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ORIGINAL ARTICLE

TNF-β +252 A>G (rs909253) polymorphism is independently associated with presence of autoantibodies in rheumatoid arthritis patients

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Abstract

The *TNF-β* +252 A>G (rs909253) polymorphism has been associated with a risk of development of rheumatoid arthritis (RA) and could influence plasma tumor necrosis factor alpha (TNF-α) levels. The aim of the present study was to evaluate the association between the *TNF-β* +252 A>G polymorphism with plasma TNF-α levels, the presence of autoantibodies, and the susceptibility for RA. This cross-sectional study included 261 patients with RA and 292 controls. The polymorphism was studied using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Soluble TNF-α and receptors swere measured by multiplex assay. Rheumatoid factor (RF) and anticyclic citrullinated peptide antibodies (anti-CCP) were measured using immunoassay. No differences were observed in allele frequency and genotype distribution among patients and controls. The presence of RF ($p=0.020$) and anti-CCP ($p=0.001$) increased 4.23-fold and 8.13-fold, respectively, in patients with B1 allele (B1/B2 + B1/B1 genotypes) independently of demographic, clinical, and inflammatory markers. Among patients with B1/B2 + B1/B1 genotypes, higher TNF-α levels were associated with positive RF ($p=0.040$), anti-CCP ($p=0.011$), or both ($p=0.038$). In patients carrying B1 allele, the increased sTNFR1 together with RF or anti-CCP or both explained about 39.0% the variations in TNF-α level. However, in B2/B2 genotype, the presence of those autoantibodies was not associated with TNF-α level. Our findings indicate that the *TNF-β* +252 A>G polymorphism was not associated with RA susceptibility and TNF-α plasma levels. However, B1 allele was associated with the presence of autoantibodies. In addition, interaction between the presence of B1 allele and autoantibodies was associated with the increase of plasma TNF-α level in RA patients.

Keywords Rheumatoid Arthritis · TNF-β +252 A>G polymorphism · rs909253 · Rheumatoid factor · Tumor necrosis factor alpha · Anticyclic citrullinated peptide antibodies

Abbreviations

ANOVA	Analysis of variance
anti-CCP	Anticyclic citrullinated peptide antibodies
CRP	C-reactive protein
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
hsCRP	High-sensitivity C-reactive protein
HLA	Human leukocyte antigen
IL	Interleukin
Ln	Natural logarithmic
LTA	Lymphotoxin A
n	Number
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor

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SEM	Standard of main
sTNFR-1	Soluble TNF receptor 1
sTNFR-2	Soluble TNF receptor 2
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TNF-α	Tumor necrosis factor-alpha
TNF-β	Tumor necrosis factor-β

Introduction

Rheumatoid arthritis (RA) is one of the most common inflammatory autoimmune diseases. It is characterized by persistent synovitis, systemic inflammation and production of autoantibodies [1]. Although the precise etiology has not been established yet, it is believed that the tendency to develop RA may be genetically inherited. Also, environmental factors, such as smoking and infections, may cause the malfunction of the immune system in genetically predisposed individuals [2, 3]. Genes within the human leukocyte antigen (HLA) region remain the most powerful disease risk genes in RA [4] and genetic variance within HLA contributes to almost 50% of the genetic susceptibility for RA [5].

Both rheumatoid factor (RF) and anticyclic citrullinated peptide antibodies (anti-CCP) are diagnostic biomarkers in RA, and concomitant evaluation of RF and anti-CCP represents the most powerful prognostic biomarker for RA [6, 7]. These autoantibodies have been shown to be associated with progression and joint destruction [8–10] and also associated with increased tumor necrosis factor-alpha (TNF-α) in in vitro [11, 12] and in vivo studies [13, 14]. TNF-α, which is the main mediator of inflammation in RA, is a potent pro-inflammatory cytokine upregulated in the joints and associated with the disease activity [13, 15–17].

Tumor necrosis factor-β (TNF-β), also known as lymphotoxin A, is a close homologue of TNF-α [18]. Both cytokines are recognized by the same widely distributed cellular TNF receptors, such as TNFR1 and TNFR2, and consequently they have many similar effects [16]. The TNF-α gene is located on chromosome 6, between HLA-B and HLA-DR genes, and polymorphisms in the *TNF-α* and *TNF-β* have been described to regulate its production [19–21]. A polymorphism at position +252 within the first intron of the *TNF-β* (rs909253) consisting of a guanine base characterizes the B1 allele, whereas an adenine base characterizes the B2 allele. The B1 allele is less frequently found than the B2 allele, and is associated with higher TNF-α and TNF-β production [20–22].

The *TNF-β* +252 A>G polymorphism has been associated with a risk of development of several autoimmune diseases, such as systemic lupus erythematosus [20, 23], multiple sclerosis [24], vitiligo [25], scleroderma [26], and primary Sjögren's syndrome [27]. The role of this polymorphism on the RA susceptibility is unclear [28–34], and TNF-α levels and its association with autoantibodies has been scarcely studied [28, 29]. Therefore, the aim of the present study was to evaluate the association between the *TNF-β* +252 A>G polymorphism with plasma TNF-α levels, the presence of autoantibodies, and the susceptibility for RA.

Subjects and methods

Subjects

This cross-sectional study included 261 patients with RA, and 297 healthy individuals from the same geographic area as a control group. The patients were recruited at the Rheumatology Ambulatory of the University Hospital of Londrina, Paraná, Brazil. The patients were classified according to the RA classification criteria [35]. Disease activity status was determined using Disease Activity Score in 28 joints based on C-reactive protein (DAS28-CRP) and erythrocyte sedimentation rate (ESR) (DAS28-ESR) [36]. Information about lifestyle and medical history were obtained at clinical evaluation. None of the subjects was receiving a specific diet. None of the participants in the study presented heart, renal, thyroid, hepatic, gastrointestinal, oncological or other autoimmune diseases, and none had a clinically evident infection or was receiving estrogen replacement therapy. Patients treated with TNF-inhibitors were excluded from analysis of TNF-α and its receptors.

Anthropometric measurements

Body mass index (BMI) was calculated as weight (kg) divided by height (cm) squared. The ethnicity was self-reported as Caucasian and non-Caucasian (Asiatic, Black, and Afro-Brazilian) [37]. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. The Ethical Committee of the University of Londrina, Paraná, Brazil approved all procedures involving human subjects. Written informed consent was obtained from all patients (CAAS: 06405812.1.0000.5231).

Biochemical and immunological biomarkers

Peripheral blood samples were collected with ethylenediaminetetraacetic acid (EDTA) as anti-coagulant, and without anticoagulant, to obtain the buffy coat and plasma, and serum, respectively. Samples were stored at − 80 °C freezer until used. White blood cell counts and ESR were determined using hematological autoanalyzer's. Serum levels of CRP were determined using high-sensitivity (hsCRP) and ferritin with chemiluminescence microparticle assay (Architect, Abbott Laboratory, Abbott Park, IL, USA). RF

titers were measured using a turbidimetric assay (C8000, ABBOTT, Architect Abbott Laboratories, Abbott Park, IL, USA) and the results were expressed as U/mL. Anti-CCP were assayed using the chemiluminescence microparticle immunoassay (Architect, Abbott Laboratory, Abbott Park, IL, USA), and the results were expressed as U/mL.

TNF-α, soluble TNFR-1 (sTNFR-1) and sTNFR-2 determination

Plasma TNF-α, sTNFR-1, and sTNFR-2 levels were determined by Human Magnetic 3-plex Custom kit, a TNF Superfamily Panel that detects these three molecules at the same time (Novex Life Technologies, Frederick, United States of America) for Luminex® platform.

TNF-β +252 A>G genetic polymorphism

The genomic DNA was extracted from the buffy coat using a commercial DNA extraction kit (Biometrix Diagnóstica, Curitiba, Brazil) according to the manufacturer's instructions. A 782 base-pair (bp) fragment of the *TNF-β* was amplified using polymerase chain reaction (PCR) as previously reported [38], and the primers used were determined according to the GenBank number X02911 as follow: primer sense, 5′ CCG TGC TTC GTG CTT TGG GAC TA 3′ and primer antisense, 5′ AGA GGG GTG GAT GCT TGG GTT TC 3′ (Invitrogen™, Life Technologies, Carlsbad, CA, USA). PCR conditions were performed in a thermocycler (PCR Sprint-Thermo Hybaid™, Biosystems, Barcelona, Spain) that comprised 5 min denaturation at 94 °C for initial denaturation; 37 cycles of 45 s at 94 °C for denaturation, 45 s at 67 °C for the annealing and 45 s at 72 °C for the elongation; and 10 min at 72 °C for final elongation. PCR products were completely digested with *Nco*I enzyme (Invitrogen, Life Technologies, Carlsbad, CA, USA) for 4 h at 37 °C. The *Nco*I genotypes were identified by restriction fragment length polymorphism analysis in a 3% agarose gel electrophoresis (70 V, 70 min) and stained with ethidium bromide. The B1 allele includes a restriction site for *Nco*I and results in 196- and 586-bp fragments after digestion, and the B2 allele (lacking the restriction site for *Nco*I) results in a fragment with 782 bp. The heterozygous genotype B1/B2 results three fragments (782, 586, and 196 bp).

Statistics

Analysis of contingency tables (χ^2 test) was employed to check the associations between categorical variables and diagnostic groups. The Kolmogorov–Smirnov test was used to assess normality of distribution. Logarithmic natural (Ln) transformation of continuous data was used in the analysis when the variables were not normally distributed or when there was heterogeneity of variance (as assessed with the Levene test. We assessed the differences in continuous variables between groups using analysis of variance (ANOVAs). Categorical variables were expressed as absolute number (*n*) and percentage (%) and continuous variables were expressed as mean ± standard error of mean (SEM). The association between inflammatory markers and RA was evaluated using binary logistic regression analysis controlled for covariates that may confound the association of interest. Automatic stepwise binary logistic regressions analyze were employed to delineate the most significant variables that are associated with positive RF and anti-CCP. To delineate the predictors of TNF-α levels, we carried out 3 different automatic stepwise linear regression (1# positive RF, 2# positive anti-CCP and 3# positive RF/anti-CCP) according *TNF-β* +252 A>G polymorphism. All statistical analyses were performed using IBM SPSS windows version 24. Tests were 2-tailed and an alpha level of 0.05 indicated statistically significant results.

Results

Table 1 shows the characteristics of patients with RA versus controls. Patients with RA were older ($p = 0.008$), had higher BMI ($p < 0.001$) and higher frequency of smoking ($p = 0.002$) than controls. Therefore, we have adjusted the results for possible effects of age, BMI, and smoking by entering the variables as additional factors or covariates in multivariate analyses. There were no significant differences in sex ($p = 0.101$) and ethnicity ($p = 0.112$) between both study groups. The outcome of ANOVAs performed on the different inflammatory markers showed that RA patients presented higher ESR ($p < 0.001$), hsCRP ($p < 0.001$), ferritin ($p = 0.019$), and TNF-α levels ($p = 0.010$) than controls. After correction, ESR, hsCRP, and TNF-α remained significantly increased in RA patients ($p < 0.001$).

The genotype distribution of *TNF-β* +252 A>G polymorphism in controls and patients were consistent with those expected from the Hardy–Weinberg equilibrium ($p > 0.05$). In RA patients, we identified 42.1% B2/B2 homozygotes, 47.9% B1/B2 heterozygotes, and 10.0% B1/B1 homozygotes, resulting in allele frequencies of 66.0% and 34.0% for B2 and B1 alleles, respectively. No differences were observed in allele frequency and genotype distribution when evaluated in an additive or in a dominant model, among patients and controls before and after correction by age, BMI and smoking ($p > 0.05$). The dominant model allows to examine the association between B1 allele and biomarkers; therefore, this model was used for sequential analysis (B2/B2 vs. B1/B2 + B1/B1) (Table 1).

Table 2 shows the characteristics of RA patients according the *TNF-β* +252 A>G polymorphism. Both groups did not differ in relation to sex ($p = 0.433$), ethnicity ($p = 0.123$),

Table 1 Socio-demographic variables and genotype frequencies of *TNF-β* +252 A>G polymorphism in patients with rheumatoid arthritis (RA) and controls

	Control ($n=292$)	RA ($n=261$)	*df*	F/X^2	*p*	p^a
Age (years)*	51.66 (1.02)	55.07 (0.72)	1/551	7.084	0.008	–
Sex (F/M)	229/63	219/42	1	2.694	0.101	–
Ethnicity (C/NC)	224/68	185/ 76	1	2.661	0.109	–
BMI (kg/m^2)	26.24 (0.28)	28.06 (0.36)	1/530	15.89	<0.001	–
Smoking (%)	19 (7.0)	41 (15.8)	1	10.060	0.002	–
Leuko (cells/uL)	6726 (197)	7641 (552)	1/364	1.104	0.294	0.333
ESR (mm/H)*	12.44 (0.81)	23.65 (20.21)	1/380	34.925	<0.001	<0.001
hsCRP (mg/L)*	3.12 (0.26)	9.83 (0.96)	1/540	48.505	<0.001	<0.001
Ferritin (ng/L)*	129.50 (8.36)	159.70 (9.75)	1/537	5.573	0.019	0.266
TNF-α (pg/mL)*	11.34 (5.35)	58.57 (12.45)	1/154	6.850	0.010	<0.001
TNF-β genotypings						
B1 Alelle (%)	201 (34.4)	177 (34.0)	1/1105	0.031	0.858	0.839
B2 Alelle (%)	383 (65.6)	345 (66.0)				
B1/B1 (%)	31 (10.6)	26 (10.0)	2	0.064	0.968	0.861
B1/B2 (%)	139 (47.6)	125 (47.9)				
B2/B2 (%)	122 (41.8)	110 (42.1)				
Dominant model						
B2/B2 (%)	122 (41.8)	110 (42.1)	1	0.008	0.931	0.824
B1/B2 + B1/B1 (%)	170 (58.2)	151 (57.9)				

Data were expressed as absolute number (*n*) and percentage (%) or mean and standard error of the mean (SEM)

[a]Adjusted for age, BMI and smoking

BMI body mass index, *Leuko* leukocytes, *ESR* erythrocyte sedimentation rate, *hsCRP* high sensitive C reactive protein, *TNF-α* tumor necrosis factor α, *TNF-β* tumor necrosis factor β, B1/B1 homozygous genotype for the allele B1 (with guanine at position +252 in the first intron of the TNF-β gene); B2/B2 homozygous genotype for the allele B2 with adenine at position +252 in the first intron of the TNF-β gene; B1/B2 heterozygous genotype

*Data were processed in natural logarithm (Ln) transformation

age ($p=0.254$), BMI ($p=0.891$), smoking ($p=0.450$), disease duration ($p=0.785$), and therapy ($p>0.05$). In univariate analyses, genotypes evaluated by dominant model were not associated with disease activity evaluated by DAS28-ESR ($p=0.759$) or DAS28-CRP ($p=0.922$), and inflammatory markers such as leukocytes ($p=0.358$), ESR ($p=0.619$), hsCRP ($p=0.754$), ferritin ($p=0.295$), TNF-α ($p=0.222$), sTNFR1 ($p=0.632$), and sTNFR2 ($p=0.783$) in patients with RA ($p>0.05$). However, patients carrying B1 allele presented higher RF levels ($p=0.020$) and higher frequency of anti-CCP positivity ($p=0.042$).

In order to examine the positivity of RF and anti-CCP we have carried out 2 automatic binary logistic regressions analysis (forward stepwise) with the presence of autoantibodies as dependent variables and *TNF-β* polymorphism, demographic (e.g. age and sex), clinical parameters (smoking, BMI, therapy, DAS28), and inflammatory markers (leukocytes, ESR, hsCRP, ferritin, TNF-α, sTNFR1, TNFR2) as explanatory variables (Table 3). In the first regression we analyzed the presence of RF, and showed that smoking ($p=0.025$, OR 4.26), positive anti-CCP ($p<0.001$, OR 14.30), and *TNF-β* allele B1 ($p=0.020$, OR 4.23) were associated with RF ($p<0.001$, Nagelkerke = 0.385; 75.0% of all cases were correctly classified with a sensitivity of 64.4% and a specificity of 84.3%); when *TNF-β* allele B1 was excluded from the model, 71.9% of all cases were correctly classified with a sensitivity of 71.1% and a specificity of 72.5%. In the second regression, we analyzed the presence of anti-CCP, and showed that positive RF ($p<0.001$, OR 12.97) and *TNF-β* allele B1 ($p=0.001$, OR 8.13) were associated with that autoantibody ($p<0.001$, Nagelkerke = 0.397; 71.9% of all cases were correctly classified with a sensitivity of 47.8% and a specificity of 94.0%); when *TNF-β* allele B1 was excluded from the model, 71.9% of all cases were correctly classified with a sensitivity of 69.6% and a specificity of 74.0%.

TNF-α plasma levels were analyzed in patients according to the presence of autoantibodies and *TNF-β* +252 A>G polymorphism (Fig. 1). Patients with a positive RF (A) or anti-CCP (B) or both test (C) and B1/B2 + B1/B1 genotypes showed higher plasma TNF-α levels ($p=0.040$, $p=0.011$, $p=0.038$, respectively) than patients with the absence of RF or anti-CCP. On the other hand, there were no differences in TNF-α levels according autoantibody positivity in patients

Table 2 Demographic, clinical, and inflammatory markers according to *TNF-β* +252 A>G polymorphism in rheumatoid arthritis (RA) patients

	B2/B2 ($n=110$)	B1/B2 + B1/B1 ($n=151$)	*df*	F/X^2	*p*
Age (years)	56.12 (0.97)	54.45 (1.03)	1/258	1.306	0.254
Sex (F/M)	90/20	129/22	1	0.615	0.433
Ethnicity (C/NC)	80/30	105/46	1	0.313	0.575
BMI (kg/m^2)	28.12 (0.53)	28.02 (0.49)	1/254	0.019	0.891
Smoking (%)	26 (17.2)	15 (13.8)	1	0.570	0.450
Disease duration (years)*	12.32 (0.96)	12.06 (0.81)	1/256	0.075	0.785
DAS 28 ESR	3.72 (0.13)	3.77 (0.12)	1/253	0.094	0.759
DAS 28 CRP*	3.38 (0.13)	3.40 (0.12)	1/253	0.010	0.922
Anti-CCP (positive)	83 (55.0)	71 (67.6)	1	4.136	0.042
Anti-CCP (U/mL)*	161.66 (29.20)	197.47 (55.46)	1/256	1.788	0.182
RF (positive)	53 (49.1)	80 (53.0)	1	0.385	0.535
RF (U/mL)*	75.67 (12.08)	149.88 (24.15)	1/257	5.466	0.020
Leuko (cells/mm^3)	7389 (276)	6956 (186)	1/257	0.848	0.358
ESR (mm/H)*	22.76 (1.90)	24.29 (1.68)	1/255	0.248	0.619
hsCRP (mg/L)*	10.94 (1.72)	9.01 (1.08)	1/258	0.098	0.754
Ferritin (ng/mL)*	168.32 (14.57)	153.43 (13.12)	1/257	1.101	0.295
TNF-α (pg/mL)*	52.54 (8.90)	62.19 (19.25)	1/102	1.510	0.222
sTNFR1 (pg/mL)*	1159.86 (82.19)	1179 (1069.76)	1/103	0.034	0.632
sTNFR2 (pg/mL)	1368 (130.17)	1314 (90.20)	1/103	0.076	0.783
Treatment					
Prednisone (%)	77 (70.0)	102 (67.5)	1	0.177	0.647
Metrotexate (%)	72 (65.5)	102 (67.5)	1	0.126	0.723
TNF-inhibitor (%)	27 (24.5)	37 (24.5)	1	0.000	0.994
Leflunomide (%)	46 (41.8)	61 (40.4)	1	0.053	0.818
Antimalarials (%)	37 (33.6)	62 (41.1)	1	1.490	0.222

Data were expressed as absolute number (*n*) and percentage (%) or mean and standard error of the mean (±SEM)

TNF-β tumor necrosis factor β; B1/B1 homozygous genotype for the allele B1 (with guanine at position +252 in the first intron of the TNF-β gene); B2/B2 homozygous genotype for the allele B2 with adenine at position +252 in the first intron of the TNF-β gene; B1/B2 heterozygous genotype; *BMI* body mass index, *Leuko* leukocytes, *RF* rheumatoid factor, *anti-CCP* anti-cyclic citrullinated peptide, *ESR* erythrocyte sedimentation rate, *hsCRP* high sensitive C reactive protein, *TNF-α* tumor necrosis factor α, *sTNFR1* tumor necrosis factor receptor 1, *sTNFR2* tumor necrosis factor receptor 2

*Data were processed in natural logarithm (Ln) transformation

Table 3 Results of binary logistic regressions analyses (forward stepwise) with rheumatoid factor (RF) and/or anti-cyclic citrullinated peptide (Anti-CCP) as dependent variable and demographic, clinical, inflammatory, and *TNF-β* +252 A>G polymorphism as explanatory variables

Dependent variables	Explanatory variables	SEM	Wald	*p*	OR (95% CI)
RF (+)	Smoking	0.648	5.003	0.025	4.263 (1.196–15.187)
	Anti-CCP (+)	0.619	18.467	<0.001	14.305 (4.251–40.136)
	TNF-β +252 G>A	0.621	5.401	0.020	4.231 (1.254–14.279)
Anti-CCP (+)	RF (+)	0.597	18.426	<0.001	12.974 (4.026–41.814)
	TNF-β +252 G>A	0.616	11.570	0.001	8.139 (2.432–27.245)

SEM standard error of the mean, *OR* odds ratio, *CI* confidence interval, *TNF-β* tumor necrosis factor β, B1/B1 homozygous genotype for the allele B1 (with guanine at position +252 in the first intron of the TNF-β gene); B2/B2 homozygous genotype for the allele B2 with adenine at position +252 in the first intron of the TNF-β gene; B1/B2 heterozygous genotype; *RF* rheumatoid factor, *anti-CCP* anti-cyclic citrullinated peptide, *TNF-α* tumor necrosis factor α

with B2/B2 genotype ($p>0.05$). sTNFR1 and sTNFR2 levels were not associated with RF (A) anti-CCP (B), and positive RF/anti-CCP (C) in both genotypes ($p>0,05$). The p value was adjusted for sex, age, BMI, and smoking.

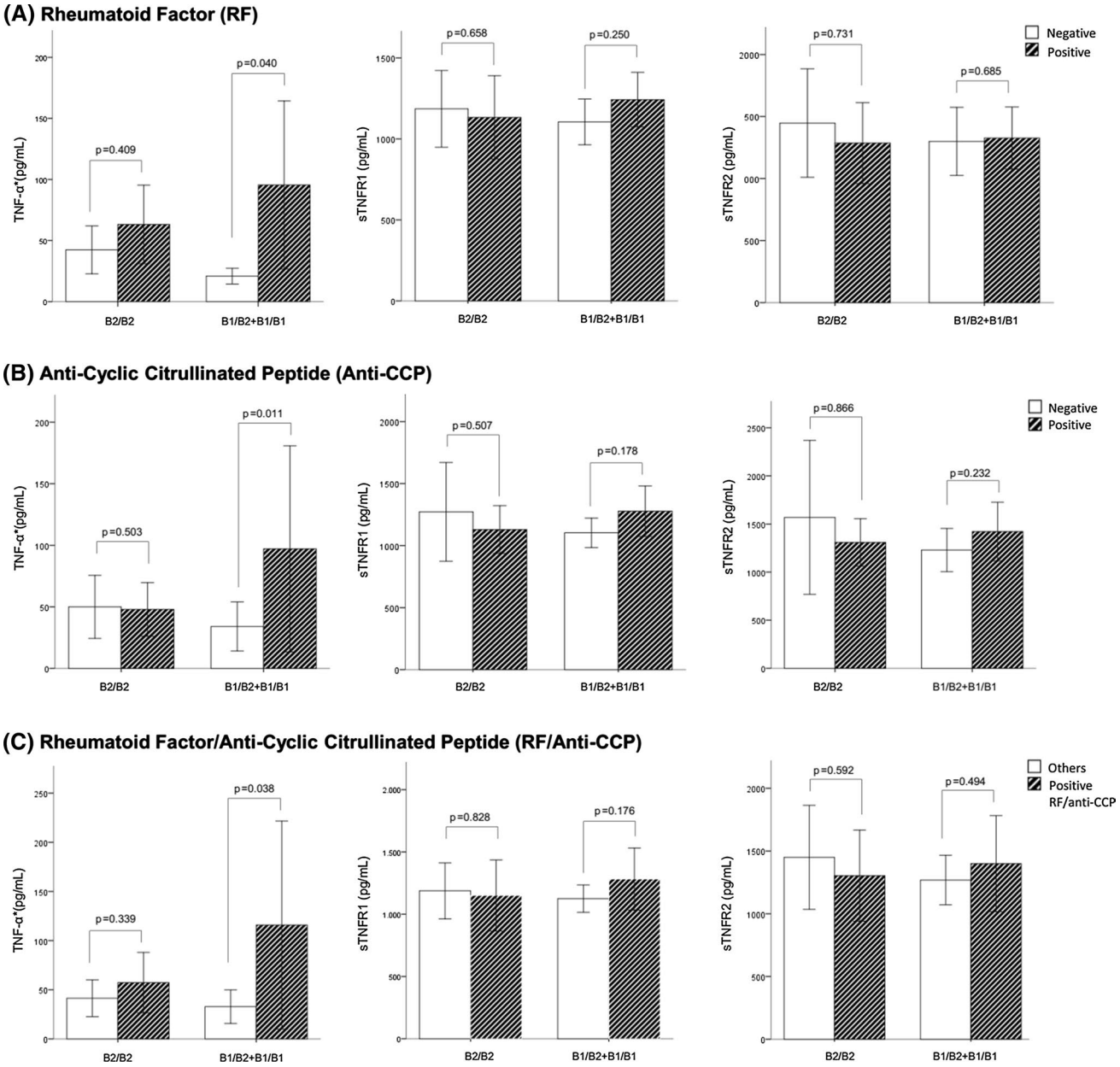

Fig. 1 Tumor necrosis factor-alpha (TNF-α), soluble receptor 1 (sTNFR1), and sTNFR2 plasma levels according *TNF-β* +252 A>G polymorphism and the presence of autoantibodies. Data expressed in mean and 95% confidence interval

Furthermore, to delineate the effect of the autoantibodies and the most important variables that affect TNF-α plasma levels according to genotype, we have carried out three automatic stepwise linear regression analyses with TNF-α as dependent variables and the demographic (e.g. age and sex), clinical (BMI, extra articular manifestation, DAS28), and inflammatory markers data as explanatory variables (Table 4). We added positive RF (#1), anti-CCP (#2) and RF/anti-CCP (#3) in explanatory variables in three different models. Among B1/B2 + B1/B1 patients, increased sTNFR1 together with positive RF, positive anti-CCP or both explained about 39.0% of the variations in TNF-α levels (37.0%, $p = 0.033$; 39.7%, $p = 0.009$; and 42.8%, $p = 0.002$, respectively). Demographic and clinical data did not contribute to TNF-α level in these models. Although in patients with B2/B2 genotype the presence of autoantibodies did not contribute to plasma TNF-α level, DAS28 was associated with this cytokine (data not shown).

Table 4 Results of stepwise linear regression analyses with TNF-α as dependent variable and presence of rheumatoid factor and/or anti-cyclic citrullinated peptide (anti-CCP) and demographic, clinical, and inflammatory data as explanatory variables according *TNF-β* +252 A>G polymorphism

	Genotyping	Dependent	Explanatory variables			*F*	*df*	*p*	R^2 (%)
			Variables	*T*	*p*				
#1	B2/B2	TNF-α*	DAS28 CRP*	2.632	0.040	5.729	1/35	0.007	20.4
	B1/B2 + B1/B1	TNF-α*	sTNFR1*	5.38	< 0.001	18.19	2/56	< 0.001	37.2
			RF (+)	2.186	0.033				
#2	B2/B2	TNF-α*	DAS28 CRP*	3.260	0.002	10.657	1/35	0.002	21.2
	B1/B2 + B1/B1	TNF-α*	sTNFR1*	5.309	< 0.001	29.642	2/55	< 0.001	39.7
			Anti-CCP (+)	2.705	0.009				
#3	B2/B2	TNF-α*	DAS28 CRP*	3.265	0.002	10.657	1/35	0.002	21.2
	B1/B2 + B1/B1	TNF-α*	sTNFR1*	5.609	< 0.001	22.699	2/55	< 0.001	42.8
			RF (+) Anti-CCP (+)	3.272	0.002				

TNF-β tumor necrosis factor β; B1/B1 homozygous genotype for the allele B1 (with guanine at position +252 in the first intron of the TNF-β gene); B2/B2 homozygous genotype for the allele B2 with adenine at position +252 in the first intron of the TNF-β gene; B1/B2 heterozygous genotype; *TNFα* tumor necrosis factor α, *RF* rheumatoid factor, *anti-CCP* anti-cyclic citrullinated peptide, *sTNFR1* tumor necrosis factor receptor 1

*Data are processed in Ln transformation

#1 Positive FR. #2 Positive anti-CCP. #3 Positive FR and anti-CCP

Discussion

The present study evaluated the association between the *TNF-β* +252 A>G polymorphism with plasma TNF-α levels, the presence of autoantibodies, and the susceptibility for RA. Our data demonstrated that the B1 allele, in heterozygosis or in homozygosis, of *TNF-β* +252 A>G polymorphism was not associated with RA susceptibility and plasma TNF-α levels. However, patients with B1 allele showed a 4.23 and 8.13-fold increase in the presence of RF and anti-CCP, respectively, independently of demographic, clinical, and inflammatory markers. Moreover, only patients carrying B1 allele with positive RF and/or positive anti-CCP showed higher plasma TNF-α level.

To our knowledge, this is the first study to investigate the association between the *TNF-β* +252 A>G polymorphism with RA susceptibility in Brazilian populations. The results of the association between RA susceptibility and *TNF-β* +252 A>G have been conflicting in different populations. The absence of association of this single nucleotide polymorphism (SNP) with RA susceptibility obtained in the present study is in agreement with studies carried out in Belgian [33] and Spanish populations [32]. In addition, a meta-analysis performed by Zhang et al. [39] showed that *TNF-β* +252 A>G polymorphism was not significantly associated with RA isn allele, dominant, recessive, and additive models. Similarly, stratification by ethnicity verified no association between this polymorphism and RA under all models in Caucasians and non-Caucasians. However, other studies showed positive association between B1 allele and RA susceptibility [29, 30]. On the other hand, the B2 allele was associated with RA susceptibility in Portuguese [22], Egyptian [31], and Japanese populations [34]. This discrepancy could be explained by different sample sizes and genetic differences among populations.

Both autoantibodies, RF and anti-CCP, are regarded as serological markers of RA and the specificity for RA can be further increased by combining the presence of these two antibodies [6, 7]. Furthermore, RF and anti-CCP can be found very early, and even may precede clinical symptoms of RA by years. In addition, RF and anti-CCP are biomarkers of worse prognosis and are important to define treatment [40]. In the present study, we demonstrated that B1 allele (B1/B2 + B1/B1 genotypes) contributes to the presence of autoantibodies in RA patients, independently of demographic, clinical, and inflammatory markers. Previously, only two studies evaluated the association between *TNF-β* +252 A>G polymorphism with RF and anti-CCP positivity in RA patients, and reported that B1 allele or B1/B1 genotype were not associated with these autoantibodies [28, 29]. No study has reported association between *TNF-β* +252 A>G polymorphism and autoantibodies in autoimmune diseases [20, 23]. In addition, our data demonstrated that in models performed to predict RF and anti-CCP positivity, the presence of B1 allele improved the specificity in 11.8% and 20.0%, respectively. Interestingly, B1 allele and smoking present similar OR with the presence of RF (4.23 and 4.26, respectively). Cigarettes are known as an important environmental factor associated with an enhanced frequency and incidence of RA [41].

Autoantibodies have been suggested to increase disease activity due to the immune complex formation and subsequent increases in production of pro-inflammatory cytokines [11, 12]. Several studies have shown that positive RF is

associated with more aggressive forms of the disease, presumably because RF has direct effects on osteoclast genesis and chondrocyte activation [8, 42], whereas the presence of anti-CCP is an independent predictor of radiological damage and progression [9, 10].

Plasma TNF-α levels changes according to the *TNF-β* +252 A>G polymorphism were not found in the present study. However, only patients carrying B1 allele (B1/B2 + B1/B1 genotypes) and with RF and/or anti-CCP presented higher TNF-α levels than patients with negative autoantibodies. Meanwhile, in patients with the B2 allele the positivity of these autoantibodies did not alter plasma TNF-α level. In a small Egyptian cohort, RA patients with B1/B1 genotype presented higher TNF-α levels compared to B2/B2 genotype, but this result may be analyzed with caution, because the genotypic frequencies were not in Hardy–Weinberg equilibrium, and the B1/B1 genotype was obtained in only six patients [43]. In addition, a previous study showed that B1/B1 genotype was associated with higher CRP levels, but not with TNF-α, sTNFR1, or interleukin (IL)-6 levels in RA patients [22].

This is the first study to show that the interaction between *TNF-β* +252 A>G polymorphism and RF and/or anti-CCP may be modulated by plasma TNF-α levels, independently of disease activity. Furthermore, we verified by automatic linear regression the most important variables which affect this cytokine, and only in patients carrying B1 allele, the autoantibodies contributed to TNF-α plasma levels. Among B1/B2 + B1/B1 patients, increased sTNFR1 together with positive RF and/or anti-CCP explained about 39.0% the variations in TNF-α levels (37.0%, 39.7% and 42.8% in relation to RF, anti-CCP, and RF/anti-CCP models, respectively). This might suggest the possible association of the given polymorphism with these antibodies. Altogether, the results of the current study allow suggesting that the presence of B1 allele with the concomitant production of RF and anti-CCP may propitiate a microenvironment which favors TNF-α production.

Patients with high levels of anti-CCP have high levels of proinflammatory molecules, such as TNF-α and IL-6 [44]. In addition, the concomitant presence of RF and anti-CCP was associated with more severe erosive bone damage [45], increased disease activity and higher TNF-α, IL-6, IL-1β, IL-12 and IL-17 levels [13]. Recently, Takeuchi et al. [14] showed that TNF-α level was the only baseline characteristic that positively correlated with both RF and anti-CCP.

The findings of the present study should be interpreted in the context of its limitations. First, the ethnicity of the subjects was self-reported. Second, the study design was based on a single basal TNF-α and receptors measurement, and a specific SNP determination, which precludes the assessment of how other factors may impact on the complex relationship between TNF-β genotype, TNF-α and receptors plasma levels, and their interaction with RA and autoantibodies. Third, the study does not allow causal relationships to be inferred. However, the present study also has several strengths. This study combines a multivariate statistical approach, allowing controlling for many possible sources of nuisance, with inclusion of a larger number of participants and controls, which were recruited in the same catchment area.

In conclusion, our findings indicate that the *TNF-β* +252 A>G polymorphism was not associated with RA susceptibility and plasma TNF-α levels in Brazilian patients. However, allele B1 was associated with the presence of autoantibodies and the progression of disease. In addition, this is the first study to show that the interaction among the presence of B1 allele and autoantibodies was associated with TNF-α increased plasma levels in RA patients.

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Author's contribution FAM, DFA, MABL, ERDA, TF, and NLM performed the laboratory analysis; TMVI and NTC: enhanced patient care; DFA and ANCS: performed the statistical analysis; FAM, DFA, ER, MABL, ERDA and ANCS: did the study design, discussed and interpreted the results obtained the results; ID and ANCS: they wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent All the participants included in this study provided written informed consent.

References

1. Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. Lancet. 2010;376:1094–108.
2. Isaacs JD. The changing face of rheumatoid arthritis: sustained remission for all? Nat Rev Immunol. 2010;10:605–11.
3. Li S, Yu Y, Yue Y, Zhang Z, Su K. Microbial infection and rheumatoid arthritis. J Clin Cell Immunol. 2013;4:174.
4. Weyand CM, Goronzy JJ. Association of MHC and rheumatoid arthritis: HLA polymorphisms in phenotypic variants of rheumatoid arthritis. Arthritis Res. 2000;2:212.
5. Saad MN, Mabrouk MS, Eldeib AM, Shaker OG. Identification of rheumatoid arthritis biomarkers based on single nucleotide

polymorphisms and haplotype blocks: a systematic review and meta-analysis. J Adv Res. 2016;7:1–16.
6. Sun J, Zhang Y, Liu L, Liu G. Diagnostic accuracy of combined tests of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis: a meta-analysis. Clin Exp Rheumatol. 2014;32:11–21.
7. Nishimura K, Sugiyama D, Kogata Y, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med. 2007;146:797–808.
8. Aletaha D, Alasti F, Smolen JS. Rheumatoid factor determines structural progression of rheumatoid arthritis dependent and independent of disease activity. Ann Rheum Dis. 2013;72:875–80.
9. Forslind K, Ahlmén M, Eberhardt K, Hafström I, Svensson B. Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP). Ann Rheum Dis. 2004;63:1090–5.
10. Rönnelid J, Wick MC, Lampa J, et al. Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression. Ann Rheum Dis. 2005;64:1744–9.
11. Laurent L, Anquetil F, Clavel C, et al. IgM rheumatoid factor amplifies the inflammatory response of macrophages induced by the rheumatoid arthritis-specific immune complexes containing anticitrullinated protein antibodies. Ann Rheum Dis. 2015;74:1425–31.
12. Clavel C, Nogueira L, Laurent L, et al. Induction of macrophage secretion of tumor necrosis factor α through Fcγ receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen. Arthritis Rheum. 2008;58:678–88.
13. Sokolove J, Johnson DS, Lahey LJ, et al. Rheumatoid factor as a potentiator of anti-citrullinated protein antibody-mediated inflammation in rheumatoid arthritis. Arthritis Rheumatol. 2014;66:813–21.
14. Takeuchi T, Miyasaka N, Inui T, et al. High titers of both rheumatoid factor and anti-CCP antibodies at baseline in patients with rheumatoid arthritis are associated with increased circulating baseline TNF level, low drug levels, and reduced clinical responses: a post hoc analysis of the RI. Arthritis Res Ther. 2017;9(1):1–11.
15. Matsuno H, Yudoh K, Katayama R, et al. The role of TNF-alpha in the pathogenesis of inflammation and joint destruction in rheumatoid arthritis (RA): a study using a human RA/SCID mouse chimera. Rheumatology (Oxford). 2002;41:329–37.
16. Croft M, Siegel RM. Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases. Nat Rev Rheumatol. 2017;13:217–33.
17. Petrovic-Rackov L, Pejnovic N. Clinical significance of IL-18, IL-15, IL-12 and TNF-measurement in rheumatoid arthritis. Clin Rheumatol. 2006;25:448–52.
18. Posch PE, Cruz I, Bradshaw D, Medhekar BA. Novel polymorphisms and the definition of promoter "alleles" of the tumor necrosis factor and lymphotoxin α loci: inclusion in HLA haplotypes. Genes Immun. 2003;4:547–58.
19. El-Tahan RR, Ghoneim AM, El-Mashad N. TNF-α gene polymorphisms and expression. Springerplus. 2016;5:1508.
20. Umare VD, Pradhan VD, Rajadhyaksha AG, Patwardhan MM, Ghosh K, Nadkarni AH. Impact of TNF-α and LTα gene polymorphisms on genetic susceptibility in Indian SLE patients. Hum Immunol. 2017;78:201–8.
21. Messer G, Spengler U, Jung MC, et al. Polymorphic structure of the tumor necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human TNF-beta gene correlates with a variant amino acid in position 26 and a reduced level of TNF-beta production. J Exp Med. 1991;173:209–19.
22. Santos MJ, Fernandes D, Caetano-Lopes J, et al. Lymphotoxin-α 252 G%3eA polymorphism: a link between disease susceptibility and dyslipidemia in rheumatoid arthritis? J Rheumatol. 2011;38:1244–9.
23. Parks CG, Pandey JP, Dooley MA, et al. Genetic polymorphisms in tumor necrosis factor (TNF)-α and TNF-β in a population-based study of systemic lupus erythematosus: associations and interaction with the interleukin-1α-889 C/T polymorphism. Hum Immunol. 2004;65:622–31.
24. Kallaur AP, Oliveira SR, Simão ANC, et al. Tumor necrosis factor beta (TNF-β) NcoI polymorphism is associated with multiple sclerosis in Caucasian patients from Southern Brazil independently from HLA-DRB1. J Mol Neurosci. 2014;53:211–21.
25. Laddha NC, Dwivedi M, Gani AR, Mansuri MS, Begum R. Tumor necrosis factor B (TNFB) genetic variants and its increased expression are associated with vitiligo susceptibility. PLoS ONE. 2013;8:e81736.
26. Pandey JP, Takeuchi F. TNF-α and TNF-β gene polymorphisms in systemic sclerosis. Hum Immunol. 1999;60:1128–30.
27. Bolstad AI, Le Hellard S, Kristjansdottir G, et al. Association between genetic variants in the tumour necrosis factor/lymphotoxin α/lymphotoxin β locus and primary Sjögren's syndrome in Scandinavian samples. Ann Rheum Dis. 2012;71:981–8.
28. Al-Rayes H, Al-Swailem R, Albelawi M, Arfin M, Al-Asmari A, Tariq M. TNF-α and TNF-β gene polymorphism in Saudi rheumatoid arthritis patients. Clin Med Insights Arthritis Musculoskelet Disord. 2011;4:55–63.
29. Panoulas VF, Nikas SN, Smith JP, et al. Lymphotoxin 252A%3eG polymorphism is common and associates with myocardial infarction in patients with rheumatoid arthritis. Ann Rheum Dis. 2008;67:1550–6.
30. Karray EF, Bendhifallah I, Benabdelghani K, Hamzaoui K, Zakraoui L. Tumor necrosis factor gene polymorphisms and susceptibility to rheumatoid arthritis in regional Tunisian population. J Infect Dis Immun. 2011;3:30–5.
31. Saad MN, Mabrouk MS, Eldeib AM, Shaker OG. Genetic case-control study for eight polymorphisms associated with rheumatoid arthritis. PLoS ONE. 2015;10:1–15.
32. Vinasco J, Beraún Y, Nieto A, et al. Polymorphism at the TNF loci in rheumatoid arthritis. Tissue Antigens. 1997;49:74–8.
33. Vandevyver C, Raus P, Stinissen P, Philippaerts L, Cassiman JJ, Raus J. Polymorphism of the tumour necrosis factor beta gene in multiple sclerosis and rheumatoid arthritis. Eur J Immunogenet. 1994;21:377–82.
34. Takeuchi F, Nabeta H, Hong GH, et al. The genetic contribution of the TNFa11 microsatellite allele and the TNFb + 252*2 allele in Japanese RA. Clin Exp Rheumatol. 2005;23:494–8.
35. Aletaha D, Neogi T, Silman AJ, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis. 2010;69:1580–8.
36. Prevoo MLL, Van'T Hof MA, Kuper HH, Van Leeuwen MA, Van De Putte LBA, Van Riel PLCM. Modified disease activity scores that include twenty-eight-joint counts development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 1995;38:44–8.
37. IBGE. Characteristics of the population and households: results of the universe. Charact. Popul. Households Results Universe. 2011. https://www.ibge.gov.br/english/estatistica/populacao/censo2010/caracteristicas%7B_%7Dda%7B_%7Dpopulacao/

default%7B_%7Dcaracteristicas%7B_%7Dda%7B_%7Dpopulacao.shtm. Accessed 8 Feb 2015.
38. Delongui F, Grion CMC, Watanabe MAE, et al. Association of tumor necrosis factor β genetic polymorphism and sepsis susceptibility. Exp Ther Med. 2011;2:349–56.
39. Zhang C, Zhao MQ, Liu J, et al. Association of lymphotoxin alpha polymorphism with systemic lupus erythematosus and rheumatoid arthritis: a meta-analysis. Int J Rheum Dis. 2015;18:398–407.
40. Rantapää-Dahlqvist S, De Jong BAW, Berglin E, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48:2741–9.
41. Chang K, Yang SM, Kim SH, Han KH, Park SJ, Shin JII. Smoking and rheumatoid arthritis. Int J Mol Sci. 2014;15:22279–95.
42. Redlich K, Smolen JS. Inflammatory bone loss: pathogenesis and therapeutic intervention. Nat Rev Drug Discov. 2012;11:234–50.
43. Shaker OG, Alnoury AM, Hegazy GA, El Haddad HE, Sayed S, Hamdy A. Redutase, fator de crescimento transformador B1 E linfotoxina-A E susceptibilidade À artrite reumatoide. Rev Bras Reumatol. 2016;56:414–20.
44. Vázquez-Del Mercado M, Nuñez-Atahualpa L, Figueroa-Sánchez M, et al. Serum levels of anticyclic citrullinated peptide antibodies, interleukin-6, tumor necrosis factor-α, and C-reactive protein are associated with increased carotid intima-media thickness: a cross-sectional analysis of a cohort of rheumatoid arthritis patients without cardiovascular risk factors. Biomed Res Int. 2015;2015:1–10.
45. Hecht C, Englbrecht M, Rech J, et al. Additive effect of anticitrullinated protein antibodies and rheumatoid factor on bone erosions in patients with RA. Ann Rheum Dis. 2015;74:2151–6.