

**BRIEF REPORT**

# Anti-Neutrophil Extracellular Trap Antibodies in Antiphospholipid Antibody-Positive Patients: Results From the Antiphospholipid Syndrome Alliance for Clinical Trials and InternatiOnal Networking Clinical Database and Repository

Yu Zuo,<sup>1</sup>  Sherwin Navaz,<sup>1</sup> Alex Tsodikov,<sup>2</sup> Katarina Kmetova,<sup>3</sup>  Lyndsay Kluge,<sup>1</sup> Amala Ambati,<sup>1</sup> Claire K. Hoy,<sup>1</sup> Srilakshmi Yalavarthi,<sup>1</sup> Danieli de Andrade,<sup>4</sup> Maria G. Tektonidou,<sup>5</sup>  Savino Sciascia,<sup>6</sup>  Vittorio Pengo,<sup>7</sup> Guillermo Ruiz-Irastorza,<sup>8</sup>  H. Michael Belmont,<sup>9</sup>  Maria Gerosa,<sup>10</sup> Paul R. Fortin,<sup>11</sup> Guilherme Ramires de Jesus,<sup>12</sup>  D. Ware Branch,<sup>13</sup> Laura Andreoli,<sup>14</sup>  Esther Rodriguez-Almaraz,<sup>15</sup> Michelle Petri,<sup>16</sup>  Ricard Cervera,<sup>17</sup> Rohan Willis,<sup>18</sup> David R. Karp,<sup>19</sup>  Quan-Zhen Li,<sup>20</sup> Hannah Cohen,<sup>21</sup> Maria Laura Bertolaccini,<sup>22</sup> Doruk Erkan,<sup>23</sup>  and Jason S. Knight,<sup>1</sup>  on behalf of the Antiphospholipid Syndrome Alliance for Clinical Trials and InternatiOnal Networking

**Objective.** This study aimed to elucidate the presence, antigen specificities, and potential clinical associations of anti-neutrophil extracellular trap (anti-NET) antibodies in a multinational cohort of antiphospholipid (aPL) antibody-positive patients who did not have lupus.

**Methods.** Anti-NET IgG/IgM levels were measured in serum samples from 389 aPL-positive patients; 308 patients met the classification criteria for antiphospholipid syndrome. Multivariate logistic regression with best variable model selection was used to determine clinical associations. For a subset of the patients (n = 214), we profiled autoantibodies using an autoantigen microarray platform.

**Results.** We found elevated levels of anti-NET IgG and/or IgM in 45% of the aPL-positive patients. High anti-NET antibody levels are associated with more circulating myeloperoxidase (MPO)-DNA complexes, which are a biomarker of NETs. When considering clinical manifestations, positive anti-NET IgG was associated with lesions affecting the white matter of the brain, even after adjusting for demographic variables and aPL profiles. Anti-NET IgM tracked with complement consumption after controlling for aPL profiles; furthermore, patient serum samples containing high levels of anti-NET IgM efficiently deposited complement C3d on NETs. As determined by autoantigen microarray, positive testing for anti-NET IgG was significantly associated with several autoantibodies, including those recognizing citrullinated histones, heparan sulfate proteoglycan, laminin, MPO-DNA complexes, and nucleosomes. Anti-NET IgM positivity was associated with autoantibodies targeting single-stranded DNA, double-stranded DNA, and proliferating cell nuclear antigen.

**Conclusion.** These data reveal high levels of anti-NET antibodies in 45% of aPL-positive patients, where they potentially activate the complement cascade. While anti-NET IgM may especially recognize DNA in NETs, anti-NET IgG species appear to be more likely to target NET-associated protein antigens.

**INTRODUCTION**

Antiphospholipid syndrome (APS) is a systemic autoimmune disease that is a leading acquired cause of both thrombosis and

late-term pregnancy loss (1). Roughly one-third of APS cases are diagnosed in patients with other autoimmune diseases such as lupus, with the remainder presenting as a stand-alone syndrome called primary APS. Current classification criteria for APS

The Antiphospholipid Syndrome Alliance for Clinical Trials and InternatiOnal Networking registry was created using REDCap, which was provided by the Clinical and Translational Science Center at Weill Cornell Medical College (grant UL1-TR000457).

<sup>1</sup>Yu Zuo, MD, Sherwin Navaz, BS, Lyndsay Kluge, Amala Ambati, MD, Claire K. Hoy, BS, Srilakshmi Yalavarthi, MS, Jason S. Knight, MD, PhD: Department of Internal Medicine, Division of Rheumatology, University of

Michigan, Ann Arbor; <sup>2</sup>Alex Tsodikov, PhD: School of Public Health, University of Michigan, Ann Arbor; <sup>3</sup>Katarina Kmetova, MS: Department of Internal Medicine, Division of Rheumatology, University of Michigan, Ann Arbor, and Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia; <sup>4</sup>Danieli de Andrade, MD, PhD: University of São Paulo, São Paulo, Brazil; <sup>5</sup>Maria G. Tektonidou, MD, PhD: National and Kapodistrian University of Athens, Athens, Greece; <sup>6</sup>Savino Sciascia, MD, PhD: University

seek persistently positive testing for anticardiolipin (aCL) antibodies, anti- $\beta_2$ -glycoprotein I (anti- $\beta_2$ GPI) antibodies, or lupus anticoagulant; these laboratory criteria should also be accompanied by either a thrombotic event (arterial, venous, and/or microvascular) or specific types of pregnancy morbidity (1). While thrombosis and pregnancy morbidity are the traditional hallmarks of APS, a variety of “extra-criteria” clinical phenotypes are also commonly seen in daily practice. Such potentially morbid manifestations include thrombocytopenia, hemolytic anemia, cardiac valve disease, livedo reticularis/racemosa, aPL-associated nephropathy, seizures, brain white matter lesions as determined by magnetic resonance imaging, and cognitive dysfunction. There are few, if any, biomarkers to predict which aPL-positive patients are at risk for these extra-criteria features that typically do not respond to treatment with anticoagulant medications (1).

While the pathophysiology that propels APS remains incompletely understood, potential aPL-mediated mechanisms that have been described previously include the activation of endothelial cells, monocytes, platelets, coagulation factors, and complement proteins (1). Neutrophil extracellular traps (NETs)—prothrombotic and proinflammatory webs of nuclear DNA, histones, and microbicidal proteins extruded by activated neutrophils—have recently received increased attention as contributors to thromboinflammation in APS (2). It appears that aPL, especially those recognizing  $\beta_2$ GPI, can engage the neutrophil surface, where they circumvent normal homeostatic mechanisms to trigger the release of NETs (2). Interestingly, beyond traditional aPL, we recently found in a small single-center study that some APS patients also develop anti-NET antibodies that impair NET clearance with the potential to amplify inflammatory responses (3). Unaddressed to date are the antigen specificities of anti-NET antibodies and the extent to which these antibodies could serve as biomarkers that would add value to traditional criteria aPL.

The Antiphospholipid Syndrome Alliance for Clinical Trials and InternatiOnal Networking (APS ACTION) was founded in 2010. It is an international research consortium that supports large-scale multicenter clinical studies in individuals who are persistently aPL-positive (4). Here, we sought to elucidate the presence, clinical associations, and antigen specificities of anti-NET antibodies in this large multinational cohort. We focused on those

individuals who did not have other underlying systemic autoimmune diseases such as lupus.

## PATIENTS AND METHODS

More detailed protocols can be found in the Supplementary Materials and Methods, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.42489>. This study complied with all relevant ethical regulations and was approved by the University of Michigan institutional review board (project no. HUM00200466).

**Human samples.** Serum samples from 389 individuals were collected upon enrollment into APS ACTION (Supplementary Table 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42489>). Of these, 175 samples were from the European core lab, 129 were from the North American core lab, and 85 were from the South American core lab.

**Generation of NETs.** Human neutrophils were isolated as we have previously described (5). Purified neutrophils were resuspended in RPMI culture media supplemented with L-glutamine and 3% fetal bovine serum (all from Gibco). Next, neutrophils were seeded into each well of a 6-well plate at a density of  $1 \times 10^7$ . To induce NETosis, neutrophils were cultured for 4 hours at 37°C and 5% CO<sub>2</sub> in the presence of 500 nM phorbol 12-myristate 13-acetate (Sigma). Following incubation, NETs (both in supernatant and adherent to the plate) were collected and centrifuged at 450g for 10 minutes at 4°C to pellet large cellular debris. The cell-free NETs-rich supernatants were collected, and NETs were pelleted by centrifuging at 16,000g for 10 minutes at 4°C. The NET pellet was washed once with ice-cold phosphate buffered saline (PBS) at 16,000g for 10 minutes at 4°C before resuspending in ice-cold PBS at a concentration corresponding to  $2 \times 10^7$  neutrophils per milliliter of PBS.

**Anti-NET IgG and IgM enzyme-linked immunosorbent assays (ELISAs).** Briefly, a high-binding 96-well enzyme immunoassay/radioimmunoassay plate was coated overnight at 4°C with micrococcal nuclease-digested NETs diluted to a 5  $\mu$ g/ml

of Turin, Turin, Italy; <sup>7</sup>Vittorio Pengo, MD: Padova University Hospital, Padova, Italy; <sup>8</sup>Guillermo Ruiz-Irastorza, MD: Hospital Universitario Cruces, University of the Basque Country, Bizkaia, Spain; <sup>9</sup>H. Michael Belmont, MD: New York University Langone Medical Center, New York; <sup>10</sup>Maria Gerosa, MD, PhD: University of Milan, Milan, Italy; <sup>11</sup>Paul R. Fortin, MD: CHU de Québec-Université Laval, Québec, Canada; <sup>12</sup>Guilherme Ramires de Jesus, MD: Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil; <sup>13</sup>D. Ware Branch, MD: University of Utah and Intermountain Healthcare, Salt Lake City; <sup>14</sup>Laura Andreoli, MD, PhD: University of Brescia, Brescia, Italy; <sup>15</sup>Esther Rodriguez-Almaraz, MD: Hospital Universitario 12 de Octubre, Madrid, Spain; <sup>16</sup>Michelle Petri, MD: Johns Hopkins University School of Medicine, Baltimore, Maryland; <sup>17</sup>Ricard Cervera, MD: Hospital Clinic de Barcelona, Barcelona, Spain; <sup>18</sup>Rohan Willis, MD: University of Texas Medical Branch, Galveston; <sup>19</sup>David R. Karp, MD, PhD: Department of Internal Medicine, Division of Rheumatic Disease,

University of Texas Southwestern Medical Center, Dallas; <sup>20</sup>Quan-Zhen Li, MD, PhD: Department of Immunology, Immune Phenotyping Core Facility, University of Texas Southwestern Medical Center, Dallas; <sup>21</sup>Hannah Cohen, MD: Haemostasis Research Unit, Department of Haematology, University College London, London, UK; <sup>22</sup>Maria Laura Bertolaccini, PhD: King's College London British Heart Foundation Centre of Excellence, London, UK; <sup>23</sup>Doruk Erkan, MD: Barbara Volcker Center for Women and Rheumatic Disease, Hospital for Special Surgery, Weill Cornell Medicine, New York, New York.

Author disclosures are available online at <https://onlinelibrary.wiley.com/doi/10.1002/art.42489>.

Address correspondence via email to Yu Zuo, MD, MSCS, at [yzu@umich.edu](mailto:yzu@umich.edu); or to Jason S. Knight, MD, PhD, at [jsknight@umich.edu](mailto:jsknight@umich.edu).

Submitted for publication August 30, 2022; accepted in revised form February 28, 2023.

concentration in 0.05M bicarbonate buffer. The plates were blocked with 4% bovine serum albumin for 2 hours at room temperature. Serum samples (diluted 1:100 in blocking buffer) were then added to individual wells. After a 1.5-hour incubation at 37°C, NET-bound autoantibodies were detected with a horseradish peroxidase-conjugated secondary antibody (anti-IgG or anti-IgM). Each experimental sample was compared to a corresponding well containing the same sample but in which no NETs were plated; this created an individual background value for each sample, which was subtracted to obtain the final result. The schematic illustration of the protocol in Figure 1A was created using BioRender software.

**Quantification of antibodies, MPO-DNA complexes, and circulating complements.** Circulating anti-double-stranded DNA (anti-dsDNA) and anti-single-stranded DNA (anti-ssDNA) were measured using ELISA kits (catalog nos. 3100, 3105, and 3115; Alpha Diagnostic) according to the manufacturer's instructions. MPO-DNA complexes were measured as previously described (6). Circulating complements C3 and C4 were measured using ELISA kits (catalog nos. NBP2-60619 and NBP2-60618; Novus Biologicals) according to the manufacturer's instructions. The immunofluorescence microscopy protocol was described previously (6).

**Autoantigen microarray.** Autoantibody profiling was conducted using an autoantigen microarray platform developed by Dr. Quan-Zhen Li at the University of Texas Southwestern Medical Center, and data were analyzed using Genepix Pro software version 7.0 (see the Supplementary Materials and Methods for a detailed description, <http://onlinelibrary.wiley.com/doi/10.1002/art.42489>).

## RESULTS

### Anti-NET antibodies in a multinational population.

We obtained serum samples from 389 persistently aPL-positive patients recruited from centers in Europe, North America, and South America. The mean age at the time of sampling was 51 years, and 70% of the patients were female (Supplementary Table 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42489>). Of these patients, 308 had primary APS and 81 patients had persistently positive aPL without "criteria" APS manifestations or another systemic autoimmune disease diagnosis. Additionally, 47 patients met 3 out of 11 American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus but did not have classifiable lupus (7). Utilizing a custom ELISA platform that we developed (Figure 1A), we measured anti-NET IgG and IgM antibodies in 389 aPL-positive patients alongside 40 healthy controls. As compared with the healthy controls, elevated levels of anti-NET IgG and IgM were detected in patients with both thrombotic and obstetric APS; levels were also

high in aPL-positive patients without any criteria manifestations (Figures 1B and C). Based on a positive threshold set at the 99th percentile for healthy-control samples, 61 patients (16%) had high anti-NET IgG activity, while 155 patients (40%) had high anti-NET IgM activity; 41 patients (11%) had positive results on testing for both anti-NET IgG and IgM. Overall, at least 1 of these tests was positive in 175 patients (45%).

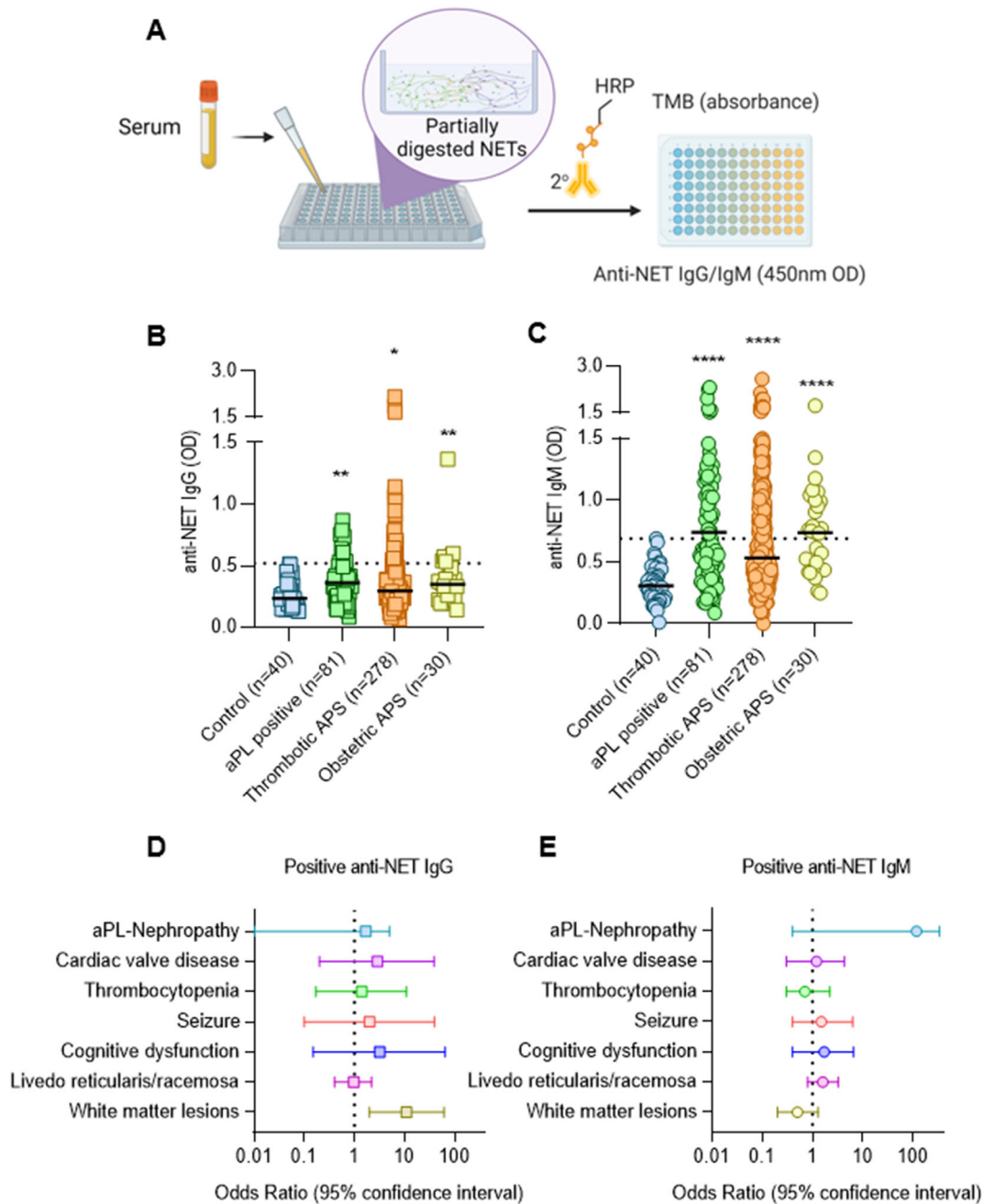
There was a strong positive relationship between anti-NET IgG and anti-NET IgM ( $r = 0.52$ ,  $P < 0.0001$ ). While no significant difference was seen between lupus anticoagulant-positive and lupus anticoagulant-negative patients ( $P = 0.32$  for anti-NET IgG,  $P = 0.72$  for anti-NET IgM), anti-NET activities did demonstrate positive correlations with levels of other traditional aPL ( $r = 0.21$  and  $P < 0.0001$  for anti-NET IgG versus anti- $\beta_2$ GPI IgG;  $r = 0.19$  and  $P = 0.0001$  for anti-NET IgG versus aCL IgG;  $r = 0.18$  and  $P = 0.0007$  for anti-NET IgM versus anti- $\beta_2$ GPI IgM; and  $r = 0.53$  and  $P < 0.0001$  for anti-NET IgM versus aCL IgM). While none of the participants had classifiable lupus (7), 217 participants (56%) were reported by the participating centers to be positive for antinuclear antibodies (ANAs); ANA titers were not available.

We tested anti-dsDNA IgG and IgM in all APS ACTION samples. We observed a modest positive correlation between anti-NET IgG and anti-dsDNA IgG ( $r = 0.22$ ,  $P < 0.0001$ ), and a stronger correlation between anti-NET IgM and anti-dsDNA IgM ( $r = 0.45$ ,  $P < 0.0001$ ). Targeted testing of anti-ssDNA IgG was performed for 80 APS ACTION participants ( $r = 0.18$ ,  $P = 0.1$ ). Among 389 APS ACTION patients, 127 were receiving treatment with hydroxychloroquine at the time of enrollment. The use of hydroxychloroquine was not associated with differences in anti-NET antibody levels (Supplementary Figure 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42489>). A circulating marker of NET release, MPO-DNA complexes, was also quantified in all 389 patients. Anti-NET IgG and IgM were both found to be positively correlated with MPO-DNA complexes ( $r = 0.12$  and  $P = 0.02$  for IgG, and  $r = 0.16$  and  $P = 0.0012$  for IgM).

### Association of anti-NET antibodies with clinical manifestations of extra-criteria APS.

We next asked whether the presence of anti-NET IgG or IgM might add to traditional aPL in the risk stratification of aPL-positive patients, especially as it relates to extra-criteria manifestations. We found that positive testing for anti-NET IgG was significantly associated with brain white matter lesions assessed by magnetic resonance imaging after adjusting for age, sex, ethnicity, and aPL profiles (odds ratio 11 [95% confidence interval 1.9–62]) (Figures 1D and E). No significant association was found between anti-NET IgM and any extra-criteria manifestations.

**Association of anti-NET antibodies with complement activation.** Work by our group and others has revealed that one function of anti-NET antibodies in patients with lupus (8), patients



**Figure 1.** Measurement of anti-neutrophil extracellular trap (anti-NET) antibodies in 389 antiphospholipid (aPL) antibody-positive patients. **A**, Schematic illustration of the anti-NET antibody enzyme-linked immunosorbent assay. **B** and **C**, Anti-NET IgG and IgM were measured in the indicated groups; no patients in this cohort had lupus. Patients were grouped based on no criteria manifestations (aPL positive), a history of thrombotic manifestations (47 of these 278 patients also had a history of obstetric manifestations), or a history of obstetric manifestations without a history of vascular thrombosis. Levels of anti-NET IgG and IgM at 450-nm optical density (OD) were compared to controls using the Kruskal-Wallis test corrected with Dunn's multiple comparison test: \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\*\* =  $P < 0.0001$ . Horizontal black lines indicate medians and dashed lines indicate the 99th percentile cut-offs. **D** and **E**, Association between positive anti-NET IgG (**D**) or IgM (**E**) and various extra-criteria APS clinical manifestations were assessed using multivariate logistic regression adjusted for demographic variables (age, sex, ethnicity) and aPL profiles.

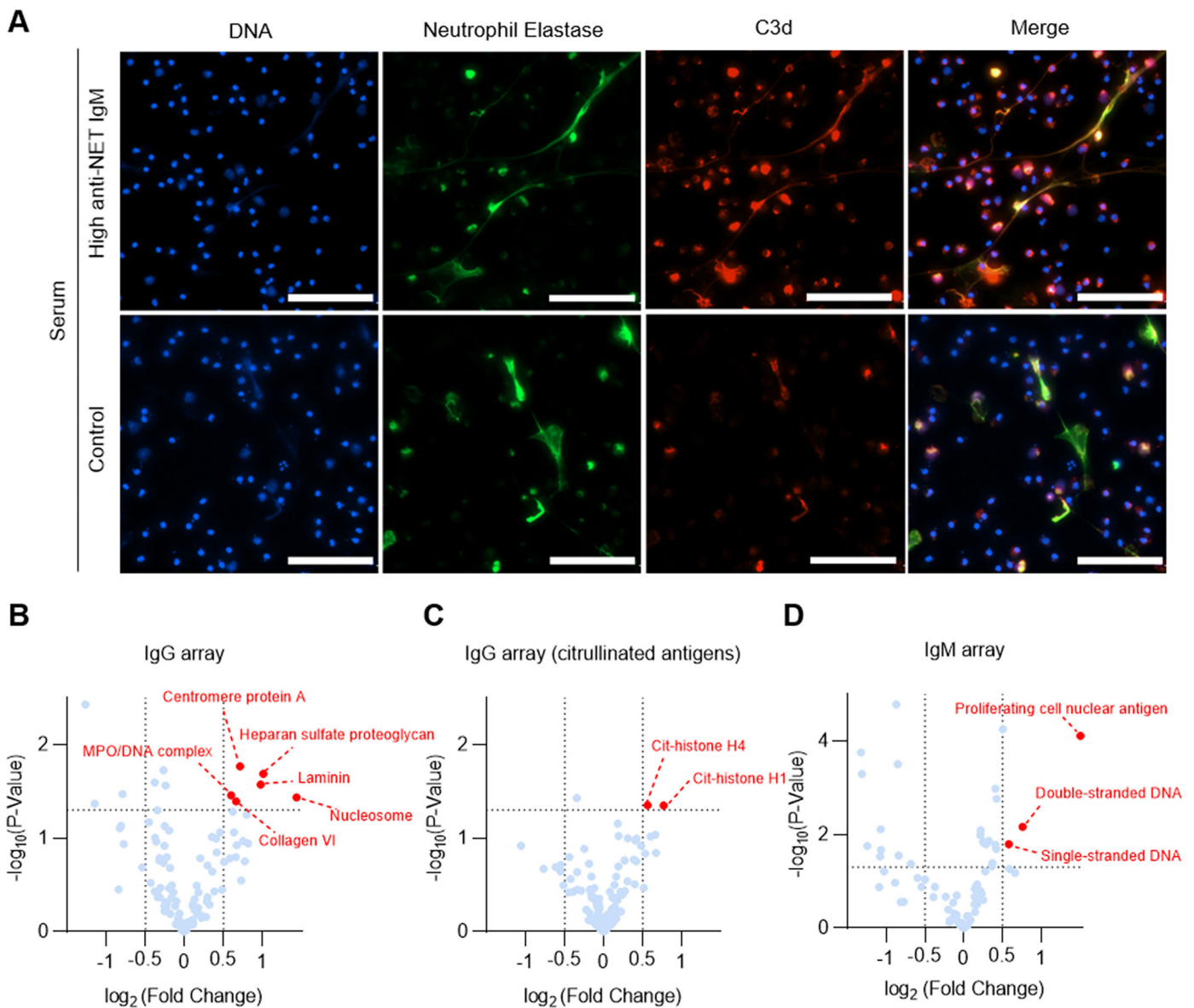
with APS (3), and patients with COVID-19 (6) is to impair NET degradation, which may present a fertile platform for complement activation (9). We next asked whether anti-NET antibodies were associated with complement activation. We measured circulating C3 and C4 among aPL-positive patients. In an unadjusted model,

both anti-NET IgG and IgM were inversely correlated with complements C3 ( $r = -0.12$  and  $P = 0.02$  for IgG, and  $r = -0.18$  and  $P = 0.0005$  for IgM) and C4 ( $r = -0.18$  and  $P = 0.0003$  for IgG, and  $r = -0.25$  and  $P < 0.0001$  for IgM); however, after adjusting for aPL profiles, only the associations between anti-NET IgM and

complement levels persisted ( $r = -0.12$  and  $P = 0.03$  for C3, and  $r = -0.13$  and  $P = 0.03$  for C4). To look at the potential effect of anti-NET IgM on NET-mediated complement activation, we incubated serum (having either high or low anti-NET IgM activity) with NETs and evaluated complement C3d deposition. Compared with control serum samples, serum samples with high anti-NET IgM activity more efficiently deposited complement C3d on NETs (Figure 2A).

**Antigen specificities of anti-NET antibodies.** In pursuit of the antigen specificity of anti-NET antibodies, we designed a 120-antigen microarray panel with a selection of 65 NET-associated

antigens and 55 other autoantigens implicated in human systemic autoimmune diseases (Supplementary Table 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42489>). An on-chip citrullination process was performed to citrullinate 120 autoantigens using a peptidylarginine deiminase (PAD) cocktail that contained a mixture of 4 PAD isoforms (PAD1, PAD2, PAD3, PAD4). Using both the noncitrullinated and the citrullinated arrays, we characterized the serum of 214 aPL-positive patients. Positive testing for anti-NET IgG was significantly associated with the following autoantibodies: Centromere protein A (a histone H3 variant), citrullinated-histone H1 and H4, collagen VI, heparan sulfate proteoglycan, laminin, MPO–DNA complexes, and



**Figure 2.** Function and potential antigen specificities of anti-NET antibodies. **A**, Complement C3d decorating NETs. Control neutrophils were stimulated with phorbol 12-myristate 13-acetate to generate NETs, which were then incubated with serum from patients with high (top panels) anti-NET IgM or from healthy controls; scale bars = 100 microns. **B–D**, Autoantibody microarray comparing anti-NET IgG-positive and anti-NET IgG-negative samples (**B** and **C**), and anti-NET IgM-positive and anti-NET IgM-negative samples (**D**). Volcano plots demonstrate (in red) antigen specificities that were significantly enriched in the anti-NET-positive groups. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.42489/abstract>.

nucleosomes. Meanwhile, anti-NET IgM positivity associated with autoantibodies recognizing ssDNA, dsDNA, and proliferating cell nuclear antigen (a DNA clamp) (Figures 2B–D). To validate the microarray findings, we selected 2 identified antigens (heparan sulfate proteoglycan and nucleosome) and developed ELISAs. We then assessed levels of anti-heparan sulfate proteoglycan IgG and anti-nucleosome IgG among 40 aPL-positive patients. High levels of anti-heparan sulfate proteoglycan IgG and antinucleosome IgG were detected in patients who were also positive for anti-NET IgG (Supplementary Figure 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42489>).

## DISCUSSION

In this study, we found high levels of anti-NET IgG and/or IgM in almost 50% of the aPL-positive patients, none of whom had been diagnosed with another systemic autoimmune disease such as lupus. Both anti-NET IgG and IgM tracked with high levels of circulating NETs. Clinically, positive testing for anti-NET IgG was able to identify patients with brain white matter lesions. Meanwhile, anti-NET IgM tracked with complement consumption as measured by low complement C3 and C4; furthermore, APS patient serum with high levels of anti-NET IgM enhanced C3d deposition on NETs. The presented data also suggested that anti-NET IgG activity in aPL-positive patients was more likely to be driven by reactivity with protein antigens such as histones, as opposed to the DNA component of NETs. At the same time, anti-NET IgM activity appeared to be more likely to target DNA or the associated DNA clamp known as proliferating cell nuclear antigen.

Several studies have provided evidence that NET-targeting antibodies are present in various autoimmune diseases where they may help propel disease pathogenesis. For example, one interesting study demonstrated that serum from lupus patients with IgG activity against NETs degraded NETs poorly, and such patients were more likely to have lupus nephritis (8). In a small, single-center study of primary APS patients, our group found high anti-NET activity in some APS patients at levels similar to or higher than those seen in lupus patients (3). In that study, we also showed that high levels of anti-NET antibodies protected NETs from nuclease digestion in serum (3). In the current study, we not only validated the presence of anti-NET antibodies among traditional APS patients in a large, multinational cohort, but also demonstrated for the first time that anti-NET antibodies were detectable in many aPL-positive patients who did not have classifiable APS features. Such anti-NET antibodies may help identify patients at risk for certain extra-criteria manifestations such as brain white matter lesions.

While complement activation has been suggested to play an important role in the pathophysiology of the thrombotic and obstetric complications of APS, the mechanisms by which the complement system is activated in APS are incompletely understood (10). Existing studies, typically focused on anti- $\beta_2$ GPI IgG,

have suggested that aPL can bind to C1q, activating the classical complement pathway. However, the predominant subclass of anti- $\beta_2$ GPI and aCL among APS patients is IgG2, which has a relatively weak ability to activate complement as compared with IgG1 and IgG3 (10). NETs have long been suggested to serve as a fertile platform for complement activation. For example, complement C3, C1q, factor B, and properdin (complement-activating glycoprotein) have all been shown to decorate the surface of NETs (9,11). Moreover, NET-associated proteins such as MPO and cathepsin G can directly bind to and activate properdin, thereby promoting complement activation (12). Potentially fueling this vicious cycle, C1q also appears to shield NETs from clearance by DNase (9). Along similar lines, high anti-NET activity may stabilize NETs, which can then serve as a scaffold for complement activation. We were intrigued to find that the IgM isotype of anti-NET antibodies was most strongly associated with complement cascade activation. One recent study demonstrated that IgM can directly interact with C1q and C4b to form a complex on liposomal membranes (13), where the assembled structure can then promote complement-mediated inflammatory responses (13). It is certainly possible that anti-NET IgM can interact with NET-bound C1q and other complement factors and further promote complement activation.

Prior to this work, nothing was known about the antigen specificities of anti-NET IgG and IgM among APS patients. To address this critical question, we designed a custom autoantigen microarray including 120 carefully selected antigens from a bank of over 500 human autoantigens. Priority was given to antigens associated with NETs in the literature. For example, heparan sulfate proteoglycans are well-known cell surface and extracellular matrix components (14,15) that have been detected in NETs, where they potentially modulate antimicrobial activities (15). Utilizing this microarray platform, we identified specific antigen targets that were significantly enriched in individuals positive for either anti-NET IgG or IgM. Among those, several interesting targets were identified, including heparan sulfate proteoglycan, laminin, and nucleosomes. Studies in lupus suggest that nucleosomes can bind to glomerular capillaries, mesangial matrix membranes, and glomerular basement membranes via their high affinity for glomerular constituents such as heparan sulfate and laminin (16,17). Antibodies targeting nucleosomes thereby contribute to lupus nephritis pathogenesis in murine models (16–18). One can speculate that reactivity of anti-NET antibodies with some of these antigens might contribute to endothelial activation in APS. While the role of aPL in activating endothelial cells has been demonstrated both *in vitro* and *in vivo*, most of these studies have focused on the activation of APS endothelium by traditional aPL, such as anti- $\beta_2$ GPI (19). Our data suggest that, beyond aPL, anti-NET antibodies with reactivities against constituents of the endothelium might also contribute to APS endothelial activation integral to inflammation and thrombosis. We can speculate that anti-NET antibody-mediated endothelial dysfunction might

disrupt the integrity of the blood brain barrier, thereby promoting an influx of inflammatory proteins into the cerebral parenchyma and provoking brain white matter lesions. Future mechanistic studies looking at the role of anti-NET antibodies in this and other thromboinflammatory phenotypes of APS are warranted.

It is well known that a subgroup of primary APS patients can eventually develop classifiable lupus (20). One cohort study of 128 primary APS patients followed up for 8 years found that 13% developed lupus or lupus-like disease (21). A clinically actionable biomarker for the identification of this subgroup of primary APS patients is lacking. Interestingly, recent advancements in understanding lupus pathogenesis have suggested that exaggerated NET release and impaired NET clearance promote lupus autoantigen expansion, lupus-specific autoantibody production, and organ-damaging type I interferon response (22). In this study, we found that many primary APS patients or aPL carriers have NET-stabilizing anti-NET antibodies, which are associated with several serologic markers of lupus, such as anti-dsDNA and antinucleosome antibodies. While we cannot draw definite conclusions on the value of anti-NET antibodies in identifying lupus-prone primary APS patients, we can speculate that these functional anti-NET IgG/IgM could play a role in the evolution in some primary APS patients toward full-blown lupus.

Our study does have some limitations. Due to the cross-sectional design, we are not able to evaluate the durability of anti-NET antibodies in aPL-positive patients. Moreover, the interesting association between anti-NET IgG and brain white matter lesions is hypothesis-generating but will need to be confirmed in other cohorts. While we did not see a positive association between the presence of anti-NET antibodies and other microvascular complications of APS, such as aPL-associated nephropathy, only 10 patients with this complication were present in the cohort, which hinders the detection power in our model. Furthermore, while we have assembled a large multinational cohort of aPL-positive patients, only 2% of the studied patients were Black. As disparities in healthcare are rightfully receiving increased attention, the impact of race and ethnicity on the frequency of anti-NET antibodies and associated risk with extra-criteria manifestations remains to be elucidated. While we have identified various specific antibodies that correlate with anti-NET activity in preliminary studies, the functions and clinical significances of those specific autoantibodies need further evaluation. In particular, it will be important to compare the functional role of DNA-targeting anti-NET antibodies versus those that recognize non-DNA antigens.

In summary, we have further defined a new class of autoantibodies among aPL-positive patients that may promote complement activation and identify certain extra-criteria APS manifestations. Anticoagulation with vitamin K antagonists remains the current mainstay of therapy for APS. While anticoagulation is reasonably efficacious in preventing aPL-associated thrombosis, it often has no bearing on extra-criteria manifestations of APS such as cardiac, renal, and neurologic complications. Future studies on

the role of anti-NET antibodies as clinically relevant biomarkers—which might lead to preemptive immunomodulatory therapy in some patients—are warranted.

## ACKNOWLEDGMENTS

We thank JoAnn Vega, CCRC, for her administrative support as the Antiphospholipid Syndrome Alliance for Clinical Trials and International Networking (APS ACTION) Global Lead Coordinator. We also want to thank all of the APS ACTION members.

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Zuo had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Zuo, Knight.

**Acquisition of data.** Zuo, Navaz, Tsodikov, Kmetova, Kluge, Ambati, Hoy, Yalavarthi, Andrade, Tektonidou, Sciascia, Pengo, Ruiz-Irastorza, Belmont, Gerosa, Fortin, Ramires de Jesus, Branch, Andreoli, Rodriguez-Almaraz, Petri, Cervera, Willis, Cohen, Bertolaccini, Erkan, Knight.

**Analysis and interpretation of data.** Zuo, Navaz, Tsodikov, Kmetova, Kluge, Ambati, Hoy, Yalavarthi, Karp, Li, Knight.

## REFERENCES

- Garcia D, Erkan D. Diagnosis and management of the antiphospholipid syndrome. *N Engl J Med* 2018;378:2010–21.
- Tambralli A, Gockman K, Knight JS. NETs in APS: current knowledge and future perspectives. *Curr Rheumatol Rep* 2020;22:67.
- Zuo Y, Yalavarthi S, Gockman K, et al. Anti-neutrophil extracellular trap antibodies and impaired neutrophil extracellular trap degradation in antiphospholipid syndrome. *Arthritis Rheumatol* 2020;72:2130–5.
- Erkan D, Sciascia S, Bertolaccini ML, et al. Antiphospholipid syndrome alliance for clinical trials and international networking (APS ACTION): 10-year update. *Curr Rheumatol Rep* 2021;23:45.
- Ali RA, Gandhi AA, Meng H, et al. Adenosine receptor agonism protects against NETosis and thrombosis in antiphospholipid syndrome. *Nat Commun* 2019;10:1916.
- Zuo Y, Yalavarthi S, Navaz SA, et al. Autoantibodies stabilize neutrophil extracellular traps in COVID-19. *JCI Insight* 2021;6.
- Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;40:1725.
- Hakkim A, Furnrohr BG, Amann K, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A* 2010;107:9813–8.
- Leffler J, Martin M, Gullstrand B, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol* 2012;188:3522–31.
- Chaturvedi S, Brodsky RA, McCrae KR. Complement in the pathophysiology of the antiphospholipid syndrome. *Front Immunol* 2019;10:449.
- Yuen J, Pluthero FG, Douda DN, et al. NETosing neutrophils activate complement both on their own NETs and bacteria via alternative and non-alternative pathways. *Front Immunol* 2016;7:137.

12. O'Flynn J, Dixon KO, Krol MC, et al. Myeloperoxidase directs properdin-mediated complement activation. *J Innate Immun* 2014;6:417–25.
13. Sharp TH, Boyle AL, Diebold CA, et al. Insights into IgM-mediated complement activation based on in situ structures of IgM-C1-C4b. *Proc Natl Acad Sci U S A* 2019;116:11900–5.
14. Marques C, Reis CA, Vives RR, et al. Heparan sulfate biosynthesis and sulfation profiles as modulators of cancer signalling and progression. *Front Oncol* 2021;11:778752.
15. Xu D, Olson J, Cole JN, et al. Heparan sulfate modulates neutrophil and endothelial function in antibacterial innate immunity. *Infect Immun* 2015;83:3648–56.
16. Kramers C, Hylkema MN, van Bruggen MC, et al. Anti-nucleosome antibodies complexed to nucleosomal antigens show anti-DNA reactivity and bind to rat glomerular basement membrane in vivo. *J Clin Invest* 1994;94:568–77.
17. Mjelle JE, Rekvig OP, Fenton KA. Nucleosomes possess a high affinity for glomerular laminin and collagen IV and bind nephritogenic antibodies in murine lupus-like nephritis. *Ann Rheum Dis* 2007;66:1661–8.
18. Muller S, Dieker J, Tincani A, et al. Pathogenic anti-nucleosome antibodies. *Lupus* 2008;17:431–6.
19. Pierangeli SS, Colden-Stanfield M, Liu X, et al. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. *Circulation* 1999;99:1997–2002.
20. Chen HH, Lin CH, Chao WC. Risk of systemic lupus erythematosus in patients with anti-phospholipid syndrome: a population-based study. *Front Med (Lausanne)* 2021;8:654791.
21. Gomez-Puerta JA, Martin H, Amigo MC, et al. Long-term follow-up in 128 patients with primary antiphospholipid syndrome: do they develop lupus? *Medicine (Baltimore)* 2005;84:225–30.
22. Yu Y, Su K. Neutrophil extracellular traps and systemic lupus erythematosus. *J Clin Cell Immunol* 2013;4.

## APPENDIX A: THE ANTIPHOSPHOLIPID SYNDROME ALLIANCE FOR CLINICAL TRIALS AND INTERNATIONAL NETWORKING MEMBERS

Members of the Antiphospholipid Syndrome Alliance for Clinical Trials and International Networking are as follows: Guillermo Pons-Estel, Bill Giannakopoulos, Steve Krilis, Guilherme de Jesus, Roger Levy, Flavio Signorelli, Danieli Andrade, Gustavo Balbi, Ann E. Clarke, Leslie Skeith, Paul R. Fortin, Lanlan Ji, Zhouli Zhang, Chengde Yang, Hui Shi, Stephane Zuilvy, Denis Wahl, Maria G. Tektonidou, Cecilia Nalli, Laura Andreoli, Angela Tincani, Cecilia B. Chighizola, Maria Gerosa, Pierluigi Meroni, Vittorio Pengo, Chunyan Cheng, Giulia Pazzola, Savino Sciascia, Silvia Fodai, Massimo Radin, Stacy Davis, Olga Amengual, Tatsuya Atsumi, Imad Uthman, Maarten Limper, Philip de Groot, Guillermo Ruiz-Irastorza, Amaia Ugarte, Ignasi Rodriguez-Pinto, Ricard Cervera, Roberto Ríos-Garcés, Giuseppe Barilaro, Jose Pardos-Gea, Esther Rodriguez Almaraz, Maria Jose Cuadrado, Maria Angeles Aguirre Zamorano, Chary Lopez-Pedraza, Bahar Artim-Esen, Murat Inanc, Maria Laura Bertolaccini, Hannah Cohen, Maria Efthymiou, Munther Khamashta, Ian Mackie, Giovanni Sanna, Jason Knight, Yu Zuo, Michelle Petri, Rebecca K. Leaf, Robert Roubey, Thomas Ortel, Emilio Gonzalez, Rohan Willis, Nina Kello, Michael Belmont, Steven Levine, Jacob Rand, Medha Barbhuiya, Doruk Erkan, Jane Salmon, Michael Lockshin, Ali A. Duarte Garcia, and D. Ware Branch.