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BRIEF REPORT

Anti–Neutrophil Extracellular Trap Antibodies and Impaired Neutrophil Extracellular Trap Degradation in Antiphospholipid Syndrome

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Objective. The release of neutrophil extracellular traps (NETs) by hyperactive neutrophils has recently been recognized to play an important role in antiphospholipid syndrome (APS). This study was undertaken to evaluate autoantibodies targeting NETs in patients with primary APS, and to determine their potential functions and clinical associations.

Methods. We measured global anti-NET activity in 76 patients with primary APS, 23 patients with systemic lupus erythematosus without antiphospholipid antibodies (aPL), 11 patients with a history of unprovoked venous thrombosis without aPL, and 44 healthy controls. The ability of APS sera to degrade NETs was also assessed.

Results. We found markedly elevated levels of anti-NET IgG and IgM in patients with primary APS compared with healthy controls (for IgG, mean ± SD optical density 0.55 ± 0.34 versus 0.33 ± 0.17; for IgM, mean ± SD optical density 0.76 ± 0.51 versus 0.26 ± 0.23). This anti-NET activity did not correlate with levels of traditional aPL and was relatively stable over time. Mechanistically, anti-NET antibodies (especially of the IgG isotype) impaired the ability of patient sera to degrade NETs ($r = 0.4$, $P = 0.003$). Levels of anti-NET IgM inversely correlated with complement C4 ($r = 0.4$, $P = 0.019$). Clinically, anti-NET antibodies associated with certain APS clinical manifestations, and in particular recurrent venous thrombosis (odds ratio 4.3; $P = 0.002$). Interestingly, anti-NET antibody levels also appeared to be associated with unprovoked venous thrombosis in the general population (for IgM, mean ± SD optical density 0.67 ± 0.34 versus 0.26 ± 0.23).

Conclusion. Our data indicate high levels of anti-NET antibodies in patients with primary APS, which may impair NET clearance and activate the complement cascade. These findings may ultimately enable more effective risk stratification.

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune thrombo-inflammatory disease manifested by arterial, venous, and/or microvascular thrombosis, as well as pregnancy loss (1). Current classification criteria require positivity for ≥1 traditional antiphospholipid antibody (aPL), including anticardiolipin, anti–β_2-glycoprotein I (anti-β_2GPI), and lupus anticoagulant, in the setting of a thrombotic event or pregnancy loss. While the pathophysiology of APS remains incompletely understood, aPL-mediated mechanisms that may contribute

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to it include activation of endothelial cells, monocytes, platelets, coagulation factors, and complement proteins (1).

Activated neutrophils—and, in particular, neutrophil extracellular trap (NET) formation—have recently received increased scrutiny as drivers of arterial, venous, and microvascular thrombosis (2). NETs are a meshwork of DNA, histones, and microbicidal proteins released from activated neutrophils via a program called NETosis. Neutrophils presumably deploy NETs to trap and kill pathogens (3); however, NETs may also be key players in the pathophysiology of thrombophilic disease states such as cancer, APS, heparin-induced thrombocytopenia, and coronavirus disease 2019 (COVID-19) (2,4,5). In APS, aPL engage the neutrophil surface, circumvent normal homeostatic mechanisms, and directly trigger NET release (5). Indeed, even when assessed between thrombotic events, patients with APS have higher levels of circulating NETs than healthy controls, and APS neutrophils have a reduced threshold for spontaneous NETosis when cultured ex vivo (5). Furthermore, neutrophils from patients with APS appear to have increased adhesive potential, dependent upon the activated form of integrin Mac-1, which may potentiate NET release (6). In mouse models of aPL-mediated large-vein thrombosis, depletion of neutrophils, disruption of neutrophil–endothelium interactions, and digestion of NETs are all protective (7).

Intriguingly, one small study has suggested that patients with primary APS have circulating IgG species that bind to NETs (8). However, quantitation, function, and prognostic significance of this anti-NET activity has not been thoroughly investigated. In this study we sought to evaluate anti-NET IgG and IgM antibodies in patients with primary APS, and to determine potential functions and clinical associations.

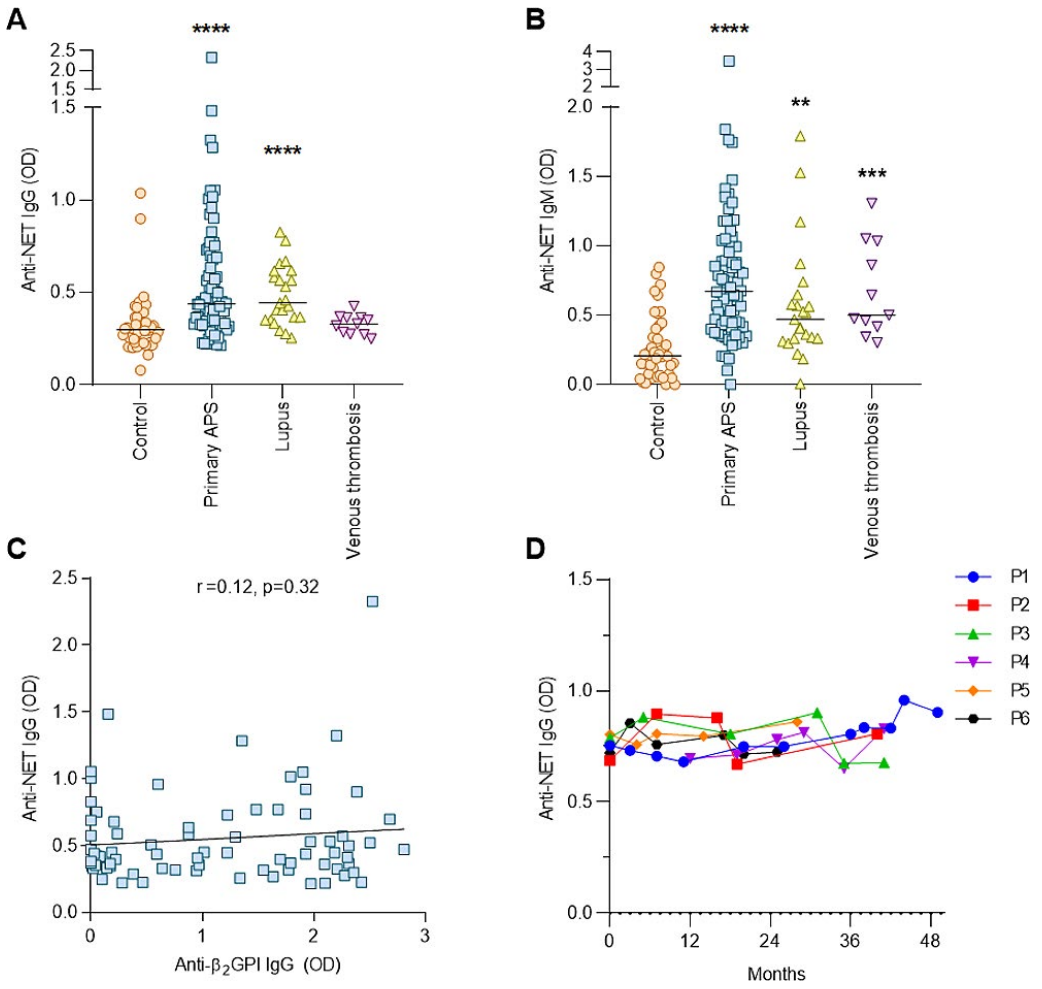

Figure 1. High levels of anti–neutrophil extracellular trap (anti-NET) antibodies among patients with primary antiphospholipid syndrome (APS). **A** and **B**, Levels of anti-NET IgG (**A**) and anti-NET IgM (**B**) antibodies at 450-nm OD in healthy controls, patients with primary APS, patients with systemic lupus erythematosus without antiphospholipid antibodies, and patients with unprovoked venous thrombosis without antiphospholipid antibodies. Symbols represent individual subjects; horizontal lines show the median. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ versus controls, by Kruskal-Wallis test. **C**, Relationship between anti-NET IgG and anti–β_2-glycoprotein I (anti-β_2GPI) IgG, assessed by Spearman's correlation test and linear regression. **D**, Anti-NET IgG levels over time in 6 patients with primary APS who had 2–4 years of follow-up in our clinic.

PATIENTS AND METHODS

The study included 2 cohorts of patients: a retrospective cohort and a prospective cohort. The retrospective cohort included patients with primary APS (n = 76), patients with systemic lupus erythematosus (SLE) without aPL (n = 23), and patients with unprovoked venous thrombosis without aPL (n = 11), as well as healthy controls (n = 44). Plasma samples obtained from these subjects were used for anti-NET IgG/IgM measurement. The prospective cohort included patients with APS (n = 37) and healthy controls (n = 17). Serum samples from these subjects were used for NET degradation assay and anti-NET enzyme-linked immunosorbent assay (ELISA). Additional details on the patients, as well as human neutrophil purification, generation of NETs, partial digestion of NETs and quantification of protein, anti-NET IgG/IgM ELISAs, NET degradation assay, and immunofluorescence microscopy, are provided in the Supplementary Methods, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract.

RESULTS

Global anti-NET activity in primary APS. Utilizing an ELISA platform, we measured anti-NET IgG and IgM antibodies in 76 patients with primary APS, 23 patients with SLE without aPL, 11 patients with a history of unprovoked venous thrombosis without aPL, and 44 healthy controls. The clinical characteristics of these patients are described in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract. Markedly elevated levels of anti-NET IgG and IgM were detected in patients with primary APS compared with healthy controls (for IgG, mean ± SD optical density 0.55 ± 0.34 versus 0.33 ± 0.17; for IgM, mean ± SD optical density 0.76 ± 0.51 versus 0.26 ± 0.23) (Figures 1A and B). High levels were also seen in patients with SLE without aPL (Figures 1A and B). While anti-NET IgG was not significantly elevated in patients with unprovoked venous thrombosis without aPL (Figure 1A), those patients did have higher levels of anti-NET IgM (for IgM, mean ± SD optical density 0.67 ± 0.34 versus 0.26 ± 0.23) (Figure 1B). We next considered whether anti-NET activity might correlate with levels of traditional aPL; however, no such correlation was detected with either anti-β_2GPI IgG/IgM (Figure 1C and Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract) or lupus anticoagulant (data not shown).

To determine the stability of anti-NET antibodies over time, we identified 6 patients with primary APS who had serial plasma banked over 2–4 years of follow-up in our clinic. Anti-NET IgG, in particular, showed stability over time (Figure 1D and Supplementary Figure 2, *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract). No significant differences were observed in the levels of anti-NET IgG or IgM between APS patients who were receiving hydroxychloroquine and those who were not (Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract). In summary, elevated levels of anti-NET IgG and IgM antibodies were present in patients with primary APS. These antibodies do not correlate with traditional aPL testing and appear to be stable over time in some patients.

Anti-NET antibodies decorate NETs generated by different stimuli. Anti-NET activity was assessed by immunofluorescence microscopy. When NETs were incubated with plasma from patients with high levels of anti-NET IgG, antibodies robustly decorated NET strands (Figure 2). It has been suggested that NETs

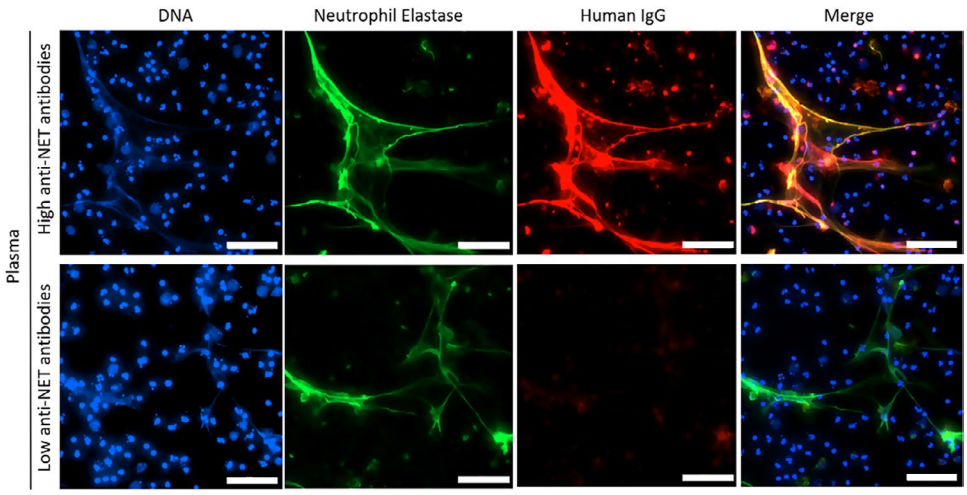

Figure 2. Anti–neutrophil extracellular trap (anti-NET) antibodies decorate NETs. Control neutrophils were stimulated with phorbol 12-myristate 13-acetate to generate NETs, which were then incubated with plasma from patients with high levels of anti-NET IgG antibodies (top) or patients with low levels of anti-NET IgG antibodies (bottom). Bars = 200μ.

generated by different stimuli may have different protein composition (9). For example, phorbol 12-myristate 13-acetate (PMA) induces NETosis through an NADPH oxidase–dependent pathway, and may have lower content of citrullinated histones compared with calcium ionophore–induced NETs (10). We therefore examined whether there was a difference in anti-NET activity in the context of these different stimuli. Notably, we found a strong correlation between APS samples tested in a PMA-NET ELISA and those tested in an ionophore-NET ELISA (r = 0.72, $P < 0.0001$ for anti-NET IgG; r = 0.71, $P < 0.0001$ for anti-NET IgM) (Supplementary Figure 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract). In summary, detection of anti-NET activity appears to be relatively independent of the stimulus used to trigger NETosis.

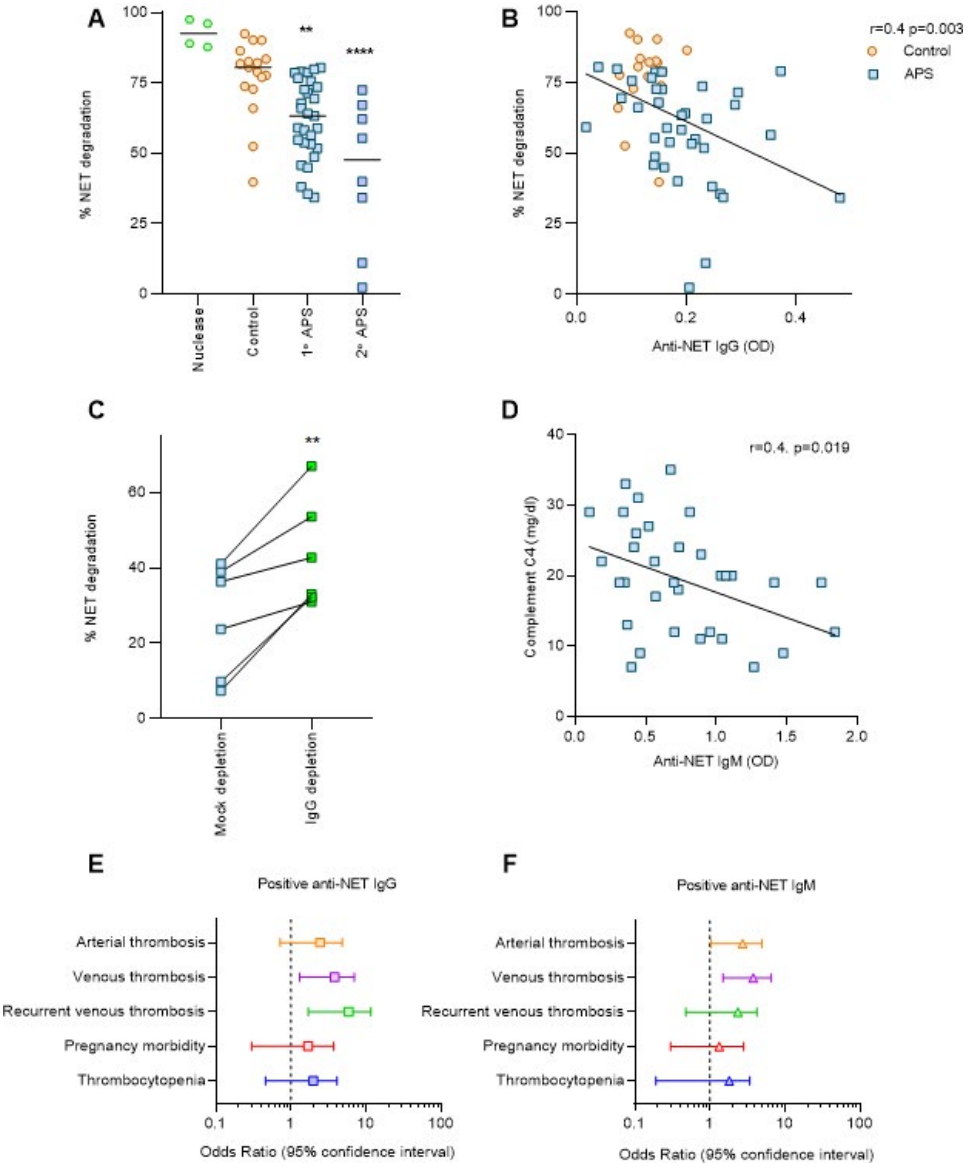

Figure 3. Correlation of anti–neutrophil extracellular trap (anti-NET) antibodies with impaired NET degradation. **A**, Percentage of NET degradation in patients with primary or secondary antiphospholipid syndrome (APS). NET release from control neutrophils was triggered with phorbol 12-myristate 13-acetate. Fresh NETs were then incubated with sera from patients with primary APS, patients with secondary APS, or healthy controls. Some samples were treated with micrococcal nuclease as a positive control. Symbols represent individual subjects; horizontal lines show the median. ** = $P < 0.01$; **** = $P < 0.0001$ versus controls, by one-way analysis of variance. **B**, Correlation between NET degradation and anti-NET IgG level, assessed by Spearman's correlation and linear regression. **C**, Percentage of NET degradation in APS serum samples (from 6 unique patients) depleted of total IgG, compared with mock-depleted samples. ** = $P < 0.01$ by paired *t*-test. **D**, Correlation between anti-NET IgM and complement C4 levels, assessed by Spearman's correlation and linear regression. **E** and **F**, Association between anti-NET IgG antibodies (**E**) or anti-NET IgM antibodies (**F**) and various clinical manifestations in 154 individuals with primary APS, systemic lupus erythematosus without antiphospholipid antibodies, unprovoked venous thrombosis without antiphospholipid antibodies, or no known disease, determined by univariate logistic regression.

Relationship of anti-NET activity to NET degradation and complement levels. Given that impaired NET degradation had been detected in some patients with SLE (11), we next examined whether anti-NET antibodies might impact the ability of APS sera to degrade NETs. Indeed, sera from both patients with primary APS and patients with secondary APS (Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract) showed impaired NET degradation compared with healthy controls (Figure 3A). Importantly, high levels of anti-NET IgG correlated with decreased NET degradation (Figure 3B), while depletion of IgG partially restored degradation (Figure 3C). As NETs and associated antibodies are potential activators of the complement system (12), we were interested in whether patients with high anti-NET activity might have evidence of smoldering complement activation. We found a correlation between high levels of anti-NET IgM and depressed complement C4 (Figure 3D), whereas no significant correlation was detected for anti-NET IgG (Supplementary Figure 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract). In summary, high levels of anti-NET IgG are associated with an impaired ability to degrade NETs, while anti-NET IgM may contribute to complement consumption.

Association of anti-NET activity with clinical manifestations. Finally, we were interested in whether anti-NET antibodies might associate with APS-related clinical manifestations such as thrombosis and pregnancy loss. After setting a positive cutoff 2 standard deviations above the mean for healthy controls, univariate logistic regression was performed to evaluate clinical associations among individuals with primary APS (n = 76), SLE (n = 23), or unprovoked venous thrombosis (n = 11); 44 healthy controls were also included in the analysis (Figures 3E and F). Twenty patients with primary APS (26.3%), 4 patients with SLE (17.4%), and 2 healthy controls (4.5%) had positive anti-NET IgG. For anti-NET IgM, 36 patients with primary APS (47.4%), 5 patients with SLE (21.7%), 4 patients with venous thrombosis (36.4%), and 3 healthy controls (6.8%) tested positive. We found that both anti-NET IgG and IgM were significantly associated with venous thrombosis (for IgG, odds ratio [OR] 3.1, $P = 0.008$; for IgM, OR 3.2, $P = 0.001$). Positive anti-NET IgG was also significantly associated with recurrent venous thrombosis (OR 4.3, $P = 0.002$), while anti-NET IgM was significantly associated with arterial thrombosis (OR 2.2, $P = 0.046$).

When we further limited the analysis to only patients with primary APS, we interestingly found that those patients with recurrent venous thrombosis had significantly higher levels of anti-NET IgG than all other patients with primary APS (Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41460/abstract). In summary, anti-NET IgG and IgM are associated with APS clinical manifestations, and anti-NET IgG may especially predict patients with the severe phenotype of recurrent venous thrombosis.

DISCUSSION

NETs are being increasingly recognized for their role in the pathogenesis of various thromboinflammatory conditions. For example, it has been suggested that increased NET formation, presence of anti-NET antibodies, and impaired NET clearance are associated with SLE disease activity and organ damage (13). To date, anti-NET antibodies have been little studied in primary APS. In this study, we found that high levels of anti-NET IgG not only predict an impaired ability of APS sera to clear NETs, but are also strongly associated with venous thrombosis (especially in patients with the severe phenotype of recurrent venous thrombosis). Whether their impact on NET degradation is by shielding NETs from DNase or direct antagonism of DNase requires further investigation. Notably, we also found elevated levels of anti-NET antibodies in SLE patients, which is interesting given that patients with active SLE are at high risk for thrombotic events (14).

The intersection of NETs, complement, and coagulation is an area of increasing study (15). NETs can directly activate complement (15), which in turn promotes coagulation via effects on tissue factor and platelets (15). At the same time, activated complement can promote NET formation, and NETs themselves then serve as a scaffold for thrombosis formation (15). It has been reported that sera from patients with active SLE that do not degrade NETs normally have low levels of complement C3 and C4, indicative of complement system activation (12). In the present study we found that high levels of anti-NET IgM were significantly correlated with depressed complement C4. It is therefore possible that anti-NET antibodies are important orchestrators of the potentially complex relationship between NETs, complement, and coagulation in thromboinflammatory disease. Future studies seem warranted to elucidate the mechanistic relationship between anti-NET activity and complement activation.

Given that aPL are regularly detected in individuals who never develop clinical manifestations of APS, it is challenging to make decisions regarding primary thrombosis prophylaxis without a reliable strategy for risk stratification. Clinically relevant biomarkers that predict thrombotic risk and allow sub-phenotyping of asymptomatic aPL carriers and patients with APS are desperately needed. The data presented here suggest that anti-NET antibodies have potential as a new class of clinically relevant biomarkers that will more effectively risk stratify and sub-phenotype aPL-positive individuals.

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. Knight 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, Yalavarthi, Gudjonsson, Kahlenberg, McCune, Bockenstedt, Karp, Knight.

Acquisition of data. Zuo, Yalavarthi, Gockman, Madison.

Analysis and interpretation of data. Zuo, Yalavarthi, Knight.

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Errata

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In the article by Meesilpavikkai et al in the May 2019 issue of *Arthritis & Rheumatology* (Efficacy of Baricitinib in the Treatment of Chilblains Associated With Aicardi-Goutières Syndrome, a Type I Interferonopathy [pages 829–831]), it was incorrectly stated that baricitinib was initiated at a daily dose of 2 mg/kg. The statement should have read "Baricitinib treatment was initiated at a daily dose of 2 mg."

In the article by Li et al in the July 2020 issue of *Arthritis & Rheumatology* (Association of Visceral Adiposity With Pain but Not Structural Osteoarthritis [pages 1103–1110]), the vertical axis labels on the second and third panels of Figures 1–4 were incorrect. They should have read "Visceral Fat, cm^2" and "Subcutaneous Fat, cm^2."

We regret the errors.