Journal of Autoimmunity 82 (2017) 31–40

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Diagnostic potential of NETosis-derived products for disease activity, atherosclerosis and therapeutic effectiveness in Rheumatoid Arthritis patients

C. Pérez-Sánchez [a, 1], P. Ruiz-Limón [a, c, 1], M.A. Aguirre [a, b, c], Y. Jiménez-Gómez [a, c], I. Arias-de la Rosa [a], M.C. Ábalos-Aguilera [a], A. Rodriguez-Ariza [a], M.C. Castro-Villegas [a, b], R. Ortega-Castro [a, b], P. Segui [a], C. Martinez [d], R. Gonzalez-Conejero [d], S. Rodríguez-López [e], J.A. Gonzalez-Reyes [e], J.M. Villalba [e], E. Collantes-Estévez [a, b, c], A. Escudero [a, b], N. Barbarroja [a, 2], Ch. López-Pedrera, MSc, PhD. [a, b, *, 2]

[a] Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Spain
[b] Unidad de Gestión Clínica Reumatología, Hospital Universitario Reina Sofía, Córdoba, Spain
[c] Departamento de Medicina (Medicina, Dermatología y Otorrinolaringología), Universidad de Córdoba, Spain
[d] Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, Spain
[e] Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Campus de Excelencia Internacional Agroalimentario, ceiA3, Córdoba, Spain

ARTICLE INFO

Article history:
Received 17 January 2017
Received in revised form
21 April 2017
Accepted 21 April 2017
Available online 29 April 2017

ABSTRACT

Objectives: 1) To assess the association of NETosis and NETosis-derived products with the activity of the disease and the development of cardiovascular disease in RA; 2) To evaluate the involvement of NETosis on the effects of biologic therapies such as anti-TNF alpha (Infliximab) and anti-IL6R drugs (Tocilizumab).
Methods: One hundred and six RA patients and 40 healthy donors were evaluated for the occurrence of NETosis. Carotid-intimae media thickness was analyzed as early atherosclerosis marker. Inflammatory and oxidative stress mediators were quantified in plasma and neutrophils. Two additional cohorts of 75 RA patients, treated either with Infliximab (n = 55) or Tocilizumab (n = 20) for six months, were evaluated.
Results: NETosis was found increased in RA patients, beside myeloperoxidase and neutrophil elastase protein levels. Cell-free nucleosomes plasma levels were elevated, and strongly correlated with the activity of the disease and the positivity for autoantibodies, alongside inflammatory and oxidative profiles in plasma and neutrophils. Moreover, ROC analyses showed that cell-free nucleosomes levels could identify RA patients showing early atherosclerosis with high specificity.
RA patients treated either with IFX or TCZ for six months exhibited decreased generation of NETs. Concomitantly, clinical parameters and serum markers of inflammation were found reduced. Mechanistic *in vitro* analyses showed that inhibition of NETs extrusion by either DNase, IFX or TCZ, further abridged the endothelial dysfunction and the activation of immune cells, thus influencing the global activity of the vascular system.
Conclusions: NETosis-derived products may have diagnostic potential for disease activity and atherosclerosis, as well as for the assessment of therapeutic effectiveness in RA.

1. Introduction

Rheumatoid Arthritis (RA) is a complex autoimmune disease with many associated co- morbidities, including cardiovascular disease (CVD). Several mechanisms have been proposed to be involved in the development of premature atherosclerosis and CVD

* Corresponding author. IMIBIC/Reina Sofia University Hospital/University of Córdoba, Avda. MenéndezPidal s/n, E-14004, Córdoba, Spain.
E-mail address: rosario.lopez.exts@juntadeandalucia.es (Ch. López-Pedrera).
[1] CP-S and PR-L shared first authorship.
[2] NB and CL-P shared last authorship.

http://dx.doi.org/10.1016/j.jaut.2017.04.007
0896-8411/

in this autoimmune condition, and a key role for white blood cells in these processes has been suggested. Thus, it has been demonstrated that autoimmune-mediated activation of leukocytes leads to the production of cytokines and mediators of inflammation, oxidative stress and endothelial dysfunction, which coordinately drive the development of atherosclerosis [1,2].

Among the immune cells, neutrophils are well established players in host defense and acute inflammation. They are known for their antimicrobial function, being essential effectors against infections. In addition, they can impact several immune related functions, from leukocyte recruitment and T-cell regulation to thrombosis and autoimmunity [3]. Neutrophil effects can be communicated either by direct contact with other cells and/or through the secretion of inflammatory mediators.

Apart from the generation of reactive oxygen species (ROS) and the release of microbicidal molecules from granules, other antimicrobial activity called NETosis has recently been described. In this process, neutrophils extrude a meshwork of chromatin fibers or NETs (neutrophil extracellular traps) that include histones, antimicrobial peptides and oxidant-generating enzymes, such as neutrophil elastase (NE), myeloperoxidase (MPO), NADPH oxidase (NOX) and nitric oxide synthase (NOS) [4–6]. Initially NETosis was placed within the context of innate immune responses to infections; however, recent evidence suggests that these structures also seem to be the centre of several pathologic states, since they can promote tissue damage, thrombosis, atherosclerosis, and autoimmunity. Thus, neutrophils have recently been revealed as proatherogenic cells. NETs have been detected in atherosclerotic lesions in both mice and humans [7]. Moreover, the oxidant activity of the enzymes of NETs has been strongly associated with CVD [8–10]. Hence, MPO represents a major source of reactive oxidants, and its activity is linked to plaque rupture and oxidation of high-density lipoproteins. NOX activity leads to the production of damaging oxidative species, superoxide anion and H_2O_2. Finally, NOS can generate NO, which in turn can combine with NOX-generated superoxide anion to produce reactive nitrogen species or be dismutated into H_2O_2, leading to HOCl or NO_2, which are also potential oxidizers of HDL. In addition, other proteins embedded in NETs, such as cathelicidins, are able to recruit monocytes into arteries, and stimulate dendritic cells in plaque lesions, promoting generation and aggravation of the atherosclerotic process [11]. Additionally, a recent study has shown that hypercholesterolemia, one of the main factors inducing atherosclerosis, can generate NETs *in vitro* [12].

In the context of autoimmune diseases, it has been shown that NETs significantly increase the autoantigenic burden, so that most of the proteins in these structures, including MPO, or peptidil-arginine-deiminase 4 (PADI4) act as potent autoantigens for autoantibody generation.

Studies have demonstrated enhanced NETosis in circulating and synovial fluid neutrophils from RA patients, associated with elevated ROS production and oxidant-generating enzymes, which significantly increase the inflammatory response [13,14]. Those studies further showed that NETosis-derived products in the serum of RA patients had a diagnostic potential. Thus, the quantitation of NETosis-derived products, such as cell-free DNA in serum, might be a useful complementary tool to discriminate between healthy donors and RA cases.

Yet, to date no study has evaluated the role of NETosis on the development of CVD in RA patients. Thus, we undertook this work to assess the association of NETosis and NETosis-derived products with the development of atherosclerosis and CVD in RA patients. The involvement of NETosis on the effects of biologic drugs such as anti-TNFalpha (i.e. Infliximab, IFX) and anti-IL6-receptor (i.e. Tocilizumab, TCZ) drugs in these patients was further evaluated.

2. Materials and methods

2.1. Patients

One hundred and six RA patients and forty healthy donors were included in the study (during a period of 24 months) after obtaining approval from the ethics committee of the Reina Sofia Hospital from Cordoba (Spain). Two more cohorts of RA patients, treated either with TCZ (162 mg/mL subcutaneous per week, N = 20) or with IFX (IFX; 3 mg/kg/day intravenous infusion at times 0, 2 and 6 weeks, and every 8 weeks thereafter, N = 55) for six months were also studied.

All the RA patients fulfilled the American College of Rheumatology criteria for the classification of RA [15]. Patients and healthy donors provided written informed consent. None of the healthy controls had a history of other autoimmune diseases, atherosclerosis or thrombosis. Clinical and laboratory parameters of the RA patients and the healthy donors included in the study are displayed in Table 1.

Clinical and laboratory parameters of both cohorts of RA patients before and after treatments are displayed in Fig. 3. Those patients were evaluated clinically and analytically at baseline (T1) and after 6 months of treatment (T2). Clinical assessment included swollen joint count (SJC), tender joint count (TJC), visual analog scale of pain (VAS; range 1–100 mm) of patient and clinician, health assessment questionnaire (HAQ) and number of DMARDs associated with either TCZ or IFX treatment. Serological evaluation

Table 1
Clinical details of the Rheumatoid Arthritis patients and Healthy donors recruited to the study.

	Rheumatoid Arthritis patients (n = 106)[a]	Healthy donors (n = 40)[a]	p value
Clinical parameters			
Female/male, n/n	75/31	27/13	0.075
Age, y	57.04 ± 11.88	45.37 ± 7.98	0.001*
Evolution time, y	12.33 ± 8.93	–	
DAS28	3.49 ± 1.52	–	
Rheumatoid Factor positive, %	54.7%	0%	
Anti-CCPsantibodies positivity, %	75.4%	0%	
Pathologic CIMT, %	27/66 (40%)	0%	
Obesity, %	9.3%	0%	
Diabetes mellitus, %	9.3%	0%	
BMI	26.4 ± 4.5	24.8 ± 2.8	0.131
Hypertension, %	25.9%	0%	
Menopause, %	56.6%	17%	
Smoker, %	21.3%	13.3%	0.211
Laboratory parameters			
Total cholesterol, mg/dL	206.2 ± 35.49	201 ± 29.9	0.519
HDL-cholesterol, mg/dL	53.96 ± 15.59	59.83 ± 14.88	0.108
Triglicerides, mg/dL	122.57 ± 62.85	83 ± 40.19	0.005*
Apolipoprotein A, g/L	146.29 ± 29.33	155.71 ± 29.45	0.203
Apolipoprotein B, g/L	88.76 ± 23.37	81.76 ± 19.62	0.218
CRP, mg/dL	14.33 ± 22.53	1.15 ± 1.4	0.001*
ESR, mm/hr	24.36 ± 15.19	9.25 ± 6.74	0.001*
Treatments			
Corticosteroids, %	54.6%	0%	
Antimalarials,%	16%	0%	
NSAIDS, %	96%	0%	
Metotrexate, %	81.3%	0%	
Leflunomide	26.6%	0%	
Vitamin D	62.6%	0%	

* Indicates significant differences *vs* Healthy donors.
[a] Values are mean ± SD. DAS = Disease activity score; anti-CCPs = Anti-cyclic citrullinated proteins; ESR = Erythrocyte sedimentation rate; CRP= C reactive protein; RF = Rheumatoid factor; CIMT = Carotid intimae media thickness; BMI= Body Mass Index; NSAIDS = non-steroid anti-inflammatory drugs.

included analysis of rheumatoid factor (RF), anti-cyclic citrullinated peptide antibodies (anti-CCPs), C-reactive protein (CRP, mg/L) and erythrocyte sedimentation rate (ESR, mm/h).

In those patients, blood samples were obtained before the start and at the end of the treatment. To avoid blood composition changes promoted by diet and circadian rhythms, samples were always collected in the early hours in the morning and after a fasting period of 8 h.

2.2. Blood sample collection and assessment of biological parameters

Plasma and serum samples, and purified neutrophils and mononuclear cells, were obtained from peripheral venous blood samples. Assessment of biological parameters, including inflammatory mediators [i.e. monocyte chemotactic protein (MCP-1), tissue plasminogen activator (tPA), and soluble P-selectin], was performed as described elsewhere [16] (see online supplementary methods).

2.3. B-mode ultrasound IMT measurements

All patients and controls underwent B-mode ultrasound imaging for CIMT (carotid intimate media thickness) measurements as previously described [16]. For further details, see supplementary methods.

2.4. RNA isolation and quantitative real-time reverse transcriptase PCR

Total RNA from neutrophils, HUVECs and mononuclear cells was extracted using TRI Reagent (Sigma, St Louis, Missouri, USA) following the manufacturer's recommendations. Genes related to inflammation and NETosis development, were evaluated as described elsewhere [16]. See supplementary methods for further details.

2.5. Determination of oxidative stress biomarkers in plasma and purified neutrophils

Oxidative stress biomarkers such as superoxide anion and hydrogen peroxide, as well as mitochondrial membrane potential ($\Delta\psi m$), were analyzed in neutrophils using a dual-laser FACSCalibur (Becton Dickinson). *See* supplementary methods for details.

2.6. NETs immunohistochemical staining and quantification, scanning electron microscopy, Neutrophil elastase (NE) and myeloperoxidase (MPO) protein expression, and detection of cell-free nucleosomes

For NETs quantification, neutrophils isolated from healthy donors or RA patients were stained with NE and DAPI (as nuclear staining). Recorded images in a Nikon Eclipse-Ti-S fluorescent microscope were then analyzed using the IMAGE-J software. Results were expressed as percentage of NETs. For scanning electron microscopy, neutrophils were fixed in glutaraldehyde 2.5% in cacodylate buffer 0.1 M (pH 7.2), washed, and dehydrated in a series of ethanol. Afterwards, neutrophils were gold shadowed using a High Vacuum Coater Leica EM ACE600. Samples were viewed and photographed using a JEOL JSM-7800F Field Emission Scanning Electron Microscope.

To evaluate NE and MPO protein expression, neutrophils were incubated either with FITC human anti-MPO or with human anti-NE primary antibody, followed for Alexa Fluor conjugated secondary antibody. Then, cells were washed and acquired on the flow cytometer FACSCalibur. Nucleosomes were measured by using the Human Cell Death Detection ELISAPLUS (Roche Diagnostics, Basel, Switzerland) following the manufacturer's recommendations. Quantification of nucleosomes was performed by photometrical determination of the absorbance at 405 nm, using as reference wavelength 492 nm.

See supplementary methods for further details.

2.7. Determination of concentration and activity of serum deoxyribonuclease I (DNase-I)

The quantitative determination of DNase-I was performed in serum from RA patients and healthy donors using a Human deoxyribonuclease I, DNase-I ELISA Kit (Biomatik, Wilmington, Delaware, USA) according to the manufacturer's instructions.

To assess DNase-I activity, a bioassay for DNA degradation was developed. Briefly, a dark 96-well plate was coated with salmon DNA (0.6 ng/μl) (Sigma, St Louis, Missouri, USA) and 100 μl of RA patients or HD (30% in DNase buffer) were added and incubated for 60 min at 37 °C. DNA content was quantified by staining with 1 μM Sytox Green-dye (Thermo Fisher, Waltham. MA, USA) -for 15 min at room temperature in the dark- and analyzed by fluorometry -fluorescence ratios 485/535 nm- (Infinite F200 Pro, Tecan, Switzerland). As reference control, salmon DNA (0.6 ng/μl) was incubated in the absence of serum. Results were expressed as percentage of DNA degradation.

2.8. In vitro studies

Neutrophils were isolated from healthy donors, and cultured in RPMI 1640 containing 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 250 pg/ml fungizone (Bio-Whittaker/MA Bioproducts, Walkersville, Maryland, USA) at 37 °C in a humidified 5% carbon dioxide (CO_2) atmosphere.

Purified neutrophils were treated for 6 h with serum obtained from 5 RA patients either, in the presence or in the absence of IFX (100 μg/ml) or TCZ (20 μg/ml) at concentrations that correspond to the therapeutic doses. RA patients used for *in vitro* studies were selected among those showing moderate-high activity (DAS28 4.17 ± 0.6), taking synthetic DMARDs, and not having any biologic DMARDs. They all were female, between 36 and 69 years old, with CRP range from 5 to 15 mg/ml and ESR range from 7 to 30 mm/h.

Then, expression of oxidative and inflammatory mediators in neutrophils and levels of cell-free nucleosomes in supernatants of cell cultures were quantified.

To evaluate the precise effects of IFX and TCZ on the oxidative and inflammatory profile of vascular and immune cells promoted by NETs, neutrophils from healthy donors were treated with serum from the above cited RA patients or healthy donors in the presence or in the absence of IFX or TCZ (at concentrations that correspond to the therapeutic doses) for 3 h. Then, supernatants from those cultures were added to either HUVECs or PBMCs -isolated from healthy donors- and further incubated for 6 h. Superoxide production and expression of various adhesion molecules and prothrombotic/proinflammatory mediators were analyzed.

Likewise, to evaluate the specific effects of cell-free DNA on the RA sera-induced athero-thrombotic profile of vascular and immune cells, neutrophils from healthy donors were treated -for 6 h- with sera from the above cited RA patients or healthy donors, which had been pre-incubated in the presence or in the absence of 15 U/ml DNase I (NZY DNase I, NZYTech - Genes & Enzymes, Lisbon, Portugal) for 2 h at 37 °C. Then, HUVECs or PBMCs -isolated from healthy donors- were treated with the supernatants from those cultures (also pre-incubated with 15 U/ml DNase I) for 18 h. Expression of various adhesion molecules and prothrombotic/

proinflammatory mediators were analyzed.

2.9. Statistical analysis

All data were expressed as mean ± SD. Statistical analyses were performed with SSPS 19.0 (SPSS Inc., Chicago, IL, USA). Following normality and equality of variance tests, clinical characteristics were compared using paired Student's *t*-test or alternatively by a nonparametric test (Mann-Whitney rank sum test). Paired samples within the same subjects were compared by Wilcoxon signed-rank test. Differences among groups of treatment were analyzed by repeated measures ANOVA. Correlations were assessed by Spearman's rank correlation. Differences were considered significant at $P < 0.05$.

Receiver operating characteristic (ROC) curve analyses, plotting the true positive rate (sensitivity) vs. the false positive rate (1-specificity) at various threshold settings were performed, and the areas under the curve (AUCs) were calculated with SPSS for various parameters. P values < 0.05 were considered statistically significant.

3. Results

3.1. Inflammatory and oxidative stress status in RA patients

An oxidative and inflammatory status was demonstrated at both plasma and cellular levels in RA patients, including the overexpression of MCP-1, the tissue plasminogen activator (tPA) and soluble P-Selectin in plasma (Supplementary Figs. S1A–C), as well as reduced total antioxidant capacity and NO levels in plasma, along with increased levels of nitrotyrosine and increased number of neutrophils with altered mitochondrial membrane potential (Supplementary Figs. S1D–H) among others.

3.2. RA patients exhibited enhanced NETs generation

Neutrophils from RA patients exhibited spontaneous NETs formation in relation to HD, as assessed by both, fluorescence microscopy (78 ± 4 vs 59 ± 3%; P = 0.02) and scanning electron microscopy (Fig. 1A and B). Increased NETs extrusion was associated with enhanced NE and MPO expression (Fig. 1C and D). Cell-

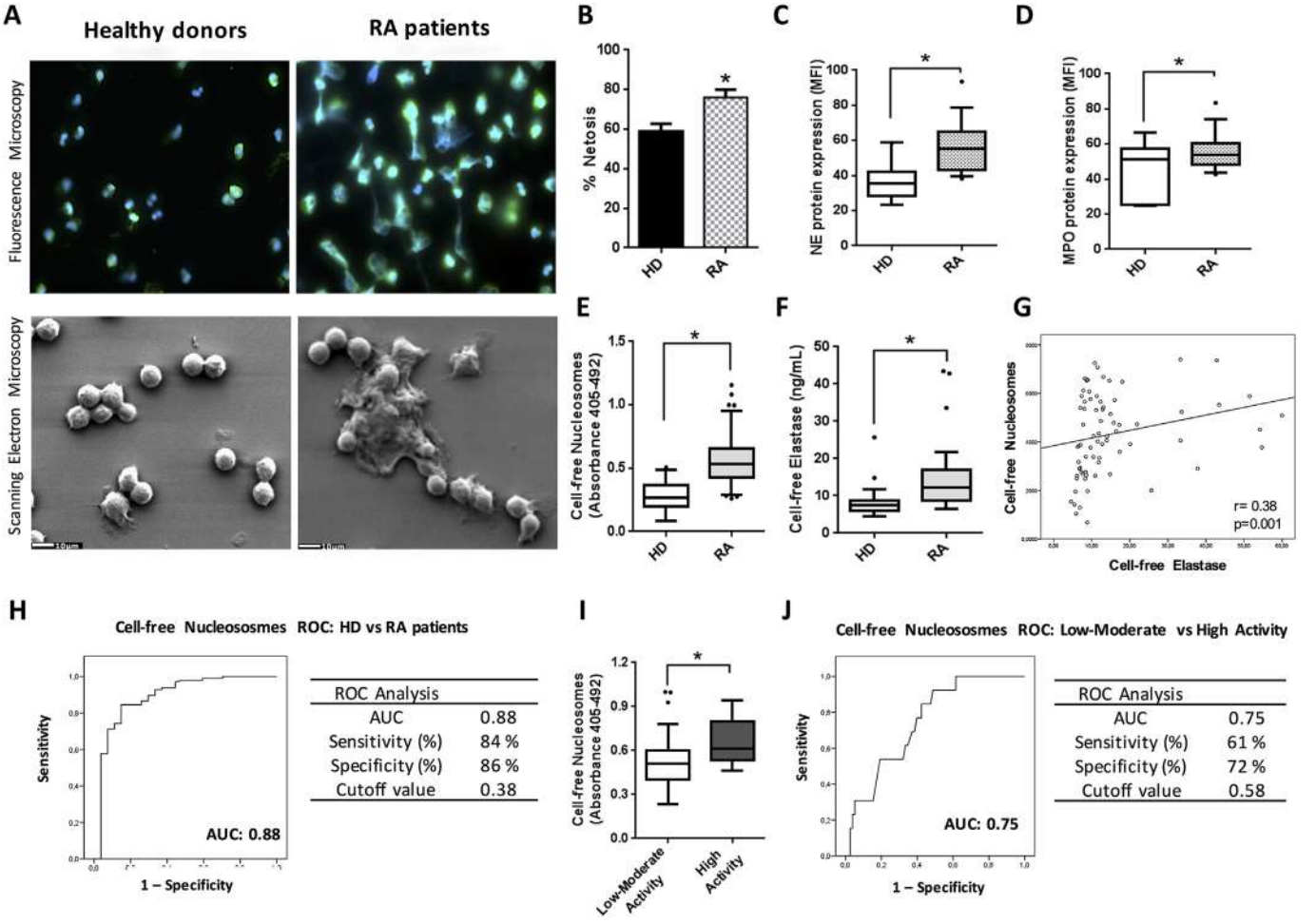


Fig. 1. Enhanced Netosis and elevated Netosis-derived products in RA patients, and diagnosis value. (A) Representative micrographs of neutrophil extracellular traps (NETs) from RA patients and healthy donors. NETs were visualized by using a Nikon Eclipse-Ti-S fluorescence microscope 20x objective. Scanning electron micrographs of NETs indicate the increase in NETosisof RA-derived neutrophils. Scale bar 10 μm. (B) Quantification of NETs. Results were expressed as percentage of NETs. Graph bars represent the mean percentage of NETs ± SEM of 20 randomly selected fields. (C,D) Intracellular NE and MPO protein expression in neutrophils from RA patients and healthy donors measured by flow cytometry and expressed as median fluorescence intensity (MFI) (*) $P < 0.05$. (E,F) Concentration of cell-free nucleosomes and cell-free elastase in serum. (G) Cell-free nucleosomes correlated positively with cell-free Elastase levels. r values of Spearman's rank correlation and P values of their null hypothesis are shown. (H) ROC curve analysis of cell-free nucleosomes to assess the accuracy of this parameter as biomarker of disease for RA patients. (I) Cell-free nucleosomes levels increased in RA with high disease activity. (*) indicates significant differences *vs* RA patients with low-moderate activity of the disease ($p < 0.05$). (J) ROC curve analysis of cell-free nucleosomes to assess the accuracy of this parameter as biomarker of disease activity for RA patients.

free nucleosomes, as well as cell-free Elastase plasma levels, were found significantly increased in RA patients in relation to HD (0.55 ± 0.18 *vs* 0.28 ± 0.1; $P = 0.0001$, and 17.3 ± 1.84 *vs* 9.01 ± 1.07 ng/mL; $P = 0.001$, respectively) (Fig. 1E and F). Moreover, a significant positive correlation was demonstrated among both parameters (Fig. 1G).

Subsequently, multiple linear regression analysis was used to evaluate the influence of age and diagnosis (RA patients *vs* HD) in NETosis-related parameters. After multiple linear regression analysis, only diagnosis was statistically proven to act as a confounding variable (Supplementary Table 1).

ROC-curve analysis, performed to assess the accuracy of cell-free nucleosomes as biomarkers of disease for RA patients, further showed that their plasma levels could identify RA patients with high specificity and sensitivity (Fig. 1H). In addition, cell-free neucleosomes were found increased in RA patients showing high-disease activity (DAS28 above 5.1) in relation to those with low or moderate activity (DAS28 between 2.6 and 5.1) (0.65 ± 0.04 *vs* 0.51 ± 0.01; $P = 0.01$) (Fig. 1 I). ROC-curve analysis further showed that cell-free nucleosomes could identify RA patients with high disease activity (Fig. 1 J).

3.3. Impaired DNase activity in RA patients

A trend to increased DNase-I levels was found in sera from RA patients in relation to HD (10.04 ± 12.75 vs 5.8 ± 8.38 ng/mL; $P = 0.112$). However, the analysis of DNase-I activity demonstrated a significant reduction of DNA degradation capacity (Supplementary Fig. S2), suggesting that impaired DNase activity might contribute to the increase in cell free nucleosomes present in RA patients, thus influencing their inflammatory profile. Moreover, reduced DNAse activity was inversely related to both, a higher activity of the disease and the presence of a pathologic CIMT (Supplementary Fig. S2).

3.4. Direct relationship among cell-free nucleosomes levels and clinical, inflammatory, and oxidative stress parameters in RA patients

Correlation and association studies demonstrated that cell-free nucleosomes levels strongly correlated with clinical parameters, such as the activity of the disease (DAS28), CRP and ESR. A direct relationship with the RA autoimmune profile was also verified, including the positivity for Rheumatoid Factor (RF) and for anti-CCPs antibodies. Moreover, parallel analyses showed that total IgG production did not correlate with NETosis-related parameters (data not shown), thus suggesting a specific mechanism for anti-CCPs. A significant relationship with altered expression levels of oxidative stress mediators at both plasma and cellular levels was also demonstrated (Fig. 2A–K). In the same way, cell-free Elastase levels were linked to the activity of the disease, CRP and ESR, as well as to the positivity for RF production (Supplementary Fig. S3). High levels of cell-free nucleosomes were further related to the presence of a pathologic CIMT (Fig. 2L). ROC-curve analysis demonstrated that cell-free nucleosomes levels could identify RA patients showing early atherosclerosis with high specificity and sensitivity (Fig. 2M).

3.5. Clinical response to anti-TNFα and to TCZ therapies

Within the cohort of RA patients treated with IFX, according to DAS28 response [17], 90.9% of patients were high or moderate responders to anti-TNFα/DMARDs combination therapy, while a 9.1% of patients were no responders. At six months of therapy most of the clinical parameters evaluated (including TJC, SJC, and HAQ) improved significantly, along with disease activity (DAS28). In addition, several autoimmune and serological parameters (such as RF, CRP, ESR) were found significantly reduced. (Fig. 3A).

On the other hand, in the cohort of patients treated with TCZ, 18 RA patients (90%) showed high or moderate response to anti-IL6R/DMARDs therapy, while 2 patients (10%) were no responders. Resembling RA patients treated with IFX, at 6 months of therapy, most of the clinical parameters evaluated (including TJC, SJC, and HAQ) improved significantly. Several autoimmune and serological parameters (such as RF, CRP, ESR) were found similarly reduced (Fig. 3D).

3.6. Anti-TNFa and anti-IL6R therapies in RA patients decreased NETosis and downregulated their inflammatory profile

A decreased release of cell-free nucleosomes, along with reduced cell-free Elastase levels, were detected in the serum of RA patients after six months of treatment with IFX (0.132 ± 0.04 *vs* 0.44 ± 0.11; $P = 0.01$ and 10.8 ± 1.07 *vs* 15.7 ± 1.07 ng/mL; $P = 0.002$, respectively) (Fig. 3B and C). Hence, cell-free nucleosomes and cell-free Elastase levels were found downregulated in the serum of a cohort of RA patients treated for six months with TCZ (0.12 ± 0.05 *vs* 0.28 ± 0.08; $P = 0.04$, and 8.49 ± 1.3 *vs* 13.01 ± 1.4 ng/mL; $P = 0.02$, respectively) (Fig. 3E and F). Concomitantly, clinical parameters and serum markers of inflammation were found reduced after treatment with any of those inhibitors (Fig. 3A and D).

In vitro, plasma from RA patients promoted a significant increase in DNA fibers extrusion, which was prevented by both IFX and TCZ, as demonstrated by fluorescence microscopy and scanning electron microscopy (Fig. 4A and B). In parallel, supernatants from cell cultures displayed down regulated levels of cell-free nucleosomes and cell-free elastase after combined treatments with both drugs (Fig. 4C and D). Intracellular NE and MPO levels, induced in neutrophils by RA serum, were also reduced in combined treatments with IFX or TCZ (Fig. 4E and F). Accordingly, the enlarged superoxide production of neutrophils from healthy donors, along with the upregulated pro-atherothrombotic profile in neutrophils, promoted by treatment with serum from RA patients, were downregulated by effect of IFX or TCZ (Supplementary Fig. S4).

3.7. Inhibition of NETs release by in vitro DNase or IFX and TCZ treatments modulates the oxidative and atherothrombotic profile of vascular and immune cells

Inhibition of NETs production by combined treatment of neutrophils with serum of RA patients plus IFX or TCZ further promoted the normalization of vascular and immune cells activities. Thus, while supernatants of neutrophils treated with serum from RA patients induced the over-expression of adhesion molecules, and prothrombotic and proinflammatory mediators in both HUVECs and PBMCs, combined treatments with either IFX or TCZ significantly reduced those effects (Fig. 5A and B). These effects were mimicked by DNase administration, so that pre-incubation of RA serum with DNase abrogated the production of prothrombotic and proinflammatory mediators in vascular and immune cells (Supplementary Fig. S5), thus suggesting that RA patients' serum was a significant trigger of NET-mediated inflammation. That data further emphasizes the importance of NETosis on the establishment of an athero-thrombotic status in RA patients, and the inhibitory effects of both processes by biologic drugs.

4. Discussion

Previous studies indicated that RA-derived neutrophils were more prone to undergo NETosis, and that NETs themselves could

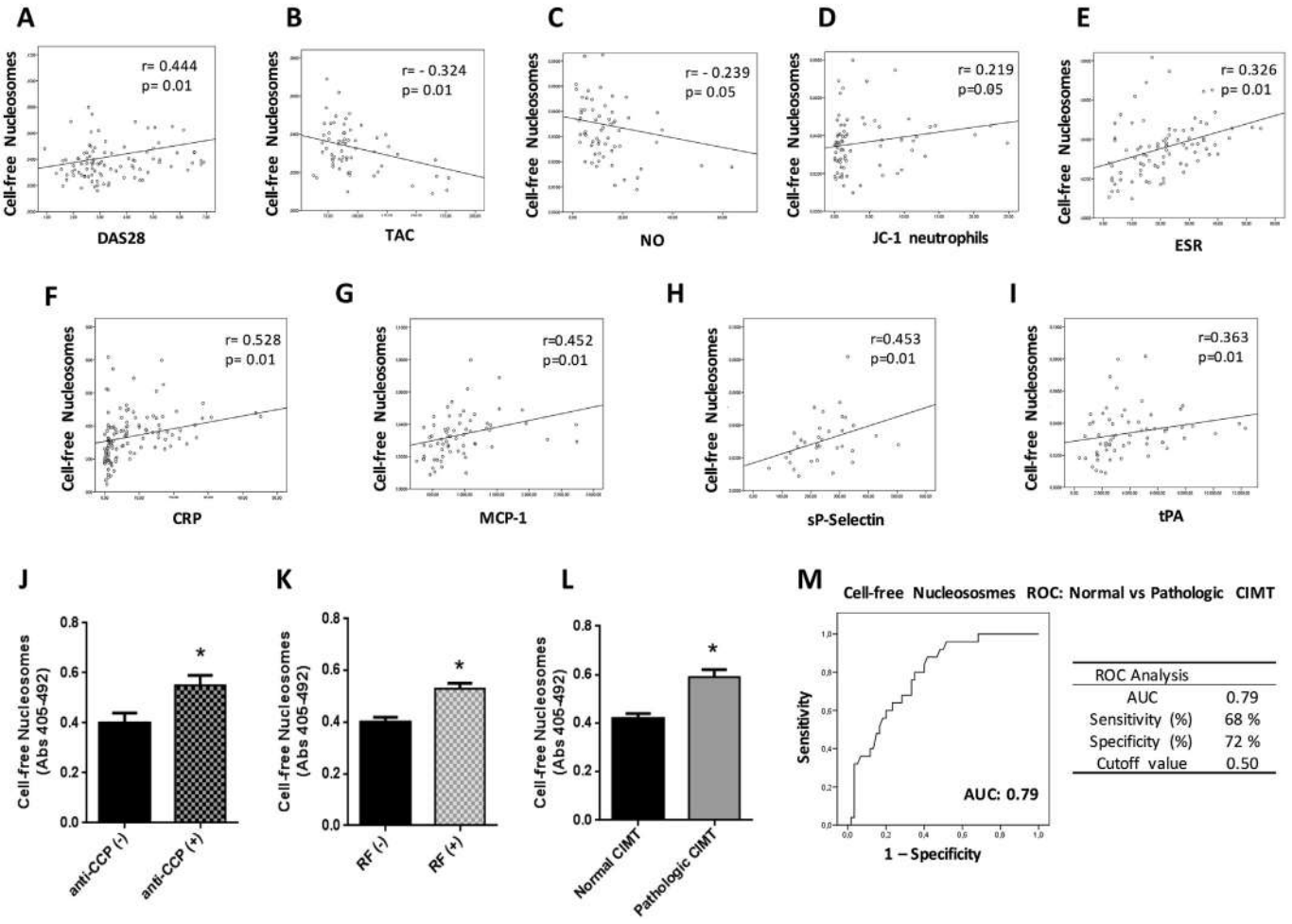


Fig. 2. Relationship among cell-free nucleosomes levels and clinical, inflammatory, and oxidative stress parameters in RA patients. Cell-free nucleosomes levels correlated significantly with DAS28 (A), oxidative stress mediators such as total antioxidant capacity (TAC) (B), nitric oxide (NO) (C), and percentage of neutrophils with altered mitochondrial membrane potential (JC-1) (D). A significant relationship was also found between high levels of cell-free nucleosomes and various inflammatory parameters, including ESR (E), CRP (F), MCP-1 (G), sP-selectin (H) and tPA (I). r values of Spearman's rank correlation and P values of their null hypothesis are shown. Positivity for auto antibodies (RF and anti-CCPs) in RA patients was found further related to high levels of cell-free nucleosomes (J,K), which were as well associated with the presence of a pathologic increase of CIMT (L).(*) indicates significant differences either *vs* patients negative for autoantibodies or *vs* patients with normal CIMT ($p < 0.05$). ROC-curve analysis, showing that cell-free nucleosomes levels could identify RA patients showing early atherosclerosis with high specificity (M).

contribute to the generation of auto-antigens or be the targets of autoantibodies [14], confirming a fundamental role of this process in the underlying etiology of RA.

The present study firstly corroborated that NETosis is enhanced in RA neutrophils, and that elements associated with the extrusion of NETs are significantly enhanced in RA patients. In addition, we extended these observations by examining the relationship among NETosis-derived products, such as cell-free DNA, and clinical and autoimmune parameters, inflammatory mediators, and oxidative stress markers related to CVD in RA patients, thus identifying NETs as relevant players in both, the activity of the disease and the physiopathology of athero-thrombosis in this autoimmune condition.

Furthermore, we demonstrated a direct effect of biologic therapies, including anti-TNFα and anti-IL6R drugs in inhibiting NETosis, thus preventing toxic side effects of NETs in inflammation and CVD in RA patients. Underlying effects of NETosis-derived products, their modulation by biologic therapies, and their effects on endothelial and immune cells have been further established.

A number of studies have shown that, in addition to their properties as cytotoxic cells, neutrophils contribute to the cytokine and chemokine cascades that accompany inflammation [18]. The present study further suggests that increased extrusion of NETs in RA might potentiate that effects, and contribute not only to the severity of the disease, but also to the development of atherothrombosis. Thus, correlation studies indicated a direct relationship among cell-free DNA levels and cellular and circulating proinflammatory and oxidative stress mediators. Moreover, ROC-curve analysis showed that cell-free DNA levels were strongly associated with the presence of a pathologic CIMT, the clearest sign of atherosclerosis.

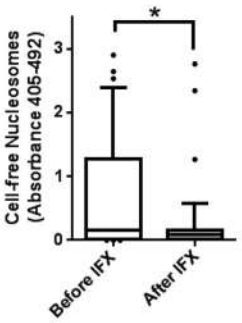
Our data further demonstrated reduced activity of serum DNase-I, which lead to an impaired DNA degradation and was inversely related to both, increased activity of the disease and presence of atheromatous plaques. As suggested in other autoimmune conditions [19–21], this reduction in serum DNAse-I activity might contribute to the accumulation of NETs, thus influencing the development of the disease and the establishment of an atherothrombotic status in RA patients.

These hypotheses were supported as well by *in vitro* studies, on which the extrusion of NETs induced by serum of RA patients promoted a significant activation of immune cells such as lymphocytes and monocytes, which expressed increased amounts of proinflammatory and prothrombotic mediators. The impact of NETs

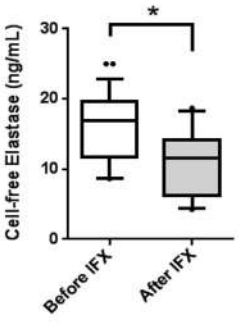
A

	RA patients (n=55)	RA patients (n=55)	p value
Clinical assessments	Baseline	IFX	
Female/male, n/n	48/55		
Age, y	54.25±10.20		
Evolution time, y	10.53±7.25		
Swollen joints (n)	11.63±4.16	2.96±1.83	<0.001
Tender joints (n)	15.65±4.78	5.35±2.37	<0.001
DAS28	5.74±0.57	3.41±0.77	<0.001
VAS	8.14±0.95	3.68±1.40	<0.001
HAQ	2.13±0.29	1.01±0.36	<0.001
Serological assessments			
Anti-CCP levels	113.71±22.84	116.23±30.45	0.801
Rheumatoid Factor levels	158.36±43.39	89.16±37.62	<0.001
CRP, mg dl^{-1}	3.89±0.28	1.59±0.16	<0.001
ESR, mm h^{-1}	56.40±17.32	28.00±17.72	<0.001

B



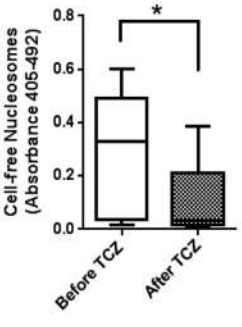
C



D

	RA patients (n=20)	RA patients (n=20)	p value
Clinical assessments	Baseline	TCZ	
Female/male, n/n	16/4		
Age, y	47.8 ± 2.30		
Evolution time, y	7.6 ± 1.76		
Swollen joints (n)	6.14 ± 1.01	0.71 ± 0.47	0.001
Tender joints (n)	19.00 ± 4.58	8.00 ± 2.93	0.045
DAS28	4.25 ± 0.18	2.90 ± 0.42	0.021
VAS	71.5 ± 3.94	48.33 ± 3.34	0.001
HAQ	1.54 ± 0.31	1.04 ± 0.33	0.046
Serological assessments			
Anti-CCP levels	761.09 ± 240.19	762.54 ± 251.44	0.991
Rheumatoid Factor Levels	90.84 ± 25.22	54.22 ± 13.99	0.041
CRP, mg dl^{-1}	25.40 ± 6.09	4.22 ± 0.70	0.014
ESR, mm h^{-1}	13.29 ± 6.08	0.46 ± 0.18	0.045

E



F

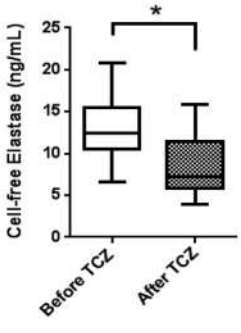


Fig. 3. Changes in NETs extrusion by effect of *in vivo* IFX and TCZ therapies in RA patients. (A) As expected, disease activity (DAS-28) and serum markers of immunity (RF) and inflammation, including CRP and ESR, were found reduced after IFX treatment. (B and C) Decreased release of cell-free nucleosomes and cell-free elastase in the serum of RA patients after six months of treatment with IFX.Boxes indicate the interval between the 25th and 75th percentiles and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 × interquartile ranges (IQR). Closed circles indicate data points outside 1.5 x IQR. $^{*}P < 0.05$(D,E and F)TCZ treatment promoted parallel effects to IFXon disease activity and serum markers of immunity and inflammation (D), as well as on levels of cell-free nucleosomes and cell-free elastase (E,F). Boxes indicate the interval between the 25th and 75th percentiles and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 × interquartile ranges (IQR). Closed circles indicate data points outside 1.5 x IQR. $^{*}P < 0.05$.

was also amplified by the simultaneous presence of ROS, which might directly affect inflammatory activity.

The structural and functional integrity of the endothelium is crucial in maintaining vascular homeostasis and preventing atherosclerosis. Patients with RA have an increased risk of developing endothelial dysfunction and premature CVD. NETs formation is increased in RA and has been proposed to contribute to endothelial damage, but the mechanism remains unclear [22]. In our hands, serum from RA patients promoted increased extrusion of NETs which, in turn, induced aberrant activation of HUVECs, thus supporting that NET formation plays a role in the endothelial dysfunction associated to RA. In conjunction, those studies supported a clear influence of NETs on the proinflammatory and prothrombotic activities of the vascular system in RA patients.

Inhibiting NETosis would be beneficial to prevent toxic side effects of NETs in inflammation, and reduce occurrence of pathological atherosclerosis and thrombosis. Several potential new targets for either the prevention of thrombosis or enhancement of thrombolysis have been described. Thus, recent clinical trials showed that aspirin, long regarded as an antiplatelet therapy, prevents venous thromboembolism recurrence [23,24].Interestingly, aspirin can also inhibit NETosis *in vitro* [25]. In the same way, the widely used anticoagulant heparin dismantles NETs [26] and prevents histone-platelet interactions [27], thus likely decreasing NETs-driven thrombosis. A recent study further demonstrated that various anti-inflammatory and CVD drugs change the ability of neutrophils to form NETs (i.e. N-acetylcysteine, ketoprofen and ethamsylate) [28].

Yet, to our knowledge, the present work is the first study that evaluated the effects of biologic therapies, such as anti-TNFα inhibitors and anti-IL6R inhibitors, in inhibiting NETosis and preventing toxic side effects of NETs in inflammation and CVD in the context of RA. Anti-TNFa therapy has significantly improved the outlook for patients suffering from RA. In addition, those that fail to respond to that treatment, frequently show optimal therapeutic response to alternative approaches, including those blocking IL-6R over expression, such as TCZ. With that premise, we evaluated in two new cohorts of RA patients the *in vivo* effects of both IFX and TCZ on the prevention of NETosis. Both drugs significantly reduced the extrusion of NETs, in parallel to an efficient downregulation of the proinflammatory profile of RA patients. Mechanistic studies further demonstrated that both drugs, *in vitro*, not only inhibited the release of NETs, but also influenced notably the activity of neutrophils as well as the NET-induced oxidative and atherothrombotic profile of vascular and immune cells.

Tumor necrosis factor alpha (TNFα) blockade is to date the most effective therapy for RA. Numerous reports have demonstrated the mechanistic effects of TNFα inhibitors on various cell types and

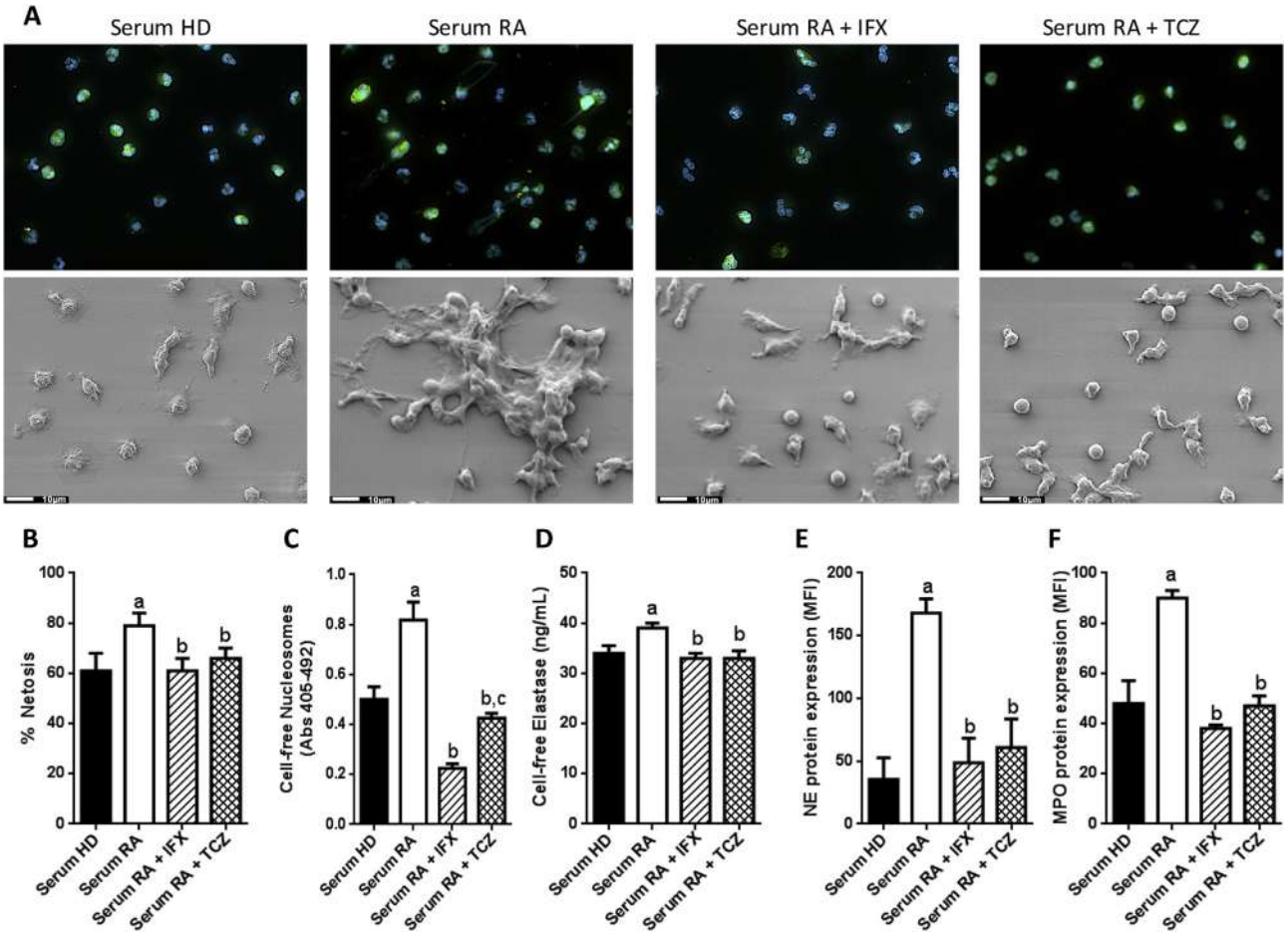


Fig. 4. Effects of *in vitro* IFX and TCZ treatments on serum RA-induced Netosis. (A)Treatment of neutrophils with serum from RA patients promoted increased DNA fibres extrusion, as demonstrated by fluorescence microscopy and by scanning electron microscopy. NETs were visualized by a Nikon Eclipse-Ti-S fluorescence microscope, 20x objective. Results were validated by scanning electron micrographs. Scale bar 10μm.(B) The number of cells positive for both, neutrophil elastase and nuclear staining (DAPI), were considered a NET and digitally recorded to prevent multiple counts. The percentage of NETs was calculated as the average of five to six fields (×40) normalized to total number of cells. Results were expressed as percentage of NETs. Graph bars represent the mean percentage of NETs ± SEM of 20 randomly selected fields.Statistically significant differences ($p < 0.05$) *vs* serum HD (a), *vs* serum RA (b), and *vs* serum RA+IFX.(C,D) Serum from RA patients promoted increased levels of cell-free nucleosomes and cell-free elastase, as well as increased intracellular protein NE and MPO (E,F) which were prevented by combined treatment with either IFX or TCZ. Graph bars represent the mean ± SEM. Statistically significant differences ($p < 0.05$) were found *vs* serum HD (a), serum RA (b), and serum RA+IFX (c).

mediators involved in the pathogenesis of RA. Thus, these agents can influence antigen presentation and self-reactive T cell activation via their effects on dendritic cells; increase the numbers and functional capacity of Treg cells to suppress effector T cells; reduce cell infiltration at the sites of inflammation by modulating cell migration and inducing their apoptosis; reduce pro-inflammatory cytokine production; modulate the functions of endothelial cells and osteoclasts; and even affect bone marrow microenvironment [29]. In the same way, the inhibition of IL-6R by effect of TCZ has been reported to be associated to increased natural killer and regulatory T cells and decreased Th1 and Th17; endorsement of an anti-inflammatory phenotype on monocytes/macrophages; induction of transient neutropenia without affecting neutrophils functions associated with host defense; improvement of endothelial dysfunction, measured by impaired flow mediated dilatation and aortic stiffness determined by pulse wave velocity [30–33].

As a complement of previous reports, the present study further delineates the influence of both inhibitors on the activity of neutrophils, including the inhibition of NETosis, as well as their relevance on the regulation of the immune and vascular systems in the context of RA. Thus, in our hands, although underlying molecular mechanisms of action of both inhibitors have been shown to be divergent, they converged on their effects on neutrophils activity, which in turn, contributed to their global anti-inflammatory and anti-thrombotic effects in RA patients. The proven relationship among both cytokines (so that one can modulate the expression of the other) [34], and the facts that they can be activated by related autoimmune mechanisms (i.e, antiCCPs) [16], and have comparable clinical behavior on RA patients, might explain that similarity.

In vivo treatment of RA patients with IFX or TCZ caused a significant decrease on disease activity and inflammatory mediators, along with a significant decrease in DNA release into extracellular space. Mechanistic *in vitro* analyses further showed inhibition of NETs extrusion, as well as reduced ROS production, and a decline of the inflammatory profile of neutrophils, which consecutively influenced the dysfunction promoted in endothelial and peripheral blood immune cells by supernatants of cultured neutrophils. Therefore, this study demonstrated a modulatory effect of TCZ and IFX on various neutrophilic functions (most probably promoted by auto-immune antibodies and inflammatory mediators present in serum of RA patients with high disease activity status), mostly including NETosis, but probably not only restricted to it, that noticeably seems to influence the pro-atherothrombotic activity of the disease.

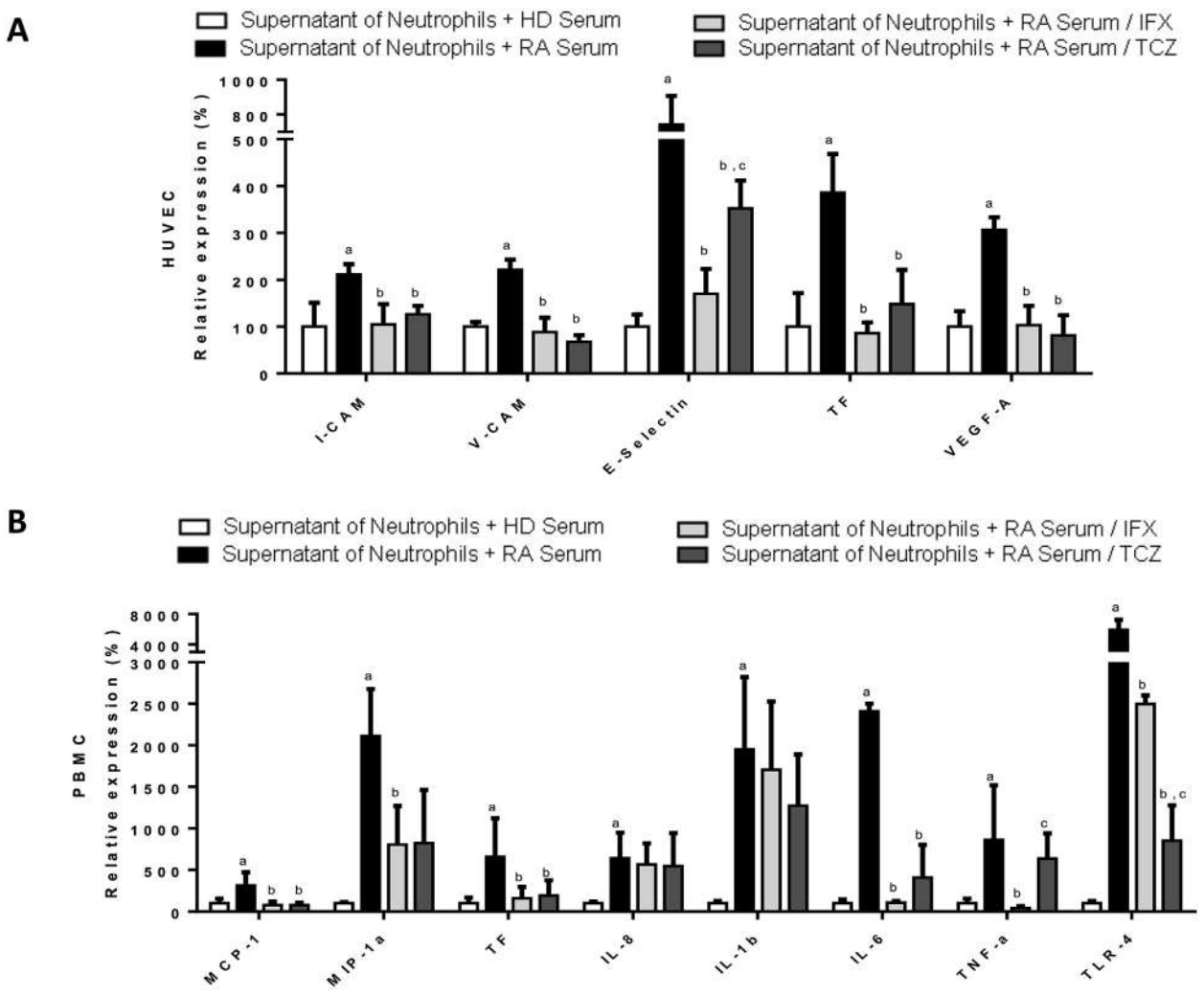


Fig. 5. Effects of *in vitro* inhibition of both NETs release and neutrophils activity by IFX and TCZ treatments on the pro-atherothrombotic profile of vascular and immune cells. Neutrophils were treated with serum of RA patients or healthy donors (HD) in the presence or in the absence of IFX or TCZ. Then, supernatants of those cultures were added to endothelial cells (HUVECs) or peripheral blood mononuclear cells (PBMCs). Bar graphs represent relative expression levels of genes related to adhesion molecules, pro-thrombotic and pro-inflammatory mediators in HUVECs (A) or PBMC (B). Significant differences at $p < 0.05$; (a) indicates significant differences *vs* HD serum; (b) indicates significant differences *vs* RA serum; (c) indicates significant differences *vs* IFX ($p < 0.05$).

5. Conclusions

Taken together, our overall data suggest that: 1) Elements associated with the extrusion of NETs are significantly enhanced in RA patients; 2) NETosis-derived products, such as cell-free DNA, strongly correlated with clinical parameters, inflammatory and oxidative stress markers; 3) Biological therapies, such as TCZ and IFX, have a significant effect on NETosis inhibition, which paralleled the reduction of both, disease activity and the expression of key inflammatory mediators. 4) *In vitro*, inhibition of NETs extrusion by either, DNase, TCZ or IFX abridged the endothelial dysfunction and the activation of circulating immune cells, thus influencing the global activity of the vascular system.

Thus, NETosis-derived products might have diagnostic potential for disease activity and atherosclerosis, as well as for assessment of therapeutic effectiveness in RA patients. All presented results can be helpful in the consideration of the medical application of analyzed drugs in RA patients, where the impact on NETosis processes may influence the progression of the disease, its co-morbidities, and their therapeutic response.

Competing interests

None.

Funding

This work was supported by grants from the 'Junta de Andalucía' (CTS-7940) and the Instituto de Salud Carlos III (PI15/01333 and PI14/00253, and RIER RD16/0012/0015), Ministerio de Economía y Competitividad, Spain (co-financed by FEDER, European Union "Unamanera de hacer Europa"). CL-P was supported by a contract from the Spanish Junta de Andalucía.

Author contributions

MCA-A, IA-R, CM, and RG-C developed the *in vivo* assays, performed the experiments and solved technical problems. MAA, AE-C, MCC-V and EC-E followed up with patients and contributed useful discussion and suggestions. CP-S, PR-L, NB and CL-P formed the hypothesis, directed and coordinated the project, designed the

experiments, analyzed the data and wrote the manuscript. RO-C and PS performed clinical analysis and contributed useful suggestions. SR-L, JAG-R and JMV were involved in developing electron microscope methods and discussing related results. YJ-G and ARA performed statistical analysis and discussed results.

Acknowledgements

We thank all patients and healthy subjects for their participation in the study. We also thanks Roche Pharma, S.A. for its collaboration. The authors thank the personnel from the Servicio Centralizado de Apoyo a la Investigación (SCAI; University of Córdoba) for technical support with the scanning electron microscope.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2017.04.007.

References

[1] F. Khan, B. Galarraga, J.J. Belch, The role of endothelial function and its assessment in rheumatoid arthritis, Nat. Rev. Rheumatol. 6 (5) (2010) 253–261.
[2] S. Skeoch, I.N. Bruce, Atherosclerosis in rheumatoid arthritis: is it all about inflammation? Nat. Rev. Rheumatol. 11 (7) (2015) 390–400.
[3] T.N. Mayadas, F. Rosetti, T. Ernandez, S. Sethi, Neutrophils: game changers in glomerulonephritis? Trends Mol. Med. 16 (8) (2010) 368–378.
[4] V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria, Sci. (New York, N.Y.) 303 (5663) (2004) 1532–1535.
[5] T.A. Fuchs, U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann, A. Zychlinsky, Novel cell death program leads to neutrophil extracellular traps, J. Cell Biol. 176 (2) (2007) 231–241.
[6] V. Brinkmann, B. Laube, U. Abu Abed, C. Goosmann, A. Zychlinsky, Neutrophil extracellular traps: how to generate and visualize them, J. Vis. Exp. JoVE (36) (2010).
[7] R.T. Megens, S. Vijayan, D. Lievens, Y. Döring, M.A. van Zandvoort, J. Grommes, C. Weber, O. Soehnlein, Presence of luminal neutrophil extracellular traps in atherosclerosis, Thromb Haemost 107 (3) (2012) 597–598.
[8] B. Shao, S. Pennathur, J.W. Heinecke, Myeloperoxidase targets apolipoprotein A-I, the major high density lipoprotein protein, for site-specific oxidation in human atherosclerotic lesions, J. Biol. Chem. 287 (9) (2012) 6375–6386.
[9] K.K. Griendling, Novel NAD(P)H oxidases in the cardiovascular system, Heart (British Card. Soc. 90 (5) (2004) 491–493.
[10] M. Tsutsui, H. Shimokawa, Y. Otsuji, N. Yanagihara, Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases, Pharmacol. Ther. 128 (3) (2010) 499–508.
[11] Y. Döring, M. Drechsler, S. Wantha, K. Kemmerich, D. Lievens, S. Vijayan, R.L. Gallo, C. Weber, O. Soehnlein, Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice, Circul Res. 110 (8) (2012) 1052–1056.
[12] M.L. Liu, M. Bashir, K. Williams, V. Werth, Cholesterol loading induces neutrophil extracellular traps, and atorvastatin attenuates this effect, Arthritis Rheumatol. 66 (2014) S530.
[13] R. Khandpur, C. Carmona-Rivera, A. Vivekanandan-Giri, A. Gizinski, S. Yalavarthi, J.S. Knight, S. Friday, S. Li, R.M. Patel, V. Subramanian, P. Thompson, P. Chen, D.A. Fox, S. Pennathur, M.J. Kaplan, NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis, Sci. Transl. Med. 5 (178) (2013) 178ra40.
[14] C. Sur Chowdhury, S. Giaglis, U.A. Walker, A. Buser, S. Hahn, P. Hasler, Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility, Arthritis Res. Ther. 16 (2014) R122.
[15] D. Aletaha, T. Neogi, A.J. Silman, J. Funovits, D.T. Felson, C.O. Bingham 3rd, N.S. Birnbaum, G.R. Burmester, V.P. Bykerk, M.D. Cohen, B. Combe, K.H. Costenbader, M. Dougados, P. Emery, G. Ferraccioli, J.M. Hazes, K. Hobbs, T.W. Huizinga, A. Kavanaugh, J. Kay, T.K. Kvien, T. Laing, P. Mease, H.A. Ménard, L.W. Moreland, R.L. Naden, T. Pincus, J.S. Smolen, E. Stanislawska-Biernat, D. Symmons, P.P. Tak, K.S. Upchurch, J. Vencovský, F. Wolfe, G. Hawker, 2010 rheumatoid arthritis classification criteria: an american College of rheumatology/european league against rheumatism collaborative initiative, Arthritis Rheumat 62 (9) (2010) 2569–2581.
[16] N. Barbarroja, C. Pérez-Sanchez, P. Ruiz-Limon, C. Castro-Villegas, M.A. Aguirre, R. Carretero, P. Segui, Y. Jimenez-Gomez, M. Sanna, A. Rodriguez-Ariza, E. Collantes-Estevez, A. Escudero, C. López-Pedrera, Anticyclic citrullinated protein antibodies are implicated in the development of cardiovascular disease in rheumatoid arthritis, Arterioscl, Thromb, Vasc. Biol. 34 (12) (2014) 2706–2716.
[17] G. Wells, J.C. Becker, J. Teng, M. Dougados, M. Schiff, J. Smolen, D. Aletaha, P.L. van Riel, Validation of the 28-joint Disease Activity Score (DAS28) and European League against Rheumatism response criteria based on C-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate, Ann. Rheumat Dis. 68 (6) (2009) 954–960.
[18] H.L. Wright, R.J. Moots, S.W. Edwards, The multifactorial role of neutrophils in rheumatoid arthritis, Nat. Rev. Rheumatol. 10 (10) (2014) 593–601.
[19] I. Kim, N.W. Hur, H.D. Shin, B.L. Park, H.S. Cheong, S.C. Bae, Associations of DNase IV polymorphisms with autoantibodies in patients with systemic lupus erythematosus, Rheumatol. Oxf. Engl. 47 (7) (2008) 996–999.
[20] S. Belguith-Maalej, H. Hadj-Kacem, N. Kaddour, Z. Bahloul, H. Ayadi, DNase1 exon2 analysis in Tunisian patients with rheumatoid arthritis, systemic lupus erythematosus and Sjögren syndrome and healthy subjects, Rheumatol. Int. 30 (1) (2009) 69–74.
[21] M. Dittmar, C. Bischofs, N. Matheis, R. Poppe, G.J. Kahaly, A novel mutation in the DNASE1 gene is related with protein instability and decreased enzyme activity in thyroid autoimmunity, J. Autoimmun. 32 (1) (2009) 7–13.
[22] P.C. Grayson, M.J. Kaplan, At the Bench: neutrophil extracellular traps (NETs) highlight novel aspects of innate immune system involvement in autoimmune diseases, J. Leukoc. Biol. 99 (2) (2016) 253–264.
[23] C. Becattini, G. Agnelli, A. Schenone, S. Eichinger, E. Bucherini, M. Silingardi, M. Bianchi, M. Moia, W. Ageno, M.R. Vandelli, E. Grandone, P. Prandoni, WARFASA Investigators, Aspirin for preventing the recurrence of venous thromboembolism, N. Engl. J. Med. 366 (21) (2012) 1959–1967.
[24] T.A. Brighton, J.W. Eikelboom, K. Mann, R. Mister, A. Gallus, P. Ockelford, H. Gibbs, W. Hague, D. Xavier, R. Diaz, A. Kirby, J. Simes, ASPIRE Investigators, Low-dose aspirin for preventing recurrent venous thromboembolism, N. Engl. J. Med. 367 (21) (2012) 1979–1987.
[25] M.J. Lapponi, A. Carestia, V.I. Landoni, L. Rivadeneyra, J. Etulain, S. Negrotto, R.G. Pozner, M. Schattner, Regulation of neutrophil extracellular trap formation by anti-inflammatory drugs, J. Pharmacol. Exp. Ther. 345 (3) (2013) 430–437.
[26] T.A. Fuchs, A. Brill, D. Duerschmied, D. Schatzberg, M. Monestier, D.D. Myers Jr., S.K. Wrobleski, T.W. Wakefield, J.H. Hartwig, D.D. Wagner, Extracellular DNA traps promote thrombosis, Proc. Natl. Acad. Sci. U. S. A. 107 (36) (2010) 15880–15885.
[27] T.A. Fuchs, A.A. Bhandari, D.D. Wagner, Histones induce rapid and profound thrombocytopenia in mice, Blood 118 (13) (2011) 3708–3714.
[28] M. Zawrotniak, A. Kozik, M. Rapala-Kozik, Selected mucolytic, anti-inflammatory and cardiovascular drugs change the ability of neutrophils to form extracellular traps (NETs), Acta biochim. Pol. 62 (3) (2015) 465–473.
[29] M. Wong, D. Ziring, Y. Korin, S. Desai, S. Kim, J. Lin, D. Gjertson, J. Braun, E. Reed, R.R. Singh, TNFalpha blockade in human diseases: mechanisms and future directions, Clin. Immunol. Orl. Fla.) 126 (2) (2008) 121–136.
[30] A. Alunno, M. Manetti, S. Caterbi, L. Ibba-Manneschi, O. Bistoni, E. Bartoloni, V. Valentini, R. Terenzi, R. Gerli, Altered immunoregulation in rheumatoid arthritis: the role of regulatory T cells and proinflammatory Th17 cells and therapeutic implications, Mediat. Inflamm. 2015 (2015) 751793.
[31] J.A. Obeng, A. Amoruso, G.L. Camaschella, D. Sola, S. Brunelleschi, L.G. Fresu, Modulation of human monocyte/macrophage activity by tocilizumab, abatacept and etanercept: an in vitro study, Eur. J. Pharmacol. 780 (2016) 33–37.
[32] H.L. Wright, A.L. Cross, S.W. Edwards, R.J. Moots, Effects of IL-6 and IL-6 blockade on neutrophil function in vitro and in vivo, Rheumatol. Oxf. Engl. 53 (7) (2014) 1321–1331.
[33] A.D. Protogerou, E. Zampeli, K. Fragiadaki, K. Stamatelopoulos, C. Papamichael, P.P. Sfikakis, A pilot study of endothelial dysfunction and aortic stiffness after interleukin-6 receptor inhibition in rheumatoid arthritis, Atherosclerosis 219 (2) (2011) 734–736.
[34] F. Schaper, S. Rose-John, Interleukin-6: biology, signaling and strategies of blockade, Cytokine & growth factor Rev. 26 (5) (2015) 475–487.