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ORIGINAL ARTICLE

Increased levels of neutrophil extracellular trap remnants in the serum of patients with rheumatoid arthritis

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Abstract

Aims: Neutrophil extracellular traps (NETs) comprise a unique form of non-apoptotic cell death exhibited by neutrophils, which occurs in a stepwise process termed NETosis. It has been postulated that NETosis plays an important role in the pathogenesis of autoimmune disorders. The aim of this study was to evaluate serum levels of NET remnants in patients with rheumatoid arthritis (RA), as well as potential associations between NET remnants and indicators of RA.

Methods: Serum levels of myeloperoxidase (MPO)-DNA complexes (NET remnants) were examined in 74 RA patients and 50 healthy controls using a modified enzyme-linked immunosorbent assay. Associations between the levels of these complexes and indicators of RA were then statistically evaluated.

Results: RA patients exhibited significantly higher levels of MPO-DNA complexes than the healthy controls, and these levels were associated with increased neutrophil counts and positivity for rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA). Among the 63 ACPA-positive RA patients examined, those with ACPA titers > 1600 U/mL showed significantly increased MPO-DNA levels. Receiver operating characteristic analysis determined that the area under the curve for all 74 RA patients was 0.798, with a sensitivity of 91.9% and a specificity of 56.0%, while that for the ACPA-negative patients was 0.891, with a sensitivity and specificity of 81.8% and 84.0%, respectively.

Conclusions: The results of this study suggest that the disease status of RA is associated with increased NETosis. In particular, evaluation of serum MPO-DNA levels may comprise a useful complementary tool for discriminating RA patients from healthy individuals.

Key words: anti-citrullinated protein/peptide antibodies (ACPA), neutrophil, neutrophil extracellular traps (NETs), rheumatoid arthritis, rheumatoid factor (RF).

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic inflammatory autoimmune disease of unknown cause. The hallmark feature of RA consists of inflammation of synovial joints leading to pain, swelling, stiffness and loss of function, and the blood of these patients frequently harbors an abnormal spectrum of autoantibodies that are specific to self-structures. A variety of immune cells and molecules mediate the immunopathological process involved in the pathogenesis of this disease. In particular, neutrophils play a multifactorial role in RA by exerting cytotoxic effects via the release of degradative enzymes and reactive oxygen species, contributing to the cytokine and chemokine cascades that accompany inflammation, and modulating the immune response via cell-cell interactions.[1,2]

Neutrophil extracellular traps (NETs) are networks of extracellular structures composed primarily of the

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nuclear and cytoplasmic contents of neutrophils, including DNA, histones and myeloperoxidase (MPO), which were first described by Brinkmann *et al.* in 2004.[3] NETs are produced by neutrophils via a novel form of non-apoptotic cell death, in a stepwise process termed NETosis. Since their discovery, the role of NETs in autoimmune diseases has garnered significant attention. For example, recent studies suggest that aberrant formation of NETs may contribute to the pathogenesis of systemic lupus erythematosus (SLE),[4–7] psoriasis,[8] small vessel vasculitis (SVV),[9,10] gouty arthropathy[11] and RA.[12–14] These studies indicated that the histones and other antigens that are released into the extracellular matrix during this process could trigger autoimmune reactions, leading to the production of autoantibodies. Moreover, recent evidence suggests that NETosis results in the release of certain antigens into the blood, which lead to the production of anti-citrullinated protein/peptide antibodies (ACPA) in RA patients. Indeed, it has been suggested that citrullinated histone 4 (H4) from activated neutrophils and NETs is a target of antibodies in RA, and the formation of ACPA is thought to be a pathogenic event in RA development.[13] The secretion of self-antigens via NETosis in the context of inflammatory stimuli provides a novel approach for boosting autoimmune responses. Further studies indicate that NETs are not only a source of autoantigens, but can mediate inflammatory cascades involved in the pathogenesis of RA.[14,15]

The aim of the present study was to evaluate the serum levels of NET remnants (MPO-DNA complexes) in patients with RA and to evaluate possible associations between the levels of NET remnants and indicators of RA.

MATERIALS AND METHODS

Study population

Serum samples were obtained from 74 RA patients (64 female and 10 male; mean age = 51.3 ± 11.0 years) and 50 healthy controls (43 female and seven male; mean age = 49.8 ± 11.7 years). Patients were defined as having RA by a specialized rheumatologist, according to the American College of Rheumatology classification criteria for RA.[16] Patients who presented with a separate autoimmune disease or inflammatory condition were excluded from the RA cohort. Serum samples were stored at −80°C until analysis. There were no significant differences between the test and control groups with respect to sex or age.

In addition to sex, age and disease duration, the following demographic and clinical data were collected for each of the 74 RA patients: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, rheumatoid factor (RF) levels, presence of ACPA, neutrophil count, neutrophil-to-lymphocyte ratio (NLR), percentage of neutrophils, and disease status (active or remission). RF and CRP levels were determined using the immunonephelometric method; values of > 20 mg/L for CRP and of > 8 mg/L for RF were considered positive. ACPA were detected using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Immunoscan CCPlus; Euro-Diagnostica, Malmo, Sweden), according to the manufacturer's recommendations. The cut-off value for a positive reaction was set at 25 U/mL, as suggested by the manufacturer. ESR was measured by the Westergren method; normal values for men and women were considered ≤ 15 mm/h and ≤ 20 mm/h, respectively. Peripheral blood cell analysis was conducted using a blood cell counter (Sysmex Cell Counter NE-8000; TOA Medical Electronics Co., Ltd., Kobe, Japan). NLR values were calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. Active RA was defined as the presence of six or more joints that were tender or painful (out of 68 joints examined) and six or more swollen joints (out of 66 joints examined), and either an ESR greater than 28 mm/h (Westergren method) or a CRP level exceeding 7 mg/L.[17] For these analyses, patients were evaluated by two specialized rheumatologists. The demographic and clinical characteristics of the 74 RA patients are presented in Table 1.

Because our study was designed to utilize serum samples taken after routine tests, written informed consent was not obtained from the recruited patients. Instead, the subjects were informed of the nature of the project, and verbal informed consent was obtained and recorded by the physician who explained the study procedure. The study protocol and verbal consent document were approved by the Ethics Committee of Xiangya Hospital, Central South University, where the study was performed.

NET remnant-specific ELISA analysis

MPO-DNA complexes were detected by ELISA, as described previously but with minor modifications.[9,18–20] Briefly, a 96-well Nunc MaxiSorp immunoplate (Nalge Nunc International, Rochester, NY, USA) was coated with a monoclonal mouse anti-human MPO antibody (266–6K1, Santa Cruz Biotechnology, Dallas, TX, USA) diluted in coating buffer (included in the Cell

Table 1 Demographic data and disease indicators for the 74 rheumatoid arthritis patients included in this study

Characteristics	Descriptions
Sex (F/M)	64/10
Mean age (years)	51.3 ± 11.0
Disease duration (years)	10.0 ± 11.6
ESR (mm/h)	60.1 ± 29.4
ESR increase (%)	44.6%
CRP (mg/L)	7.8 (1–173)
CRP increase (%)	45.9%
RF (IU/mL)	93.8 (20–3120)
RF-positive (%)	77.0%
ACPA-positive (%)	86.5%
Neutrophil count (10^9/L)	4.3 ± 1.6
Lymphocyte count (10^9/L)	1.5 (0.7–7.2)
NLR	2.5 (0.8–9.0)
Active disease (%)	27.0%

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; IU, international units; ACPA, anti-citrullinated peptide antibodies; NLR, neutrophil-to-lymphocyte ratio.

Death Detection Kit; Roche, Basel, Switzerland) at a concentration of 5 μg/mL and incubated at 4°C overnight. After washing three times, the uncoated sites were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 37°C for 1 h. The microwells were again washed, and serum samples diluted 1:10 in 1% BSA/PBS-Tween 20 (PBST) were added to distinct wells. The plate was incubated at 4°C overnight. After washing, a commercial horseradish peroxidase (HRP)-labeled anti-human DNA monoclonal antibody (component no. 2 of the Cell Death Detection Kit, Roche) was added to each well, and the plate was incubated at room temperature for 2 h. The plate was again washed and then developed using the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, followed by a 2N sulfuric acid stop solution (both from the Cell Death Detection Kit, Roche). The optical density (OD) of each well was subsequently measured at a wavelength of 405 nm (OD_{405}), with 490 nm used as a reference wavelength. All samples were assayed in duplicate and the results were expressed as mean $OD_{405/490}$ values. Considering there is no standard for MPO-DNA complex, we used one patient's serum with a weakly positive value ($OD_{405/490}$ = 0.368) to serve as the positive control. The inter-assay variability less than 20% was considered acceptable.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software for Windows (SPSS Statistics, Inc., Chicago, IL, USA). For normally distributed data, the results were expressed as means ± standard deviations (SD), and differences between groups were assessed using Student's *t*-tests. For data lacking a normal distribution, the results were expressed as medians (ranges), and the differences between groups were assessed by Mann–Whitney *U*-test analysis. Receiver operating characteristic (ROC) curves were generated, and the area under the curve (AUC), along with corresponding standard error of the mean (SEM) values, were calculated. *P*-values < 0.05 were considered statistically significant.

RESULTS

Distribution of MPO-DNA complexes in patients and controls

The distributions of the levels of MPO-DNA complexes in RA patients and in the healthy control population are presented in Figure 1. Notably, RA patients exhibited significantly higher (median = 0.063; range = 0.041–2.388) levels of MPO-DNA complexes than healthy individuals (median = 0.049; range = 0.032–0.140).

MPO-DNA levels can serve as a marker for discriminating RA patients from healthy controls

ROC analysis was conducted to explore whether the levels of MPO-DNA complexes could be utilized for discriminating RA from the healthy controls. The AUC value for the 74 RA patients was 0.798. When the cut-

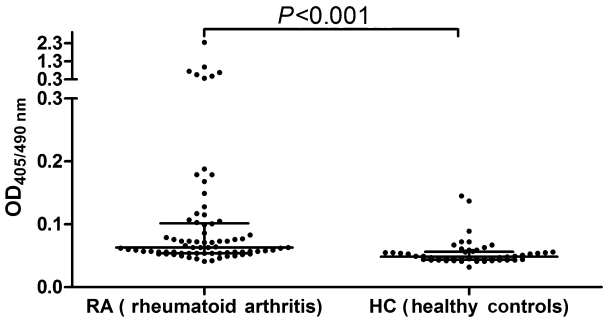

Figure 1 Distribution of the serum levels of myeloperoxidase (MPO)-DNA complexes among patients with rheumatoid arthritis (RA) and healthy controls. The serum levels of MPO-DNA complexes were measured in 74 RA patients and 50 healthy controls using a modified enzyme-linked immunosorbent assay (ELISA). For these analyses, MPO-DNA levels were estimated by measuring the mean optical density (OD) of each sample at 405 nm (OD_{405}), using the OD_{490} value as a reference. Solid bars represent the median with interquartile ranges.

off was set at $OD_{405/490}$ = 0.050, the ROC AUC translated into a sensitivity of 91.9%, with a specificity of 56.0%, for differentiating between RA cases and healthy controls (Fig. 2a). Meanwhile, we also conducted ROC analysis for MPO-DNA levels in ACPA-negative patients. The AUC value for these patients was 0.891, and when the cut-off was set at $OD_{405/490}$ = 0.062, the ROC AUC translated into a sensitivity of 81.8%, with a specificity of 84.0%, for differentiating between ACPA-negative cases and healthy controls (Fig. 2b).

Associations between the serum levels of MPO-DNA complexes and RA disease parameters

Next, we analyzed the cohort of 74 RA patients to detect associations between the serum levels of MPO-DNA complexes and RA clinical or laboratory variables. We detected significantly lower MPO-DNA levels in ACPA-positive patients (n = 63) than in ACPA-negative patients (n = 11; P = 0.044; Fig. 3a). Likewise, RF-positive RA patients (n = 57) exhibited lower MPO-DNA levels than RF-negative RA patients (n = 17; P = 0.030; Fig. 3b). Meanwhile, of the 63 ACPA-positive RA patients examined, 28 had markedly elevated ACPA titers (> 1600 U/mL). Notably, these patients exhibited higher MPO-DNA levels than those with moderate antibody titers (between 25 and 1600 U/mL; P = 0.022; Fig. 3c). Furthermore, we detected significantly higher levels of MPO-DNA complexes among the patients with neutrophil counts greater than 6.0×10^9/L, compared with those showing lower neutrophil counts, in both the RA (n = 74; P = 0.001; Fig. 4a) and ACPA-positive RA (n = 63; P = 0.001; Fig. 4b) groups. Conversely, there were no significant associations between serum MPO-DNA levels and other clinical and laboratory variables, including disease activity.

DISCUSSION

In the present study, we adopted the definition of NET remnants as complexes of DNA and MPO, as suggested by previously published studies.[9,18–20] Notably, we detected increased levels of NET remnants in the serum of patients with RA. Moreover, the associations between NET remnants and indicators of RA observed in this study further indicate a connection with RF, ACPA and neutrophils, thereby suggesting that NETosis might be involved in RA pathogenesis.

Emerging evidence indicates that NET formation plays a key role in the pathogenesis of various autoimmune disorders. In particular, there has been a great deal of progress in our understanding of the etiology of RA, including the discovery that neutrophil NETosis might be involved in this process.[21,22] In a recent study, Khandpur *et al.* detected increased levels of NETs in the tissues, peripheral blood (PB), and synovial fluid (SF) of RA patients, and discovered that these increases correlated with systemic inflammatory factors.[12] Subsequently, Sur Chowdhury *et al.* extended these finding by demonstrating that the sera of RA patients exhibited elevated levels of the principal components of NETs, including MPO, neutrophil elastase (NE), and cell-free nucleosomes, indicating enhanced NET extrusion during clot formation in these patients, which has potential clinical applications.[14] Consistent with these findings, we found that RA patients exhibited significantly higher levels of two principal components of NETs (i.e., MPO-DNA complexes) than the healthy controls, which

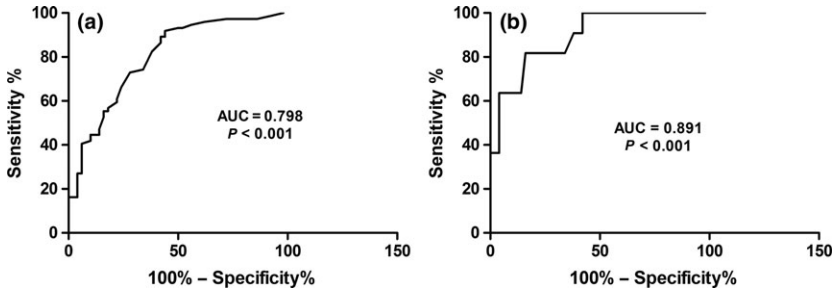

Figure 2 Receiver operating characteristic (ROC) analysis of serum myeloperoxidase (MPO)-DNA levels among rheumatoid arthritis (RA) patients, anti-citrullinated peptide antibody (ACPA)-negative RA patients, and healthy controls. (a) The ROC curve generated for the 74 RA patients examined had an area under the curve (AUC) value of 0.798. When the cut-off was set at an optical density (OD) at 405/490 nm ($OD_{405/490}$) of 0.050, the ROC AUC translated to a sensitivity of 91.9%, with a specificity of 56.0%, for differentiating between RA cases and healthy controls. (b) The ROC curve for the 11 ACPA-negative patients had an AUC of 0.891. When the cut-off was set at $OD_{405/490}$ = 0.062, the ROC AUC translated into a sensitivity of 81.8%, with a specificity of 84.0%, for differentiating between ACPA-negative cases and healthy controls.

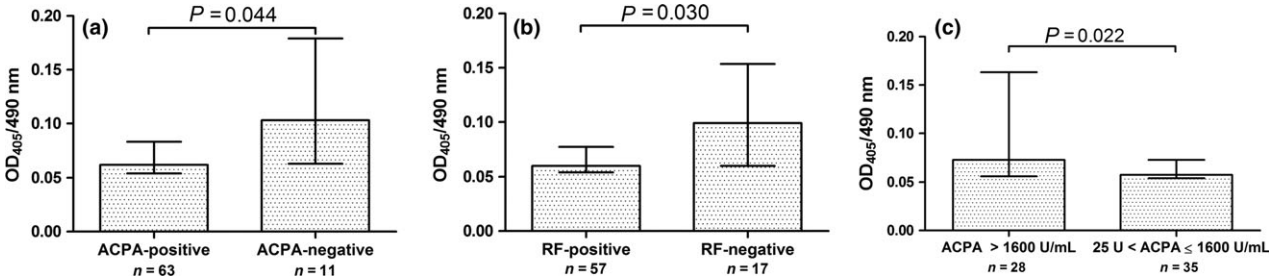

Figure 3 Association between the serum levels of myeloperoxidase (MPO)-DNA complexes and autoantibodies. Serum levels of MPO-DNA complexes were measured in the 74 RA patients using a modified enzyme-linked immunosorbent assay (ELISA). For these analyses, MPO-DNA levels were estimated by measuring the mean optical density (OD) of each sample at 405 nm (OD_{405}), using the OD_{490} value as a reference. (a–c) Graphic depictions of the differences in MPO-DNA levels between patient groups, as assessed by Mann–Whitney *U*-test. Bars represent the median MPO-DNA values with interquartile ranges. (a and b) Rheumatoid arthritis (RA) patients (n = 74) that tested positive for (a) anti-citrullinated peptide antibody (ACPA; n = 63) and (b) rheumatoid factor (RF; n = 57) exhibited significantly lower MPO-DNA levels than ACPA-negative (n = 11; P = 0.044) and RF-negative (n = 17; P = 0.030) patients, respectively. (c) The 28 (out of 63) ACPA-positive RA patients that presented with markedly high titers of ACPA (> 1600 U/mL) exhibited significantly higher MPO-DNA levels than those presenting with moderate (between 25 and 1600 U/mL; P = 0.022) antibody titers.

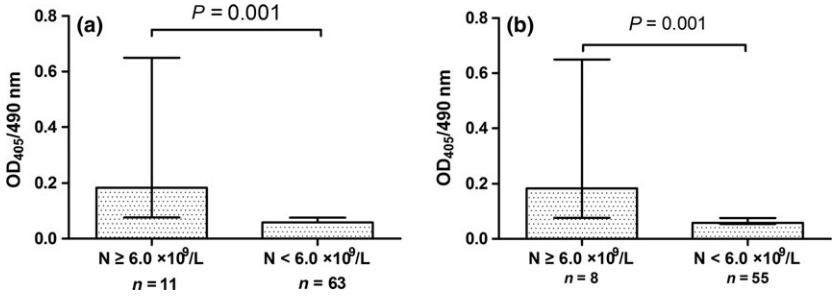

Figure 4 Serum levels of myeloperoxidase (MPO)-DNA complexes were associated with neutrophil counts in both rheumatoid arthritis (RA) patients and anti-citrullinated peptide antibody (ACPA)-positive RA patients. Serum levels of MPO-DNA complexes were measured in 74 RA patients using a modified enzyme-linked immunosorbent assay (ELISA). For these analyses, MPO-DNA levels were estimated by measuring the mean optical density (OD) of each sample at 405 nm (OD_{405}), using the OD_{490} value as a reference. (a and b) Graphic depictions of the differences in MPO-DNA levels between patient groups, as assessed by Mann–Whitney *U*-test. Bars represent median MPO-DNA values with interquartile ranges. Patients exhibiting elevated neutrophil counts ($N \geq 6.0 \times 10^9$/L) were associated with significantly higher serum levels of MPO-DNA complexes than patients with lower neutrophil counts ($N < 6.0 \times 10^9$/L) in both the (a) RA (n = 74; P = 0.001) and (b) ACPA-positive RA patient groups (n = 63; P = 0.001).

supports the conclusion that neutrophils are activated and prone to NET formation during the development and progression of RA. Recently, researchers indicated that plasma NETs remnants were elevated in a human model of lipopolysaccharide-induced inflammation, using a newly established ELISA based on citrullinated histone H3. The findings further support that inflammation may be one of the reasons for the elevated NETosis in RA.[23]

Detection of NETosis-derived products can be utilized to differentiate RA patients from healthy controls. Indeed, Sur Chowdhury *et al.* found that detection of cell-free nucleosomes showed higher diagnostic efficacy for RA than NE or MPO, with a sensitivity and specificity of 91% and 92%, respectively.[14] Additionally, our results indicate that MPO-DNA complexes also have the potential to serve as markers for discriminating RA. Indeed, we believe that MPO-DNA complexes are more representative of NETosis than any single component produced during this process. Although the specificity of MPO-DNA detection was only 56.0% among all RA patients tested, this marker showed considerable sensitivity (91.9%). Also, while ACPA is an accepted diagnostic and prognostic marker for RA, efficient diagnostic indicators of RA in ACPA-negative patients are still urgently needed. Notably, via ROC analysis, we

found that evaluation of MPO-DNA levels has potential to recognize ACPA-negative RA patients (AUC = 0.891), with an acceptable sensitivity of 81.8% and specificity of 84.0%. As such, MPO-DNA levels may comprise a useful complementary indicator for the RA patients that test negative for ACPA. However, a limitation of these data is that only 11 ACPA-negative patients were included in this study. Thus, these results must be validated in a more systematic study using a larger patient population.

By evaluating associations between MPO-DNA complexes and clinical or experimental indicators within our RA cohort, we found that MPO-DNA complexes were significantly differentially distributed between ACPA-positive and -negative patients, as well as among RF-positive and -negative patients. Specifically, patients that tested positive for ACPA or RF exhibited significantly lower levels of MPO-DNA complexes than the ACPA- and RF-negative patients, respectively. Nevertheless, because ACPA-positive RA patients are genetically and clinically distinct from ACPA-negative RA patients, we further evaluated associations between MPO-DNA levels and clinical/experimental indicators in the 63 ACPA-positive RA individuals included in this study. Patients with high ACPA titers (>1600 U/mL) showed higher levels of MPO-DNA complexes than the patients with moderate titers (between 25 and 1600 U/mL; $P = 0.022$). However, notably, these results contrast with previously published data: Khandpur *et al.* detected significant correlations between the percentage of netting neutrophils within the PB and serum levels of ACPA, ESR and CRP,[12] while Sur Chowdhury *et al.* observed a trend toward elevated serum nucleosome levels in ACPA-positive, compared to ACPA-negative, patients.[14] Meanwhile, in the ACPA-positive patients evaluated in this study, a significant association was found between ACPA titers and MPO-DNA levels. Although each of these three studies aimed at evaluating the influence of NETosis on RA, distinct concepts (percentages of netting neutrophils in PB, cell-free nucleosomes and MPO-DNA complexes) were adopted, resulting in poor comparability. Another reason for the variation in these results might be heterogeneity among the study cohorts, including distinct ACPA levels, genetic backgrounds, environmental factors and/or therapies. Nevertheless, consistent with our data that MPO-DNA levels were lower in RA patients without pathogenic antibodies (i.e., ACPA and RF) than those with, a recent study also detected a similar relationship between the levels of NET remnants and the pathogenic autoantibodies (MPO-ANCA) in patients with active small vessel vasculitis.[10] These findings may further obscure our understanding of the involvement of NETosis in the progression of autoimmune diseases. In any case, the connection between RA-specific autoantibodies and the formation of NETs is likely intricate.

Notably, while neutrophils are the direct source of NETs, we failed to detect a correlation between neutrophil counts and the levels of MPO-DNA complexes in RA patients. However, upon dividing the patients in half according to a neutrophil count cut-off of 6.0×10^9/L, a significantly different distribution of MPO-DNA levels was observed. Specifically, RA patients with neutrophil counts $\geq 6.0 \times 10^9$/L showed significantly higher levels of MPO-DNA complexes than the patients with lower neutrophil counts ($< 6.0 \times 10^9$ neutrophils/L) in both the RA ($n = 74$) and ACPA-positive RA ($n = 63$) patient groups. These data indicate that elevated numbers of neutrophils within the blood results in enhanced NETosis.

CONCLUSION

In conclusion, we detected increased levels of MPO-DNA complexes in patients with RA than in healthy individuals. Moreover, measurement of MPO-DNA levels can be used for discriminating RA, particularly ACPA-negative patients, from the healthy cases. While MPO-DNA levels appear to be positively associated with ACPA titers, RF levels and neutrophil counts, these associations might be blurred by various disease-related factors, resulting in controversial results between studies. Thus, we hypothesize that NETs not only serve as a source of autoantigens, but also contribute to the pathogenesis of RA through an intricate and precise process regulated by a series of bioactive factors. However, in this study, we used MPO-DNA complexes to represent NETs according to the definition, or referenced published studies. We think that MPO-DNA complexes are one of the forms of evaluating NETs, and the methodology of the assay must be standardized. On the basis of improved methodology, the clinical value of MPO-DNA complexes for ACPA-negative RA patients should be validated using a larger study population. Additionally, prospective studies are needed to clarify the precise relationships between NETosis, and disease indicators and disease progression.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Wei Wang. Performed the experiments: Wanchan Peng. Analyzed the data: Xingwang Ning. Contributed reagents/materials/analysis tools: Wei Wang. Wrote the paper: Wei Wang.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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