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ORIGINAL ARTICLE

Differential distribution of vitamin D receptor (*VDR*) gene variants and its expression in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disorder that displays an important genetic background. Vitamin D_3 (VD_3) through its receptor (VDR) plays an important immunomodulatory role in autoimmune misbalance, being capable of modulating immune responses. Genetic alterations in *VDR* gene may contribute to an altered risk in SLE development and clinical manifestations. We investigated *VDR* SNPs (single nucleotide polymorphisms) frequencies in 128 SLE patients and 138 healthy controls (HC) and mRNA differential expression in 29 patients and 17 HC regarding SLE susceptibility as well as clinical features. We observed that rs11168268 G allele (OR = 1.55, $p = .01$) and G/G genotype (OR = 2.69, $p = .008$) were associated with increased SLE susceptibility. The rs2248098 G allele and A/G and G/G genotypes were associated to lower SLE susceptibility (OR = 0.66, $p = .01$; OR = 0.46, $p = .01$; OR = 0.44, $p = .02$, respectively). Regarding clinical features, we observed lower risk for: rs11168268 A/G genotype and nephritis (OR = 0.31, $p = .01$); rs4760648 T/T genotype and photosensitivity (OR = 0.24, $p = .02$); rs1540339 T/T genotype and antibody anti-dsDNA (OR = 0.19, $p = .015$); rs3890733 T/T genotype and serositis (OR = 0.10, $p = .01$). We identified a significant downregulation in *VDR* expression levels when compared patients and controls overall ($p = 1.04e^{-7}$), in *Cdx-2* A/G and G/G ($p = .008$ and $p = .014$, respectively) and in patients with nephritis ($p = .016$)

Our results suggested that *VDR* SNPs influence upon SLE susceptibility and in particular clinical features, acting on mRNA expression in SLE patients overall and the ones with nephritis.

KEYWORDS
genetic variants, nephritis, SLE, VDR

1 | INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder featured by different clinical manifestations (Mok & Lau, 2003). Indeed, SLE clinical heterogeneity led to the establishment of 11 criteria by the American College of Rheumatology (ACR), in which 4 are simultaneously needed for the disease's formal diagnosis (Hochberg, 1997; Tsokos, 2011). SLE hallmark is the over production of autoantibodies, which leads to deposition of antibody-containing immune complexes throughout the body featuring tissue and organ damage (Silva et al., 2013). Additionally, a common feature in most of autoimmune diseases is a strong sex bias and especially in SLE this discrepancy is increased towards woman in childbearing age (Yan et al., 2012). According to the Brazilian Society of Rheumatology, epidemiology data in Brazil, estimates around 65,000 people with lupus, the majority being women with 1 affected in every 1700 (Sociedade Brasileira de Reumatologia, 2020).

Several clinical complications are observed in patients with SLE, with emphasis on lupus nephritis (LN), one of the most severe manifestations of this disease that results in a glomerulonephritis caused by an inflammatory response to endogenous immunogenic chromatin (Anders et al., 2020).

As a multifactorial disease, SLE presents an active interplay from many altered genes, particularly the ones involved with immune response regulation, responsible for disease's establishment and maintenance (Iruretagoyena et al., 2015; Mok & Lau, 2003). The steroid hormone vitamin D (VD_3) has as primary function calcium homeostasis and bone metabolism (Veldurthy et al., 2016), however recent studies have been reported as a pleiotropic regulator of human physiology and immune system modulation (Di Rosa et al., 2011). In fact, VD_3 has emerged as a potent immunosuppressive hormone, interfering with T regulatory (Th) cell functions and modulation which may be a key mechanism in SLE's development (Kamen & Tangpricha, 2010). Besides that, SLE Brazilian patients present overall low levels of vitamin D (Eloi et al., 2017).

VD_3 exerts its actions through interaction with its specific receptor named Vitamin D Receptor (VDR), which is widely spread throughout several organs, tissues and noteworthy, in all immune cells (Wang et al., 2012). *VDR* is located on chromosome 12 (12q13.11) and encloses several single nucleotide polymorphisms (SNPs), which can modulate VDR levels and activity (Silva et al., 2013). The SNPs described in the *VDR* are mainly in the promoter regions close to the f and c sites of exon 1, between exons 2 and 9 and in the 3′UTR region (Figure 1). The most frequently *VDR* polymorphisms in the literature are: *Cdx2* (G > A), *FokI* (C > T), *BsmI* (A > G), *EcoRV* (G > A), *ApaI* (G > T) and *TaqI* (T > C) (Uitterlinden et al., 2004). Polymorphisms in the *VDR* gene can alter both gene function and expression, thus leading to altered VD action. Since vitamin D levels has already been associated to inflammatory diseases including SLE (Wöbke et al., 2014), attention in its role on disease's pathogenesis has dramatically grown.

Therefore, considering VD_3 a key regulator in immune system, we aimed to evaluated the TagSNPs: rs11168268, rs2248098, rs1540339, rs4760648 and rs3890733 and functional SNPs: rs2228570 (FokI) and rs11568820 (Cdx-2) (Figure 1a), in SLE Northeast Brazilian patients in order to understand their consequence in our studied subject group, since associations were previously described with different consequences in other populations (Table 1). We also assessed *VDR* mRNA levels in order to evaluated gene expression profile in these patients, according its clinical manifestations (LN, photosensitivity, antibody anti-dsDNA and serositis) and also comparing different genotypes from rs11568820 SNP (Cdx2).

2 | MATERIAL AND METHODS

2.1 | Study design, location and subjects

We performed a case-control study to perform a genetic association analysis and an experimental study with a quantitative case-control approach to expression analysis. Genotyping patient group was composed by 128 females SLE patients (mean age 37.1 years ± 10.5) diagnosed according to the ACR (Hochberg, 1997). All patients were selected from the Division of Rheumatology from a hospital in the metropolitan region of Recife, Pernambuco, Brazil. Patients were classified according to the criteria of the American College of Rheumatology (ACR) (Hochberg, 1997) and SLICC (cumulative organic damage index (SLICC/ACR) or SLEDAI (disease activity index) according to patient status. For clinical and laboratory evaluation of SLE patients was collected the following data: photosensitivity, malar or discoid rashes, oral ulcers, serositis (pleuritis, pericarditis), arthritis, neuropsychiatric disorder (seizures, headache, psychosis), haematological alterations (haemolytic anaemia, leucopenia, lymphopenia, thrombocytopenia), presence of anti-double-strand DNA antibody (anti-ds-DNA), presence of antinuclear antibodies (ANA) and nephritic disorder. Nephritic disease was evaluated on laboratory parameters, specifically changes in urine summary and 24 h proteinuria, as follows: persistent proteinuria (>0.5 g/day or 3+) or abnormal cylindruria. The health control (HC) group consisted by 138 healthy females (mean age 33.5 years ± 13.4). The exclusion criteria were autoimmune, renal, chronic inflammatory disease or infection diseases. Subjects were chosen randomly in the population and matched for sex, age, ethnic group and same geographical area of the patients. Clinical and laboratorial characteristics are available at Table 2.

For assessing *VDR* gene expression levels we sampled 29 SLE patients (clinical and laboratorial confirmation) and 17 individuals as controls randomly selected. The inclusion and exclusion criteria were the same of genotyping study. SLE patients and healthy controls also denied any calcium or vitamin D replacement in the past two years. To evaluate the correlation of *VDR* mRNA levels and SLE activity, we assessed the SLEDAI mean to obtain the activity profile of SLE group.

2.2 | VDR association study

Genomic DNA was isolated from peripheral blood samples using DNA Wizard Genomic DNA Purification Kit (Promega, Madison, WI)

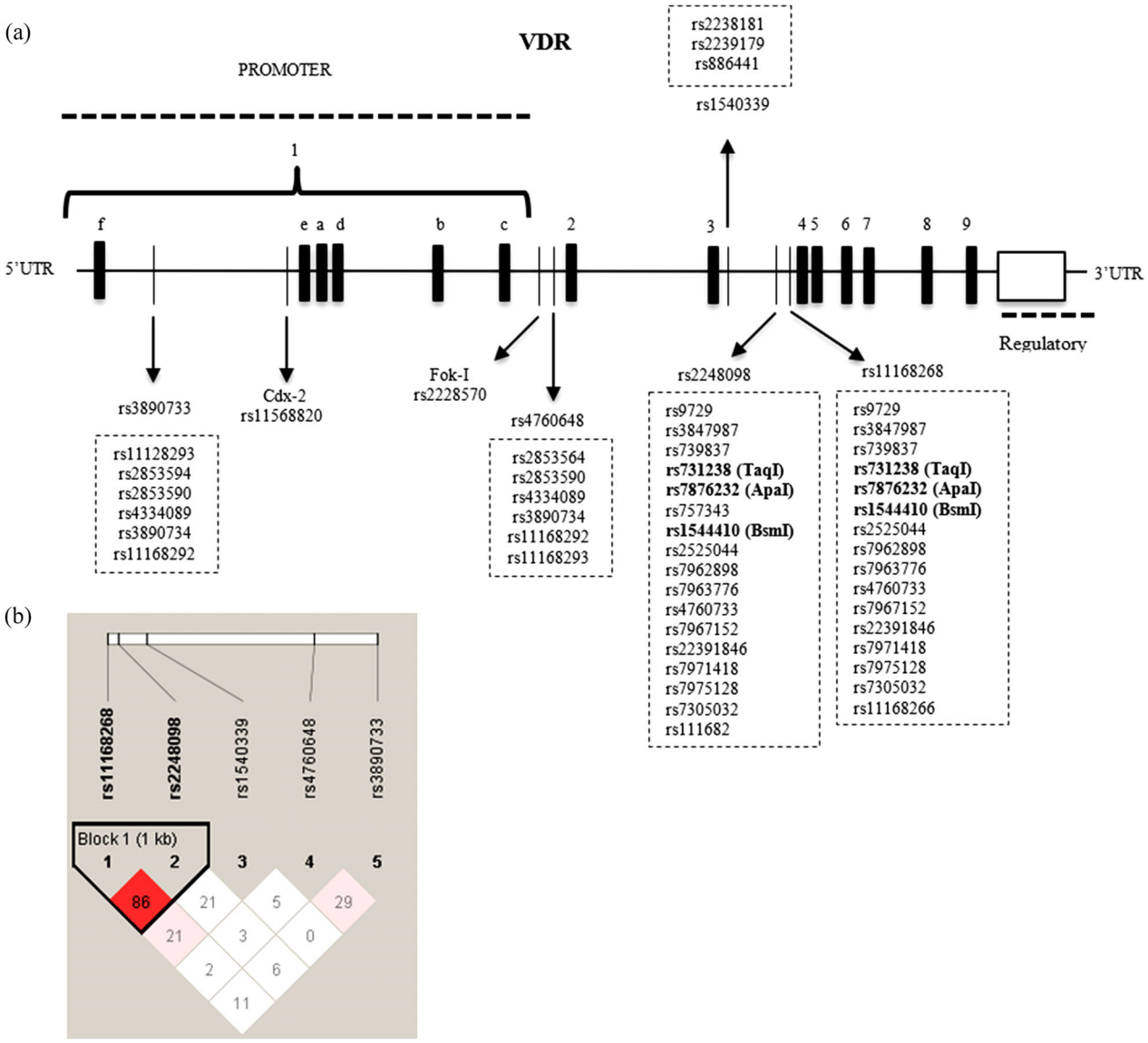

FIGURE 1 a) VDR gene schematic structure. The arrows indicate the position of all the assessed SNPs and the doted boxes indicate all the tagged SNPs. (b) Haplotype graphical representation. Linkage Disequilibrium (LD) plot among the studied polymorphisms, whereas in a *D*' values. The marked red box represents the LD shows (*D*' = 0.86) between the rs11168268 and rs2248098, demonstrating a strong linkage disequilibrium. Graph from Haploview Software

according to manufacturer's protocol. Polymorphisms were selected using the SNPBrowser software 4.0 (Applied Biosystems, Foster City, CA) and HapMap database (http://hapmap.ncbi.nlm.nih.gov/). We selected TagSNPs: rs11168268, rs2248098, rs1540339, rs4760648, rs3890733 (TagSNPs are representative SNPs in a gene region by linkage disequilibrium) (LD) (Stram, 2004) and rs2228579 (Fok1) and rs11568820 (Cdx-2), SNPs with functional impact. All selected SNPs presented at least 10% Minimum Allele Frequency (MAF) in CEU and YRI populations and covered most of *VDR* gene (Figure 1a).

Genotyping was evaluated by Taqman Probes® (Applied Biosystems, Foster City, CA) using the ABI7500 Real-Time PCR platform (Applied Biosystems, Foster City, CA). Allelic discrimination followed as recommended by the manufacturer and analysed using the SDS software 2.3 (Applied Biosystems, Foster City, CA).

2.3 | VDR gene expression study

RNA isolation was performed using the Qiagen Whole Blood RNAse kit, as described in manufacturer's instructions. The RNA integrity was performed by agarose gel electrophoresis and quantification by Nanodrop 2000 (Thermo Scientific USA). SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA) was performed for cDNA synthesis using for each sample a standard input of 500 ng from total RNA for reaction of 20 μl of cDNA. Oligo(dT) was used as primers in all samples.

The mRNA levels were determined for the target gene *VDR* and the reference genes *GAPDH* and *β-Actin* was used for data normalization (*VDR*: Hs00172113_m1, *GAPDH*: Hs02758991_g1, *ACTB*: Hs 99999903_M1). Expression assays were performed on ABI 7500

TABLE 1 Most frequents SNPs assessed according populations

VDR SNP	Function	Author (reference)	Country (ethnicity)	Population (cases/controls)	Relevant results
rs1544410 (*BsmI*)	Located on intron 8 (A > G or B > b). Could affect mRNA stability and *VDR* gene expression. Could generate an alteration in the splice sites for mRNA transcription or a change in the intron regulatory elements of VDR.	Ozaki et al., 2000	Japan (Asian)	58/87	SLE and allele B ($p < .0001$) Nephritis and allele b ($p = .03$)
		Huang et al., 2002	Taiwan (Asian)	47/90	SLE and allele B ($p < .0001$)
		Luo et al., 2012	China (Han Chinese)	337/239	SLE and allele B ($p = .031$) Nephritis and allele B ($p = .027$)
rs2228570 (*FokI*)	Located on exon 2, generates a non-synonymous polymorphism with a change of C > T (also called F > f), resulting in a change of threonine to methionine. The presence of the restriction site FokI C allele (F allele), generates a new start codon (ATG) 9 bp after the common starting site, which translates to a shorter truncated VDR protein of 424 amino acids with more transactivation capacity as a transcription factor than the wild type full-length VDR A isoform (VDRA) with 427 amino acids.	Luo et al., 2011	China (Han Chinese)	271/130	SLE and allele F ($p = .001$)
		Carvalho et al., 2015	Portugal (Caucasian)	170/192	CT genotype and higher SLICC value ($p = .031$)
		Salimi et al., 2019	Southeast Iranian	1027/139	CT genotype and higher SLE susceptibility ($p = .02$)
rs7975232 (*ApaI*)	Located on intron 8 (A > C also called A > a), does not change the amino acid sequence of the VDR protein, therefore could affect mRNA stability and the gene expression of VDR;	Salimi et al., 2019	Southeast Iranian	1027/139	No association
rs731236 (*TaqI*)	Located on the exon 9 (C > T also called T > t) and generates a synonymous change of the isoleucine amino acid in the coding sequence, therefore it does not change the encoded protein, but it could influence the stability of the mRNA.	Carvalho et al., 2015	Portugal (Caucasian)	170/192	TT genotype and higher SLICC value ($p = .046$)
		Salimi et al., 2019	Southeast Iranian	1027/139	Tt genotype and higher SLE susceptibility ($p = .0002$)
rs11168268	TagSNP	Silva et al., 2013	Brazil (Southeast)	158/190	Cutaneous alterations ($p = .036$)

(Continues)

TABLE 1 (Continued)

VDR SNP	Function	Author (reference)	Country (ethnicity)	Population (cases/controls)	Relevant results
rs3890733	TagSNP	Silva et al., 2013	Brazil (Southeast)	158/190	Arthritis ($p = .001$)
rs2248098	TagSNP	Silva et al., 2013	Brazil (Southeast)	158/190	Immunological alterations ($p = .040$)
rs4760648	TagSNP	Silva et al., 2013	Brazil (Southeast)	158/190	Antibody anti-dsDNA ($p = .036$)

platform (Applied Biosystems, Foster City, CA, USA). Relative quantity (RQ) of *VDR* mRNA was measured by quantification cycle (Cq) values obtained for *VDR* and each of the endogenous reference genes from all samples. Then, the mean value for each gene in each group was used to calculate *VDR* mRNA levels using ΔCq as quantification method (Livak & Schmittgen, 2001). We performed all qPCR assays in technical triplicates.

2.4 | Statistical analysis

SNPStats tool (http://bioinfo.iconcologia.net/SNPstats) was used for calculate allele and genotype frequencies and Hardy–Weinberg equilibrium, and Fisher's exact test was used to the statistical significance of difference in allele and genotype frequencies. After Bonferroni's Correction, a $p < .071$ was considerate statistically significant for SNP association study. For haplotype associations and linkage disequilibrium analysis (LD) was used Haploview Software (version 4.2). The multivariate analysis logistic regression was performed to investigate the association between the qualitative variables and dependent variable binary: SLE risk and ACR clinical phenotypes. The open-source R Studio 4.1.2 (www.r-project.org) was used for all statistical analyses. The post hoc power analysis was performed in the G*Power 3.1.9.4 software and the results were included at Table 2.

The statistical tests applied to gene expression analyses were: Shapiro–Wilk, to verify the sample's distribution, and Student's *t*-test and one-way ANOVA for analysis of variance, considering as statistically significant in both $p < .05$ in a 95% confidence interval (95% CI).

3 | RESULTS

The *VDR* allelic and genotypic frequencies from all assessed SNPs were in Hardy–Weinberg equilibrium in SLE patients and HC, except for patients' group in rs2228570 (FokI). The frequencies distribution presented significantly differed between SLE patients and HC in three out of the seven assessed SNPs namely: rs11168268, rs2248098 and rs2228570 as shown in Table 3.

For the rs11168268 (A > G) SNP, the G allele (OR = 1.55, CI = 1.08–2.23, $p = .01$) and G/G genotype (OR = 2.69, CI = 1.24–6.01, $p = .008$) were associated to increased SLE susceptibility. In the other hand for rs2248098 (A > G) SNP, the G allele (OR = 0.66, CI = 0.46–0.94, $p = .01$) and A/G (OR = 0.46, CI = 0.24–0.86, $p = .01$) and G/G (OR = 0.44, CI = 0.20–0.93, $p = .02$) genotypes were associated to lower SLE susceptibility, as shown in Table 3. For the rs2228570 (C > T) SNP, the frequency of C allele, C/T and C/C genotypes was increased in controls when compared to patients (OR = 0.19, $p = 2.7 \times 10^{-16}$; OR = 0.14, $p = 1.55 \times 10^{-7}$; OR = 0.05, $p = 1.77 \times 10^{-13}$, respectively), as shown in Table 3. However, this last result regarding rs2228570 is biased once the patient's group did not present H-W equilibrium.

TABLE 2 Clinical and laboratorial features from the SLE patients studied

Clinical/laboratorial characteristics	*n* (%)
Photosensitivity	80 (62.5%)
Malar Rash	77 (60.16%)
Discoid Rash	22 (17.19%)
Oral ulcers	27 (21%)
Serositis	28 (21.88%)
Arthritis	92 (71.8%)
Neuropsychiatric disorder	11 (8.5%)
Nephritic disorder	65 (50.7%)
Haematological alterations	87 (67.9%)
Antinuclear factor positive (FAN)	128 (100%)
Antibody anti DNA (anti ds-DNA)	33 (25.7%)

For the SNPs rs4760648, rs1540339 and rs11568820, no significant difference in allelic and genotypic distribution was observed (Table 3).

Regarding *VDR* polymorphisms and clinical and laboratorial characteristics we report association between following SNPs and clinical features: rs11168268 A/G genotype (OR = 0.31, CI = 0.11–0.8, $p = .01$) with lower nephritis susceptibility; rs4760648 T/T genotype (OR = 0.24, CI = 0.05–0.9, $p = .02$) with diminished photosensitivity; rs1540339 T/T genotype (OR = 0.19, CI = 0.04–0.78, $p = .015$) with less frequency of antibody anti-dsDNA and rs3890733 T/T genotype (OR = 0.10, CI = 0.002–0.81, $p = .01$) with lesser serositis development, as seen in Table 4. Multivariate analysis results are demonstrated at Table 5.

Haplotype analysis was performed to assess linkage disequilibrium among the tested TagSNPs. We observed LD between TagSNPs rs11168268 and rs2248048 (*Dt'* = 0.86) as shown in Figure 1b. Even though we identified a haplotype combination, no association was observed to SLE or its clinical features susceptibility (data not shown).

We performed a relative gene expression assay to evaluate the mRNA levels from *VDR* in SLE patients as well as in healthy controls. We observed that overall *VDR* gene expression was downregulated in patients (−10.51 FC, $p = 1.04e^{-7}$) when compared to HC (Figure 2a). We also analysed whether the Cdx-2 (rs11568820) genotypes (A/G-G/G) influence *VDR* gene expression in SLE patients. Our analyses indicated that the A/G and G/G genotypes decrease *VDR* mRNA levels (−9.6, $p = .008$ and −12.6 FC, $p = .014$, respectively) when compared to A/A genotype (Figure 2b).

When assessing *VDR* gene expression and altered risk for SLE clinical manifestations, we found a differential expression in patients with nephritis (−5.7 FC, $p = .016$; Figure 2c). We also found a differential expression in patients with skin alterations (+1.3 FC, $p = .587$), but the data comparison is not statistically significant. The differential expression reported with mRNA levels from *VDR* and clinical manifestations are showed in Figure 2a–c. Other clinical features were analysed, but they did not present enough sampling power to be included in the expression analyses.

4 | DISCUSSION

In present study, we observed an association between rs11168268 and rs2248098 and SLE development, where rs11168268 (A > G) SNP, the G allele and G/G genotype conferred risk for SLE; and rs2248098 (A > G) SNP, the G allele, as well the A/G and G/G genotypes conferring lower susceptibility to SLE. Interestingly, although these SNPs were not associated with SLE itself, it had already been previously reported as associated to clinical features in a southeast Brazilian population study (Silva et al., 2013).

In our study, a strong linkage disequilibrium was observed between rs11168268 and rs2248098. Interesting, Cavalcanti et al. (2016) also verified significant linkage disequilibrium between these same SNPs ($D' = 0.91$, $r^2 = .72$), corroborating our data ($D' = 0.86$).

VDR plays a key role regulating vitamin D pathway and its physiological importance in immune modulation relates it to several immune disorders, including SLE (Kamen et al., 2006; Wöbke et al., 2014). In our study the presence of C allele as well C/T and C/C genotypes from FokI SNP indicated a lower risk of SLE susceptibility. FokI display a cytosine to thymine change (C > T) creating a methionine codon three codons latter, which in turn, leads to a final protein with 424 amino acids (aa) shorter than the one with the T allele, with 427aa. In fact, the shorter variant (C) seems to interact more strongly to the transcription factor II B (TFIIB) compared to the longer one. Therefore, it seems that the VDR shorter protein may be more efficient than the longer one in activating vitamin D pathway (Dzhebir et al., 2016). Besides the statistical association, it is important to mention that in our population for this SNP, the patient's group was out of Hardy–Weinberg equilibrium.

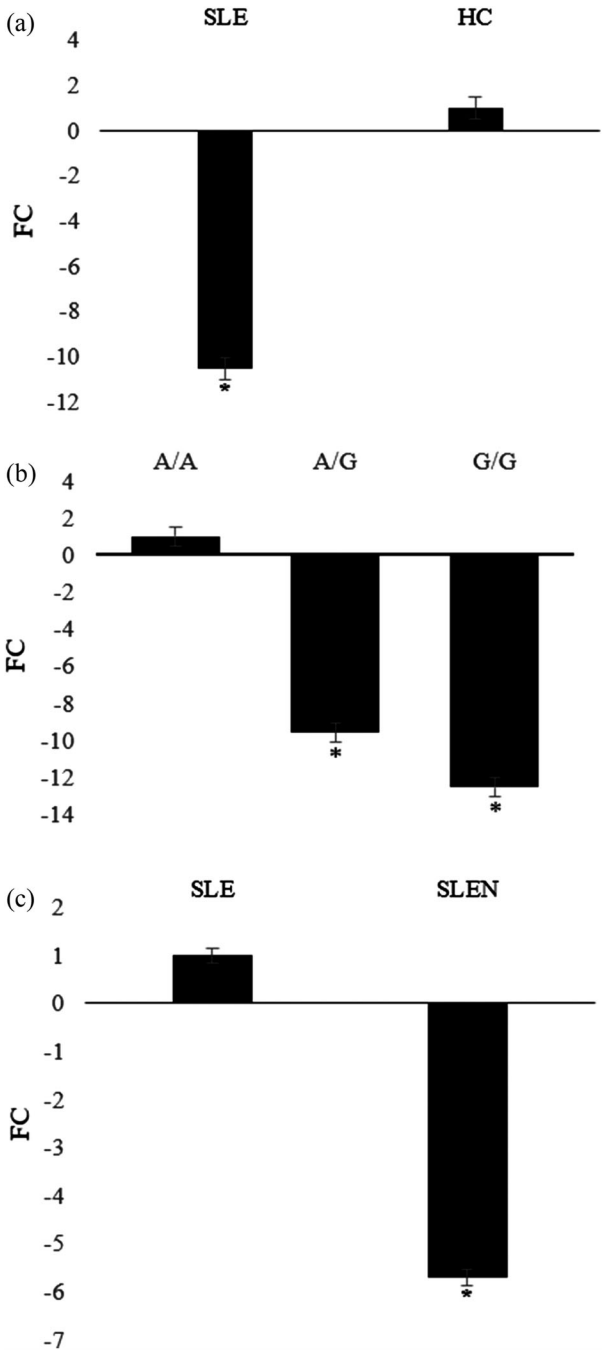

FIGURE 2 a) VDR expression graph comparing SLE patients and HC. (b) VDR expression graph of SLE patients among from Cdx-2 SNP genotypes (A/A $n = 6$; A/G $n = 10$; G/G $n = 5$). (c) VDR expression graph of SLE patients or SLE nephritis (SLEN). The results were normalized using *GAPDH* and *ACTB* as endogenous references. SLE: patients with Systemic lupus erythematosus ($n = 29$); HC: Healthy controls ($n = 17$); SLEN: patients with SLE and nephritis ($n = 12$); FC: fold-change. $^{*}p < .05$

Two previous *VDR* association studies were performed in Brazilian populations. The first one, by Monticielo et al. (2012), was performed in a south Brazilian population and included, amongst others, the two most studied *VDR* SNPs: *BsmI* and *FokI*. However, the authors did not find statistically significant differences in genotype and allelic

TABLE 3 Allelic and genotypic VDR SNPs and TagSNPs frequencies from all assessed SLE patients and HC

SNP ID	HC	SLE patients	OR (95%CI)	*p* Value
rs3890733	**N = 138**	**N = 127**		
Allele				
C	194 (70%)	169 (67%)	1.00	
T	82 (30%)	85 (33%)	1.18 (0.81–1.74)	.39
Genotype				
CC	69 (50%)	63 (49.6%)	1.00	
CT	56 (40.6%)	43 (33.9%)	0.84 (0.48–1.46)	.84
TT	13 (9.4%)	21 (16.5%)	1.76 (0.76–4.17)	.17
rs11568820	**N =109**	**N = 115**		
Allele				
G	124 (57%)	132 (57%)		
A	94 (43%)	98 (43%)	0.97 (0.66–1.44)	.92
Genotype				
GG	33 (30.3%)	40 (34.8%)		
AG	58 (53.2%)	52 (45.2%)	0.74 (0.39–1.39)	.36
AA	18 (16.5%)	23 (20%)	1.05 (0.45–2.45)	1.00
rs2228570	**N = 108**	**N = 107**		
Allele[a]				
T	71 (33%)	154 (72%)	1.00	
C	**145 (67%)**	**60 (28%)**	**0.19 (0.12–0.30)**	**2.7×10^{-16}***
Genotype[a]				
TT	12 (11.1%)	60 (56.1%)	1.00	
CT	**47 (43.5%)**	**34 (31.8%)**	**0.14 (0.06 - 0.32)**	**1.55×10^{-7}***
CC	**49 (45.5%)**	**13 (12.2%)**	**0.05 (0.02–0.13)**	**1.77×10^{-13}***
rs4760648	**N = 138**	**N = 127**		
Allele				
C	151 (55%)	132 (52%)	1.00	
T	125 (45%)	122 (48%)	1.11 (0.78–1.59)	.54
Genotype				
CC	37 (26.8%)	31 (24.4%)	1.00	
CT	77 (55.8%)	70 (55.1%)	1.08 (0.58–2.0)	.88
TT	24 (17.4%)	26 (20.5%)	1.29 (0.58–2.86)	.57
rs1540339	**N = 138**	**N = 128**		
Allele				
C	193 (70%)	176 (69%)	1.00	
T	83 (30%)	80 (31%)	1.05 (0.71–1.55)	.77
Genotype				
CC	63 (45.6%)	62 (48.4%)	1.00	
CT	67 (48.5%)	52 (40.6%)	0.78 (0.46–1.34)	.37
TT	8 (5.8%)	14 (10.9%)	1.77 (0.63–5.24)	.25

(Continues)

TABLE 3 (Continued)

SNP ID	HC	SLE patients	OR (95%CI)	*p* Value
rs2248098	**N = 138**	**N = 127**		
Allele[a]				
A	127 (46%)	143 (56%)	1.00	
G	**149 (54%)**	**111 (44%)**	**0.66 (0.46–0.94)**	**.01***
Genotype[a]				
AA	26 (18.8%)	43 (33.9%)	1.00	
AG	**75 (54.4%)**	**57 (44.9%)**	**0.46 (0.24–0.86)**	**.01***
GG	**37 (26.8%)**	**27 (21.3%)**	**0.44 (0.20–0.93)**	**.02***
rs11168268	**N = 138**	**N = 127**		
Allele[a]				
A	176 (64%)	135 (53%)	1.00	
G	**100 (36%)**	**119 (47%)**	**1.55 (1.08–2.23)**	**.01***
Genotype[a]				
AA	54 (39.1%)	41 (32.3%)	1.00	
AG	68 (49.3%)	53 (41.7%)	1.02 (0.57–1.83)	1.00
GG	**16 (11.6%)**	**33 (26%)**	**2.69 (1.24–6.01)**	**.008***

*$p < .05$; Values in bold are the results with association (significant *p*). The SNPs used in the study are in italics.
[a]Power > 0.8.

frequencies between SLE patients and healthy individuals. The other study performed by our research group in a southeast Brazilian cohort and even though we did not find any association to SLE itself, we reported association to cutaneous manifestations, arthritis, immunological alterations and antibody anti-dsDNA (Silva et al., 2013).

In relation to *VDR* SNPs and clinical manifestations, the study found statistically significant association with antibody anti-dsDNA (rs1540339), photosensitivity (rs4760648), nephritis (rs11168268) and serositis (rs3890733).

In our study, the presence of T/T genotype of rs1540339 SNP is associated with lower frequency of antibody anti-dsDNA presence. Corroborating with our findings, anti-dsDNA is an important marker to evaluate the disease activity in SLE patients. Studies have shown that SLE patients present vitamin D deficiency when compared to the general population. SLE patients with VD_3 deficiency presents increased disease's activity and raised anti-dsDNA levels, which strengthen VD_3 role as an immune modulator in autoimmune diseases (Mok et al., 2012).

Our results showed that the presence of rs4760648 T/T genotype confers a lower susceptibility to photosensitivity development and are in agreement with Silva et al (2013) that identified in southeast Brazilian population the same association. Photosensitivity is an important clinical manifestation in SLE patients and contributes to poor life quality of these individuals (Klein et al., 2011). Lesions caused by photosensitivity in SLE patients are characterized by increased epidermal apoptosis and infiltrate of inflammatory cells like dendritic cells in the dermis (Kim & Chong, 2013). The immunoregulation promoted by VD_3 in immune cells recruitment and cytokine liberation may play a crucial role in SLE patient response to lesions caused by ultraviolet (UV) exposure (Correa-Rodríguez et al., 2021).

The most frequent and severe clinical finding in SLE patients is LN (Tang et al., 2017). LN is an important condition and major risk factor for morbidity and mortality in SLE patients (Almaani et al., 2017). In our study, we found a significant association between LN and the A/G genotype of rs11168269 SNP. Corroborating with our findings, the TagSNP rs11168269 tags the *BsmI*, already reported as associated to LN lower susceptibility. Located on intron 8, *BsmI* represents the change of adenine for guanine (A > G), also called for B > b (BB, Bb and bb genotypes). The SNP *BsmI* may affect mRNA stability and *VDR* gene expression, altering in the splice sites in mRNA transcription or a change in intronic regulatory elements of VDR (Luo et al., 2011; Luo et al., 2012). The LN pathogenesis is not completely elucidated. Low levels of Vitamin D may play a role in SLE progression and nephritis development. On the other hand, VD_3 supplementation may prevent renal involvement by lessening proteinuria risk, a frequent condition in LN patients (Yu et al., 2019).

We also found an association between rs3890733 T/T genotype and lower susceptibility to serositis. Serositis is an inflammation of serous membranes and a significant cause of morbidity in SLE patients (Liang et al., 2017). Located at promoter region, rs3090733 is a TagSNP that tags another six SNPs by linkage disequilibrium. The rs4334089 is tagged by rs3090733 and its variant genotype A/A is associated to lower risk to upper respiratory infection (URI) development. It is hypothesized that the presence of this variant would improve the inflammatory response performed by the VD_3/VDR complex (Jolliffe et al., 2018). Although the literature lacks association studies correlating *VDR* and clinical features in SLE such as serositis, a study conducted by Luo et al. (2012), with SLE patients from Chinese population and *VDR* SNPs found a relation between *ApaI* and *BsmI* polymorphisms with serositis and also an increased risk to SLE development considering combined genotype Aa-bb.

TABLE 4 Genotypes from VDR SNVs and TagSNVs associated with SLE clinical manifestations

SNV	Clinical Feature	Genotype	OR	95%CI	*p* Value
rs1540339 (C > T)	Anti-dsDNA	TT	0.19	0.04–0.78	.015
rs3890733 (C > T)	Serositis	TT	0.10	0.002–0.81	.01
rs11168268 (A > G)	Nephritis	AG	0.31	0.11–0.8	.01
rs4760648 (C > T)	Photosensitivity	TT	0.24	0.05–0.9	.02

TABLE 5 Multivariate analysis using as dependent variables SLE susceptibility and ACR clinical phenotypes and as independent variables seven the SNPs analysed

SNP	Genotype	Dependent variables	Exponential value	*p* Value
rs2228570	C/T	SLE susceptibility	3.06×10^{5}	6.92×10^{-11}
	T/T	SLE susceptibility	4.45×10^{4}	.00584
rs4760648	C/T	Serositis	0.086	.009
		Neurological alterations	0.179	.0316
	T/T		0.082	.0123
rs11168268	A/G	Antibody anti-dsDNA	0.121	.0208
rs1540339	C/T	Discoid rash	−3.663	.0313
		Photosensitivity	−6.495	.0408
		Nephritis	0.204	.0402
		Antibody anti-dsDNA	0.184	.0276
rs3890733	C/T	Neurological alterations	0.1705	.0214
		Antibody anti-dsDNA	0.121	.014
rs11568820	G/G	Arthritis	−32.402	.0014
	A/G	Nephritis	0.149	.045

Complex diseases as SLE presents several variants in specific genes, as VDR, which provides diverse clinical phenotypes, raising a challenge in identifying genetic variations associated simultaneously with correlated traits. Multivariate analysis should be done to detect independent predictors of different clinical phenotypes. The multivariate analysis performed in the present study (Table 5) found statistically significant association with SLE susceptibility and rs2228570 SNP (FokI). The analysis was also performed using ACR clinical characteristics directly related to the accumulation of immune complexes such as photosensitivity (rs1540339), serositis (rs4760648), neurological alterations (rs4760648, rs3890733), antibody anti-dsDNA (rs11168268, rs1540339, rs3890733), discoid rash (rs1540339), nephritis (rs1540339, rs11568820/Cdx-2) and arthritis (rs11568820/Cdx-2). Inflammatory process is one of the most important roles on SLE's pathogenesis, and vitamin D levels has already been associated to its modulation (Iruretagoyena et al., 2015; Wöbke et al., 2014). In addition, a recent study shows vitamin D levels are associated with SLE activity and DNA damage growth (Correa-Rodríguez et al., 2021).

Accessing *VDR* expression levels, we found a downregulation (−10.51 FC) in SLE patients comparing with HC group. VD_3/VDR complex plays an important role in immune cells as monocytes, macrophages, dendritic, T and B cells (Wang et al., 2012). SLE patients are deficient or insufficient in vitamin D levels compared with healthy controls, where 1,25(OH)2 D_3 serum levels and *VDR* mRNA expression in peripheral blood were decreased in SLE patients and it could inhibit the activation of $CD4^+$ T cells and suppress the immune response in SLE (Xiao et al., 2016). VD_3 inhibits the action of activated B cells and induces their apoptosis. B cells, on the other hand, express mRNAs for proteins involved in VD_3 activity, including *VDR*, which consequently is regulated by vitamin D levels (Chen et al., 2007), in concordance with our results.

In addition, when *VDR* gene expression was analysed in SLE patients according rs11568820 (Cdx-2), a downregulation was observed in patients with the genotype G/A and G/G, when compared to A/A genotype, indicating that, the G allele decreases *VDR* mRNA levels in these individuals. The Cdx-2 polymorphism is located at *VDR* promoter region and consists in change of adenine to guanine, potentiating the binding strength between VDR and its transcriptional complex (Ralston & Rossant, 2008; Savory et al., 2009).

When assessing *VDR* expression and risk for SLE clinical manifestations our analyses showed that patients with skin alterations as malar rash, discoid rash and photosensitivity presents an upregulation of *VDR* mRNA levels (+1.3 FC), however this data was not statistically significant. Individuals with SLE show increased cutaneous manifestations in response to UV light exposure that induces apoptosis with subsequent

immune complexes formation, justifying inflammation and skin lesions (Bijl & Kallenberg, 2006).

We also report a downregulation of *VDR* expression in patients that present nephritis (−5.7 FC). Interestingly, several studies report significant associations of *VDR* SNPs with LN (Luo et al., 2012, Emerah & El-Shal, 2013; Mostowska et al, 2013; Ozaki et al, 2000). Being ours the first one to bring up an expression data from nephritis in SLE patients, our results agree with the fact that $1,25(OH)_2D_3$ upregulates *VDR* gene expression in kidney cells (Andress, 2006; Healy et al., 2003; Healy et al., 2005) and since SLE patients with nephritic disorders have significantly lower vitamin D levels (Sumethkul et al., 2013), it justifies the *VDR* downregulation detection. The kidney is one of the main organs processing pro-forms of inactive vitamin D into active forms (1,25a-OH vitamin D) (Veldurthy et al., 2016) when its function is impaired, it may influence upon vitamin D levels, contributing to deficiency. Therefore, due to our sample limitation we suggest that further studies needed to be performed in other population to better elucidate the VDR role in LN.

Our results support *VDR* polymorphisms and mRNA expression levels associated to SLE and some clinical features, particularly nephritis. We also assessed in SLE patients according *Cdx-2* genotype, which indicated a downregulation when compared to healthy individuals. To the better of our knowledge, this was the first and only study to evaluate almost the completed *VDR* gene SNPs by linkage disequilibrium. Our main limitation is the relatively small sample size from SLE patients' group and not following the functional analysis from the associations detected, falling into the main gap of all genetic association studies. Thus, these findings reinforce the VDR key role in SLE and its clinical features.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Vitamin D receptor gene polymorphisms influence on clinical profile and bone mineral density at different skeletal sites in postmenopausal osteoporotic women

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Abstract

Bone remodeling is marked by bone synthesis and absorption balance, and any altered dynamic in this process leads to osteoporosis (OP). The interaction of hormonal, environmental and genetic factors regulate bone metabolism. Since vitamin D displays a classic role in bone metabolism regulation, acting through vitamin D receptor (VDR), the genetic variants within *VDR* were the first ones associated with bone density and remodelling. Therefore, we investigated whether three single nucleotide polymorphisms (SNPs) within *VDR* were associated with OP differential susceptibility and clinical profile from postmenopausal versus healthy women from Northeast Brazil. Genetic association study enrolling 146 postmenopausal osteoporotic women as the patient group and 95 healthy age-matched women as the control group. We assessed three SNPs within *VDR* (rs11168268, rs1540339 and rs3890733), considering the clinical profile of all patients. Our results showed an association of rs11168268 G/G genotype with higher bone mineral density (BMD) mean for the total hip (A/A = 0.828 ± 0.09; A/G = 0.081 ± 0.13; G/G = 0.876 ± 0.12, $p = .039$), and the rs3890733 T/T genotype was associated with increased OP risk in patients below 60 years old (odds ratio [OR] = 5.12, 95% confidence interval [CI]= 1.13–23.27, $p = .012$). The rs1540339 T/T genotype was associated with protection for individuals with low melanin deposition when compared to the high melanin deposition group (OR = 0.24, 95%CI = 0.06–0.94, $p = .029$). Additionally, 61% of patients presented deficient vitamin D serum levels. The SNP rs11168268 G/G was associated with a significantly increased mean total hip BMD in patients OP, highlighting this SNP and its relationship with BMD.

KEYWORDS
osteoporosis, SNP, VDR, vitamin D

1 | INTRODUCTION

Osteoporosis (OP) is an essential skeletal condition characterized by low bone mass, reduced bone strength and increased risk of fractures. The condition results from bone synthesis and reabsorption misbalance, regulated by endocrine, environmental and genetic factors (Brent Richards et al., 2012; Chandra & Rajawat, 2021). The multiple aetiologies of decreased bone mineral density (BMD) and metabolic bone

diseases development create major confounding factors, leaving the exact aetiology of OP poorly understood (He et al., 2015).

OP is a current concern in public health worldwide, and its prevalence increases as people live longer. Additionally, it affects both genders and displays bias towards postmenopausal women. The condition presents a genetic background characterized by the polygenic influence and many gene variations associated with low BMD and possibly facilitating fractures (Conti et al., 2015; Lovšin et al., 2018). Different endocrine pathways involved in skeletal ageing with BMD loss process have been described, leading to new possibilities for prognosis and effectiveness in disease therapies (Chandra & Rajawat, 2021; Larsson & Fazzalari, 2014; Y. Zhang et al., 2014).

Vitamin D is a secosteroid hormone with a major source in the skin, synthesized when 7-dehydrocolesterol reacts with ultraviolet B (UVB) light. The active form of vitamin D, known as D_3, acts through its receptor—vitamin D receptor (VDR)—and has a significant function in calcium (Ca) absorption and equilibrium, being the natural modulator of bone homeostasis (Goltzman, 2018; Holick, 2004; Y. Y. Zhang et al., 2003). *VDR*, located at chromosome 12 (12q12–q14) and displaying 14 exons, is a member of the nuclear receptor family of transcription factors, regulating essential bone metabolism genes such as osteocalcin, osteopontin and factor nuclear kappa B (NF-κB) receptor (Haussler et al., 2010; He et al., 2015). *VDR* is a highly polymorphic gene with several polymorphisms described and the first gene known to be associated with bone density, remodelling and turnover (Conti et al., 2015; He et al., 2015).

VDR polymorphisms were first associated with OP because vitamin D and its metabolites play a significant role in the Ca absorption pathway and bone metabolism. However, most of the studied single nucleotide polymorphisms (SNPs; *TaqI*, *BmsI* and *ApaI*) within *VDR* are in non-coding regions or with no known function, except for *FokI* and *Cdx-2* (Mohammadi et al., 2014; Yang et al., 2020). Nevertheless, they have been associated with several pathologies from systemic autoimmune disorders, such as systemic lupus erythematosus to cancer, such as melanoma (Carvalho et al., 2015; Shahbazi et al., 2013; Zeljic et al., 2014).

For its role in bone metabolism and homeostasis, we evaluated whether the TagSNPs rs1168268, rs1540339 and rs3890733, covered by linkage disequilibrium in most *VDR* genes, were associated with postmenopausal OP in women. Additionally, we assessed all patients' vitamin D serum levels, BMD, clinical features and their relation to the *VDR* SNPs investigated.

2 | METHODS AND SUBJECTS

2.1 | Subjects

In this study, 146 osteoporotic women were enrolled based on clinical and laboratory diagnosis, all postmenopausal (mean age at diagnosis 59, SD ± 3.91, ranging from 50 to 65 years old). All patients were recruited from the Rheumatology Division at Clinical Hospital, Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil. Menopause was defined according to the World Health Organization (WHO) criteria as amenorrhea for at least 1 year in women over 45 years old without any other pathological or physiological cause.

Since Brazilian populations are ancestrally genetically heterogeneous, it is not appropriate to divide them into different groups, such as Caucasian or African-derived (Coelho et al., 2015). However, skin melanin deposition is essential to evaluate vitamin D levels and their consequences. Therefore, we arbitrarily divided the OP patients into two main groups: group I—patients with moderate to high melanin deposition (dark to very dark skin) and group II—patients with very low and low melanin deposition (fair and fair light skin tone).

In the control group, 95 postmenopausal non-osteoporotic age-matched (mean age 57, SD ± 3.96, ranging from 49 to 64) women were enrolled. All individuals from the control group presented no medical history of secondary OP on physical examination and laboratory tests. Additionally, no subjects from the study were on hormone replacement therapy. In the absence of a fragility fracture, BMD by dual-energy x-ray absorptiometry was used to diagnose OP or osteopenia according to the WHO classification (Kanis & Kanis, 1994). In addition, plain x-rays of the dorsal-lumbar spine (LS) and hip were performed to diagnose osteoporotic fractures. All information was obtained directly from the patient's assessment and medical records.

All the participants provided written informed consent approved by the local Research Ethics Committee (CEP/CCS/UFPE No. 513/11), according to the 1964 Helsinki Declaration.

2.2 | Measurement of 25-hydroxyvitamin D_3 (25(OH)D) serum levels and BMD

25(OH)D were determined by LIAISON Chemiluminescent Immunoassay (CLIA) (DiaSorin, Stillwater, MN, USA). Normal 25(OH)D serum levels were defined as values ≥ 30 ng/mL, insufficiency as values 20–30 ng/mL and deficiency as values < 20 ng/mL, according to Yamada et al. (2001).

Measurement by the Dual Energy X-ray Absorptiometry (Lunar Corporation, Madison, WI, USA) was performed at the LS from L1 to L4 anteroposteriorly and the total hip, including the femoral neck, Ward's triangle and trochanter. The results are expressed in g/cm^2 and T-score. We used a local database (reference population aged 20 to 29 years) to calculate the T-score. The mean (± SD) of normal values for women was 1.085 g/cm^2 (± 0.1) at the LS, 0.913 g/cm^2 (± 0.12) at the femoral neck and 0.316 g/cm^2 (± 0.07) at the distal radius. The in vivo precision error of the equipment employed in the study expressed in percentage coefficient of variation (%CV = SD + mean BMD of repeated measurements) was 0.9% for the LS on the anteroposterior view and 1.2% for the femoral neck.

2.3 | SNPs selection and VDR genotyping

Genomic DNA was isolated from 5 mL of whole blood using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), following the protocol according to the manufacturer's

guidelines. Polymorphisms were selected using the SNPBrowser software version 4.0 (Applied Biosystems, Foster City, CA, USA) and the HapMap database (http://hapmap.ncbi.nlm.nih.gov/). We selected three TagSNPs rs11168268, rs1540339 and rs3890733, tagging some of the most studied polymorphisms, namely, *Taq-I*, *Bsm-I*, *Apa-I*, and considering the 10% minimum allele frequency in Caucasian (Nothen European from Utah-CEU) and African-derived (Yoruba in Ibadan-YRI) subpopulation according to National Center for Biotechnology Information. A TagSNP is a representative SNP in a particular genome region, presenting high linkage disequilibrium with other polymorphisms within or not a gene (Stram, 2004). The list of all tagged SNPs by the ones assessed herein is shown in Supporting Information Data 1.

Genotyping was performed with commercially available fluorogenic allele-specific Taqman Probes (Applied Biosystems, Foster City, CA, USA) using the ABI7500 Real-Time PCR system (Thermo Fisher, Madison, WI, USA). Allelic discrimination was followed as recommended by the manufacturer and analysed using the SDS software version 2.3 (Applied Biosystems, Foster City, CA, USA).

2.4 | Statistical analysis

Allelic and genotypic frequencies and Hardy–Weinberg equilibrium were performed using the SNPStats tool (available online: http://bioinfo.iconcologia.net/SNPstats). The exact Fisher test was applied to determine the statistical significance of all comparisons. Haploview Software version 4.2 was used for haplotype associations. 'SNPassoc' R software package version 2.12.2, developed for genetic studies, was used for evaluating the association between SNPs and postmenopausal OP susceptibility and all clinical features (González et al., 2007). The multivariate analysis logistic regression was performed to investigate the association between the variables and dependent variables binary: SNPs and Ca, vitamin D and BMD of total hip and femoral neck in IBM SPSS statistic software version 18.0 (IBM Corp, Armonk, NY, USA). *p*-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Clinical characterization of the OP patients

In total, 146 patients with postmenopausal OP were included in our analysis. Additionally, all the clinical and laboratory findings and anti-OP therapy from the patients' group are depicted in Table 1, with a mean level of 25(OH)D of 27.99 ng/mL. Low serum levels of 25(OH)D were observed in 89/146 (60.95%) and vitamin D deficiency in 68/146 (45.58%). Furthermore, 9/146 (6.2%) presented historical fractures, whereas 137/146 (93.8%) patients had no fractures (Table 1).

3.2 | *VDR* allelic and genotyping frequencies

VDR allelic and genotypic frequencies from the selected SNPs were in Hardy–Weinberg equilibrium in OP patients and healthy controls, and all assessed frequencies are shown in Table 2. No association was identified when assessing the patients and control groups overall. However, we identified associations by subgroups when stratifying the patient's group by clinical features and SNPs presence.

When comparing patients below and above 60 years old, the SNP rs3890733 T/T genotype was associated with the group of individuals below 60 years old (odds ratio [OR] = 5.16, 95% confidence interval [CI] = 1.1–24.1, $p = .043$ and OR = 5.12, 95%CI = 1.13–23.27, $p = .012$) in the codominant and recessive model, respectively (Table 3).

TABLE 1 Clinical features from all assessed osteoporotic postmenopausal women.

Characteristic	*N* (%)
Moderate to high skin melanin deposition	84 (57.5)
Low to very low skin melanin deposition	62 (42.5)
Age (range)	59 (50–65)
Mean of years since menopause (range)	11 (2–27)
Mean of body mass index mean (range)	26.24 (18.37–41.65)
Obesity (%)	
Present	87 (59.6)
Absent	59 (40.4)
Smoking (%)	55 (37.7)
Smoking duration, years, mean (range)	20 (5–45)
Mean bone mineral density in g/cm^2 (range)	
Femoral neck	0.739 (0.482–0.987)
Total hip	0.828 (0.518–1.182)
Lumbar spine (LS)	0.768 (0.484–0.923)
Site of osteoporosis (OP)[a] (%)	
Femoral neck	26 (17.8)
Total hip	16 (10.96)
LS	142 (97.26)
Osteoporotic fractures (%)	
Present	9 (6.2)
Absent	137 (93.8)
Vitamin D serum levels (%)	
Low serum level of 25-hydroxyvitamin D_3	89 (60.95)
Vitamin D insufficiency	21 (14.38)
Vitamin D deficiency	68 (45.58)

[a]Some patients presented OP in more than one site.

3.3 | *VDR* SNPs and clinical features from postmenopausal osteoporotic patients

Moreover, no association was found between *VDR* polymorphisms and vitamin D levels. Regarding BMD, we observed a statistically significant higher BMD mean of total hip among patients for the SNP rs11168268 G/G genotype when compared with A/G genotype (A/A = 0.828 ± 0.09; A/G = 0.081 ± 0.13; G/G = 0.876 ± 0.12, $p = .039$; Supporting Information Data 2).

TABLE 2 Allelic and genotypic distribution from all TagSNPs assessed within vitamin D receptor OP patients and controls.

Polymorphism	Patients *N* (%)	Controls *N* (%)	OR (95% CI)	*p*-value
rs11168268				
Allele	**140**	**92**		
A	159 (57%)	113 (61%)		
G	121 (43%)	71 (39%)	1.21 (0.81–1.80)	.33
Genotype				
A/A	48 (34.28%)	35 (38.04%)	1.00	
A/G	63 (45%)	43 (46.74%)	1.07 (0.57–1.99)	.88
G/G	29 (20.72%)	14 (15.22%)	1.52 (0.65–3.56)	.33
rs1540339				
Allele	**142**	**84**		
C	204 (72%)	119 (71%)		
T	80 (28%)	49 (29%)	0.95 (0.61–1.48)	.83
Genotype				
C/C	73 (51.41%)	41 (48.81%)	1.00	
C/T	58 (40.85%)	37 (44.05%)	0.88 (0.48–1.60)	.66
T/T	11 (7.74%)	6 (7.14%)	1.03 (0.32–3.65)	1.00
rs3890733				
Allele	**141**	**88**		
C	197 (70%)	126 (72)		
T	85 (30%)	50 (28%)	1.08 (0.70–1.68)	.75
Genotype				
C/C	74 (52.48%)	47 (53.41%)	1.00	
C/T	49 (34.75%)	32 (36.36%)	0.97 (0.52–1.80)	1.00
T/T	18 (12.77%)	9 (10.23%)	1.27 (0.49–3.48)	.66

Abbreviations: CI, 95% confidence interval; OR, odds ratio; *p*-value.

TABLE 3 Patient stratification analysis according to age: Above and below 60 years old and genotype distribution.

rs3890733	>60 years N = 50 (%)	<60 years N = 91(%)	OR, CI and *p*-value
Codominant			
C/C	29 (58%)	45 (49.5%)	OR = 1
C/T	19 (38%)	30 (33%)	OR = 1.02,CI = 0.49–2.13
T/T	02 (4%)	16 (17.5%)	OR = 5.16,CI = 1.1–24.10, ***p* = .043**[a]
Recessive			
C/C + C/T	48(96%)	75(82.4%)	OR = 1
T/T	02(4%)	16(17.6%)	OR = 5.12,CI = 1.13–23.27, ***p* = .012**[a]

Abbreviations: CI, 95% confidence interval; OR, odds ratio; *p*-value.
[a]Statistically significant *p*-value.

We also evaluated haplotype combinations in our data, but no association either to OP susceptibility or gene–gene interactions (epistasis) was detected in this study (Supporting Information Data 3). However, when considering the *VDR* SNPs and skin deposition melanin in the patients' group, SNP rs1540339 T/T genotype was associated with low susceptibility for individuals from group II (fair and fair light skin tone) in both codominant and recessive models (OR = 0.2, 95%CI = 0.05–0.8, $p = .043$ and OR = 0.24, 95%CI = 0.06–0.94, $p = .029$; Supporting Information Data 4).

Multivariate analysis of genotypes with clinical features such as Ca, vitamin D and BMD of total hip and femoral neck has not presented statistically significant results (data not shown).

4 | DISCUSSION

This study assessed the TagSNPs rs3890733, rs11168268 and rs1540339 within *VDR* and their role in OP susceptibility and its traits. *VDR* polymorphisms have been associated with both OP susceptibility and BMD in several populations (Horst-Sikorska et al., 2013; Mohammadi et al., 2014; Pouresmaeili et al., 2013; Singh et al., 2013; Wang et al., 2021).

Age-related bone loss is usually asymptomatic, with different genetic and environmental influences on BMD in several sites throughout the skeleton (Pouresmaeili et al., 2013). However, we identified TagSNP rs3890733 T/T genotype association with an increased risk of presenting OP at a younger age (< 60 years old) in both codominant and dominant analysis, which could relate to a more intense loss in bone turnover over the years due to the fact that in the first 10 years after menopause, women show a marked loss of bone mass (Finkelstein et al., 2008). Estrogen is one of the main elements in physiological bone remodelling. Its deficiency after menopause causes increased production of cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF), which increase the half-life of the osteoclast and the differentiation of pre-osteoclastic cells in mature osteoclasts. The lack of the hormone also decreases the number of osteoblasts and osteocytes, which impairs the detection of microdamage and immediate repair of bone mass (Pacifici, 1996; Rahnama et al., 2013).

The SNP rs1168268 G/G was associated with increased BMD in this study. Noteworthy, this particular SNP tags the extensively studied SNP *BsmI* (rs1544410). The *BsmI* is an intronic variant with unknown protein consequence; however, it is in strong linkage disequilibrium with the polyA variable number of tandem repeats in the 3′ untranslated region (3'UTR) (Ingles et al., 1997). Therefore, it may influence *VDR* transcript (Uitterlinden et al., 2004), which in turn enhances vitamin D proper function and leads to increased BMD. So, the G/G genotype could be an attenuating factor for patients with hip OP. Therefore, this TagSNP could improve the BMD in these patients. Furthermore, it covers three well-recognized SNPs (Apa-I, Bsm-I and Taq-I) that may potentially influence the stability of RNAm at the VDR gene (Chen et al., 2020). Some studies observed an association between the presence of these restriction sites (Apa-I, Bsm-I and Taq-I) with increased BMD, higher peak bone mass and even a decrease of bone loss in Iranian, Turkish, Chinese and Dutch populations (Creatsa et al., 2011; Jakubowska-Pietkiewicz et al., 2012; Li et al., 2012; Özaydin et al., 2010; Pouresmaeili et al., 2013; Qin et al., 2004). However, the finding of this study is unusual in the Brazilian population.

The TagSNP rs1540339 T/T genotype was associated with lower susceptibility to OP in group II (very low to low melanin skin deposition), compared to group I (moderate to high melanin skin deposition). Our data disagree with those of Horst-Sikorska, which show that decreased susceptibility to OP and its fractures are substantially lower in African-derived subjects—which usually present moderately high melanin when compared to Caucasian and Asian-derived origin, with very low to low melanin skin deposition (Horst-Sikorska et al., 2013). This particular TagSNP rs1540339, located in the intron 4 within *VDR* and has no precise function, has previously been associated by our research group with Type I Diabetes (De Azevêdo Silva et al., 2013) and tags three other SNPs rs2239181, rs2239179 and rs886441. At the same time, the rs2239179 has been associated with lower susceptibility to melanoma in a Caucasian-derived population (Ogbah et al., 2013). Melanoma is a type of cancer strongly influenced by sun exposure, and melanin is known to act as a biological protector against UV light damage to DNA (Böhm et al., 2005). Therefore, Caucasian-derived individuals with less melanin than African-derived with moderate to high melanin may display advantages in vitamin D production but with increased UV light exposure consequences (Lupsa & Insogna, 2015).

In this study, we observed that 61% of all patients presented low vitamin D levels, which agrees with most studies showing that low vitamin D status is a risk factor for BMD loss in postmenopausal women (Chang & Lee, 2019; van der Wielen et al., 1995). Estrogen has an essential role in increasing the activity of the enzyme responsible for activating vitamin D; therefore, declining estrogen levels during menopause could lead to vitamin D deficiency (LeBlanc et al., 2014). Furthermore, even though 25-hydroxyvitamin D serum level of 20 ng/mL seems to be enough for homeostasis of bone metabolism, it is considered low for the non-classical roles of vitamin D, leading to other pathologies (Lupsa & Insogna, 2015).

Low vitamin D levels are known to contribute to reducing BMD, increasing the risk of fractures and falls in the elderly. However, in this study, the absence of fractures was more prevalent (93.8%) than its presence. In addition, the patient recruitment was carefully carried out to age-matched healthy controls. Therefore, it is probable that the younger the OP patient, the lower the risk of falling with subsequent fractures. Nevertheless, low vitamin D levels were still inversely associated with increased parathyroid hormone, alkaline phosphatase and osteocalcin levels in postmenopausal women leading to accelerated bone mass loss and low BMD (Capatina et al., 2014; Lips et al., 2001).

In summary, our data show that *VDR* variants are associated with higher BMD and increased susceptibility to OP in patients under 60 years old. Although this study has a limited number of samples and a '*p*-value' close to borderline statistical significance, it is the first study that evaluated SNPs in linkage disequilibrium in a Brazilian population, which emphasizes the role of VDR in the clinical features of the disease in this population.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Inflammation Research

ORIGINAL RESEARCH PAPER

Polymorphisms and expression of inflammasome genes are associated with the development and severity of rheumatoid arthritis in Brazilian patients

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Abstract

Objective In the present study, we analyzed the possible association of inflammasome gene variants and expression to rheumatoid arthritis (RA)'s development and severity in the Brazilian population.

Materials and methods Thirteen single nucleotide polymorphisms within six inflammasome genes (*NLRP1, NLRP3, NLRC4, AIM2, CARD8, CASP1*) as well as *IL1B* and *IL18* genes in two different Brazilian populations (from Northeast and Southeast Brazil) were analyzed. We also evaluated inflammasome gene expression profile in resting and LPS + ATP-treated monocytes from RA patients and healthy individuals. For genetic association study, 218 patients and 307 healthy controls were genotyped. For gene expression study, inflammasome genes mRNA levels of 12 patients and ten healthy individuals were assessed by qPCR.

Results Our results showed that rs10754558 *NLRP3* and rs2043211 *CARD8* polymorphisms are associated with RA development (p value = 0.044, OR = 1.77, statistical power = 0.999) and severity measured by Health Assessment Questionnaire (HAQ) (p value = 0.03), respectively. Gene expression analyses showed that RA patients display activation of *CASP1, IL1B* and *IL1R* genes independently of LPS + ATP activation. In LPS + ATP-treated monocytes, *NLRP3* and *NLRC4* expressions were also significantly higher in patients compared with controls.

Conclusions The first reported results in Brazilian populations support the role of inflammasome in the development of RA.

Keywords SNPs · Autoimmunity · Prognostic and monocytes

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Introduction

Rheumatoid arthritis (RA) is a complex and chronic inflammatory disease associated with progressive joint destruction, disabling and systemic complications. It is characterized by proliferation of synovial tissue, autoantibody production and systemic alterations such as cardiovascular, pulmonary and dermatological [1, 2]. RA is a disorder with an important sex bias, substantially more affecting women from 30 to 50 years old [2, 3]. Even though RA etiology remains unclear, it depends upon the interaction between genetic and environmental factors. The genetic component has a pivotal role in RA etiology with several genes contributing in disease´s triggering [2, 4]. The majority of genetic variations associated with RA development are within immune response-related genes, with the human leukocyte antigen (HLA) variants being the most well-known genetic risk factors [4].

The pathogenesis of RA encompasses abnormal innate and adaptive immune responses, being tumor necrosis factor and interleukin (IL)-6 the main pro-inflammatory cytokines involved in RA pathogenesis [5, 6]. High levels of IL-1β, secreted by monocytes, macrophages and dendritic cells, have also been involved in RA, revealing an important role in bone resorption and cartilage destruction [7]. Indeed, the therapeutic inhibition of IL-1 reduces signs and symptoms of RA as well as radiological damage. Animal models of RA, such as collagen-induced arthritis and antigen-induced arthritis, also respond to IL-1 inhibition [8, 9], underlining the importance of this cytokine in disease's development.

IL-1β secretion is induced by the activation of inflammasomes, which are multiprotein complexes capable of promoting the processing and maturation of IL-1β. Assembly of inflammasomes depends upon the activation of intracellular receptors belonging to Nod-like receptors and PHIN receptors families, such as NLR family pyrin domain-containing 1 (NLRP1), NLR family pyrin domain-containing 3 (NLRP3), NLR family CARD domain-containing protein 4 (NLRC4) and absent in melanoma 2 (AIM2), the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) and the effector protein caspase-1. When sensing pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP), the intracellular receptors recruit the adaptor protein ASC, which triggers the cleavage of pro-caspase-1 into caspase-1. Activated caspase-1 processes pro-IL-1β and pro-IL-18 cytokines into their mature and secreted form. Inflammasome activation is strictly regulated by transcriptional mechanisms (i.e., NF-kB-dependent transcription of *NLRP3, AIM2, IL1B*), post-transductional modifications (i.e., ubiquitination, nitrosylation), as well as by endogenous proteins, such as caspase recruitment domain-containing protein 8 (CARD8), which specifically inhibit NLRP3-inflammasome activation. Pro-IL-1β production is dependent on NF-kB role, being the cytokine secretion a two-stage process [10–12].

Mutations in inflammasome genes may cause rare autoinflammatory disorders characterized by constitutively elevated level of IL-1β contributing to the systemic inflammatory presentation [13]. Polymorphisms in inflammasome genes have been associated to more common chronic inflammatory disorders, such as systemic lupus erythematosus [14], psoriasis [15], Crohn´s disease [16, 17], celiac disease [18, 19], type 1 diabetes [18, 20] and vitiligo [21]. Previous studies reported the association between *NLRP3* and/or *CARD8* and RA in different populations [22–27], and despite their heterogeneity, the results pointed out a major role of NLRP3-inflammasome dysregulation in RA pathogenesis.

Herein, we first performed in Brazilian populations a genetic association study to assess inflammasome and also *IL1B* and *IL18* gene polymorphisms and the susceptibility to RA and its clinical manifestations. Moreover, we analyzed inflammasome components genes as well as *IL1B* and *IL1R* expression profile in peripheral blood-derived monocytes from RA subjects with and without inflammasome activation in vitro.

Methods

Genotyping study

Subjects

For this study, a total of 218 RA patients and 307 healthy individuals, all from Brazil, were enrolled. The Northeastern sample comprised 128 patients (mean age 51.3 ± 11.7 years; mean age at diagnosis 42.1 ± 11.7 years; 122 females and 6 males) and 149 healthy individuals (mean age 39.2 ± 14.2 years; 122 females and 27 males) from the state of Pernambuco, Northeastern Brazil. The Southeast sample comprised 90 patients (mean age 55.7 ± 10.8 years; mean age at diagnosis 44.8 ± 13.2 years; 83 females and 7 males) and 158 healthy controls (mean age 37.4 ± 11.3 years; 76 females and 82 males) from state of São Paulo, Southeast Brazil. Patients from Northeastern Brazil were under care of the Division of Rheumatology of Hospital das Clínicas from Federal University of Pernambuco, whereas patients from Southeast Brazil were recruited from Division of Clinical Immunology of University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo. All RA patients were diagnosed according to the American College of Rheumatology (ACR) criteria [28]; control individuals were healthy blood donors without previous family history

of autoimmune diseases, as reported in appropriate questionnaire. The patients were evaluated for the presence of rheumatoid factor and bone erosions. Disease activity score in 28 joints (DAS28) [29, 30] and Health Assessment Questionnaire (HAQ) [31] were applied to patients as a measurement of disease activity and functional disability, respectively.

Aiming to maintain a good statistical power, and also to report the results as from the Brazilian population as a whole, we joined the two groups into one, and then correct the analysis by geographical origin and demographic data. The demographic and clinical features from all assessed RA patients and controls are shown in Supplementary data 1.

All procedures involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the participants provided a written informed consent approved by the local Research Ethics Committee (Southeast: HCRP 2981/2009 and Northeast: CAAE 03065312.3.0000.5208).

DNA isolation

Genomic DNA was isolated from whole blood samples, using DNA Wizard Genomic DNA Purification Kit (Promega, USA) according manufacturer's protocol or using a salting out method [32].

SNPs selection and genotyping

Thirteen SNPs within *IL1B, IL18, NLRP1, NLRP3, NLRC4, AIM2, CARD8* and *CASP1* genes were selected according to previously reported association studies [18, 19] and/or their functional effect on protein activity [16] or mRNA stability [33]. More details about all assessed SNPs are reported in Supplementary data 2.

SNPs genotyping was performed using fluorogenic allele-specific probes (Taqman Probes, Applied Biosystems, USA) on an ABI7500 sequence detection system (Applied Biosystems, USA).

Statistical analysis

Genotype distribution was compared for Hardy–Weinberg (HW) equilibrium using genotype transposer [34]. At a first sight, considering RA patients and controls independently of their geographical origin, the allele and genotype frequencies of controls and RA patients were compared using Chi-square test. Binary logistic regression was used to confirm the association between the polymorphisms and RA, adjusting for origin of sample and gender. Bonferroni's correction for multiple comparisons was applied (pBonf = observed *p* value x N, N = number of studied polymorphisms within the same gene). The possible association of the assessed polymorphisms with DAS28 and HAQ in RA patients was analyzed using the likelihood ratio test. The significance level was set at $\alpha = 0.05$ (two tailed). All statistical analyses were performed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). The eventual presence of linkage disequilibrium (LD) between polymorphisms within the same gene and the association of haplotypes with RA susceptibility were evaluated using the online software SNPStats [35]. The power was verified using G*Power software 3.1.9.2 [36].

Inflammasome genes expression profile in peripheral blood monocytes

Heparinized whole blood samples were obtained from twelve post-menopausal RA female patients (mean age 50.42 ± 13.91 years) and ten healthy post-menopausal female controls (HC) (mean age 57.5 ± 8.14 years) from metropolitan area of state of Pernambuco. None of the patients or controls had received systemic treatment recently, as corticosteroids drugs. To evaluate the correlation of inflammasome mRNA levels and RA activity, measured by DAS28, patients were stratified as high disease activity (DAS28 > 5.1), moderate disease activity (3.2 < DAS28 < 5.1), low disease activity (2.6 < DAS28 < 3.2) or disease in remission (DAS28 < 2.6) [29, 30].

Peripheral blood monocytes were isolated by adherence from PBMCs obtained by centrifugation over Ficoll–Paque (Sigma-Aldrich, USA) gradient. An amount of 5×10^6 PBMCs/well was cultured in RPMI 1640 containing 10% fetal bovine serum in 24-wells microplate (Invitrogen, Life Technology, USA). For this assay, we performed biological replicates. Monocytes were stimulated with 1 µg/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 4 h and 1 mM adenosine triphosphate (ATP; Sigma-Aldrich) for 15′. After incubation supernatants were collected for cytokines measurement, cells were lysed for mRNA isolation and gene expression analysis. RNA isolation from monocytes was performed using the RNAqueous micro kit (Ambion, Life Technologies, USA), following the manufacturer's instructions. The RNA samples were stored at − 80 °C until used and RNA integrity analysis was performed by gel electrophoresis and quantification by Nanodrop 2000 (Thermo Scientific, USA). cDNA synthesis was performed with SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Life Technology, USA).

The major inflammasome genes, *NLRP1, NLRP3, NLRC4, AIM2, ASC/PYCARD* and *CASP1*, and *IL1B* and *IL1R* genes were amplified with specific TaqMan Gene Expression Assays using the ABI 7500 SDS platform (Applied Biosystems, USA). *GAPDH* was the reference gene used for normalization. Relative quantitative expression was calculated comparing RA and healthy individuals cells (fold change (FC) = RA $2^{-\Delta Cq}$/HC $2^{-\Delta Cq}$) following the

indications by Schmittgen and Livak, 2008 [37]. Student's *t* test was applied to compare different groups.

IL-1β measurement

The IL-1β secreted by monocytes was measured with ELISA kit (R&D systems, USA) following manufacturer's protocols and results were expressed in picograms per milliliter. One-way ANOVA test with Bonferroni post-test was applied to compare IL-1β secretion in RA and healthy controls cells as well as stimulated and resting cells.

Results

Genetic association study

Genotype distributions were in Hardy–Weinberg equilibrium for the assessed SNPs with exception of *NLRP3* rs35829419 and *IL1B* rs1143643 SNPs in the controls (p value = 0.0001 and 0.0419, respectively). No association was observed among polymorphisms within *NLRP1, NLRC4, AIM2, CASP1, IL1B* and *IL18* genes and RA in studied population. Genotype and allele frequencies are shown in Table 1.

Considering RA patients and controls independently of their geographical origin, it showed a significant association between the *NLRP3* rs10754558 SNP [C > G] and RA susceptibility with a statistical power = 0.999. The C allele and C/C genotype were significantly more frequent in patients than in controls (67 vs 56%, p value = 0.005, OR = 1.59, 95% CI = 1.23–2.05; and 47 vs 32%, p value = 0.0036, OR = 2.23, 95% CI = 1.32–3.75, respectively). After applying Bonferroni's correction, the association remained statistically significant (pBonf = 0.01 and 0.007, respectively).

When we performed a binary logistic regression adjusting for gender and geographical origin of sample, the association of C/C genotype for *NLRP3* rs10754558 SNP with susceptibility to RA remained statistically significant (p value = 0.044, OR = 1.77, 95% CI = 1.23–3.09) (Table 1), suggesting that the association is not affected by gender or origin.

The association of the studied SNPs with Rheumatoid Factor production and bone erosions development was evaluated and no significant association was observed. Also, the association between SNPs and disease activity, measured by DAS28, was not detected (data not shown).

When we assessed the influence of the studied polymorphisms on disease activity (DAS28) and patient's functional disability (HAQ), the association between *CARD8* rs2043211 SNP [A > T] and a higher mean for HAQ was observed. Patients homozygous for T allele presented a higher functional disability (HAQ mean = 2.13 ± 0.20) when compared to homozygous for A allele (HAQ mean = 1.32 ± 0.11) and heterozygous patients (HAQ mean = 1.41 ± 0.10). This association was observed in both codominant [p value = 0.03, *Akaike Information Criterion* (AIC) = 309.2] and recessive model (p value = 0.01, AIC = 307.5) (Table 2).

Linkage disequilibrium was found for polymorphisms rs12150220 [A > T] and rs2670660 [A > G] within *NLRP1* (D' = 87), rs455060 [A > G] and rs212713 [T > C] within *NLRC4* (D' = 0.94) and also for polymorphisms rs1143643 [C > T] and rs1143634 [G > A] in *IL1B* gene (D' = 0.82). However, the observed haplotypes were not differentially distributed between RA patients and healthy individuals (Data not shown).

Inflammasome expression analyses

We evaluated the expression of major inflammasome genes (*NLRP1, NLRP3, NLRC4, AIM2, ASC* and *CASP1*) and of IL-1β cytokine and its receptor genes *(IL1B* and *IL1R*) in non-stimulated/resting and LPS + ATP-stimulated monocytes from twelve RA patients and ten healthy controls. First, basal as well as LPS + ATP-stimulated expression was evaluated comparing RA patients with controls. We found a statistical significant increase in the basal expression of *CASP1* (+ 499.02-fold, p value = 0.01), *IL1B* (+ 2.976-fold, p value = 0.003) and *IL1R* (+ 216.6-fold, p value = 0.013) genes in untreated monocytes from RA patients when compared with untreated monocytes from healthy individuals (Fig. 1a). In LPS + ATP-treated monocytes, *NLRP3* (+ 14.5-fold, p value = 0.002), *NLRC4* (+ 53.88-fold, p value = 0.04), *CASP1* (+ 19.1-fold, p value = 0.004), *IL1B* (+ 19.7-fold, p value = 0.004) and *IL1R* (+ 14.1-fold, p value = 0.007) genes were statistical significantly upregulated in monocytes from RA patients compared to monocytes from control individuals (Fig. 1b).

LPS + ATP-induced IL-1β secretion in monocytes from RA (LPS + ATP versus ATP: p = 0.007; LPS + ATP versus resting: p = 0.006) as well as from healthy individuals (LPS + ATP versus ATP: p = 0.002; LPS + ATP versus resting, p = 0.006). The stimulation with LPS induced a small increase in IL-1β secretion in controls; however, a greater augmentation was observed in LPS + ATP cells. In RA cells, LPS as well LPS + ATP were able to induce a similar increment in IL-1β secretion.

IL-1β secretion was incremented in RA patients compared to controls in resting (mean = 6.12 ± 10.8 vs. 2.13 ± 2.98 pg/ml), +ATP (12.6 ± 19.1 vs. 2.65 ± 3.26 pg/ml), +LPS (124.2 ± 129.7 vs. 16.12 ± 8.8 pg/ml) and in LPS + ATP cells (287.75 ± 175.63 vs. 273.7 ± 174 pg/ml, respectively). However, no statistical significance was observed between RA and healthy individuals (Fig. 2).

When we evaluated the correlation of inflammasome components and *IL1B* and *IL18* mRNA levels with RA

Table 1 Allele and genotype frequencies from RA patients and controls

SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI	SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI
NLRP1 rs12150220						*CASP1* rs572687					
A	383 (66)	288 (67)		1		G	493 (82)	359 (82)		1	
T	201 (34)	144 (33)	0.77	0.95	0.73–1.24	A	109 (18)	77 (18)	0.92	0.97	0.7–1.34
AA	132 (45)	96 (44)		1		GG	200 (66)	148 (68)		1	
AT	119 (41)	96 (44)	0.66	1.11	0.75–1.64	GA	93 (31)	63 (29)	0.72	0.92	0.61–1.37
TT	41 (14)	24 (12)	0.54	0.8	0.46–1.42	AA	8 (3)	7 (3)	0.96	1.18	0.42–3.33
NLRP1 rs2670660						*IL1B* rs1143643					
A	346 (58)	231 (55)		1		C	390 (65)	307 (71)		1	
G	248 (42)	187 (45)	0.38	1.13	0.88–1.45	T	214 (35)	127 (29)	0.05	0.75	0.57–0.99
AA	97 (33)	62 (30)		1		CC	134 (44)	111 (51)			
AG	152 (51)	107 (51)	0.71	1.1	0.72–1.68	CT	122 (41)	85 (39)	0.39	0.84	0.57–1.24
GG	48 (16)	40 (19)	0.39	1.3	0.77–2.21	TT	46 (15)	21 (10)	0.05	0.55	0.29–1.01
NLRP3 rs35829419						*IL1B* rs1143634					
C	585 (96)	427 (98)		1		G	489 (81)	347 (80)		1	
A	23 (4)	9 (2)	0.16	0.54	0.25–1.17	A	115 (19)	85 (20)	0.86	1.04	0.76–1.42
CC	284 (93)	209 (96)		1		GG	198 (65)	144 (67)		1	
CA	17 (6)	9 (4)	0.56	0.72	0.28–1.75	GA	93 (31)	59 (27)	0.56	0.87	0.58–1.31
AA	3 (1)	0 (0)	0.37	ND	ND	AA	11 (4)	13 (6)	0.35	1.63	0.71–3.73
NLRP3 rs10754558						*IL18* rs1946519					
G	269 (44)	144 (33)		1		C	165 (57)	237 (54)		1	
C	339 (56)	288 (67)	0.005[a]	1.59	1.23–2.05	A	125 (43)	199 (46)	0.54	1.1	0.81–1.51
GG	62 (20)	29 (13)		1		CC	51 (35)	66 (30)		1	
GC	145 (48)	86 (40)	0.43	1.27	0.38–0.85	AC	63 (44)	105 (48)	0.33	1.29	0.77–2.14
CC	97 (32)	101 (47)	0.0036[b, c]	2.23	1.32–3.75	AA	31 (21)	47 (22)	0.66	1.17	0.63–2.19
AIM2 rs35130877						*NLRC4* rs212713					
T	614 (100)	436 (100)		1		T	324 (53)	234 (54)		1	
G	0 (0)	0 (0)	ND	ND	ND	C	290 (47)	200 (46)	0.75	0.95	0.74–1.23
TT	307 (100)	218 (100)		1		TT	84 (27)	63 (29)		1	
TG	0 (0)	0 (0)	ND	ND	ND	CT	156 (51)	108 (50)	0.75	0.92	0.60–1.42
GG	0 (0)	0 (0)	ND	ND	ND	CC	67 (22)	46 (21)	0.8	0.92	0.54–1.55
AIM2 rs2276405						*NLRC4* rs455060					
C	597 (98)	431 (99)		1		A	403 (66)	271 (62)		1	
T	11 (2)	5 (1)	0.55	0.63	0.22–1.83	G	211 (34)	163 (38)	0.32	1.15	0.89–1.48
CC	293 (96)	213 (98)		1		AA	138 (45)	87 (40)		1	
CT	11 (4)	5 (2)	0.54	0.63	0.17–1.99	AG	127 (41)	97 (45)	0.37	1.21	0.82–1.79
TT	0 (0)	0 (0)	ND	ND	ND	GG	42 (14)	33 (15)	0.49	1.25	0.73–2.12

Table 1 (continued)

SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI	SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI
CARD8 rs2043211											
A	434 (72)	300 (69)		1							
T	168 (28)	136 (31)	0.28	1.17	0.89–1.53						
AA	157 (52)	99 (45)		1							
AT	120 (40)	102 (47)	0.13	1.35	0.94–1.94						
TT	24 (8)	17 (8)	0.87	1.12	0.57–2.2						

RA rheumatoid arthritis, *p* Chi-square test *p* value, *OR* odds ratio, *CI* confidence interval, *ND* not determined

*Due to technical issues, only 145 healthy individuals were genotyped for SNP rs1946519

[a]*p* value after Bonferroni correction = 0.01

[b]*p* value after Bonferroni correction = 0.007

[c]Binary logistic regression, adjusted by sex and origin of sample: $p = 0.044$, OR = 1.77, 95% CI = 1.23–3.09

activity, measured by DAS28, no statistical significance was observed (Data not show).

Discussion

In the last years, substantial information has emerged connecting deregulated inflammasome signaling to inflammatory diseases. In this study, we evidenced for the first time the association between SNPs in inflammasome genes and RA development in the Brazilian population. In addition, we demonstrated a dysregulated expression of some inflammasome components as well as IL-1β cytokine and its receptor genes *(IL1B* and *IL1R*) in RA patients.

The *NLRP3* rs10754558 [C > G] C allele seems to confer an augmented risk for the development of RA when in homozygosis (OR = 1.77) with a power up to 99.9%; the moderate value of OR suggest that being RA a multifactorial trait *NLRP3* SNP gives a partial contribution to diseases susceptibility. This SNP is located within the 3′UTR of *NLRP3* gene and according to the PolymiRTS Database 2.0 (http://compbio.uthsc.edu/miRSNP/) its occurrence affects the binding of miRNAs. In the presence of G allele, there is a binding site for miR-3529-3p and miR-549a, while the C allele abrogates such site, but gives rise to a binding site for miR-146a-5p, miR-146b-5p, miR-589-5p and miR-7153-5p. If and how rs10754558 SNP interferes with the binding of these miRNAs *in vivo* remains to be further elucidated. According to Hitomi et al., 2009, functional analyses of *NLRP3* rs10754558 SNP showed that allele G influences higher *NLRP3* expression (1.4-fold) by altering mRNA stability (34). The eventual effect of this 1.4-fold increasing in *NLRP3* expression on the inflammasome activation has not been investigated. The C allele was also associated to type 1 diabetes mellitus susceptibility [18] and to systemic lupus erythematosus development (personal communication, manuscript submitted), suggesting a role of this polymorphism in autoimmunity development.

When considering inflammasome SNPs in RA clinical manifestations, we observed the association between *CARD8* rs2043211 SNP [A > T] (p.C10X) and disease's severity. Patients homozygous for T allele presented a higher functional disability measured by HAQ. CARD8 interacts physically with caspase-1 and negatively regulates caspase-1-dependent IL-1β expression and nuclear factor NFκB activation [38–41]. The rs2043211 polymorphism introduces a premature stop codon (Cys > Stop), which results in the expression of a severely truncated protein [42]. The exact role of CARD8 in inflammasome biology is still unclear. It has been proposed that CARD8 acts as a modulator of NLRP3 activation or it exerts an inflammasome independent role, as NF-kB inductor [43]. CARD8 C10X variation leads to an increased secretion of IL-1β, especially in combination

Table 2 Association between *CARD8* rs2043211 SNP and a higher functional disability

	n	HAQ mean ± SD	dif	95% CI	*p*	AIC
Codominant model						
A/A	61	1.32 ± 0.11	0			
A/T	58	1.41 ± 0.10	0.09	− 0.20 to 0.37	0.03	309.2
T/T	8	2.13 ± 0.20	0.80	0.21 to 1.39		
Recessive model						
A/A–A/T	119	1.36 ± 0.07	0			
T/T	8	2.13 ± 0.20	0.76	0.19 to 1.33	0.01	307.5

dif HAQ mean difference in relation to A/A genotype, *CI* confidence interval, *p p* value, *AIC* Akaike information criterion

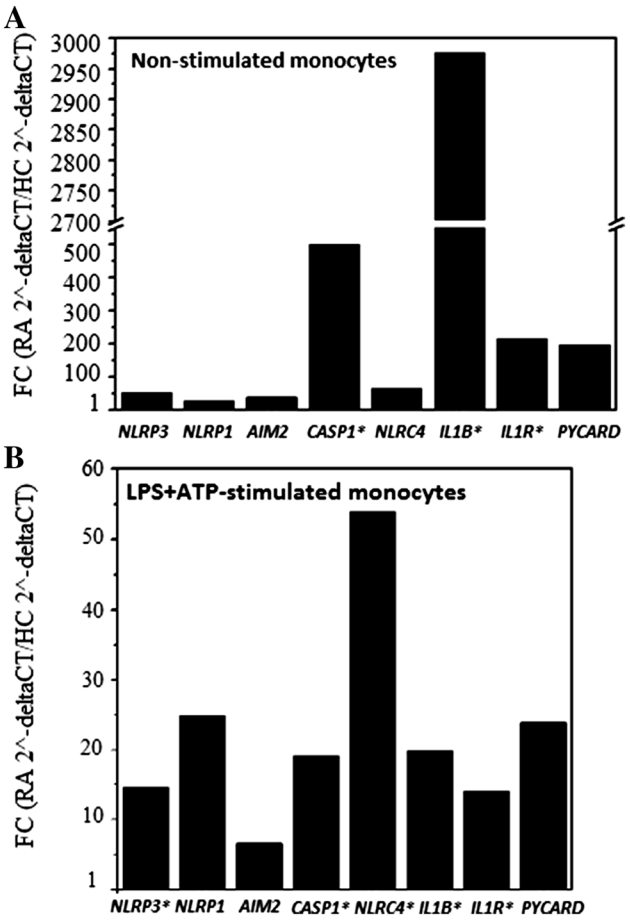

Fig. 1 *NLRP3, NLRP1, AIM2, CASP1, NLRC4, IL1B, IL1R* and *PYCARD* gene expressions in RA patients compared with healthy controls (HC). The results were normalized to *GAPDH* expression. Target gene expression in healthy controls was normalized to 1 (not reported in the graph) and fold change (FC) is reported as RA $2^{-\Delta CT}$/HC $2^{-\Delta CT}$. **a** Expression in non-stimulated monocytes. **b** Expression in LPS + ATP-stimulated monocytes. **p* value < 0.05

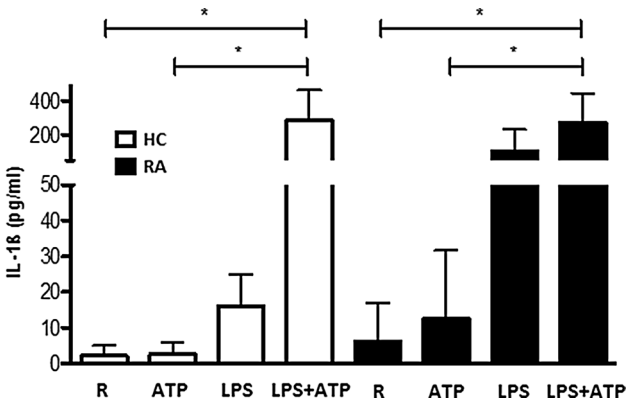

Fig. 2 IL-1β secretion in healthy controls (HC) and RA patients in non-stimulated monocytes (R); ATP-stimulated monocytes (ATP); LPS-stimulated monocytes (LPS); and LPS + ATP-stimulated monocytes (LPS + ATP). **p* < 0.05

with NLRP3 Q705K [16]. Moreover, this variation was associated to an increased induction of NFκB activity and its translocation to the nucleus [44], which leads to high constitutive levels of pro-IL-1β and tumor necrosis factor α, mediators of inflammation in RA [45]. Furthermore, NF-κB has been reported to contribute to the proliferation of synovial cells, and consequently, to bone and cartilage destruction [46, 47].

Kastbom et al., 2010 [27] and Fontalba et al., 2007 [44] also described an association between *CARD8* p.C10X polymorphism and RA severity in Swedish and Spanish patients, respectively. Different from our study, both studies assessed RA severity through cumulative amount DAS28 over 2 years, while we have chosen to measure the severity of the disease by the HAQ due to the fluctuation of the DAS28 during the course of the disease. However, our study corroborates the involvement of *CARD8* p.C10X polymorphism in RA severity and then its possible use as a genetic prognostic marker for RA.

The combination between the minor alleles for *NLRP3* rs35829419 (Q705K) and *CARD8* rs2043211 (p.C10X) polymorphisms was described as associated with delayed apoptosis of neutrophils [48] and RA susceptibility and severity [22]. However, it was not possible to confirm this association in our population, as the *NLRP3* A/A genotype was not found in our patients group. When we tested the

combination between the other genotypes for those polymorphisms, no significant association was observed.

Moreover, our results corroborate the findings from García-Bermúdez et al., 2013 [26], Hamad et al., 2011 [24] and Kastbom et al., 2010 [27], which demonstrated the lack of association of *CARD8* rs2043211 with RA susceptibility when individually analyzed in Spanish, French, Tunisian and Swedish populations. Despite the association between the rs2043211 SNP and RA severity, this SNP was not associated to bone erosions and rheumatoid factor in our population.

When we assessed the gene expression profile for RA patients, we found a statistical significant upregulation of *CASP1, IL1B* and *IL1R* genes in untreated monocytes from RA patients when compared with healthy individuals. This result suggests that RA patients are characterized by a chronic expression of *CASP1, IL1B* and *IL1R* genes. In LPS + ATP-treated monocytes from RA patients, the same genes were upregulated but accompanied by *NLRP3* and *NLRC4* genes. This increased expression of *CASP1, IL1B* and *IL1R* in stimulated monocytes from patients may be consequence of the previous upregulation in resting monocytes. The observed augmented expression of *NLRP3* and *NLRC4* genes in patients after stimulus prompt us to hypothesize that *NLRP3* and *NLRC4* transcriptions are dysregulated in RA patients, contributing to the establishment of the exacerbated inflammation observed in the disease. When we compared the gene expression between patients with disease in remission and patients with high disease activity, no statistical difference was observed, suggesting that the treatment of patients is efficient to control the disease symptoms but may be not able to control the deregulation of inflammasome.

Being aware that other mechanisms may influence upon IL-1β production overall, the observed differences in inflammasome components and IL-1β and its receptor genes expression between patients and healthy controls are accompanied by IL-1β secretion *ex vivo* monocytes. In all studied conditions, the RA monocytes secreted higher amounts of IL-1β. Basal secretion of IL-1β in RA monocytes was higher compared to healthy ones, indicating a constitutive secretion of pro-inflammatory cytokine.

The exact mechanisms responsible for the production and secretion of IL-1β remain unclear, but two signals are traditionally required. The first signal, in our case LPS, induces the transcription of pro-IL-1β and inflammasome subunits; the second signal promotes rapid activation of caspase-1 and then secretion of mature IL-1β [49, 50]. This second signal is provided by reduction of intracellular K + generated by ATP stimulus. Of note in both LPS-stimulated and ATP-stimulated RA monocyte, the signals individually are sufficient to induce an augment of IL-1β secretion, without the common first or second signal mediation (Fig. 2), suggesting an inflammasome complex more prone to activation in RA monocytes than in healthy ones.

Mathews et al., 2014 [23] also showed a higher *NLRP3* and *CASP1* expression in RA patients. However, in this study, the gene expression was characterized directly in peripheral blood mononuclear cells of patients. Thereby, the upregulation of these inflammasome components could be due to the abundant release of DAMPs upon tissue damage, typical in RA pathogenesis [51–53]. Differently, our study evaluated the inflammasome gene expression in RA and control individual's monocytes under the same conditions, proving indeed the dysregulated transcription of these inflammasome components.

The importance of *NLRP3* in RA pathogenesis is confirmed by the findings of increased *NLRP3* mRNA in the synovium of RA patients compared to individuals suffering from non-autoimmune osteoarthritis [25]. The NLRP3-inflammasome was described as an activator of both apoptotic and pyroptotic cell death [54]. Therefore, besides the excessive IL-1β secretion, the deregulated activation of this complex may exacerbate the cell death, contributing to the inflammatory process and its maintenance in RA disease.

Conclusion

In conclusion, herein, we provide enough data to infer that *CASP1, IL1B* and *IL1R* are activated in RA patients as well as *NLRP3* and *NLRC4* genes are dysregulated transcript upon stimulus. We also demonstrated that *NLRP3* and *CARD8* polymorphisms are associated to RA susceptibility and severity in our studied populations. These results are useful to help understanding the role of inflammasome complex in RA.

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Author contributions CAJC conducted experiments, wrote the manuscript and prepared the tables and figures; HLAC, JEA and ALMTR helped conducting the experiments and provided insightful suggestions to the manuscript; PSG, JAS, AP and SC assisted in the study design and coordination, and read, corrected and provided major suggestions to this manuscript; TSF, AD, LFRJ, ALBPD, RDRO, PLJ and EAD recruited patients and participated in data acquisition. All authors addressed important intellectual content and approved the final manuscript for publication.

Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

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ORIGINAL ARTICLE

Differential expression of the inflammasome complex genes in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder involving heterogeneous clinical manifestations and numerous susceptibility genes. Several findings evidence the critical role of inflammasomes in the predisposition to autoimmune diseases and in SLE. We investigated whether inflammasome polymorphins could affect susceptibility to develop and/or severity SLE. Moreover, differences in inflammasome activation in peripheral blood were also evaluated in SLE patients and controls. The distribution of 13 SNPs in eight inflammasome genes was evaluated. To assess inflammasome priming in peripheral blood monocytes of SLE and controls, differential expression of selected inflammasome genes and IL-1ß production was analyzed in resting condition as well as after LPS and ATP stimulation. Results showed that the gain-of-function variant rs10754558 (*NLRP3)* was significantly more frequent in SLE patients with nephritis, reinforcing the concept of a key role of *NLRP3* inflammasome not only in SLE but also especially in kidney disease. SLE monocytes in resting condition showed a higher level of IL-1ß expression and produced higher levels of IL-1ß when stimulated with LPS+ATP comparing to controls. The stimulation induced a significant expression of *NLRP1*, *AIM2*, *CASP1*, and *IL1B* genes, suggesting that the *NLRP1* inflammasome is responsible for the IL-1ß production observed in monocytes. These data emphasized once more the important contribution of inflammasome in SLE-associated inflammation.

Keywords Systemic lupus erythematosus · Polymorphisms · Inflammasome · Gene expression · Nephritis

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease that predominantly affects women of childbearing age. SLE hallmark is the generation of autoantibodies that react with self-nuclear and cytoplasmic antigens, culminating in immunocomplex deposition in several organs, inducing cell death and organ failure (Davidson and Diamond 2001; Tsokos et al. 2007). Although under intense investigations, the genetic basis of human SLE is still not fully understood (Ghodke-Puranik and Niewold 2015; Tsao 2003; Croker and Kimberly 2005).

Several studies indicate that abnormal activation of genes related to the inflammatory response, resulting in an altered activation of IL-1ß and/or NF-kB, may contribute to the pathogenesis of autoimmune disorders with a strong inflammatory component, as observed in SLE (Shinkai and TH 2008; Shaw et al. 2011a; Aksentijevich et al. 2007; Magitta et al. 2009;

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Yang et al. 2015a; Kahlenberg and Kaplan 2014a). In the last few years, the innate immune signaling complex, called inflammasome, has garnered support for a role in triggering and maintaining SLE (Kahlenberg and Kaplan 2014a). Different innate immune cytoplasmic receptors, belonging to Nod-like Receptors/NLRs (i.e., NLRP1, NLRP3, NLRC4) or PYHIN (i.e., AIM2, IFI16) families, have been described as able to assemble an inflammasome in response to pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) leading to caspase-1 activation and consequent cleavage and secretion of pro-inflammatory cytokines IL-1β and IL-18 (Shaw et al. 2011b; Man and Kanneganti 2015; Ito et al. 2014).

Several lupus-associated DAMPs (i.e., generation of reactive oxygen species due to inefficient clearance of cellular debris; impaired clearance of neutrophil extracellular trap (NET); accumulation of cytosolic self DNA) could be recognized by inflammasome receptors consequently inducing an inflammatory response (Yang and Chiang 2015).

NLRP3 inflammasome activation has been described as increase in lupus macrophages (Kahlenberg et al. 2013) and two recent studies suggested that this induction appeared to be, at least partially, caused by lupus-specific autoantibodies (Zhang et al. 2016; Shin et al. 2013a). NLRP3 inflammasome plays an important role also for the progression of SLE, contributing to the development of nephritis (Ka et al. 2015; Li et al. 2015).

Recently, it was demonstrated that the hyper-expression of NLRP3 in myeloid cells induces a severe disease in an experimental model of lupus (Lu et al. 2017). Even if NLRP3 remains the first candidate DAMPs' receptor involved in SLE pathogenesis, other inflammasome components have been pointed out as possible contributing factors. Our research group demonstrated that gain-of-function polymorphisms in the receptor *NLRP1* gene were associated to SLE and SLE-associated nephritis, rash, and arthritis (Pontillo et al. 2012). Accordingly, a deregulation not only of NLRP3 but also of NLRP1 inflammasomes has been reported in patients with SLE (Yang et al. 2014).

Accordingly, a deregulation not only of NLRP3 but also of NLRP1 inflammasomes has been reported in patients with SLE (Yang et al. 2014). Even though, the role of inflammasome in the pathogenesis of SLE should be more deeply elucidated (Yang et al. 2015b; Camargo et al. 2004; Wang et al. 2013; Wen et al. 2014). So, to better understand the possible impact of inflammasome gene disregulation in SLE development and its clinical phenotype, we analyzed a selected panel of single-nucleotide polymorphisms (SNPs) in *NLRP*1, *NLRP3*, *NLRC4*, *AIM2*, *CARD8*, *CASP1*, *IL1B*, and *IL18* genes; moreover, inflammasome activation was evaluated in monocytes from SLE patients to further characterize inflammasome profile in these individuals.

Materials and methods

Subject

We recruited 132 SLE patients (129 women/3 men, mean age 37.1 years ± 10.5) and 154 healthy controls (HC) (125 women/29 men, mean age 33.5 years ± 13.4) at the Clinical Hospital of Federal University of Pernambuco (HC-UFPE), from metropolitan region of Recife (Pernambuco, Brazil). Patients were classified according to the criteria of the American College of Rheumatology (ACR) (Hochberg 1997) and in the cumulative organic damage index (SLICC/ACR) or disease activity index (SLEDAI). The control group was composed of healthy volunteers without SLE or any other autoimmune diseases, or other problems that may impair the immune system. Subjects with diabetes mellitus, renal or hepatic dysfunction, acute or chronic inflammatory disease, cancer, and infection diseases were excluded from the study. Subjects were chosen randomly in the population, sex-, age-, and ethnicity-matched and from the same geographical area of the patients (metropolitan Recife, PE). The demographic, clinical, and laboratory profiles of patients and controls are reported in Table 1.

The following laboratory and clinical data regarding the SLE patients were collected: hematological alterations (hemolytic anemia, leucopenia, lymphopenia, thrombocytopenia), immunological alterations (Anticardiolipin, Anti-Sm, Anti-

Table 1 Demographic, clinical, and laboratory data of Brazilian case/control cohort of SLE. Data are expressed as number of individuals and percentage or means ± standard deviation. ANA: antinuclear antibody test

Characteristic	SLE ($n = 132$)	HC ($n = 154$)
Sex, male/female; *n* (%)	3 (2)/129 (98)	29 (19)/125 (81)
Age, years; mean ±SD	37.1 ± 10.5	33.5 ± 13.4
Lupus blood tests	*n* (%)	
Anti-dsDNA positiveness	37 (27%)	
ANA positiveness	114 (82%)	
Immunologic alterations[a]	44 (32%)	
Hematologic alterations[b]	82 (59%)	
Clinical manifestations	n (%)	
Cutaneous manifestations	89 (64%)	
Photosensitivity	85 (61%)	
Lupus arthritis	88 (63%)	
Oral ulcers	29 (20%)	
Nephritic disorders	58 (42%)	
Neuropsychiatric disorders[c]	12 (8%)	

[a] Anticardiolipin, anti-Sm, anti-RNP, anti-Ro/SSA, anti-La/SSB

[b] Hemolytic anemia, leucopenia, lymphopenia, thrombocytopenia

[c] Seizures, headache, psychosis

RNP, Anti-Ro/SSA, Anti-La/SSB), presence of antinuclear antibodies (ANA), presence of anti-double-strand DNA antibody (anti-ds-DNA), antiphospholipid syndrome (APS), photosensitivity, serositis (pleuritis, pericarditis), Lupus arthritis, cutaneous manifestations (malar or discoid rashes), oral ulcers, neuropsychiatric disorder (seizures, headache, psychosis), Raynaud phenomenon, and nephritic disorder. Patients were classified as having nephritic disease based on laboratory parameters, specifically changes in urine summary and 24-h proteinuria, as follows: persistent proteinuria (> 0.5 g/day or 3+) or abnormal cylindrury. All the participants provided a written informed consent approved by the local Research Ethics Committee (CAAE 03065312.3.0000.5208).

DNA isolation and genotyping

Genomic DNA from SLE patients and controls was extracted from peripheral blood using the DNA Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

Thirteen SNPs were selected within *NLRP1*, *NLRP3*, *NLRC4*, *AIM2*, *CARD8*, *CASP1*, *IL1B*, and *IL18* genes according to previously reported association studies (Pontillo et al. 2010; Pontillo et al. 2011) and/or their functional effect on protein activity or mRNA stability (Roberts et al. 2010; Hitomi et al. 2009), and minor allele frequency (MAF; > 5%) (Supplementary File 1).

SNPs were genotyped using allele-specific TaqMan assays (ThermoFisher Scientific, California, USA) and qPCR in a ABI7500 Real-Time PCR equipment (Thermo Fisher Scientific, California, USA). SDS software v2.3 (Thermo Fisher Scientific) was used for allelic discrimination.

Statistical analysis

SNPs distribution in case/control cohort as well as in SLE groups (stratified according to clinical presentation) was analyzed by multivariate association based on general linear model (GLM) adjusted for confounders variables (age, sex, race) using statistical program R package "SNP-association" version 1.5–2. Genetic analyses were performed taking into account co-dominant, dominant, recessive, and overdominant models for all SNPs. The Haploview software was used to investigate the linkage disequilibrium (LD) and to derive the haplotypes. A significant threshold of $p = 0.004$ was assumed after Bonferroni correction for multiple SNPs analysis ($p = 0.05/n$; $n = 13$ SNPs).

Inflammasome gene expression assays

Subjects

We investigated the relative mRNA expression of *IL-1β*, *NLRP1*, *NLRP3*, *NLRC4*, *AIM2*, *ASC/PYCARD*, and *CASP1* genes in peripheral blood–derived monocytes among subjects within the case/control study (10 SLE patients and 10 healthy individuals) and if their expression differed between the basal condition and after LPS+ATP stimulation.

All the patients selected for gene expression study had a remission for SLE. The healthy individuals were sex-, age-, and ethnicity-matched according to patients and did not present recent illness (autoimmune diseases, diabetes mellitus, renal or hepatic dysfunction, acute or chronic inflammatory disease, cancer, infection diseases) or any pharmacological treatment before blood collection.

Peripheral blood monocytes culture

Heparinized whole blood samples were obtained from ten post-menopausal SLE female patients (mean age 42.6 ± 12 years) and ten healthy post-menopausal female controls (HC) (mean age 57.5 ± 8.14 years). To establish a condition where both patients and controls ex vivo cell cultures would clear up from no reported inflammatory responses (in HC), cells were cultured overnight and after this period medium was changed. This procedure allowed cells to metabolize any ex vivo conditions before initiate inflammasome stimuli. To stimulate, we exposed 0.5×10^6 peripheral blood monocytes with 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 4 h and then with 1 mM adenosine triphosphate (ATP; Sigma-Aldrich) for 15′ in RPMI-1640 + 10% fetal bovine serum/FBS (ThermoFisher Scientific). Inflammasome genes modulation was evaluated in monocytes by real-time quantitative PCR and gene expression specific Taqman assays (Thermo Fisher Scientific).

IL-1β measurement

The secreted IL-1β was measured with ELISA (IL-1β assays, R&D systems, USA). Results were expressed in picograms per milliliter. Secretion differences between patients and controls were tested with Mann-Whitney U test with SPSS 15.0 (SPSS, Inc., Chicago, IL, USA).

Relative gene expression analysis

Total RNA was isolated using the RNAqueous micro kit (Ambion, Thermofisher Scientific, USA). RNA integrity was assessed by gel electrophoresis and quantification by Nanodrop 2000 (ThermoScientific). After retro-transcription of 0.5 μg total RNA (Super Script™ III Reverse Transcriptase (Invitrogen, Thermoscientific)), *IL1B* and selected inflammasome genes, namely *NLRP1*, *NLRP3*, *NLRC4*, *AIM2*, *ASC/PYCARD*, and *CASP1* were amplified with TaqMan® gene-specific assays and ABI Prism 7500 Real-Time PCR equipment. Gene modulation in SLE monocytes compared to HC and stimulated (LPS) versus unstimulated

resting (R) monocytes were calculated as 2exp-ΔCt ± standard deviation (fold change - FC). The SDS 2.3 software was used to obtain cycle quantification (Cq) values for relative gene expression analysis according to FC method (Schmittgen and Livak 2008). *GAPDH* was the reference gene used for normalization (ΔCt).

Statistical analysis

The comparison among expression levels of studied genes and patients and healthy control group were calculated using Student's *t* or Mann-Whitney *U* tests as appropriate. GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) was used for statistical analyses and differences were accepted as significant for p values < 0.05.

Results

Genotyping study

We genotyped 13 SNPs located at eight inflammasome genes in 132 SLE unrelated patients and 154 healthy controls. Genotypes distribution was in Hardy-Weinberg equilibrium ($p > 0.05$). None of the studied polymorphisms resulted differently distributed in cases and controls (Table 2).

Linkage disequilibrium analysis revealed that *IL1B* SNPs rs1143643 and rs1143634 were in strong LD (D' = 100), whereas *NLRP1* rs12150220 and rs2670660, as well as *NLRP3* rs10754558 and rs35829419 in moderate LD (D′ = 78 and D' = 0.86, respectively). The distribution of *IL1B*, *NLRP1*, and *NLRP3* haplotypes was not significantly different between cases and controls (Supplementary File 2).

Then we analyzed SNPs distribution according to SLE clinical presentation: none of the studied SNPs resulted significantly associated to clinical features or laboratory parameters after Bonferroni correction with the exception of *NLRP3* rs10754558 which correlated with lupus nephritis (Table 3).

SLE individuals carrying rs10754558 minor G allele were significantly more frequent ($p = 0.0004$) in patients with nephritis (0.68) than in patients without kidney involvement (0.36), according to a dominant model of inheritance (C/G + G/G; OR = 3.88; 95%CI = 1.80–8.40). This result was poorly affected by confounders variables: age, sex, and race, ($p_{adj} = 0.0005$; $OR_{adj} = 4.0$; 95%CI = 1.79–8.92).

Of note, some SNPs resulted differently distributed according to clinical or laboratory data ($p < 0.05$), however, the analysis did not reach the statistical significance after Bonferroni correction (Table 3). *NLRP1* rs2670660 was less frequent in SLE patients positive for anti-DNA antibodies (0.08 versus 0.29) according to a recessive model of inheritance (G/G; $p_{adj} = 0.007$; $OR_{adj} = 0.22$). *CARD8* rs2043211 resulted more frequent in SLE patients with cutaneous manifestations (0.15 versus 0.02) according to a recessive model of inheritance (T/T; $p_{adj} = 0.022$; $OR_{adj} = 7.34$), and less frequent in SLE with hematologic involvement (0.27 versus 0.49) according to an overdominant model of inheritance (A/T; $p_{adj} = 0.024$; $OR_{adj} = 0.37$). *IL1B* rs1143643 was more frequent in SLE patients with photosensitivity (0.67 versus 0.44) according to a dominant model of inheritance (C/T + T/T; $p_{adj} = 0.009$; $OR_{adj} = 2.76$). *NLRC4* rs455060 was more

Table 2 Association results for case/control analysis. Inflammasome SNPs genotypes distribution in SLE patients (SLE) and healthy controls (HC) was analyzed by general linear model (GLM). Genotypes distribution in patients, p value and p value adjusted for sex, age, and race are reported. p value < 0.05 are underlined

Gene	SNP ID	Genotypes	SLE ($n = 132$)	HC ($n = 154$)	p	p_{adj}
NLRP1	rs2670660	A/A-A/G-G/G	43-62-19	47-66-22	0.985	0.940
NLRP1	rs12150220	A/A-A/T-T/T	68-47-9	75-55-16	0.607	0.860
NLRP3	rs35829419	C/C-C/A-A/A	126-6-0	143-9-2	0.609	0.633
NLRP3	rs10754558	C/C-C/G-G/G	61/60/11	60/64/30	0.012	0.074
NLRC4	rs455060	A/A-A/G-G/G	58-51-16	59-69-21	0.526	
AIM2	rs2276405	C/C-C/T-TT	112-5-0	117-6-0	1.0	
AIM2	rs35130877	T/T-G/T-T/T	126-0-0	152-0-0	1.0	
CARD8	rs2043211	A/A-A/T-T/T	58-51-6	84-59-7	0.681	
CASP1	rs572687	G/G-A/G-A/A	89-32-5	95-44-3	0.439	
IL1B	rs1143643	C/C-C/T-T/T	60-58-8	67-58-14	0.496	
IL1B	rs1143634	G/G-A/G-A/A	79-28-7	87-41-6	0.555	
IL18	rs1946519	C/C-A/C-A/A	41-53-20	46-60-27	0.876	

Table 3 Association results stratified for clinical and laboratory data. Inflammasome SNPs genotypes distribution in SLE patients was analyzed according to clinical and laboratory variables by general linear model (GLM). *p* value and *p* value adjusted for sex, age, and race are reported. *p* value < 0.05 are underlined; *p* value < 0.004 are indicated in bold characters. Adjusted p values are reported within brackets

Gene	SNP ID	Clinical/laboratory data	*p* value
NLRP1	rs2670660	Anti-DNA antibodies	0.008 (0.007)
NLRP3	rs10754558	Nephritis	0.0004 (0.0005)
NLRC4	rs455060	Neurological presentation	0.122 (0.027)
CARD8	rs2043211	Cutaneous manifestations	0.022 (0.022)
CARD8	rs2043211	Hematological involvement	0.036 (0.024)
IL1B	rs1143643	Photosensitivity	0.009 (0.008)

frequent in SLE women with neurologic presentation (0.56 versus 0.20) according to a dominant model of inheritance (A/G + G/G; $p_{adj} = 0.027$; $OR_{adj} = 5.16$).

No significant association with antinuclear antibodies (ANA) was evidenced, however, a different distribution of *NLRP1* rs2670220, *CARD8* rs2043211 was observed. *NLRP1* rs2670220 was less frequent between patients with immunological alterations (0.08 versus 0.26; $p_{adj} = 0.011$; $OR_{adj} = 0.25$). Similarly, *CARD8* rs2043211 was less frequent between patients with immunological alterations (0.01 versus 0.13; $p_{adj} = 0.013$; $OR_{adj} = 0.10$) (data not shown).

Inflammasome expression analysis

Then, we questioned whether an inflammasome signature characterizes SLE patients, as reported for other autoimmune and chronic inflammatory diseases (Shin et al. 2012a). So, we first evaluated the modulation of inflammasome genes expression in peripheral blood–derived monocytes of SLE and HC individuals, and then the production of IL-1β in these cells.

To investigate the hypothesis that the threshold of NLRP inflammasome responsiveness could be affected in SLE individuals, we evaluated the inflammasome genes modulation in monocytes from SLE versus HC. In un-stimulated resting monocytes we observed a higher expression of *IL1B* (fold change: 50.79; $p = 0.0212$; $t = 2.525$; df = 18) in SLE patients compared to HC (Fig. 1); however, we did not observe any significant modulation in *NLRP3*, *NLCR4*, *ASC/PYCARD*, *AIM2*, and *CASP1* expression ($p > 0.05$).

Within each group, LPS+ATP stimulation induced a significant increased expression of *NLRP1* (fold change 5.89; $p = 0.0009$, $t = 4.205$, df = 14), *CASP1* (fold change 8.68; $p = 0.0028$, $t = 3.427$, df = 19), *IL1B* (fold change 6.37; $p = 0.0051$, $t = 3.162$, df = 19), and *AIM2* (fold change 14.57; $p = 0.0009$, $t = 4.207$, df = 14 (Fig. 2). These data are

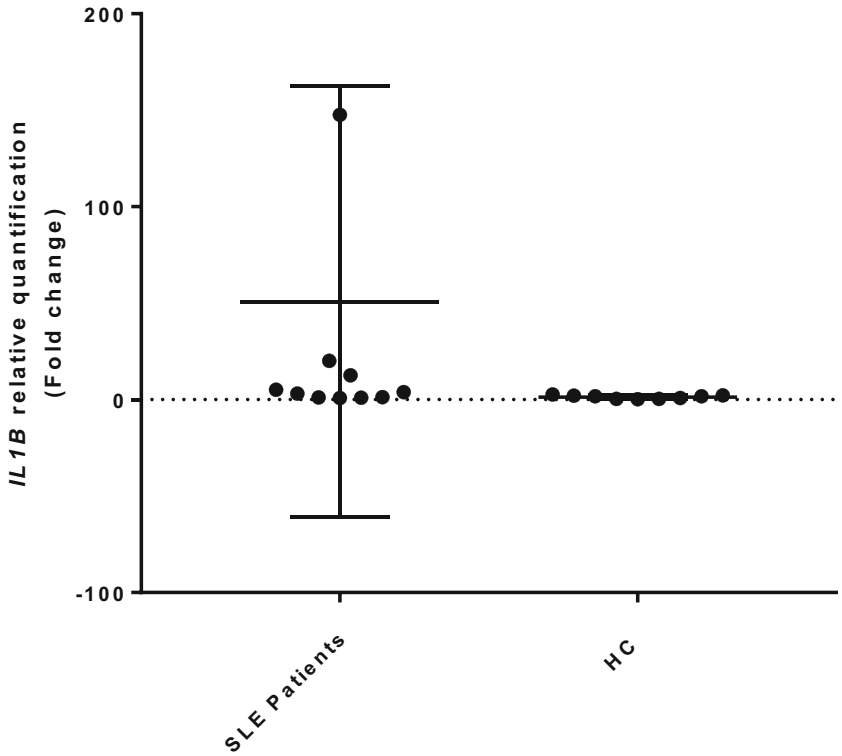

Fig. 1 Modulation of IL1B gene in monocytes isolated from SLE and HC individuals in a resting condition. A total of three out of 10 SLE individuals displayed a higher steady-state expression of *IL1B* transcripts in relation to SLE patients and HC individuals. *IL1B* relative expression (2exp-ΔCt ± standard deviation) between SLE individuals ($n = 10$) and HC (n = 10) showed statistically significant difference ($p = 0.0212$; $t = 2.525$). SLE: Systemic Lupus Erythematosus; HC: Healthy Controls

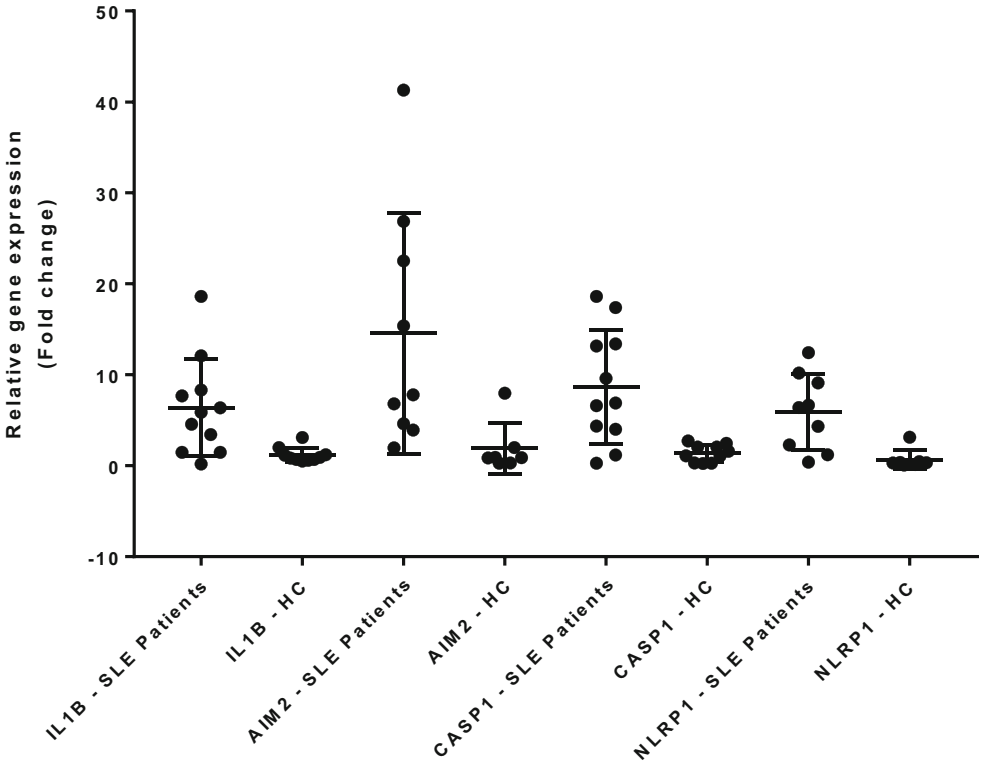

Fig. 2 Modulation of *IL1B* and inflammasome genes in monocytes isolated from SLE individuals and HC individuals with LPS+ATP stimulation. Relative expression (2exp-ΔCt ± standard deviation) between SLE individuals ($n = 10$) and HC ($n = 10$) showed statistically significant for *IL1B* ($p = 0.0051$; $t = 3.162$), *AIM2* ($p = 0.0009$; $t = 4.207$), *CASP1* ($p = 0.0028$; $t = 3.427$), and *NLRP1* ($p = 0.0009$; $t = 4.205$). SLE: Systemic Lupus Erythematosus; HC: Healthy Controls

compatible with the NLRP1 inflammasome being responsible for the IL-1ß production observed in monocytes.

As expected, monocytes from SLE patients produced higher levels of IL-1ß comparing to HC in LPS (lipopolysaccharide) stimulated monocytes as well as in LPS+ATP stimulated cells, however, only the last condition was statistically different ($p = 5.6$ exp-4) (Fig. 3). Intriguingly, this effect appeared to be emphasized in the presence of LPS+ATP, suggesting that the inflammasomes respond actively to LPS and ATP associated.

Discussion

Recent studies have reported the association of *NLRP1*, *NLRP3*, and *IL1B* genes with SLE in terms of susceptibility factors and/or disease severity modulation (Magitta et al. 2009; Pontillo et al. 2012; Wang et al. 2013; Wen et al. 2014). Studies concerning specific polymorphisms in inflammasome-related genes and the relationships with SLE susceptibility are necessary to better understand the involvement of these molecules in SLE pathology. Here, we

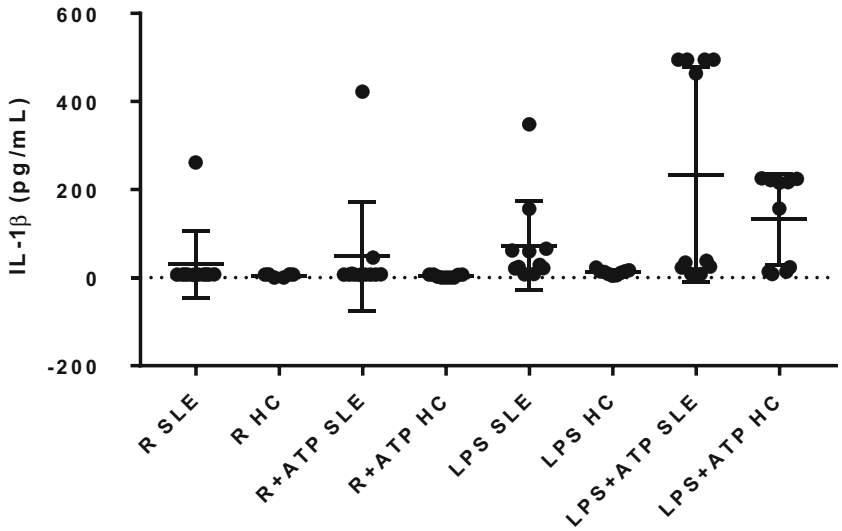

Fig. 3 Production of IL-1ß in monocytes isolated from SLE and HC individuals. Concentration of IL-1ß (pg/mL) in supernatants of monocytes from SLE individuals ($n = 10$) and HC ($n = 10$) in unstimulated/resting condition or stimulated with 1 μg/mL LPS for 4 h and 1 mM ATP for 15 min. Only LPS+ATP condition showed statistically significant difference between groups ($p = 5.6$ exp-4). SLE: Systemic Lupus Erythematosus; HC: Healthy Controls; R: Resting condition; LPS: stimulated with LPS; LPS+ATP: stimulated with LPS and ATP

demonstrated the relation between inflammasome SNPs and SLE clinical manifestation as well as a chronic expression of some inflammasome components in monocytes from SLE patients.

The previously observed association between *NLRP1* polymorphisms and SLE (Li et al. 2015) was not replicated in our study performed on a different Brazilian group of patients and controls, even if the frequency of the SNPs in the populations was similarly distributed in the Southeast of Brazil when comparing to Northeast population in Brazil. On the other hand, the *NLRP3* rs10754558 gain-of-function variant associated with augmented risk to development of lupus nephritis, which apparently is sustained by NLRP3 inflammasome expression findings in experimental model using LPS stimulated monocytes from SLE patients and HC (Shin et al. 2012a; Tsai et al. 2011; Zhao et al. 2013; Zhao et al. 2015; Kahlenberg et al. 2011; Huang et al. 2017; Fu et al. 2017).

The gene expression profile of SLE patients showed an upregulation for *IL1B* gene in resting condition and for *IL1B*, *AIM2*, *CASP1*, and *NLRP1* genes in LPS+ATP-stimulated monocytes when comparing to healthy individuals, suggesting that cells are dramatically sensitized to ligands and respond quickly for signs of stimulation.

Immune complexes formed secondary to antibody recognition of DNA or RNA antigens have been shown to stimulate inflammasome activation through upregulation of TLR-dependent activation of NF-kB and subsequent activation of the NLRP1 and NLRP3 inflammasomes, producing high amounts of IL-1β (Shin et al. 2012b, 2013b; Levandowski et al. 2013). Thereby, the upregulation of inflammasome components is expected since in autoimmune diseases there are abundant releases of DAMPs upon tissue damage, which may activate the inflammasome (Shin et al. 2013b; Kahlenberg and Kaplan 2014b). Therefore, besides the excessive IL-1β secretion, the deregulated activation of these complexes may exacerbate the cell death, contributing to the inflammatory process and its maintenance in SLE disease. Our findings suggest that SLE monocytes may be dramatically sensitized to ligands and respond faster for signs of stimulation, contributing to the establishment of the exacerbated inflammation observed in the disease.

These differences in inflammasome genes expression between patients and healthy controls are underlined by the results observed analyzing IL-1β secretion in monocytes supernatants. In all studied conditions, the SLE monocytes secreted higher amounts of IL-1β. The exact mechanisms responsible for the production and secretion of IL-1β remain unclear, but two signals are traditionally required. The first signal, in our case LPS, induces the transcription of pro-IL-1β and inflammasome subunits (Shin et al. 2012b, 2013b). One-second signal is provided by reduction of intracellular K+ generated by ATP promoting a rapid activation of caspase-1 and then enhancing secretion of mature IL-1β (Perregaux and Gabel 1998; Perregaux and Gabel 1994).

In conclusion, our results indicate that the inflammasome is an important player in lupus pathogenesis. SNPs in genes of inflammasome components are involved in the disease and a chronic expression of some of them was observed, indicating a dysfunction of this protein complex in SLE disease.

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Compliance with ethical standards

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

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T-cell specific upregulation of Sema4A as risk factor for autoimmunity in systemic lupus erythematosus and rheumatoid arthritis

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ABSTRACT

The aim of the present study was to evaluate the impact of *SEMA4A* genetic variants on expression of sema4A protein and its relation to autoimmunity development in Systemic Lupus Erythematosus and Rheumatoid Arthritis patients. A total of 541 SLE patients, 390 RA patients and 607 healthy individuals were genotyped. We also assessed *SEMA4A* mRNA expression from whole blood cells and the in vitro protein production from resting and activated T lymphocytes as well as mature dendritic cells from healthy individuals stratified according to their genotypes for SLE/RA associated *SEMA4A* variants. Our results showed that T/T genotype for rs3738581 SNP is associated with both RA and SLE development ($p = .000053$, OR = 2.35; $p = .0019$, OR = 2.07, respectively; statistical power = 100%) and also to an increased in vitro sema4A production in active T lymphocytes. Our findings are indicative of a T cell-specific upregulation of sema4A in the presence of T/T genotype, being a risk factor for SLE and RA.

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KEYWORDS
SEMA4A; systemic lupus erythematosus; rheumatoid arthritis; polymorphism; T cells

1. Introduction

Semaphorins, first described as guidance factors that assist axon pathfinding during neuronal development [1], have been shown to play important roles in heart morphogenesis [2,3], vascular growth [2,3], tumour progression [4,5], and immunoregulation [6]. This group of proteins has been divided into different subclasses I to VII according to their structure, being the classes III–VII expressed in vertebrates [7].

The Semaphorin 4 A (sema4A) is highly expressed on the surface of mature dendritic cells (DC) and active T lymphocytes, but poorly expressed on B and resting T cells membrane [8]. DC-derived sema4A showed to be essential for T cell priming as well as T-cell derived sema4A for T helper type 1 (Th1) and 17 (Th17) differentiation [9].

Sandrin-Garcia et al. [10] evaluated the expression profile of 4500 genes in patients with active and inactive systemic lupus erythematosus (SLE): a total of 156 genes were differentially expressed between patients and healthy controls. Within them, *SEMA4A* gene was upregulated (+32-fold change) in active SLE patients. Considering the imbalance of T helper cell subsets in SLE pathogenesis and the involvement of sema4A on the immune cell regulation [11], we considered the *SEMA4A* gene as a potential target gene in susceptibility to SLE and autoimmunity development.

In this study, we have investigated the hypothesis that genetic variants in *SEMA4A* result in an altered expression of sema4A and are involved in autoimmunity development. Therefore, we investigated the impact of *SEMA4A* genetic variants in SLE and rheumatoid arthritis (RA) development in Brazilian populations. Following, to functionally understand the impact of the associated genetic variants, we assessed *SEMA4A* mRNA expression in whole blood cells and the in vitro protein production from resting and activated T lymphocytes as well as mature DC cells from healthy individuals stratified according to their genotypes for SLE and RA associated polymorphism.

2. Material and methods

2.1. SEMA4A *genotyping study*

2.1.1. Subjects

For this study, we enrolled patients and controls from three Brazilian cohorts (a Northeastern cohort from state of

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Supplemental data for this article can be accessed here.

Pernambuco, a Southeastern cohort from São Paulo and a South-Brazilian cohort from Rio Grande do Sul), comprising 541 SLE patients, 390 RA patients and 607 healthy controls. The northeastern sample comprised 123 SLE patients, 118 RA patients and 158 healthy individuals from the state of Pernambuco (Northeast of Brazil). The Southeastern sample comprised 158 SLE patients, 89 RA patients and 189 healthy controls from state of São Paulo (Southeast of Brazil) and the Southern sample comprised 260 SLE patients, 183 RA patients and 260 healthy controls from state of Rio Grande do Sul (South of Brazil). Patients from Northeast of Brazil were under care of the Division of Rheumatology of Clinical Hospital from Federal University of Pernambuco, whereas patients from Southeast and South's Brazil were recruited from Division of Clinical Immunology of University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo and Division of Rheumatology of Clinical Hospital from Federal University of Rio Grande do Sul, respectively. All patients were diagnosed according to the American College of Rheumatology (ACR) criteria for the classification of SLE [12] and RA [13]. As control group, we enrolled healthy blood donors without previous family history of autoimmune diseases, as reported in an appropriate questionnaire. Clinical and laboratory data from all patients were collected from medical records and are shown in Table 1. All the participants provided a written informed consent according to the Declaration of Helsinki and approved by the local Research Ethics Committee (Southeast: CAAE 34636013.9.3001.5440 (SLE and RA), Northeast: CAAE 03065312.3.0000.5208 (SLE and RA) and South: CAAE 01731012.0.0000.5327 (SLE) and HCPA 08-366 (RA)).

Table 1. Controls and patients' characteristics.

	Northeastern	Southeastern	Southern
Controls ($n = 607$)	$n = 158$	$n = 189$	$n = 260$
Females	86.4%	51.6%	35.8%
Males	13.6%	48.4%	64.2%
Age[a]	40.8 ± 14.65	37.4 ± 11.31	42.1 ± 8.9
Rheumatoid arthritis ($n = 390$)	$n = 118$	$n = 89$	$n = 183$
Females	95.3%	92.2%	81.5%
Males	4.7%	7.8%	18.5%
Age[a]	51.2 ± 11.6	55.7 ± 10.8	64 ± 12.7
Age at diagnosis[a]	42.1 ± 11.7	44.8 ± 13.2	46 ± 13.7
Positive rheumatoid factor	71.2%	73.4%	89.7%
Bone erosions	75.8%	89.9%	86.1%
DAS28[b]	4.53 (0–7.84)	5.59 (2.87–7.89)	3.88 (0.84–7.45)
HAQ[b]	1.42 (0–3)	NA	1.22 (0–3)
Systemic lupus erythematosus ($n = 541$)	$n = 123$	$n = 158$	$n = 260$
Females	99%	94.4%	90.8%
Males	1%	5.6%	9.2%
Age[a]	37.5 ± 10.4	39.8 ± 11.9	49.7 ± 15.1
Age at diagnosis[a]	31.2 ± 7.7	NA	33 ± 14
Malar rash	58.9%	55%	56.7%
Discoid rash	17.8%	16%	13.5%
Photosensitivity	67.3%	29.4%	79.3%
Ulcers	19.6%	NA	36.5%
Arthritis	72%	42.6%	83.7%
Serositis	22.4%	25.2%	31.3%
Nephritis	50.5%	55.9%	39.9%
Neurological alterations	7.5%	20.3%	10.6%
Hematological alterations	68.2%	61.8%	70.7%
Positive ANA	93.5%	80.4%	99.5%
Anti-DNA	24.4%	19.6%	46.6%
Anti-Sm	8.42%	NA	17.8%
Antiphospholipid syndrome	5.6%	21%	6.3%
SLEDAI[b]	3.6 (0-16)	NA	3 (0-37)
SLICC[b]	0.9 (0-5)	NA	1 (0-7)

[a]Mean ± SD.
[b]Mean (minimum–maximum).
DAS28: disease activity score in 28 joints; HAQ: health assessment questionnaire; ANA: antinuclear antibody; SLEDAI: systemic lupus erythematosus disease activity index; SLICC: systemic lupus international collaborating clinics; NA: not available.

2.1.2. DNA isolation

Genomic DNA was isolated from peripheral blood samples, using DNA Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the standard protocol from the manufacturer or using a salting out method [14].

2.1.3. SNPs selection and gene genotyping

Polymorphisms were selected crossing and merging data from SNP Browser software 4.0 (Applied Biosystems, Foster City, CA) and HapMap database (hapmap.ncbi.nlm.nih.gov) in order to cover most of the *SEMA4* gene (1q22). The selected tag SNPs - rs3738581 (intronic region, near 3′UTR), rs7695 (3′UTR), rs12401573 (exonic region) and rs3738582 (intronic region)—presented at least 10% minimum allele frequency (MAF) (Figure 1).

Genotyping was performed with fluorogenic allele specific probes (Taqman Probes, Applied Biosystems, Foster City, CA), using an ABI7500 sequence detection system (Applied Biosystems, Foster City, CA). Initially, the four tag SNPs were genotyped in SLE patients and controls and then after analysis, the SLE-associated SNP (rs3738581) was genotyped in RA patients.

2.1.4. Statistical analysis

Genotype frequencies were compared for Hardy–Weinberg (HW) expectations using Genotype Transposer [15]. In a preliminary analysis, the allele and genotype frequencies from healthy controls and RA and SLE patients were compared using chi-square test. Binary logistic regression was used to evaluate the association between the polymorphisms and SLE and RA, adjusting for origin of sample and sex. Bonferroni's correction for multiple comparisons was applied when p-value was significant ($N = 4$). The significance level was set at $\alpha = .05$ (two-tailed). All statistical analyses were performed with SPSS 15.0 (SPSS, Inc., Chicago, IL). The eventual presence of linkage disequilibrium between *SEMA4A* polymorphisms and the association of haplotypes with SLE susceptibility were evaluated by using the online tool SNPStats [16]. The power of the analyses was verified using G*Power software 3.1.9.2 (Kiel University, Germany).

2.2. SEMA4 mRNA and protein expression assays

2.2.1. Subjects

We assessed the *SEMA4A* mRNA expression levels in whole blood cells and the sema4A protein levels from resting and stimulated T cell as well as mature DCs of 28 healthy

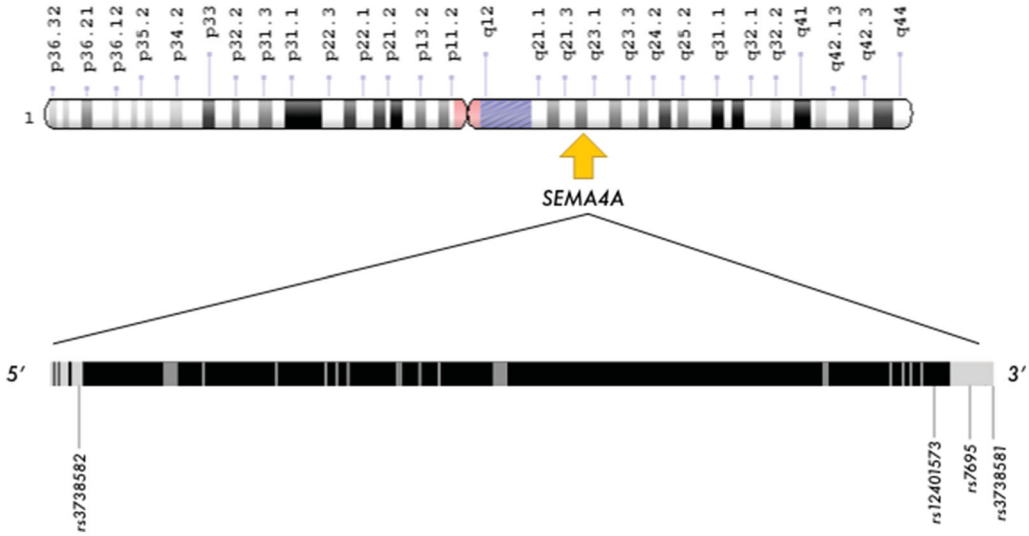

Figure 1. *SEMA4* gene (1q22) structure and studied Tag SNPs; rs3738581 (intron, near 3′UTR), rs7695 (3′UTR), rs12401573 (exon 15) and rs3738582 (intron, near 5′ UTR).

individuals stratified according to their genotypes for the SLE/RA associated *SEMA4A* polymorphism.

2.2.2. Whole blood RNA extraction and cDNA synthesis

Peripheral blood samples were collected and immediately used for RNA isolation, which was performed using the Qiagen Whole Blood RNAse kit, following the manufacturer's instructions. The samples were stored at −80 °C until used and RNA integrity analysis was performed by gel electrophoresis and quantification by Nanodrop 2000 (Thermo Scientific USA).

For cDNA synthesis, we followed the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA) standard protocol, employing for each sample a standard RNA input of 500 ng for each reaction of 20 µL of cDNA. Oligo(dT) was used as primers in all samples. cDNA was stored at −20 °C until qPCR assays.

2.2.3. mRNA expression analysis

Gene expression assays for *SEMA4A* cDNA was performed using the ABI 7500 SDS platform (Applied Biosystems, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* was the reference gene used for normalization (*SEMA4A*: Hs00223617_m1 and *GAPDH*: Hs02758991_g1). Gene expression analyses were performed with technical triplicates. The calculation of $2^{-\Delta Ct}$ was performed for each subject [17]. Student's *t*-test was applied to compare the quantitative expression between different genotypes.

2.2.4. Cells culture and immunophenotype analysis

Peripheral blood (10 mL) was collected in EDTA tube by venipuncture and isolated by density gradient using Ficoll-Paque (Sigma-Aldrich, USA). After centrifugation, the PBMCs ring was collected and washed twice in 1× PBS saline. The cell pellet was suspended in 1 mL of RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% of FBS (Fetal Bovine Serum, Sigma-Aldrich). Cells were counted by means of microscopy in a Neubauer Chamber and the viability always exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma-Aldrich, St. Louis, MO). PBMCs were culture on 96-well plate "U bottom" at a concentration of the 200,000 cells/well for subsequent staining with antibodies.

T lymphocytes were cultured in nonstimulated and stimulated conditions. For stimulation, T cells were cultured with phytohemaglutinin 1%. Following 18 h, cells were harvested and analyzed by flow cytometry.

DCs were generated from monocytes isolated by adherence after 7 days of culture. The following cytokines were used: 50 ng/mL of human granulocyte-macrophage colony stimulating factor (GM-CSF) and recombinant human IL-4 (Sigma-Aldrich, USA) for 5 days. On day 5, the DCs were maturated through 500 ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich). Following two days, DCs were harvested and analyzed by light microscopy and by flow cytometry. The phenotype of the cultured DC was confirmed by the expression of HLA-DR, CD11c, CD80 and CD86, and low levels of CD14 [18,19].

Cells were incubated with conjugated mAb or isotype controls for 30 min protected from light at room temperature. The monoclonal antibodies used were: SEMA4A-FITC, CD80—PE-CY7, CD86—APC, CD14—Texas Red, CD11c—PE and HLA-DR—PerCP-Cy5.5 for DCs; CD3—APCH7 and CD4—PerCP-Cy5.5 for resting T cells; and CD3, CD4 and CD25—FITC for stimulated T lymphocytes. All purchased from BD Biosciences, San Diego, CA. The stained cells were washed twice with PBS, fixed with 2% paraformaldehyde in PBS. Cells were kept at 4 °C and protected from light until analysis by flow cytometry. The frequency of positive cells was calculated from lymphocyte gates from PBMCs and 10,000 events were acquired by size (FSC) and granularity (SSC) using the BD *FACSAria*™ III cell-sorter flow cytometer (BD Biosciences, San Diego, CA). For DC, at least 20,000 events were acquired and analyzed. Compensation was made with non-stained and single

Table 2. *SEMA4A* rs3738581 SNP allele and genotype frequencies in controls and SLE and RA patients.

SNPs	Controls *N* (%)	SLE *N* (%)	*p*	OR	95% CI	RA *N* (%)	*p*	OR	95% CI
rs3738581									
C	780 (64.3)	596 (55.1)		OR = 1		434 (55.6)		OR = 1	
T	434 (35.7)	486 (44.9)	.000009	OR = 1.47	1.23–1.73	346 (44.4)	.00014	OR = 1.43	1.19–1.72
C/C	252 (41.5)	156 (28.8)		OR = 1		121 (31.1)		OR = 1	
C/T	276 (45.5)	284 (52.5)	.0001[a]	OR = 1.66	1.27–2.17	192 (49.2)	.011[c]	OR = 1.45	1.1–1.95
T/T	79 (13)	101 (18.7)	.0007[b]	OR = 2.06	1.42–2.99	77 (19.7)	.0003[d]	OR = 2.03	1.36–3.03
HWE	0.80	0.16				0.95			

[a]Binary logistic regression, adjusted by gender and origin of sample: $p = .00018$, OR = 1.76, 95% CI = 1.31–2.36; *p*-value after Bonferroni correction = .0007.
[b]Binary logistic regression, adjusted by gender and origin of sample: $p = .000053$, OR = 2.35, 95% CI = 1.55–3.55; *p*-value after Bonferroni correction = .0002.
[c]Binary logistic regression, adjusted by gender and origin of sample: $p = .019$, OR = 1.49, 95% CI = 1.1–2.09; *p*-value after Bonferroni correction = .076.
[d]Binary logistic regression, adjusted by gender and origin of sample: $p = .0019$, OR = 2.07, 95% CI = 1.31–3.27; *p*-value after Bonferroni correction = .0076.
SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; *p*: chi-square test *p*-value; OR: odds ratio; CI: confidence interval; HWE: Hardy–Weinberg equilibrium.

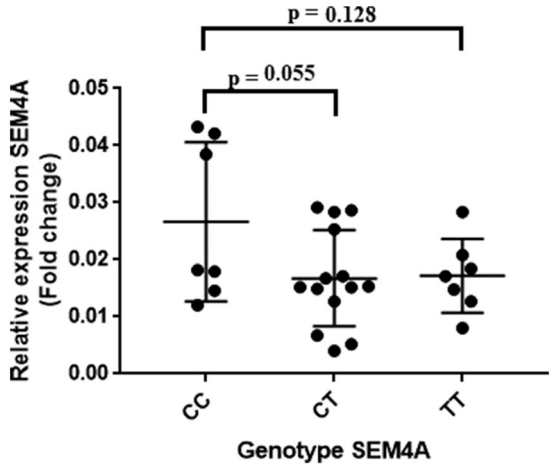

Figure 2. *SEMA4A* gene expression in 28 healthy controls stratified by genotypes (T/T genotype: 7 individuals; C/T: 14; C/C:7). The results were normalized to *GAPDH* expression. The calculation of $2^{-\Delta Ct}$ was performed for each subject. Student's *t*-test was applied to compare the quantitative expression between different genotypes.

stained controls. All analyzes were performed by FlowJo 7.5.5 (Tree Star Corporation, Ashland, OR).

All variables were tested for normality of distribution by means with the Kolmogorov-Smirnov test. Statistical analysis of data was performed using One-way ANOVA. Bonferroni's correction was applied for multiple comparisons among individuals stratified by genotypes. Data are presented as mean fluorescence intensity (MFI) of SEMA4A. The significance level was set at $\alpha = 0.05$ (two-tailed). Analyses of data were performed through the SPSS 15.0 Software Inc. (Chicago, IL).

3. Results

3.1. Genotyping study

Genotype distributions were in Hardy–Weinberg equilibrium for the assessed SNPs with exception of *SEMA4A* rs12401573 in SLE group ($p = .04$) (Supplementary Table 1).

Considering patients and controls independently of their geographical origin, we found a significant association of *SEMA4A* rs3738581 SNP [C > T] with RA and SLE susceptibility with a statistical power = 1.0 for both groups. The T allele and C/T and T/T genotypes were significantly more frequent in SLE (44.9%, *p* value = .000009, OR = 1.47, 95% CI = 1.23–1.73; 52.5%, *p*-value = .0001, OR = 1.66, 95% CI = 1.27–2.17; and 18.7%, *p*-value = .0007, OR = 2.06, 95% CI = 1.42–2.99, respectively) and RA patients (44.4%, *p*-value = .00014, OR = 1.43, 95% CI = 1.19–1.72; 49.2%, *p*-value = .011, OR = 1.45, 95% CI = 1.1–1.95; and 19.7%, *p*-value = .0003, OR = 2.03, 95% CI = 1.36–3.03, respectively) than in controls (35.7%, 45.5%, and 13%, respectively). When we performed a binary logistic regression adjusting for gender and geographical origin of sample, the association between C/T and T/T genotypes and susceptibility to SLE (*p*-value = .00018, OR = 1.76, 95% CI = 1.31–2.36; *p*-value = .000053, OR = 2.35, 95% CI = 1.55–3.55, respectively) and RA (*p*-value = .019, OR = 1.49, 95% CI = 1.1–2.09; *p*-value = .0019, OR = 2.07, 95% CI = 1.31–3.27, respectively) remained statistically significant. After applying Bonferroni's correction, the associations still remained statistically significant ($p\text{Bonf} < .05$) with exception of C/T genotype for RA group ($p\text{Bonf} = .076$). *SEMA4A* rs3738581 genotype and allele frequencies are shown in Table 2.

When we assessed the influence of *SEMA4A* polymorphisms on SLE and RA clinical and laboratorial features, the association between T/C and C/C genotypes for rs12401573 SNP [T > C] and hematological alterations in SLE patients was observed (*p*-value = .007, OR = 2.01, 95% CI = 1.21–3.32; *p*-value = .018, OR = 2.09, 95% CI = 1.14–3.84, respectively) (Supplementary Table 2). Linkage disequilibrium was not observed for the studied *SEMA4A* polymorphisms.

3.2. SEMA4A expression analyses

To determine whether SLE and RA associated *SEMA4A* rs3738581 SNP differentially affects gene expression, we compared *SEMA4A* mRNA levels of C/C healthy individuals with heterozygous (C/T) and homozygous (T/T) individuals for the risk allele in whole blood cells. Our findings indicated C/T and T/T genotypes do not impact *SEMA4A* gene regulation (*p*-value > .05) (Figure 2). To explore if any genotype-associated down or upregulation of *SEMA4A* could be cell type or stimulus dependent, we evaluated the in vitro sema4A expression in mature DCs as well as resting and active T lymphocytes. We found a significantly increased expression of sema4A on the surface of stimulated T cells ($CD3^+CD4^+CD25^+$) from individuals homozygous for the risk allele when compared to the expression of C/C

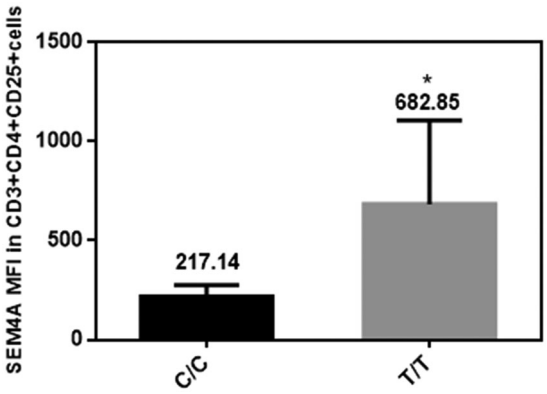

Figure 3. Sema4A production in 28 healthy controls stratified by genotypes (T/T genotype: 7 individuals; C/T: 14; C/C:7). Data are presented as mean fluorescence intensity (MFI) of sema4A. *p-value $< .05$.

T cells (MFI 682.85 ± 25.03 *vs.* MFI 217.14 ± 14.05 respectively, p-value $= .04$) (Figure 3).

4. Discussion

The development of SLE and RA autoimmunity is related to both environmental and genetic factors, the latter involving immune response–related genes variants and differential expression [20,21]. In this study we evidenced for the first time an increased sema4A expression in active T cells related to the SLE and RA-associated polymorphism rs3738581 [C > T]. The *SEMA4A* rs3738581 T allele seems to confer a similar augmented risk for the development of both RA and SLE when in homozygous condition (OR = 2.07 and 2.35, respectively) with a power of 100%. When considering *SEMA4A* SNPs in SLE clinical and laboratorial manifestations we observed the association between T/C and C/C genotypes for rs12401573 SNP [T > C] and hematological alterations.

The polymorphism rs3738581 is located within an intronic region near 3′ UTR of *SEMA4A* gene, which could interfere with the binding of miRNAs. In whole blood cells, we did not observe statistically significant changes in *SEMA4A* mRNA expression in heterozygous or homozygous healthy individuals for rs3738581 risk allele. When we evaluated the in vitro sema4A production in mature DCs and T cells, in nonstimulated and stimulated conditions, to further explore if any genotype-associated down or upregulation of *SEMA4A* could be cell type or stimulus dependent, an increased sema4A production was observed in active T lymphocytes of risk allele homozygous subjects.

Our results are indicative of a T cell-specific upregulation of sema4A after activation in the presence of T/T genotype. The risk allele in homozygous individuals may have an impact on amplified sema4A production in T cells, being a susceptibility factor for the autoimmunity development. The specific mechanism by which the sema4A manifests greater production due to T/T genotype remains unknown.

Studies on the roles of sema4A have observed this molecule as critical immune regulators in Th1 and Th17 differentiation [9]. SLE and RA are inherited as complex traits, in which no single gene variant is sufficient to cause disease. Due to the moderate value of OR, the high risk *SEMA4A* allele is not sufficient by itself to result in disease development. Rather, increased sema4A levels may contribute to the pathogenesis of autoimmunity, perhaps by functioning as an adjuvant, exacerbating Th1 and Th17 response.

It is important to point out that increased levels of sema4A have already been observed in the synovial tissue and serum of RA patients compared to osteoarthritis. Further, increased sema4A levels were also correlated with RA activity [22]. Moreover, as before mentioned, Sandrin-Garcia et al. [10] observed the upregulation of *SEMA4A* gene in active SLE patients. Wang et al. [22] observed the in vitro upregulated expression of matrix metalloproteinases and proinflammatory cytokines interleukin-1β and tumour necrosis factor (TNF)-α through the treatment with human recombinant sema4A. High levels of metalloproteinases as well as the pro-inflammatory cytokines IL-1β and TNF-α have been involved in SLE and RA pathogenesis, revealing an important role in autoimmunity and inflammation [23–27]. Sema4A is also considered a player in experimental autoimmune encephalomyelitis as increased expression of sema4A was shown to cause neuroinflammation that leads to demyelination [28]. These findings together with our results indicate a relationship between altered expression of sema4A and the beginning of autoimmunity, which may culminate in the development of autoimmune diseases such as SLE and RA.

5. Conclusion

In conclusion, our results support a role of *SEMA4A* gene in the susceptibility to RA and SLE. To the best of our knowledge, this is the first association study between *SEMA4A* SNPs and RA and SLE. We also showed an impact of rs3738581 SNP on increased sema4A expression levels and their potential correlation with SLE and RA development and worsening. Further studies in different cohorts and mechanistic studies are required to confirm and fully understand the role of SEMA4A in autoimmunity development.

Disclosure statement

The authors have declared no conflicts of interest.

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