DNA damage increase in peripheral neutrophils from patients with rheumatoid arthritis is associated with the disease activity and the presence of shared epitope

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Abstract

Objective

Neutrophils play a major role in rheumatoid arthritis (RA) pathogenesis. We aimed to evaluate if neutrophil DNA damage in RA patients is associated with the disease activity, autoantibodies status, carriage of the RA shared epitope (SE) and treatment.

Methods

DNA damage was assessed by alkaline comet assay in peripheral blood (77 patients and 55 healthy controls) and in 10 RA synovial fluid neutrophils. Evaluation of the respiratory burst of 30 patients with RA and 30 healthy controls was done.

Results

Compared to controls, RA patients exhibited increased neutrophil DNA damage. RA synovial fluid cells DNA damage was increased when compared to OA synovial fluids cells. In addition, our study shows that Anti-TNF-α therapy reduces the frequency of DNA damage. Patients with simple or double dose of shared epitope presented a higher frequency of DNA damage compared to patients without the allele. Positive correlation was found between neutrophil DNA damage and DAS-28 and ROS production.

Conclusion

Our results suggest that an increase of respiratory burst of neutrophils reflects the higher levels of DNA damage in neutrophils and a positive correlation between DNA damage and disease activity shows the importance of oxidative stress in the pathogenesis of RA.

Key words

rheumatoid arthritis, autoimmune disease, shared epitope, DNA damage, anti-TNF-α, neutrophils, burst oxidative, oxidative stress, ROS and DAS-28.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic joint inflammation, synovial hyperplasia and bone erosion (1-3). Synovial microenvironment is considerably altered due to secretion of pro-inflammatory cytokines and subsequent infiltration of inflammatory cells, facilitating pannus formation and synovial tissue infiltration into articular cartilage and bone, with eventual joint destruction (4).

Environmental and genetic factors are believed to contribute to the development of the disease (5, 6). Although genetically complex, the genes of the human leucocyte antigen (HLA) class II region at chromosome 6 have been strongly associated with susceptibility to the disease. Several HLA-DRB1 alleles (*04:01,*04:04,*04:05,*04:08,*01:01,*01:02,*10:01 and*14:02) have been associated with RA susceptibility, according to the population studied (7). All these DRB1 alleles share a highly conserved amino acid sequence between positions 70 and 74 (QKRAA, QRRAA or RRRAA) in the third hypervariable region, which forms part of the peptide-binding pocket of the HLA-DRα/β heterodimer molecule. This conserved sequence has been commonly referred to as the RA shared epitope (SE) and the presence of a double dose of SE alleles has been associated with the outcome of the disease (8, 9). In addition, certain combinations of SE-carrying alleles, particularly DRB1*04:01 and *04:04, have been associated with more severe disease as evaluated by clinical features, radiological lesion progression and the presence of extra-articular manifestations (10).

Several lines of evidence indicate that the oxidative stress plays an important role in the pathogenesis of RA (11). Increased production of reactive oxygen species (ROS) are associated with increased levels of lipid peroxidation products, degradation of hyaluronic acid by free radical mechanisms, decreased levels of ascorbic acid in serum and synovial fluid and increased breath pentane excretion (12), increased levels of lipid peroxidation products such as malondialdehyde and 4-hydroxynonenal are associated with disease activity (13), and positive a correlation between the erythrocyte sedimentation rate and these lipid products has been reported (14), supporting the idea of an unbalanced oxidant-antioxidant system in RA (15). The increased pressure in the synovial cavity, reduced capillary density, vascular changes and increased metabolic rate of synovial tissue, and the presence of locally activated leukocytes facilitate the generation of ROS (16). Increased levels of thioredoxine, a relevant marker of oxidative stress, are observed in synovial fluid from RA patients compared with patients with other forms of arthritis (17). Peripheral blood lymphocyte DNA obtained from RA patients presents increased levels of the promutagenic 8-oxohydrodeoxyguanosine (8-oxodG) (18), which is a product of oxidative damage to DNA, pointing to the genotoxic effects of oxidative stress. Activated neutrophils and macrophages present in RA synovial fluid produce ROS as a result of active processing of endocytosed material, leading to the destruction of articular cartilage (19, 20).

Oxidative stress products may induce DNA damage, which may contribute to increased mutation rates, genome instability, apoptosis and associated tissue regeneration and cell proliferation (21). Considering that: i) neutrophils play a major role in RA pathogenesis, ii) the overactivity of neutrophils is associated with increased oxidative stress and DNA damage, iii) the genetic profile of RA patients, particularly the presence SE alleles, may be associated with the magnitude of the oxidative stress, and iv) treatment features may influence the oxidative stress, in this study we evaluated DNA damage in peripheral blood and synovial fluid RA neutrophils, associating this variable with the respiratory burst, disease activity score, SE profile, and patient clinical, laboratory and treatment features.

Materials and methods

Participant recruitment

Seventy-seven women (age 41.54±9.89 years) with RA followed at the University Hospital of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, diagnosed ac-

cording to the criteria of the American College of Rheumatology (22), were studied. According to the disease activity score DAS28, 34 patients exhibited DAS28 <3.2 and 37 patients exhibited DAS28 >3.2. Synovial fluid was obtained from 10 of the patients exhibiting active disease and 7 from osteoarthritis (OA). 17 patients exhibited SE at a double dose (SE/SE), 25 at a single dose (SE/X) and 28 exhibited at SE (X/X). Regarding treatment, 7 patients were not taking drugs and the others were taking DMARDs and low doses of prednisone (<10 mg/day). 16 were taking anti-TNF-α (infliximab 3–5 mg/kg/dose), in which 3 patients with SE/SE genotype, 2 patients with SE/X genotype SE/X and 11 with X/X genotype. All of them were considered to be responders to this treatment. The demographic, laboratory and treatment features of these patients are summarised in (Table I).

The control group was composed by 55 healthy women (mean age 41.52±7.84 years), presenting no previous history of autoimmune disorders and 6 synovial fluid samples from osteoarthritis patients. The protocol of the study was approved by Ethics Committee of the University Hospital of the Faculty of Medicine of Ribeirão Preto of the University of São Paulo (Protocol no. 7075/2008), and all participants gave written informed consent before blood withdrawal.

Neutrophil isolation

Patients' blood and volunteers' blood diluted in Alsever's solution (v/v) were centrifuged at 755 g for 10 min, at room temperature (25°C) (5810R, Eppendorf, Hamburg, Germany). The platelet-rich plasma and the buffy coat – a well-defined and narrow layer over the red blood cells composed mainly of mononuclear cells – were carefully and completely removed using Pasteur pipettes with long and thin tips. The granulocyte-rich pellet was suspended in 2 vol of 2.5% (w/v) gelatin solution, prepared in 0.15 M NaCl, and pre-warmed at 37°C; the tubes were gently rotated end-over-end and, after mixing thoroughly, they were incubated for 30 min, at 37°C. The neutrophil-rich supernatant was transferred to another tube, diluted in 3 vol of 0.15 M NaCl, and centrifuged (400 g, 10 min, 25°C). To lyse the remaining red blood cells, the pellet was suspended in 0.83% NH4Cl (pH 7.2) pre-warmed at 37°C, incubated for 5 min, at 37°C, and centrifuged (400 g, 10 min, 25°C). The pellet was washed with 0.15 M NaCl (400 g, 10 min, 25 °C), suspended in 1 mL of HBSS supplemented with 1 mg mL-1 gelatin, and kept on ice until use. Neutrophils from the same patients were used to measure burst oxidative production and DNA damage quantity.

Comet assay

The alkaline version of the comet assay was used according to Singh *et al*. 1988 (23). Briefly, 10 μL of peripheral blood leukocytes or 10 μL of synovial fluid, isolated after red cell lysis, were added to 120 μL of 0.5% low melting point agarose (Sigma) at 37°C, layered on slides pre-coated with 1.5% regular agarose, covered with a coverslip, and left to stand for 10 min at 4°C. Coverslips were carefully removed and the slides immersed into a lysis solution (2.5 M NaCl; 100 mM EDTA; 10 mM Tris [pH 10]; 1% N-lauroyl sarcosine sodium; 1% Triton X-100 and 10% DMSO) overnight. Slides were then washed in PBS for 5 min, immersed in freshly prepared alkaline buffer (10N NaOH, 200 mM EDTA, pH13) and randomly distributed in a horizontal electrophoresis chamber. After a 20 min DNA unwinding period, electrophoresis was performed at 25 V and 300 mA (1 V/cm) for an additional period of 20 min. After electrophoresis, slides were rinsed three times with neutralisation buffer (0.4M Tris; pH 7.5), fixed with absolute ethanol, stained with 50 μL of 20 μg/mL ethidium bromide (Sigma) and scored under a fluorescence microscope at 400× magnification. Cells were protected from light to prevent additional DNA damage during all steps followed. One hundred cells per donor were randomly captured using a fluorescence microscope coupled to a camera (Axio Scope A1, Carl Zeiss, Jena, Germany), and images were analysed for the tail moment (TM) using the CometScore software (TriTek Corp, 2003, a free download at www.autocomet.com).

ROS production (oxidative burst) by neutrophils

ROS generation by neutrophils was assayed as described by Lucisano-Valim *et al*. (2002) (23), with modifications. Luminol (28 mM) and n-fMLP (10 mM) stock solutions were prepared in DMSO, and diluted in HBSS prior to use. Aliquots of neutrophils (1×106 cells mL-1) were incubated in the presence and absence of GM-CSF (2 ng mL-1) for 1 h, at 37°C, before adding luminol (280 μM) and one of the stimuli – i-IC (100 μg mL-1), s-IC (100 μg mL-1), or n-fMLP (1 μM) – to the reaction mixture (0.5 mL). The chemiluminescence (CL) production was measured for 10 min, at 37°C, in a luminometer (AutoLumat LB 953, EG&G Berthold, Bad Wildbad, Germany). Oxidative burst production was only possible in 30 patients out of 77.

HLA-DRB1 typing

DNA was extracted using a classical salting out procedure. The HLA-DRB1 typing at low or high resolution level was performed using polymerase chain reaction-amplified DNA hybridised with sequence-specific primers using commercially available kits (One-Lambda, Canoga Park, CA). For SE typification, 70 out of the 77 patients were studied.

Rheumatoid factor and anti-CCP antibody detection

Rheumatoid factor was detected by a commercial nephelometric assay (Boehring, Hamburg, Germany). Serum samples presenting results >10 UI/mL were considered positive. Anti-CCP2 Ig-G antibodies were detected using commercially available ELISA, containing synthetic peptides (Quanta Lite anti-CCP 2 Inova, San Diego, CA), according to the manufacturer's instructions. Serum samples presenting results >20 UI/mL were considered positive.

The detection of anti-CCP2 immunoglobulin G antibodies was performed using commercially available ELISA, containing synthetic peptides (Quanta Lite anti-CCP 2 Inova, San Diego, CA). The ELISA was performed according to the manufacturer's instructions. Serum samples presenting results >20 U/mL were considered to be positive.

Statistical analysis

The comparisons of DNA damage between patients and controls were performed using the nonparametric Mann-Whitney test (two groups) or Kruskal-Wallis (three groups) test, followed by the Dunn post-test. *p*-values <0.05 were considered significant.

Results

DNA damage in RA patients

Value of DNA damage was significantly higher in RA total leucocytes than in total leucocytes of healthy controls (Tail moment: 21.23±7.32, 4.35±3.25; $p<0.05$). Compared to healthy individuals (6.14±2.21), RA patients exhibited increased neutrophil DNA damage (35.47±11.47, $p<0.05$). The Tail Moment of cells present in the synovial fluid (64.19±9.24) was increased when compared to the synovial fluid of osteoarthritis patients (15.16±5.45, $p<0.05$), as shown in Table II.

DNA damage in RA patients regarding the clinical and genetic variations

The group of patients with score disease activity more than 3.2 had more DNA damage (44.29±8.79) than the group of patients less 3.2 score disease (26.66±5.46) $p<0.05$, (Fig. 1A) and the DNA damage was positively correlated with DAS28 (Spearman r=0.8202, $p<0.05$) (Fig. 1B). The RA group was also stratified according to treatment. Seven patients were not taking drugs and the other patients were taking methotrexate, leflunomide and anti-TNF-α therapies in different combinations. There was significant decrease in the magnitude of neutrophil DNA damage between neutrophils from patients who were making use of anti-TNF-α therapy (21.73±5.58) when compared with the other groups of treatments (untreated patients 49.52±12.31; patients treated only with MTX 38.66±10.12; patients treated only with leflunomide 33.94±8.82 and patients with the combinations MTX and leflunomide 41.67±3.55 $p<0.05$), (Fig. 2).

Significant difference in the magnitude of neutrophil DNA damage was observed between the group that does not have shared epitope (23.97±4.33) than the group that have single (41.03±856) or double dose of SE alleles (51.3±5.76) $p<0.05$) (Fig. 3). The neutrophil DNA damage was positively correlated with oxidative burst (Spearman r=0.6632, $p<0.05$) (Fig. 4). No significant associations were observed regarding the influence of other clinical and laboratory manifestations on neutrophil DNA damage like anti-cyclic citrullinated peptide antibodies and rheumatoid factor Table II.

Table I. Baseline clinical and demographics characteristics of the rheumatoid arthritis patients.

Clinical features	RA patients (n=77)	Controls (n=55)
Mean age (years) ±SD	41.54 ± 9.89	41.52 ± 7.84
Women %	100	100
Caucasian %	84	89
RF-positive %	85	/
Anti-CCP positive %	69.8	/
Means disease duration (years)	7.89 ± 5.16	/
DMARD use %	90	/
Prednisone (<10 mg/day) %	90	/
Anti-TNF use (>6 months) %	22,86	/
DAS-28 (mean ± SD)	3.37 1,64	/
Tobaco use %	5.19	/

Anti-CCP: anti-cyclic citrullinated peptide antibodies; anti-TNF: anti-tumour necrosis factor; DAS28: disease activity score; DMARDs: disease-modifying anti-rheumatic drugs; RA: rheumatoid arthritis; RF: rheumatoid factor; SD: standard deviation.

Table II. Mean DNA damage (Tail moment) in patients with rheumatoid arthritis, osteoarthritis and controls.

	DNA damage ± SD (Tail moment)
RA patients	
Total leucocyte	21.23 ± 7.32[a]
Neutrophil	35.47 ± 11.47[b]
Neutrophil Anti-CCP positive	64.19 ± 9.24[c]
Neutrophil Anti-CCP negative	27.32 ± 2.36[d]
Neutrophil RF-positive	25.85 ± 3.25
Neutrophil RF-negative	30.25 ± 2.36[e]
	31.26 ± 5.32
OA patients	
Synovial fluid cells	
Heathy controls	
Total leucocyte	4.35 ± 3.25
Neutrophil	6.14 ± 2.21

AR: rheumatoid arthritis; Anti-CCP: anti-cyclic citrullinated peptide antibodies; RF: rheumatoid factor; OA: osteoarthritis; SD: standard deviation.
a – RA total leucocytes x control total leucocytes $p<0.001$.
b – RA neutrophils x control neutrophils $p<0.001$.
c – RA synovial fluid cells x OA synovial fluid cells $p<0.001$.
d – RA neutrophil anti-CCP positive x RA neutrophil anti-CCP negative $p>0.005$.
e - RA neutrophil RF positive x RA neutrophil RF negative $p>0.005$.

Discussion

There are many lines of evidence showing that the cells of patients with autoimmune diseases are more sensitive to genotoxic stress compared to healthy individuals. Lymphocytes from patients with autoimmune diseases, including juvenile rheumatoid arthritis (JIA), systemic lupus erythematosus (LES) and Systemic sclerosis (SSc) present elevated levels of DNA damage when submitted to gamma radiation, a potent inductor of reactive oxygen species and DNA breaks. It has been reported that cells of systemic lupus erythematosus (LES) patients when submitted to ionising radiation are less efficient in repairing DNA damage, indicating an increased sensitivity to ionising radiation (25, 26).

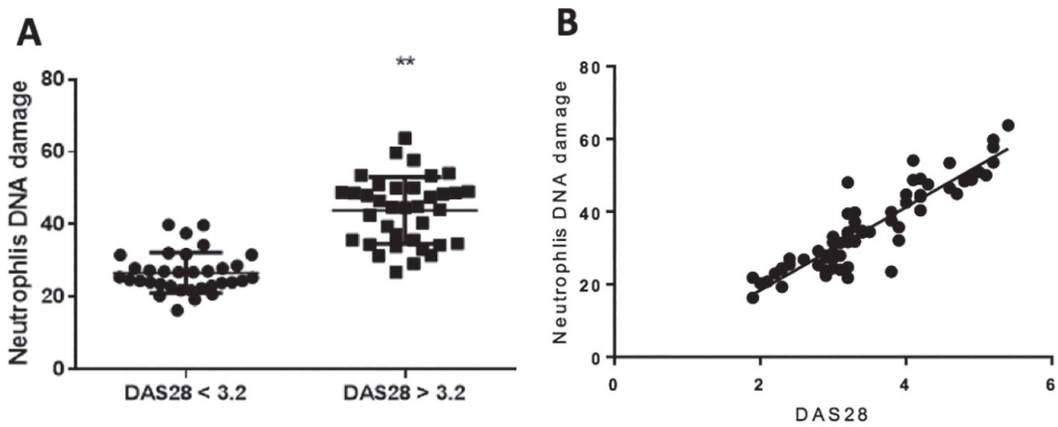

Fig. 1.
A. Increased DNA damage in neutrophils of RA patients with DAS28 >3.2 $p<0.001$.
B. Regression lines between neutrophil DNA damage and disease activity parameters (Spearman r=0.8202, $p<0.001$).

In this study, we showed that the level of DNA damage, as measured by comet assay, was from two- to nine-fold higher in RA patients compared to healthy individuals, irrespective of disease variant and of drug treatment with methotrexate, leflunomide and anti-TNF-α. In addition, we further studied one small groups of patients consisting of individuals using no drugs and no difference in the magnitude of DNA damage was observed. Taken together, one can speculate that these findings indicate that the increased DNA damage observed in RA cells can be probably caused by the disease itself rather than by drug therapy. Although the influence of DNA damage on RA pathogenesis has not been completely elucidated, the results reported in the present study, together with literature findings reporting that genomic instability may appear in RA patients even preceding clinical manifestations, can indicate that DNA damage may contribute to RA complications. Indeed, RA is associated with an increased risk of lymphoma, and the risk of solid cancer may be increased slightly in patients with RA compared with the general population (27).

Besides RA complications, DNA damage may also contribute as a co-factor to RA pathogenesis, since DNA damage and genomic instability in different chromosomal regions have been associated with increased production of reactive oxygen species in chronic inflammatory and degenerative processes, and also in autoimmune disorders (28). ROS produced in the course of cellular phosphorylation, and by phagocytic cells during oxidative bursts, exceed the physiological buffering capacity and result in oxidative stress that can damage protein, lipids, nucleic acid and matrix components (11). The ROS may attack the different types of macromolecules including DNA and impair DNA-repair mechanisms. The superoxide anion and hydrogen peroxide does not react directly with DNA, however, when reacted with the transition metals

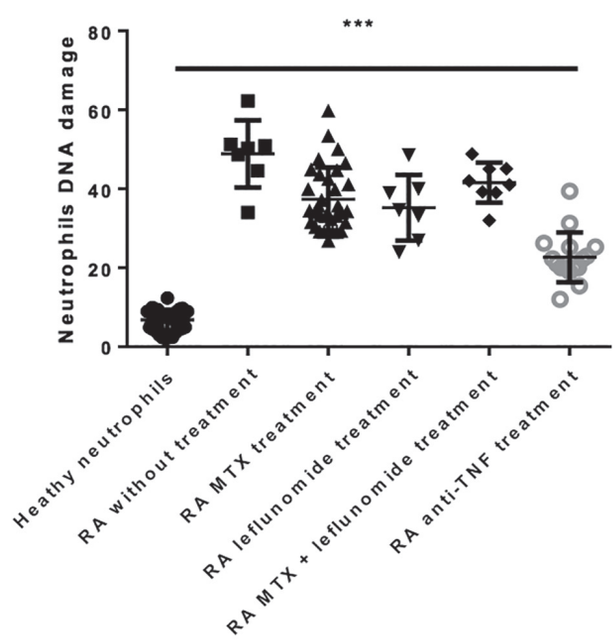

Fig. 2. Neutrophil DNA damage in RA patients taking different treatments

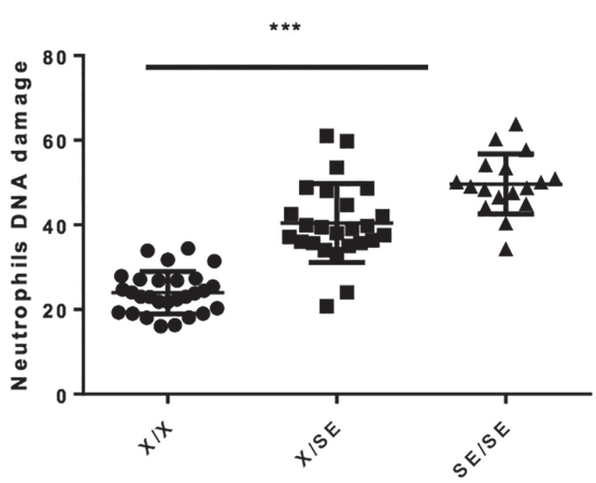

Fig. 3. Increased neutrophil DNA damage in RA patients with shared epitoped.

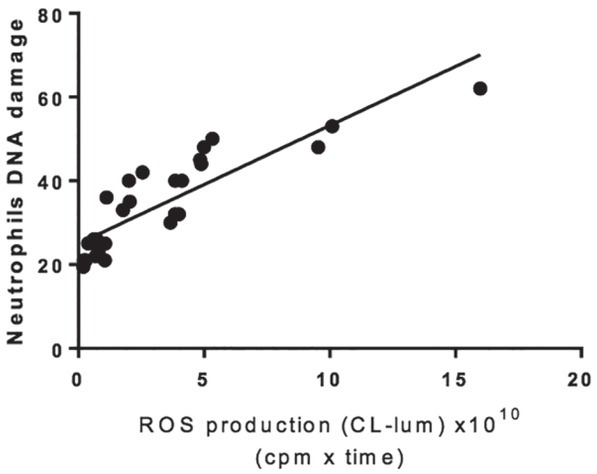

Fig. 4. Positive correlation between neutrophil DNA damage and oxidative burst.

such as Fe^{2+} or Cu^{+}, promote catalysis, converging them into hydroxyl radicals highly reactive which cause a spectrum of lesions in DNA (29).

Our findings indicated that there were important correlations between isolated neutrophil DNA damage, bursts oxidative and neutrophil DNA damage in patients with RA. Therefore, we suggest that chronic inflammation may cause increased oxidative stress and DNA damage in patients with RA. To our knowledge, this is the first report which demonstrates the association between neutrophil DNA damage and burst oxidative and neutrophil DNA damage. In RA patients, circulating neutrophils have been found to be already pre-activated (primed) (30). Observations from human studies like, rheumatoid arthritis, vasculitis, chronic obstructive pulmonary disease and inflammatory bowel disease, neutrophils and their products of activation (*e.g.* proteases and ROS) in the tissue- and organ damage are associated (31) Neutrophils promotes the systemic and local (in the synovia) oxidative stress in patients with RA (32) presenting elevated intracellular ROS production at baseline (30) and this activation is associated with the presence of IgG containing immune complexes in SF from RA patients (31). The comparison between RA-derived blood and SF neutrophils and other cells derived from other forms of arthritis revealed that RA cells on average respond more potently, thus suggesting greater disease activity (30). Recently, neutrophils from the SF of RA were found to be activated and to produce ROS intracellularly in RA, probably as a result of active processing of endocytosed material (30).

This report shows that patients taking the treatment with TNF-α blocker present less DNA damage than the patients taking disease-modifying anti-rheumatic drugs (DMARDs). The drug methotrexate (DMARDs) is considered modifier better tolerated course of disease, having the capability of reducing signs and symptoms of RA activity and improving the functional status by blocking the progression of radiographic lesions (33).

Methotrexate is commonly the most used drug in the treatment of RA and other inflammatory diseases and its action is mainly related with the inhibition of the enzyme dihydrofolate reductase. However, the mechanism of MTX immunosuppressive low dose remains uncertain (33). Another proposed mechanism is the activation of apoptosis in the early S phase of the cell cycle in activated leukocytes, probably due to lack of timidinas bases, leading to breaks in the DNA chain produced by DNA polymerase (34), although this type of signaling apoptosis induced by changing the DNA remains uncertain (35). When comparing studied parameters in the RA patients according to methotrexate intake, the levels were significantly altered in those receiving methotrexate. Extensive ROS production can significantly accelerate the process of articular cartilage damage. It is believed that many (DMARDs) affect oxidative stress (36). Methotrexate used in RA exerts its anti-inflammatory and immunosuppressive effects most likely due to induction of apoptosis through oxidative stress (37).

Leflunomide (Arava™) is a novel immunoregulatory disease-modifying anti-rheumatic drug, structurally unrelated to other immunosuppressive agents (38). It iss a non-cytotoxic inhibitor of the proliferation of mitogen-stimulated T- and B-lymphocytes *in vitro*, it is effective in several rodent autoimmune disease models and prolongs tissue graft survival in animals. To date, there is convincing evidence that the repression of de novo pyrimidine synthesis by inhibition of dihydroorotate-dehydrogenase (DHODH), the fourth enzyme in the pathway, is the predominant target of the mode of action of leflunomide (39) possibly the depletion of pyrimidine bases leads to DNA damage evidenced by increased expression of p53 and p53-dependent G1 or S-phase checkpoints prevent cells from proliferating under adverse conditions (40).

Several studies have examined the consequence of blocking inflammation pathways such as those mediated by TNF-α. Current targeted biological therapies, including anti-TNF-α inhibitors result in greater disease improvement and prevention of joint erosion. TNF-α is a proinflammatory cytokine, activating the NFk-β pathway, leading to a downstream cascade of other proinflammatory cytokines (41) and promotes DNA damage (42).

The RA synovium is enriched with proliferation and rapid activation immune cells during inflammation causes them to undergo a metabolic switch in favour of glycolysis over oxidative phosphorylation. This metabolic shift occurs in many hypoxia-associated inflammatory conditions. The activation of the transcription factor hypoxia-inducible factor (HIF)-1α in response to low pO2 modulates the activity of a number of genes and, by inducing genes that encode glucose transporters and glycolytic enzymes, HIF can promote the production of glycolytic energy (43) the fact that the potential role of hypoxia in the inflamed joint is further supported by several *in vitro* studies in which hypoxia induces key angiogenic growth factors (vascular endothelial growth factor and angiopoietins), hypoxia is

an early event, or a possible trigger factor in the pathogenesis of inflammatory arthritis (44). Precedent studies showed that patients who respond to anti-TNFα therapy restores oxygen homeostasis, as the joint tissue becomes less inflamed a corresponding reduction in hypoxia is effected (44) reducing the frequency of mitochondrial synovial mutagenesis, the formation of the lipid derived from the aldehydes, oxidative damage after three months of therapy (45, 46).

To date, the strongest association has been reported with the HLA region, in particular, with HLA–DRB1 alleles that share a similar amino acid sequence. Numerous studies have pursued clarification of this relation; however, there is no definite consensus in literature (47). We demonstrated, for the first time, that patients who had one dose or two doses of the SE allele had higher DNA damage in neutrophils from the peripheral blood interest, this association did not appear to be dose-dependent, with a similar risk in those carrying a single allele versus those carrying 2 alleles. Previous studies of the association of the SE with RA severity have demonstrated inconsistent results. The most consistent finding across different ethnic groups is of an association with radiographic erosions (48). SE acts as an allele-specific ligand that inhibits an adenosine-mediated anti-oxidative pathway in opposite cells, increasing cell vulnerability to oxidative damage. Both adenosine and the cAMP-mediated pathway have been previously shown to reduce oxidative stresses. Adenosine is a known anti-inflammatory and cytoprotective molecule secreted by injured cells in order to protect the bystander tissue and has also been shown to inhibit phorbol myristate acetate-induced intracellular production of ROS (49).

Considering our findings, we can conclude that oxidative stress plays a key role in the etiopathogenesis of RA, inducing DNA damage in neutrophil patients, especially in times of exacerbation of disease activity. We have also demonstrated that DNA damage may vary with the medication used for treating and also the condition of patients having SE allele in single or double dose, increasing DNA damage.

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