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Original article

Soluble LILRA3 is aberrantly expressed in antiphospholipid syndrome (APS) and is a potential marker of thrombotic APS

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Abstract

Objective. Leucocyte immunoglobulin-like receptor A3 (LILRA3) belongs to a family of leucocyte receptors. Our previous study reported LILRA3 transcripts were markedly upregulated in neutrophils from patients with APS. We undertook this study to investigate clinical implications of LILRA3 in APS and its potential role in APS-associated thrombosis.

Methods. Two independent cohorts were studied. The first consisted of 294 APS patients, 48 asymptomatic aPL carriers and 150 healthy controls (HCs) from Peking University People's Hospital. The second included 99 APS patients, 25 aPL carriers and 40 HCs from United States APS centres. Serum or plasma concentrations of LILRA3 and MPO-DNA complexes were measured. Additionally, 35 patients with thrombotic APS (tAPS) were evaluated to determine potential effects of immunosuppressive therapy on serum concentrations of LILRA3 and MPO-DNA complexes.

Results. Both positivity and serum concentration of LILRA3 were significantly increased in APS patients, especially in those with tAPS. LILRA3-positive tAPS patients displayed more severe thrombotic manifestations. Serum LILRA3 was positively correlated with MPO-DNA complexes in LILRA3-positive tAPS. After immunosuppressive treatment, LILRA3 and MPO-DNA complexes were consistently decreased in tAPS patients. Key findings from the Peking cohort were confirmed in the United States cohort.

Conclusion. Our study provides first evidence that LILRA3 is aberrantly expressed in APS, especially in patients with tAPS. Serum LILRA3 correlated with MPO-DNA complexes, and the two indices were consistently decreased in tAPS patients after treatment. LILRA3 may play a role in thrombosis of APS and may serve as a biomarker and/or therapeutic target in tAPS.

Key words: APS, LILRA3, clinical relevance, thrombosis

BASIC SCIENCE

Rheumatology key messages

- LILRA3 is aberrantly expressed in patients with APS, especially in thrombotic APS patients.
- LILRA3-positive APS patients demonstrated higher antiphospholipid antibody positivity and more severe thrombotic manifestations.
- Serum concentrations of LILRA3 were significantly decreased in thrombotic APS patients after immunosuppressive treatment.

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Introduction

APS is defined by the presence of aPL in patients with thrombotic complications and/or pregnancy morbidity [1]. APS can occur either as an isolated autoimmune condition known as primary APS [2] or in association with other autoimmune diseases, most often SLE, referred to as secondary APS [3, 4]. Nowadays it is generally accepted that APS can be classified into two distinct forms: thrombotic APS (tAPS) and obstetric APS (oAPS) [5, 6]. In addition to the presence of aPL, tAPS is characterized by the occurrence of venous, arterial or microvascular thrombosis. By contrast, oAPS manifests with pregnancy complications, especially late trimester growth restriction and fetal death [5, 7]. Emerging evidence has suggested that tAPS and oAPS have different underlying pathophysiologies despite being marked by similar antibody profiles [6, 8]. Overall, the clinical heterogeneity of APS indicates that more than one pathogenic process is involved, and there is an unmet need to understand which patients have the highest thrombotic risk. Neutrophils are thought to play an important role in the pathogenesis of APS. It has been revealed that aPL can directly activate neutrophils to release neutrophil extracellular traps (NETs) and promote thrombosis in many diseases including APS [9].

The leucocyte immunoglobulin-like receptors (LILRs) are a group of HLA-I receptors expressed mainly by myeloid lineage cells [10]. The LILR family consists of 11 functional genes encoding six activating (LILRA1-6) and five inhibitory (LILRB1-5) forms. LILRA3 is the only soluble receptor of the group as it lacks transmembrane and cytoplasmic domains [10]. Moreover, LILRA3 is unique as it exhibits a 6.7-kb deletion polymorphism, which leads to a nonfunctional protein due to loss of the first six out seven exons [11]. Interestingly, the frequency of the *LILRA3* 6.7-kb deletion varies widely across populations, with much higher frequencies in northeastern Asians (0.56–0.84) compared with Europeans (0.17) or Africans (0.10) [12]. We and other groups have previously demonstrated that functional *LILRA3* (i.e. no deletion) is associated with susceptibility and disease severity in multiple autoimmune diseases, including rheumatoid arthritis, SLE, primary Sjögren's syndrome, ankylosing spondylitis, Takayasu arteritis and multiple sclerosis [13–17]. More recently, Wang *et al.* reported that functional *LILRA3* is a novel genetic risk factor for adult-onset Still's disease and recombinant human LILRA3 can promote the release of NETs *in vitro* [18]. By performing a comprehensive transcriptome analysis of APS neutrophils, our group previously reported that all the activating LILR family members, including *LILRA3*, were significantly upregulated in neutrophils from United States patients with primary APS compared with HCs [19]. However, the link between LILRA3 and APS has not been otherwise investigated. In this study, we aimed to define the expression characteristics of LILRA3 in APS patients and determine the extent to which LILRA3 may play a role in APS-mediated thrombosis.

Materials and methods

Study subjects

The study subjects were recruited from two independent cohorts. One cohort of serum samples, including 294 APS patients, 48 asymptomatic aPL carriers and 150 healthy controls (HCs) was recruited from Peking University People's Hospital. Another study cohort of citrated plasma samples, including 99 patients with APS, 25 aPL carriers and 40 HCs was recruited in the United States from the University of Michigan and Mayo Clinic.

All patients were designated as having APS fulfilled the 2006 revised Sydney classification criteria for APS [1], while the aPL carriers fulfilled only the laboratory criteria. The aPL were defined according to 2006 Sydney Classification Criteria [1]. The smaller 'treatment evaluation group' included 35 out of 294 tAPS patients who received immunosuppressants in Peking University People's Hospital.

The study was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital (2020PHB087-01), the Institutional Review Board of the University of Michigan (HUM00122519) and the Institutional Review Board of Mayo Clinic (20–000476). All participants of this study gave written informed consent.

Data collection

Clinical and laboratory data were collected at the time of blood sampling. Clinical information included demographic data, medical history, thrombotic complications and morbid pregnancy events. Laboratory parameters included complete blood count, ESR, CRP, profiles of blood lipids, coagulation and immunology. The adjusted global APS score (aGAPSS) was calculated as previously described [20, 21].

Quantification of LILRA3

LILRA3 was quantified by the Human LILRA3/ILT-6/CD85e ELISA Kit (ELH-LILRA3, RayBiotech, Norcross, GA, USA), according to the manufacturer's instructions.

Quantification of MPO-DNA complexes

MPO-DNA complexes were quantified by a previously reported method [22], anti-human MPO antibody (Bio-Rad 0400–0002) were coated at 5 μg/ml at 4°C overnight and incubated with the diluted serum (1:10) at room temperature for 90 min. 10× anti-DNA antibody (1:100, the Cell Death kit) was added to each well as secondary antibody. The results were determined by measuring the absorbance at 450 nm.

Statistical analysis

Categorical and continuous variables were presented as number (%) and mean (S.D.), respectively. Categorical variables were analysed using χ^2 test. The two-tailed unpaired Student's *t* test was applied when the continuous variables were normally distributed and the nonparametric Mann–Whitney rank sum test was applied if the data

distribution was skewed. The paired *t* test was applied to evaluate the difference between the paired samples. One-way ANOVA with *post hoc* Dunnett multiple-comparisons test was performed to evaluate the difference between three data sets. Spearman's correlation analysis was used to evaluate the correlation between different continuous parameters. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS 21.0.

Results

General characteristics

A total of 294 patients with APS, 48 aPL carriers and 150 sex- and age-matched HCs were included in the first part of the study. According to the clinical manifestations of thrombotic events and/or pregnancy morbidity, APS patients were classified into three groups: tAPS ($n=174$, 59.2%), oAPS ($n=98$, 33.3%) and mixed tAPS +oAPS ($n=22$, 7.5%); given that only a few patients were present in the last group, these were not included in some analyses. According to any additional underlying autoimmune conditions [4], the APS patients were classified into primary APS and secondary APS. Baseline characteristics of the patients and controls are shown in Supplementary Table S1, available at *Rheumatology* online.

Increased rate of positivity and serum levels of LILRA3 in APS, especially in tAPS

According to the manufacturer's instructions, a serum concentration above 90 pg/ml was considered as LILRA3-positive. To validate the specificity of the ELISA kit for LILRA3 detection, we measured serum levels of LILRA3 for 32 HCs and 48 APS patients with known *LILRA3* genotypes. As shown in Fig. 1A and B, LILRA3 was not detected in patients who were homozygous for deleted *LILRA3* (*LILRA3*$^{-/-}$), whereas it was detected in almost all of the *LILRA3* allele carriers (*LILRA3*$\pm$ and *LILRA3*$^{+/+}$), supporting good specificity. We next analysed LILRA3 positivity rate and serum levels in APS patients, aPL carriers and HCs. As shown in Fig. 1C and D, both LILRA3 positivity rate and serum levels were significantly increased in APS patients compared with aPL carriers and HCs; aPL carriers did not differ from HCs. Next, we analysed the same variables according to underlying conditions. As seen in Fig. 1E, LILRA3 positivity rates were increased in both primary APS and secondary APS, but there was no significant difference between these two groups. Patients with secondary APS did have higher serum levels of LILRA3 than did patients with primary APS ($P<0.05$) (Fig. 1F). Finally, we analysed LILRA3 positivity rates and serum concentrations in groups according to clinical manifestations and found both indices were higher in tAPS compared with oAPS and the control groups (Fig. 1G, H). In summary, our results indicate that LILRA3 positivity rate and LILRA3 serum levels were increased in APS patients, especially in those individuals with tAPS.

Comparison of clinical and laboratory characteristics between LILRA3-positive and LILRA3-negative APS patients

Next, we investigated differences of clinical and laboratory parameters between LILRA3-positive and LILRA3-negative APS patients. As shown in Table 1, the incidence of thrombotic events was significantly increased in LILRA3-positive APS compared with LILRA3-negative APS (74.2% *vs* 55.2%, $P=0.001$), whereas there was no significant difference in pregnancy morbidity incidence. Furthermore, the incidences of thrombocytopenia, anaemia, aCL-positivity, low C3 and C4, elevated ESR, elevated fibrinogen, elevated D-dimer, low HDL and dyslipidaemia were also significantly higher in LILRA3-positive APS (Table 1). We analysed the laboratory parameters as quantitative/continuous variables. As seen in Table 1, platelets, haemoglobin, C3, C4 and HDL were significantly decreased in the LILRA3-positive group ($P<0.05$), whereas anti-β2GPI antibodies, ESR, CRP, D-dimer and the aGAPSS were significantly increased ($P<0.05$). The aGAPSS also positively correlated with serum levels of LILRA3 in LILRA3-positive patients ($r=0.186$, $P=0.013$) (Supplementary Fig. S1, available at *Rheumatology* online).

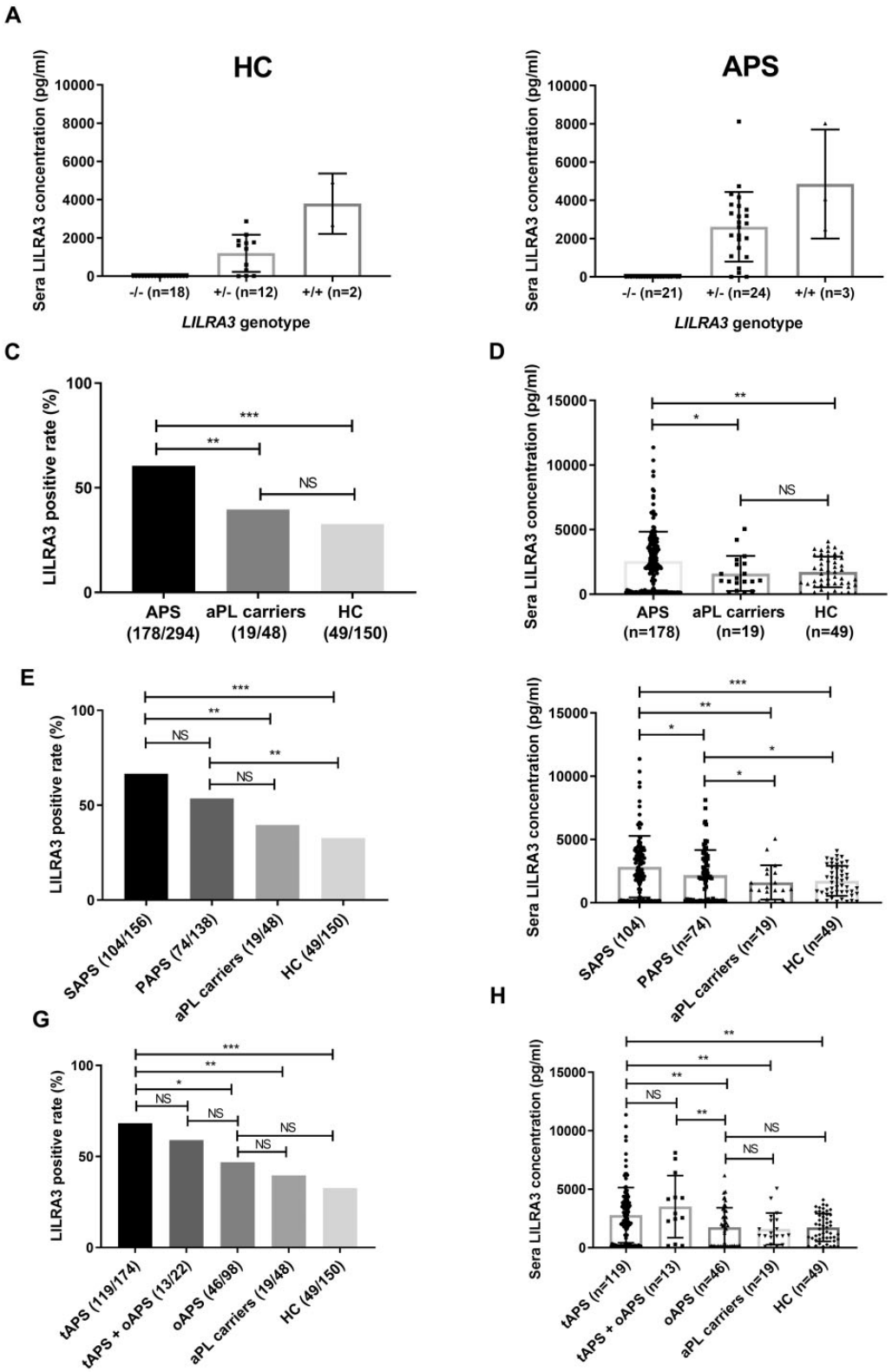
Furthermore, we investigated clinical and laboratory parameters in LILRA3-positive and LILRA3-negative APS groups according to the clinical manifestations (i.e. tAPS and oAPS). Notably, following stratification by clinical manifestations, we found the above differences between LILRA3-positive and LILRA3-negative APS groups were driven exclusively by tAPS patients (Table 1).

Next, we investigated clinical and laboratory parameters in LILRA3-positive/negative APS groups according to their underlying diagnosis (i.e. primary APS and secondary APS). Following stratification by the underlying diagnosis, we observed that the secondary APS group had more thrombotic events than that did the primary APS group (67.3% *vs* 52.2%, $P=0.008$, data not shown), which was consistent with previous studies [23–26]. Furthermore, we observed that for secondary APS, thrombotic events were significantly increased in LILRA3-positive patients as compared with LILRA3-negative patients (70.2% *vs* 61.5%, $P=0.017$). In the primary APS group, thrombotic events were also increased in LILRA3-positive patients, though the difference did not reach statistical significance (55.4% *vs* 48.4%, $P=0.373$) (shown in revised Supplementary Table S2, available at *Rheumatology* online). Extensive clinical/laboratory evaluation was detailed in the Supplementary Results available at *Rheumatology* online.

Increased anti-β2GPI and aCL positivity in LILRA3-positive APS

Next, we investigated the anti-β2GPI and aCL positivity between LILRA3-positive and LILRA3-negative APS patients. As shown in Table 2, positivity rates for anti-β2GPI, aCL and double positive for both anti-β2GPI and

Fig. 1 Increased positive rates and serum levels of LILRA3 in APS, especially in thrombotic APS



Serum levels of LILRA3 were confirmed in 32 healthy controls (HCs) and 48 antiphospholipid syndrome (APS) with known LILRA3 genotype (**A** and **B**). Rate of LILRA3 positivity and serum levels of LILRA3 in positive individuals were analysed in total APS (**C** and **D**), Primary APS (PAPS) and Secondary APS (SAPS) (**E** and **F**), thrombotic APS (tAPS) and obstetric APS (oAPS) subgroups (**G** and **H**). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; NS, not significant).

Table 1 Comparison of clinical and laboratory characteristics between LILRA3-positive and LILRA3-negative patients with total APS, tAPS and oAPS, respectively

Characteristic	Total APS			tAPS			oAPS		
	LILRA3$^+$ ($n = 178$)	LILRA3$^-$ ($n = 116$)	*P*-value	LILRA3$^+$ ($n = 119$)	LILRA3$^-$ ($n = 55$)	*P*-value	LILRA3$^+$ ($n = 46$)	LILRA3$^-$ ($n = 52$)	*P*-value
Thrombotic events	132/178 (74.2)	64/116 (55.2)	**0.001**	—	—	—	—	—	—
Morbid pregnancy	59/129 (45.7)	61/106 (57.5)	0.072	—	—	—	—	—	—
Thrombocytopenia	79/174 (45.4)	26/113 (23)	**<0.001**	59/117 (50.4)	14/55 (25.5)	**0.002**	15/44 (34.1)	8/49 (16.3)	**0.047**
Anaemia	70/174 (40.2)	27/113 (23.9)	**0.004**	56/117 (47.9)	12/55 (21.8)	**0.001**	10/44 (22.7)	12/49 (24.5)	0.842
leukopenia	20/174 (11.5)	8/113 (7.1)	0.218	13/117 (11.1)	3/55 (5.5)	0.234	4/44 (9.1)	3/49 (6.1)	0.588
Anti-β2GPI$^+$	98/162 (60.5)	62/112 (55.4)	0.396	67/106 (63.2)	30/53 (56.6)	0.421	27/44 (61.4)	28/51 (54.9)	0.525
aCL$^+$	91/164 (55.5)	45/107 (42.1)	**0.031**	71/109 (65.1)	27/51 (52.9)	0.140	15/43 (34.9)	15/48 (31.3)	0.713
LA$^+$	73/142 (51.4)	56/103 (54.4)	0.647	57/93 (61.3)	29/48 (60.4)	0.920	9/37 (24.3)	20/47 (42.6)	0.081
Elevated IgA	18/138 (13)	8/91 (8.8)	0.321	17/92 (18.5)	6/44 (13.6)	0.481	1/33 (3)	2/39 (5.1)	0.657
Elevated IgG	29/141 (20.6)	14/91 (15.4)	0.321	21/94 (22.3)	8/44 (18.2)	0.576	8/34 (23.5)	4/39 (10.3)	0.127
Elevated IgM	13/139 (9.4)	3/91 (3.3)	0.078	8/93 (8.6)	2/44 (4.5)	0.394	5/33 (15.2)	1/39 (2.6)	0.054
Low C3	76/153 (49.7)	32/98 (32.7)	**0.008**	54/104 (51.9)	19/48 (39.6)	0.157	14/36 (38.9)	10/41 (24.4)	0.171
Low C4	72/153 (47.1)	30/98 (30.6)	**0.010**	50/104 (48.1)	15/48 (31.3)	0.051	16/36 (44.4)	13/41 (31.7)	0.250
Elevated ESR	68/117 (58.1)	22/69 (31.9)	**0.001**	52/87 (59.8)	12/37 (32.4)	**0.005**	10/19 (52.6)	6/24 (25)	0.063
Elevated CRP	42/116 (36.2)	14/60 (23.3)	0.082	36/89 (40.4)	9/33 (27.3)	0.180	3/16 (18.8)	4/19 (21.1)	0.865
Prolonged PT	52/139 (37.4)	26/81 (32.1)	0.427	44/104 (42.3)	19/43 (44.2)	0.834	4/24 (16.7)	4/30 (13.3)	0.732
Prolonged APTT	44/132 (33.3)	26/78 (33.3)	1.000	35/99 (35.4)	16/40 (40)	0.607	5/22 (22.7)	6/30 (20)	0.812
Elevated FIB	35/132 (26.5)	11/78 (14.1)	**0.036**	27/99 (27.3)	5/40 (12.5)	**0.049**	5/22 (22.7)	6/30 (20)	0.812
Elevated D-dimer	71/127 (55.9)	23/70 (32.9)	**0.002**	58/96 (60.4)	11/33 (33.3)	**0.007**	7/20 (35)	8/29 (27.6)	0.580
Elevated cholesterol	10/146 (6.8)	3/84 (3.6)	0.300	6/108 (5.6)	1/45 (2.2)	0.369	3/25 (12)	1/31 (3.2)	0.205
Elevated triglycerides	50/146 (34.2)	21/84 (35)	0.144	41/108 (38)	11/45 (24.4)	0.108	5/25 (20)	9/31 (29)	0.438
Low HDL	57/146 (39)	22/84 (26.2)	**0.048**	46/108 (42.6)	13/45 (28.9)	0.113	5/25 (20)	6/31 (19.4)	0.952
Elevated LDL	14/145 (9.7)	3/84 (3.6)	0.091	10/107 (9.3)	2/45 (4.4)	0.306	3/25 (12)	0/31 0	0.166
Dyslipidaemia	86/146 (58.9)	35/84 (41.7)	**0.012**	68/108 (63.0)	18/45 (40.0)	**0.011**	10/25 (40.0)	12/31 (38.7)	0.922
PLT ($\times 10^9$/L)	152.14 ± 98.46	186.02 ± 90.86	**0.002**	136.28 ± 96.10	179.32 ± 81.24	**0.002**	195.65 ± 95.14	203.47 ± 97.24	0.797
Hb (g/L)	119.78 ± 23.62	128.32 ± 20.63	**0.003**	117.13 ± 24.33	133.01 ± 21.35	**<0.001**	128.04 ± 19.78	125.31 ± 17.40	0.221
WBC ($\times 10^9$/L)	7.19 ± 3.80	7.09 ± 3.14	0.676	7.30 ± 4.16	6.88 ± 2.97	0.918	7.38 ± 2.95	7.46 ± 3.35	0.878
Anti-β2-GPI (RU/mL)	66.19 ± 78.28	50.29 ± 73.92	**0.036**	75.34 ± 85.82	64.84 ± 92.12	0.256	48.50 ± 54.15	40.00 ± 52.46	0.190
aCL (U/mL)	26.56 ± 34.46	19.36 ± 25.28	0.110	31.42 ± 37.62	21.71 ± 25.79	0.265	15.60 ± 22.49	18.16 ± 26.34	0.712
LA	1.34 ± 0.45	1.38 ± 0.44	0.465	1.40 ± 0.46	1.47 ± 0.50	0.657	1.16 ± 0.36	1.25 ± 0.36	0.080
IgA (g/L)	2.79 ± 1.76	2.49 ± 1.43	0.276	2.94 ± 1.71	2.52 ± 1.78	0.110	2.30 ± 1.06	2.42 ± 1.05	0.799
IgG (g/L)	14.02 ± 5.49	13.41 ± 4.48	0.781	14.18 ± 5.05	13.12 ± 5.04	0.281	14.89 ± 6.79	13.49 ± 2.99	0.960
IgM (g/L)	1.50 ± 1.21	1.31 ± 0.77	0.548	1.46 ± 1.32	1.10 ± 0.79	**0.045**	1.66 ± 1.05	1.57 ± 0.71	0.684
C3 (g/L)	0.81 ± 0.29	0.92 ± 0.31	**0.003**	0.81 ± 0.30	0.92 ± 0.36	0.064	0.84 ± 0.25	0.94 ± 0.25	0.098
C4 (g/L)	0.18 ± 0.17	0.21 ± 0.10	**0.002**	0.19 ± 0.20	0.22 ± 0.13	**0.012**	0.18 ± 0.09	0.20 ± 0.07	0.298
ESR (mm/h)	39.31 ± 35.19	25.54 ± 29.87	**0.003**	40.77 ± 35.63	24.05 ± 30.88	**0.001**	36.05 ± 34.79	22.67 ± 25.11	0.573
CRP (mg/L)	21.16 ± 41.92	9.57 ± 23.69	**0.009**	23.08 ± 42.96	12.14 ± 29.70	**0.026**	19.57 ± 49.19	5.13 ± 8.07	0.829

(continued)

Table 1 Continued

Characteristic	Total APS			tAPS			oAPS		
	LILRA3$^+$ ($n = 178$)	LILRA3$^-$ ($n = 116$)	*P*-value	LILRA3$^+$ ($n = 119$)	LILRA3$^-$ ($n = 55$)	*P*-value	LILRA3$^+$ ($n = 46$)	LILRA3$^-$ ($n = 52$)	*P*-value
PT (s)	13.36 ± 5.11	14.10 ± 5.92	0.259	13.75 ± 5.63	16.01 ± 7.31	**0.021**	11.77 ± 1.77	11.31 ± 1.33	0.187
APTT (s)	39.40 ± 15.48	38.60 ± 13.26	0.985	40.31 ± 15.77	40.42 ± 15.12	0.885	34.70 ± 8.89	34.55 ± 8.90	0.846
FIB (mg/dL)	329.32 ± 127.08	326.76 ± 92.41	0.477	329.84 ± 124.34	328.28 ± 102.33	0.561	312.91 ± 107.87	331.90 ± 89.80	0.442
D-dimer (ng/mL)	934.24 ± 2616.66	374.92 ± 692.94	**0.001**	1035.33 ± 2917.79	563.65 ± 959.58	**0.041**	610.44 ± 1518.01	195.76 ± 197.86	0.204
Cholesterol (mmol/L)	4.45 ± 1.33	4.48 ± 1.01	0.440	4.37 ± 1.26	4.51 ± 1.02	0.287	4.83 ± 1.50	4.40 ± 0.94	0.311
Triglycerides (mmol/L)	1.58 ± 0.87	1.52 ± 0.89	0.318	1.66 ± 0.95	1.48 ± 0.76	0.266	1.35 ± 0.57	1.60 ± 1.13	0.993
HDL (mmol/L)	1.17 ± 0.43	1.27 ± 0.37	**0.024**	1.11 ± 0.39	1.24 ± 0.38	**0.031**	1.48 ± 0.49	1.34 ± 0.35	0.434
LDL (mmol/L)	2.69 ± 0.95	2.66 ± 0.74	0.794	2.68 ± 0.92	2.73 ± 0.76	0.463	2.76 ± 1.08	2.50 ± 0.65	0.458
aGAPSS	9.82 ± 4.46	8.54 ± 4.49	**0.017**	11.09 ± 3.75	9.58 ± 4.29	**0.020**	6.87 ± 4.48	7.63 ± 4.54	0.404

aCL: anticardiolipin antibody; aGAPSS: adjusted global anti-phospholipid syndrome score; anti-β2GP1: anti-β2-glycoprotein 1 antibody; APTT: activated partial thromboplastin time; C3: complement 3; C4: complement 4; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; FIB: fibrinogen; Hb: haemoglobin; HDL: high density lipoprotein; IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M; LA: lupus anticoagulant; LDL: low density lipoprotein; oAPS: obstetric APS; PLT: platelet; PT: prothrombin time; tAPS: thrombotic APS; WBC: white blood cell. *P*-values < 0.05 were considered statistically significant.

Table 2 Comparison of positive rate of aPL between LILRA3-positive and LILRA3-negative patients with total APS, tAPS and oAPS, respectively

Type of aPL	Total APS			tAPS			oAPS		
	LILRA3$^+$(n = 178)	LILRA3$^-$($n = 116$)	*P*-value	LILRA3$^+$($n = 119$)	LILRA3$^-$($n = 55$)	*P*-value	LILRA3$^+$($n = 46$)	LILRA3$^-$($n = 52$)	*P*-value
Anti-β2GP1$^+$	128/175 (73.1)	70/116 (55.4)	**0.022**	91/117 (77.8)	32/55 (58.2)	**0.008**	31/46 (67.4)	34/52 (65.4)	0.834
aCL$^+$	120/178 (67.4)	64/116 (55.2)	**0.034**	94/119 (79.0)	35/55 (63.6)	**0.031**	20/46 (43.5)	26/52 (50.0)	0.519
LA$^+$	108/162 (66.7)	75/115 (65.2)	0.802	82/108 (75.9)	43/55 (78.2)	0.747	16/42 (38.1)	25/51 (49.0)	0.291
Anti-β2GP1$^+$ + aCL$^+$	99/175 (56.6)	45/116 (38.8)	**0.005**	78/117 (66.7)	22/55 (40.0)	**0.001**	16/46 (34.8)	20/52 (38.5)	0.706
Anti-β2GP1$^+$ + LA$^+$	73/161 (45.3)	41/115 (35.7)	0.107	59/107 (55.1)	23/55 (41.8)	0.108	9/42 (21.4)	15/51 (29.4)	0.381
aCL$^+$+ LA$^+$	79/162 (48.8)	46/115 (40.0)	0.149	64/108 (59.3)	30/55 (54.5)	0.565	10/42 (23.8)	13/51 (25.5)	0.825
Triple aPL$^+$	62/161 (38.5)	34/115 (29.6)	0.124	50/107 (46.7)	18/55 (32.7)	0.087	8/42 (19.0)	13/51 (25.5)	0.460

aCL: anticardiolipin antibody; anti-β2GP1: anti-β2-glycoprotein 1 antibody; LA: lupus anticoagulant; oAPS: obstetric APS; tAPS: thrombotic APS. *P*-values < 0.05 were considered statistically significant.

aCL were significantly higher in LILRA3-positive APS (anti-β2GPI: 73.1% *vs* 55.4%, $P = 0.022$; aCL: 67.4% *vs* 55.2%, $P = 0.034$; anti-β2GPI + aCL: 56.6% *vs* 38.8%, $P = 0.005$, respectively). Notably, after stratification by clinical manifestations, we observed that the increased aPL positivity was driven by the tAPS group (anti-β2GPI: 77.8% *vs* 58.2%, $P = 0.008$; aCL: 79.0% *vs* 63.6%, $P = 0.031$; anti-β2GPI and aCL: 66.7% *vs* 40.0%, $P = 0.001$, respectively) and not the oAPS group (shown in Table 2). Furthermore, we found that serum levels of LILRA3 were positively correlated with the levels of aCL in all APS ($r = 0.176$, $P = 0.025$) and tAPS ($r = 0.234$, $P = 0.015$), but not oAPS ($r = -0.115$, $P = 0.462$) (Supplementary Fig. S2A–C, available at *Rheumatology* online). No significant correlations were observed for anti-β2GPI, LA, and other laboratory parameters (data not shown).

Given that anti-dsDNA positivity could play a significant role in the development of thrombosis in patients with SLE [27], we next assessed the potential association/correlation between the presence/titers of anti-dsDNA autoantibodies and the positivity/serum levels of LILRA3. As shown in Supplementary Fig. S3, available at *Rheumatology* online, there was a non-significant trend toward a positive association between titers of anti-dsDNA and levels of LILRA3. There was also no difference in serum levels of LILRA3 between anti-dsDNA-positive and anti-dsDNA-negative patients.

LILRA3-positive tAPS patients displayed more severe thrombotic manifestations

Given the above findings, we further investigated the extent to which LILRA3-positive tAPS patients associated with more severe thrombotic disease, defined by number of thrombotic events, number of thrombus sites, and types of thrombosis. As shown in Supplementary Table S3, available at *Rheumatology* online, number of thrombotic events was higher in LILRA3-positive tAPS, compared with LILRA3-negative tAPS (two or more times: 60.5% *vs* 30.9%, $P < 0.001$) (Supplementary Table S3, available at *Rheumatology* online). Similarly, number of thrombus sites was also higher in LILRA3-positive tAPS (two or more sites: 52.1% *vs* 29.1%, $P = 0.005$). In contrast, the LILRA3-negative tAPS patients had more 'one-time' (69.1% *vs* 39.5%, $P = 0.001$) and 'one-site' thrombosis (70.9% *vs* 47.9%, $P = 0.005$). Considering the types of thrombosis, we observed that the incidences of deep vein thrombosis and arterial ischemic stroke were significantly increased in LILRA3-positive tAPS (deep vein thrombosis: 68.1% *vs* 50.9%, $P = 0.030$; ischemic stroke: 52.0% *vs* 25.5%, $P = 0.035$) (Supplementary Table S3, available at *Rheumatology* online). In addition, incidence of pulmonary embolism was also higher among LILRA3-positive tAPS patients, although the difference did not reach statistical significance (pulmonary embolism: 18.5% *vs* 10.9%, $P = 0.206$). Our results indicate that LILRA3-positive tAPS patients had more severe thrombotic manifestations.

Serum concentration of LILRA3 was positively correlated with MPO-DNA complexes (NET) in LILRA3-positive tAPS

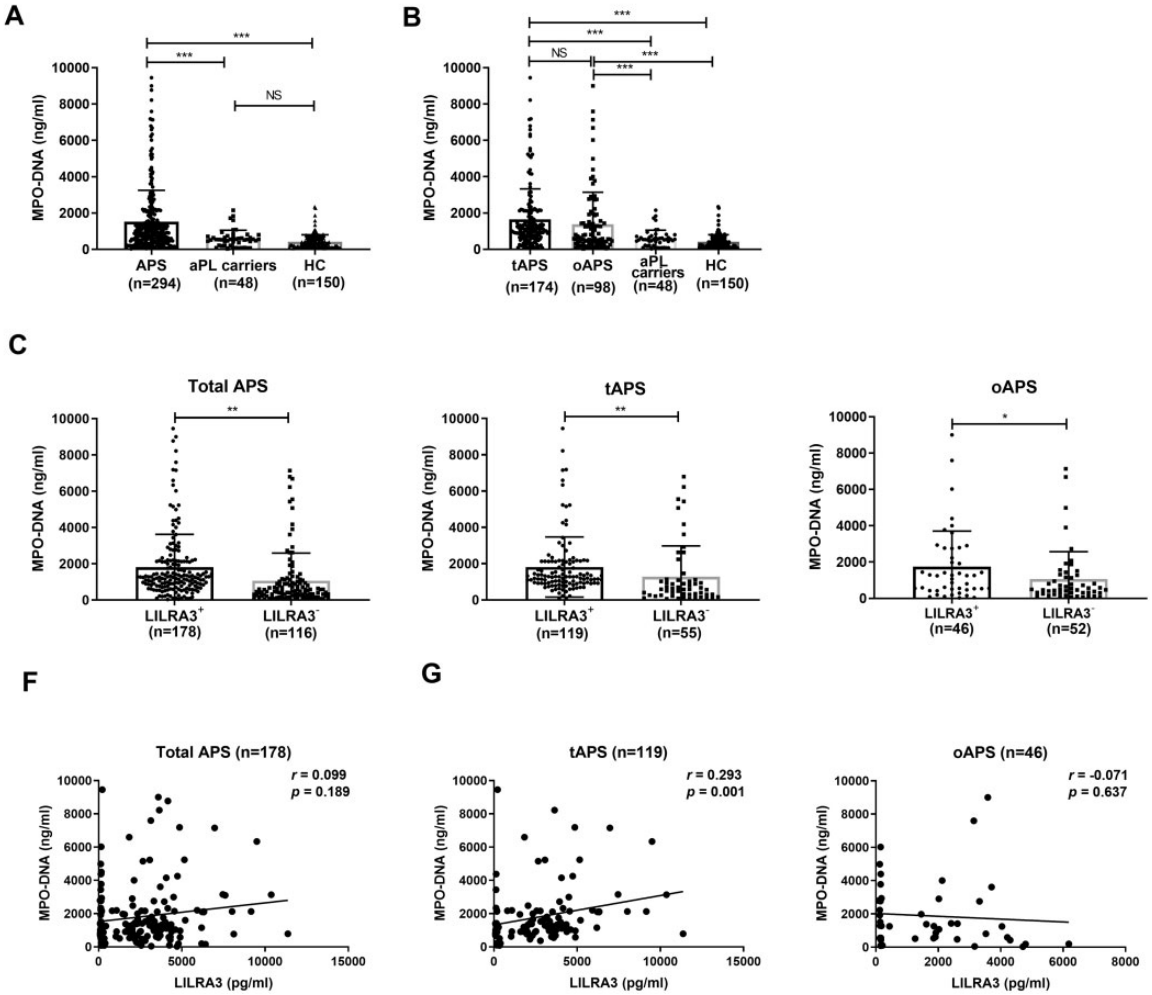
Increased NET formation may contribute to thrombosis in APS [9]. More recently, a study has reported that LILRA3 potentially triggers neutrophils to release NETs in adult-onset Still's disease [18]. To further investigate the possible link(s) between LILRA3 and thrombosis in APS patients, we measured serum MPO-DNA complexes in APS patients and HCs, as it is generally accepted that MPO-DNA complexes are highly specific for NET remnants [22, 28]. As shown in Fig. 2A, serum levels of MPO-DNA complexes were significantly increased in APS patients, compared with aPL carriers ($P < 0.001$) and HCs ($P < 0.001$). Levels of MPO-DNA complexes were increased in both tAPS and oAPS (Fig. 2B). Following stratification by LILRA3 positivity, increased levels of MPO-DNA complexes were observed in LILRA3-positive APS compared with LILRA3-negative APS ($P < 0.01$) (Fig. 2C). A similar pattern was seen in both LILRA3-positive tAPS ($P < 0.01$) and LILRA3-positive oAPS ($P < 0.05$) (Fig. 2D and E).

Next, we evaluated potential correlation between serum levels of LILRA3 and MPO-DNA complexes in LILRA3-positive patients. As seen in Fig. 2F, there was a trend toward a correlation between levels of LILRA3 and MPO-DNA complexes when all APS patients were considered ($r = 0.099$, $P = 0.189$). Notably, after stratification by clinical manifestations, a significant positive correlation was observed between levels of LILRA3 and MPO-DNA complexes in LILRA3-positive tAPS ($r = 0.293$, $P = 0.001$) (Fig. 2G) but not in oAPS ($r = -0.071$, $P = 0.637$) (Fig. 2H).

LILRA3 levels are elevated in a United States APS cohort

Next, we asked the extent to which these findings in a Chinese population (where *LILRA3* deletion is relatively common) might extend to a cohort of APS patients in which the vast majority will be LILRA3-positive. LILRA3 levels were measured in the plasma of 99 APS patients, 25 aPL carriers and 40 HCs, recruited at two academic medical centres in the United States. Of the 164 samples tested, only six (4%) had levels <90 pg/ml suggestive of likely homozygous *LILRA3* deletion. The demographics and clinical profiles of the patients are described in Supplementary Table S4, available at *Rheumatology* online. Notably, 21% of APS patients and 32% of aPL carriers had lupus; none were diagnosed with other systemic autoimmune diseases. As compared with healthy controls, LILRA3 levels were elevated in patients with both primary APS ($P < 0.001$) and secondary APS ($P < 0.05$), with a similar median in both instances (Fig. 3A). LILRA3 levels trended toward higher in aPL carriers but without reaching statistical significance ($P = 0.051$) (Fig. 3A). Of the 99 APS patients, 88 had a history of thrombosis and 20 had a history of obstetric morbidity. As expected, LILRA3 levels were elevated as

FIG. 2 Serum concentration of LILRA3 was positively correlated with MPO-DNA complexes (NETs) in LILRA3-positive tAPS



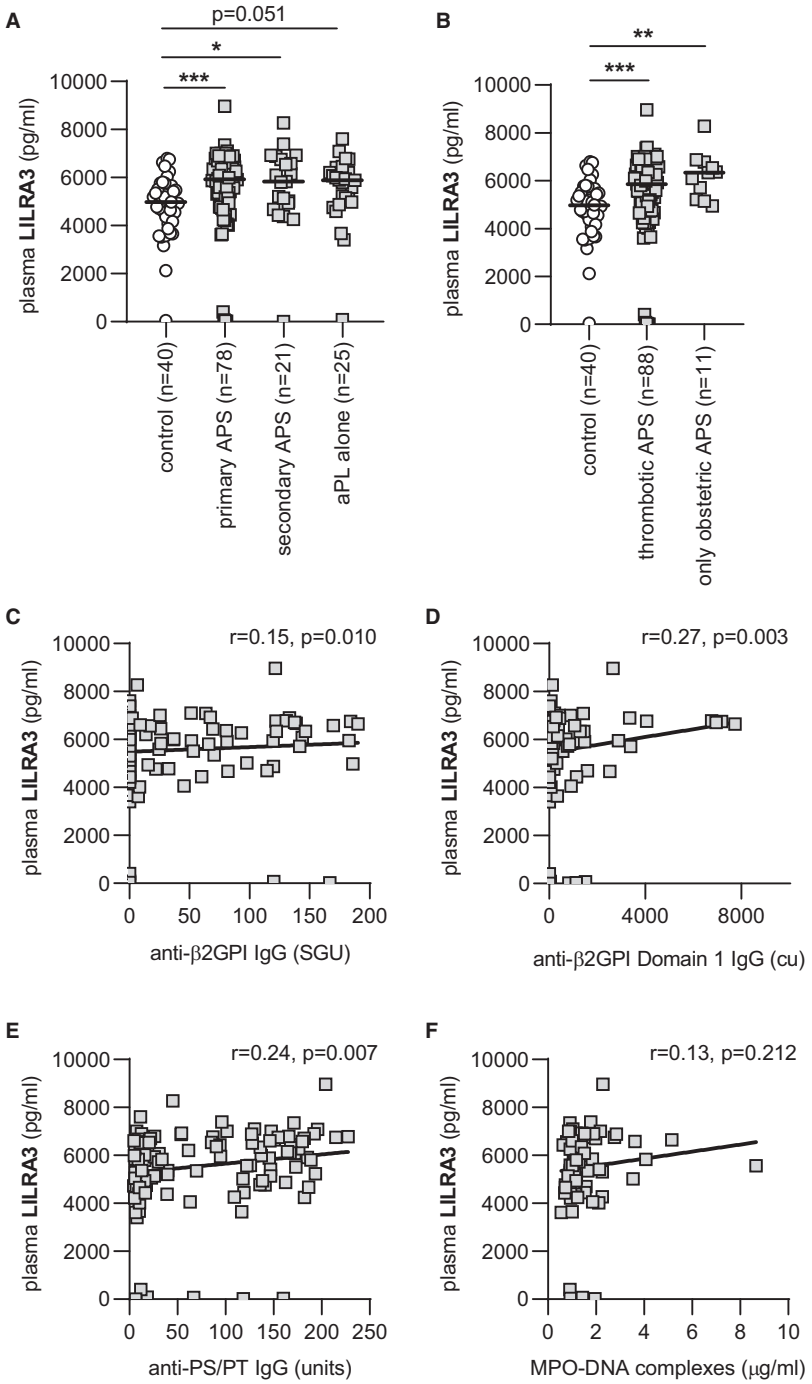
Serum MPO-DNA complex levels were analysed in total APS (**A**), tAPS and oAPS (**B**). Serum MPO-DNA complex levels were compared in LILRA3-positive and negative total APS (**C**), tAPS (**D**) and oAPS (**E**). The correlation between MPO-DNA complex and LILRA3 levels was evaluated in LILRA3-positive total APS (**F**), tAPS (**G**) and oAPS (**H**) respectively. ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$; NS, not significant).

compared with healthy controls in thrombotic APS (Fig. 3B). This was also true for the 11 patients with a history of only obstetric morbidity (Fig. 3B). Given the above observation that higher levels of certain aPL associated with higher levels of LILRA3, we measured anti-β2GPI IgG, anti-β2GPI domain 1 IgG and anti-phosphatidylserine/prothrombin (PS/PT) IgG in the 124 patients with a history of durable aPL positivity. All three antibodies demonstrated a statistically significant positive correlation with LILRA3, with the effect strongest for anti-β2GPI domain I and anti-PS/PT (Fig. 3C–E). In this cohort, we also asked whether there might be a correlation between LILRA3 and MPO-DNA complexes in thrombotic APS patients, as had been observed in the Chinese cohort. In this case, we found a positive association, but it did not reach statistical significance (Fig. 3F). In summary, APS does associate with higher levels of LILRA3 in a cohort of patients in which most had experienced a past thrombotic event and >95% were LILRA3-positive.

Serum levels of LILRA3 and MPO-DNA complexes decreased in tAPS patients after treatment

To evaluate the effect of treatment on LILRA3 and MPO-DNA complexes in tAPS, we identified 35 patients with thrombotic APS. Many of these thrombotic APS patients received immunosuppressants for refractory APS. All had serum samples available before and after treatment with blood sampling spaced by at least 3 months. Clinical characteristics and treatment are shown in Supplementary Table S5, available at *Rheumatology* online. We first analysed the possible correlation before treatment and found that serum levels

Fig. 3 LILRA3 levels are elevated in a United States antiphospholipid cohort



LILRA3 levels were measured in plasma (**A**). LILRA3 levels were sorted by clinical manifestations; the thrombosis group includes nine patients with history of both thrombosis and obstetric morbidity (**B**). Correlation between LILRA3 and IgG aPL as indicated; $n = 124$ (**C**–**E**). Correlation between LILRA3 and MPO-DNA complexes; $n = 88$ patients with thrombotic APS (**F**). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

of LILRA3 were positively correlated with MPO-DNA complexes in these tAPS patients ($r = 0.492$, $P = 0.002$) (Fig. 4A). Notably, after treatment both serum LILRA3 and MPO-DNA levels were significantly decreased ($P < 0.001$) (Fig. 4B and C), although did remain correlated with each other (Fig. 4D). Moreover, after treatment serum aCL, anti-β2GPI, ESR and CRP were also significantly decreased (Fig. 4E–H). In addition, we

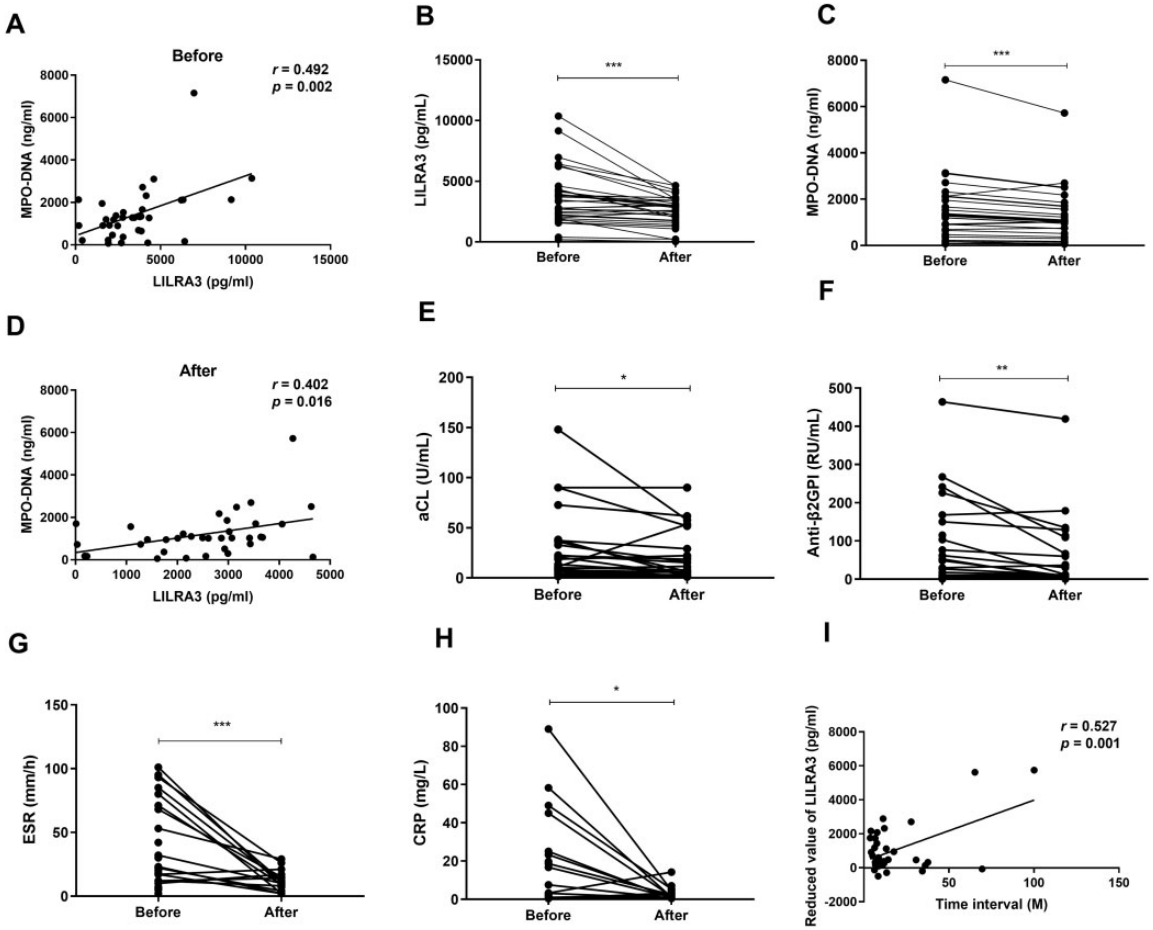
observed that the reduction of serum LILRA3 was positively correlated with the time interval of treatment ($r = 0.527$, $P = 0.001$) (Fig. 4I).

We further assessed the potential additive effect of HCQ with immunosuppressive drugs (ISDs), including MMF, CYC and rapamycin (RAPA), on LILRA3 reduction. As almost all of the patients also received anticoagulants (ACs) or in combination with corticosteroids (CSs), those variables were not considered in the analysis. As shown in Supplementary Fig. S4, available at *Rheumatology* online, concentrations of LILRA3 were decreased with either HCQ or ISDs treatment, though the reduction did not reach statistical significance in either group [**(A)** HCQ, $P = 0.061$, $n = 9$; **(B)** ISDs $P = 0.070$, $n = 9$]. By contrast, concentrations of LILRA3 were significantly decreased in patients on combined treatment with HCQ and ISDs [**(C)** HCQ + ISDs, $P = 0.004$, $n = 13$]. Taken together, these results suggest that combined treatment with HCQ and ISDs may exhibit more profound effects on reduction of LILRA3 levels as compared with either HCQ or ISDs alone.

Discussion

In the past decade, studies have suggested that tAPS and oAPS are distinct APS subgroups, perhaps mediated by similar autoantibodies but through different mechanisms [5–8]. It is now generally accepted that the pathogenesis of oAPS are mainly attributed to aPL-mediated placental inflammation, leading to morbid pregnancy events [6], whereas the pathogenesis of tAPS may be attributed to the 'two hit' hypothesis [5, 29]. The presence of aPL (first hit) causes a thrombophilic state, with clotting then happening when another procoagulant condition occurs (second hit). The second hit mainly

Fig. 4 Serum levels of LILRA3 and MPO-DNA complexes decreased in tAPS patients after treatment



The correlation between serum LILRA3 and MPO-DNA levels was analysed in 35 tAPS patients before treatment (**A**). Serum LILRA3 and MPO-DNA levels were compared before and after treatment (**B** and **C**). The correlation between serum LILRA3 and MPO-DNA levels was analysed in these patients after treatment (**D**). Serum anticardiolipin antibody (aCL), anti-β2-glycoprotein 1 antibody (anti-β2GPI), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were compared before and after treatment (**E**, **F**, **G** and **H**). The correlation between the reduced values of serum LILRA3 and the time interval of treatment was analysed (**I**). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; NS, not significant).

includes but is not limited to inflammatory responses, mechanical trauma, immobility, venous stasis and estrogen-containing contraception [29–31]. Moreover, thrombotic events occur only intermittently despite the persistent presence of aPL in some individuals [6, 7]. Thus, other inflammatory mediators may work in the pathogenesis of APS. In this study, for the first time, we report that both LILRA3 positivity rate and serum concentrations are significantly increased in APS patients, especially in tAPS patients. Incidences of thrombotic events and thrombocytopenia, concentrations of D-dimer, aGAPSS scores and aPL positivity were significantly increased in LILRA3-positive APS, more specifically in tAPS. LILRA3-positive tAPS patients displayed more severe thrombotic manifestations. Serum concentrations of LILRA3 were positively correlated with MPO-DNA complexes and both were consistently decreased after treatment in LILRA3-positive tAPS. Our data indicate that LILRA3 may play a role in the thrombosis of APS.

It is widely accepted that aPL play a key role in APS pathogenesis. We observed that the positive rates of anti-β2GPI antibodies, aCL and double positive for anti-β2GPI antibodies and aCL were significantly higher in the LILRA3-positive APS, compared with LILRA3-negative APS. Strikingly, the increased aPL positivity and the correlation between serum LILRA3 and aCL appeared only in tAPS but not in oAPS. Thus, we speculate that aPL may potentiate the production of LILRA3 in tAPS, perhaps via neutrophil activation.

Thrombocytopenia is a common clinical feature in APS patients. LILRA3-positive tAPS patients had a significantly higher incidence of thrombocytopenia than LILRA3-negative tAPS patients. The finding is consistent with two previous studies which reported that LILRA3 was associated with thrombocytopenia in SLE patients [14, 32]. Additionally, we observed that incidence of anaemia was higher in LILRA3-positive tAPS, which is in agreement with a recent study reporting that LILRA3 expression was increased and positively associated with disease severity in patients with aplastic anaemia [33]. LILRA3-positive tAPS patients had increased ESR and CRP, and decreased complement C3 and C4, indicating LILRA3 may be associated with disease activity and inflammation in tAPS. Complement activation plays an important role in thrombosis [34]. Complement C3 and C5 activation can promote aPL-mediated thrombosis [35]. Hyperlipidemia is a conventional cardiovascular risk factor of APS thrombosis [20, 36]. Interestingly, we found that LILRA3-positive tAPS patients had a lower HDL and higher incidence of dyslipidaemia. Our results are in agreement with a previous study which reported an essential splice site mutation within *LILRA3* was associated with elevated HDL, indicating LILRA3 may play a role in regulating HDL in humans [37]. Furthermore, we observed an elevated concentration of D-dimer in LILRA3-positive APS, especially in tAPS patients. D-dimer is an important molecular marker for diagnosis of thrombosis [38].

The aGAPSS is a risk score for predicting thrombotic APS and high aGAPSS is associated with recurrent thrombosis [20]. In the present study, the LILRA3-positive tAPS patients had increased aGAPSS and the aGAPSS scores were also positively correlated with serum LILRA3 levels in the LILRA3-positive patients. Furthermore, LILRA3-positive tAPS patients had much higher incidences of thrombotic events, increased number of thrombus sites, and increased incidences of deep vein thrombosis and arterial ischemic stroke. These findings provided further evidence that LILRA3 play a role in the thrombosis of APS.

In recent years, we and other groups have performed studies focusing on the role of APS neutrophils in thrombosis [9, 19, 39]. We reported elevated levels of NETs in the circulation of APS patients and demonstrated that anti-β2GPI IgG could promote NET release *in vitro* [9]. Subsequently, we confirmed the link between NETs and thrombosis by using a mouse model of APS *in vivo* [40]. By performing a transcriptome analysis in neutrophils, we found that almost all of the activating LILR family members, including *LILRA3*, were significantly upregulated in neutrophils from APS patients [19]. Wang *et al.* reported that recombinant human LILRA3 could enhance the release of NETs *in vitro* [18]. Interestingly, we observed that serum concentrations of LILRA3 were positively correlated with MPO-DNA complexes in tAPS patients. More importantly, we found serum LILRA3 and MPO-DNA complex were consistently decreased in tAPS patients after treatment. These results indicate LILRA3 may promote the release of NETs and thereby participate in the development of APS thrombosis. Future functional studies of LILRA3 are warranted to fully understand its contribution to APS thrombotic inflammation.

In summary, the present study provides the first evidence that soluble LILRA3 was aberrantly expressed in APS patients, especially in tAPS patients. LILRA3-positive tAPS patients displayed increased aPL positivity and more severe thrombotic manifestations. Serum concentrations of LILRA3 were positively correlated with MPO-DNA complexes in LILRA3-positive tAPS, and the two indices were consistently decreased in tAPS patients after treatment. LILRA3 may serve as a biomarker and a promising therapeutic target for tAPS. Going forward, it will be worthwhile to initiate a multicentre investigation with a larger number of samples, including patients with diverse ethnic backgrounds, in order to determine how consistently soluble LILRA3 and/or LILRA3-NET complexes predict the risk of APS-related thrombosis.

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Data availability statement

The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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