. Cytokines, complements, autoantibodies, activated platelets and endothelial cells, and immunological complexes are only a few examples of the infectious and non-infectious stimuli that result in the production of NETs [25]. Although NETs most likely developed to trap infections, their prothrombotic properties are now well understood [25]. Both artery and deep vein thrombi contain NETs, which stimulate platelets and clotting factors [27].

Neutrophils as well as complement were discovered to be significant mediators of fetal harm as described in noteworthy research studies that described pregnancy models of APS in the early 2000s [28]. Phagocytosis, oxidative burst, and L-selectin shedding are three mechanisms by which several human monoclonal factors have been shown to activate neutrophils in vitro **[29-31]**. The researchers discovered

that roughly 13% of APS serum samples had erroneous NET breakdown as compared to healthy serum. By introducing APS serum into NETs that had already been produced and then watching for IgG deposition, the "anti-NET antibodies" from the same study were discovered. Additionally, **Mauracher *et al.***, [32] concluded that, in patients with primary antiphospholipid syndrome, neutrophils may raise their risk of thrombosis, making them a potential target for thrombosis prevention in APS. In a recently published study, **Onuora**, [33] showed that activated neutrophils cause arterial and venous thrombosis in a mouse model of antiphospholipid syndrome (APS). This finding raises the possibility that therapies that target the pathways of neutrophil activation may prevent thrombosis in diseases like APS. Furthermore, it might be conceivable to utilize drugs like peptidyl arginine deiminase inhibitors or neutrophil elastase inhibitors to block the NETosis process [34], or potentially anti-interferon therapies to block the downstream effects of NETs, if the mechanisms of NETosis are better known [35]. Consequently, it has been possible to find new, selective medications that may be effective in treating thrombotic disorders.

Targeting neutrophils is a safer treatment approach than the currently available antithrombotic drugs via changing the activity of neutrophils and production of NET and has no impact on hemostasis. It follows that it is undeniable that the emergence of NETs is a critical component of an out-of-date innate immune system that is necessary for warding off infections and halting their spread. So, deliberate consideration and investigation are required before NET inhibition is viewed as a viable anti-thrombotic strategy because avoiding NETosis can be problematic [35]. Further research is therefore needed to determine the best method to apply this knowledge to develop focused tactics that reduce the pro-thrombotic environment while preserving our capacity to combat infections.

These were some of the study's limitations:

First, even though the sample size is quite tiny, the examinations had to be carried out using recently drawn blood samples because APS is an uncommon disease. We were unable to assess the impact of comorbidities due to the very small participant pool. Second, just one component of the role of neutrophils in thrombotic events—activation of myeloperoxidase (Mpo), matrix metalloproteinase 2, McP1, and H3cit—was examined. As a result, it would be intriguing to investigate alternate NETosis mechanisms in this instance. This is challenging, nevertheless, because of the lack of traits unique to NET generation and cell death programs.

CONCLUSION

In APS, neutrophils should still be researched as potential treatment targets. These studies must also include models for thrombosis in the microvasculature and arterial thrombosis in addition to venous thrombosis.

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- **Author contributions:** All authors read the manuscript and gave their final approval on publication.

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