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ORIGINAL ARTICLE

Relation between anti-*Porphyromonas gingivalis* antibody titers and HLA-DRB1 neutral alleles in individuals with rheumatoid arthritis

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ABSTRACT

Objective: This study aimed to determine the relation between titres of anti-*Porphyromonas gingivalis (P. gingivalis)* antibody and rheumatoid arthritis (RA) HLA-DRB1 susceptibility region associated with shared epitope (SE) using the Gregersen's and de Vries's classification methods.

Material and Methods: In this cross-sectional study, results of immunoglobulin G1 (IgG1) and immunoglobulin G2 (IgG2) anti-P. gingivalis antibodies, anti-citrullinated protein antibodies (ACPA), diagnosis for RA, and periodontal disease (PD), and a genetic study of the HLA DRB1 region were obtained from 50 patients with RA and 50 control individuals.

Results: Anti-*P. gingivalis* antibody levels and PD parameters were similar in control and RA groups. Anti-*P. gingivalis* antibodies were not associated with SE or ACPA. There was no association between ACPA and SE. However, de Vries' classification in RA patients revealed an association between the HLA DRB1 neutral alleles and higher titres of anti-*P. gingivalis* antibodies as follows: IgG1 anti-*P. gingivalis* $\geq$ 1:400 ($p = .039$); IgG2 anti-*P. gingivalis* $\geq$ 1:400 with neutral/neutral genotype (N/N), being exclusive for RA ($p = .008$); and IgG2 anti-*P. gingivalis* $\geq$ 1:200 and N/N ($p = .016$).

Conclusions: Although no association was found between SE and anti-*P. gingivalis* antibodies; according to the de Vries' classification, there was an existing association between HLA DRB1 neutral alleles, with high titres of IgG anti-*P.gingivalis* antibodies for RA, focussing on novel associations between *P.gingivalis* and RA.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that causes destruction of the joint structure, resulting in disability [1]. Although the aetiology of RA remains partially elucidated, the notion that the disease is a polygenic autoimmune disorder requiring environmental factors to manifest itself is not new; historically, infectious agents have been implicated in RA development. Scientific evidence evokes the pathogenic role of altered oral microbiota in the course and development of RA [2]. Among oral diseases, periodontal disease (PD), has been closely associated with the development of RA [3–5] because of its infectious and multifactorial inflammatory condition, which triggers the destruction of the supporting tissues of the teeth. The comorbidity of PD and RA is based on causal and non-causal pathway models; the causal theory is supported by the findings of mechanisms by which there is an influence of PD in RA and vice versa [6].

An increasing number of epidemiological and serological studies as well as animal models provide evidence that *Porphyromonas gingivalis (P. gingivalis),* being an important microbial aetiological agent of PD, could be involved in the RA onset and progression [3,5,7,8]. Such an association was made because *P. gingivalis* it is a prokaryotic species with peptidyl arginine deiminase (PAD) activity [9] and can citrullinate human fibrinogen and α-enolase [8]. Moreover, the ability of *P. gingivalis* to citrullinate its own endogenous proteins has been previously shown [8,10]. Immune response to citrullinated proteins has been essential for the early detection of RA [11], because the occurrence of anti-citrullinated protein antibodies (ACPA) is closely associated with the presence of HLA II alleles specifically with the group of HLA DRB1 alleles that encode a shared epitope (SE), which have been reported as the most important genetic risk factor for RA [12].

There have been numerous analyses focussing on the amino acid region, known as SE, and its influence on RA pathogenesis, including susceptibility, severity, and response to treatment. In our previous study, the classification methods of HLA DRβ1 by Gregersen and de Vries related to SE were considered appropriate for characterising RA in the Colombian population [13]. Gregersen's classification is a classical method that is based on preserved sequences of

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three homologous amino acid variants at positions 70–74 of the HLA DRβ1 molecule, these sequences correspond to the amino acids QKRAA, QRRAA, and RRRAA [14]. The de Vries classification considers the SE according to the amino acid positions 67, 70, 71, 73, and 74 corresponding to the LQKAA, LQRAA, and LRRAA sequences [15].

There was 100% similarity observed in the alleles' susceptibility group between the two classifications. Additionally, de Vries' classification generated two more allele groups. One of them was a protective allele group—which presents isoleucine at the 67th position or aspartic acid at the 70th position of HLA DRβ1—and all the other alleles were categorised as "neutral effect" [15]. This group of neutral effect alleles has not been biologically defined other than its categorisation of neither belonging to the RA or the healthy population.

To identify if there is a relationship between the presence of anti-*P gingivalis* antibodies and the susceptibility region in HLA DRB1 (SE), in patients with RA, we analysed the possible associations between the HLA DRB1 region according to the categorisation performed using the Gregersen and de Vries classification methods with the presence of immunoglobulin G1 (IgG1) and immunoglobulin G2 (IgG2) anti-*P gingivalis* antibody titres in RA and control individuals. IgG is the most common type of immunoglobulin found within the human serum and can be categorised in four subclasses as follows: IgG1, IgG2, IgG3, and IgG4. IgG1—the most abundant and reflects total IgG values—is mainly directed against protein antigens, IgG2 is associated with a response to infections caused by encapsulated bacteria and their polysaccharides. Considering this, both isotypes were included in the evaluation [16].

To reduce the presence of other environmental factors related to the citrullination process, smoking habit was excluded in the participants, considering that tobacco use is the most consistent for RA [17].

Materials and methods

This cross-sectional study included 50 patients diagnosed with RA according to the American College of Rheumatology 1987 [18] or 2010 [19] classification criteria and 50 control individuals. Individuals in the control group were selected from the same hospital as well as neighbours and companions of the patients, and were matched for age and sex, except for one man in the control group (Figure 1: Flowchart of the study population selection). All participants previously signed the informed consent form, thereby accepting their participation in the study. The ethics committees of the participating institutions approved the study according to the Declaration of Helsinki of the World Medical Association, Universidad Nacional de Colombia (24/10/2013-62), and Hospital Militar Central (2014-4337).

Inclusion criteria

Subjects aged 18–65 years, participants and their two previous generations in direct line born on Colombian territory, patients with RA and control individuals who had visited the Hospital Militar Central in Bogotá Colombia between May 2012 and May 2014 (Figure 1).

Exclusion criteria

Presence of diabetes, cancer, lactation, other autoimmune diseases, orthodontic treatment, presence of <6 teeth, ongoing infectious processes, history of past or current use of tobacco, intake of antibiotics in the previous 3 months, and periodontal treatment in the previous 6 months; and also for the control group: history of rheumatic diseases in the families (Figure 1).

Periodontal assessment

All candidates were subject to periodontal diagnosis according to the CDC-AAP classification criteria [20]. The clinical indices were measured by two periodontists who participated in an inter-examination calibration, according to Polson's criteria [21]. Repeated evaluations were conducted before the study on 5 randomly selected individuals in order to determine intra-examiner reproducibility. The clinical indices used were as follows: pocket depth (PD), (intra-examiner intraclass correlation coefficient (IE-ICC, 0.92–0.98)), clinical attachment loss (CAL) (IE-ICC, 0.90–0.98), bleeding on probing (BoP) (IE- kappa index 0.85–0.95), and plaque index (PI) (IE- kappa index 0.85–0.92).

IgG/IgA ACPA

The Quanta lite® CCP 3.1 IgG/IgA (INNOVA Diagnosis, San Diego, USA) kit was used following the manufacturer's instructions. The final result was measured obtaining a negative value <20 UI.

IgG1 and IgG2 anti-P. gingivalis antibody titres

Indirect solid phase enzyme linked immunosorbent assay (ELISA) was performed according to the technique previously mentioned by Bello-Gualtero et al. [22]: An indirect ELISA was performed in-house in 96-well plates†† to detect IgG1 and IgG2 antibodies against *P. gingivalis*. In the A, C, E, and G lanes, each well was coated with 5 μg of a sonicated preparation of whole *P. gingivalis* ATCC-33277 and W83 strains in carbonated phosphate-buffered saline (PBS) ‡‡32,33. To determine the final titre per sample, the other four lanes were coated with only carbonated PBS and incubated for 16 h at 4 °C. After coating, 150 μL of A solution (5% non-fat dry milk, biotin-binding protein,§§ 1% bovine serum albumin||||) was added for stabilisation and preblocking.¶¶ Each well was incubated with 150 μL of B solution (biotin## and PBS without polysorbate 20***) at room temperature for 1 h. For the serum samples, two-fold serial dilutions beginning at 1:100 were performed using 1x PBS, 1% bovine serum albumin, and 0.1% polysorbate 20, and were incubated at 37 °C for 1 h. Then, 150 μL anti-human IgG1††† or biotinylated anti-human IgG2‡‡‡ was added after a 1:4,000 dilution in

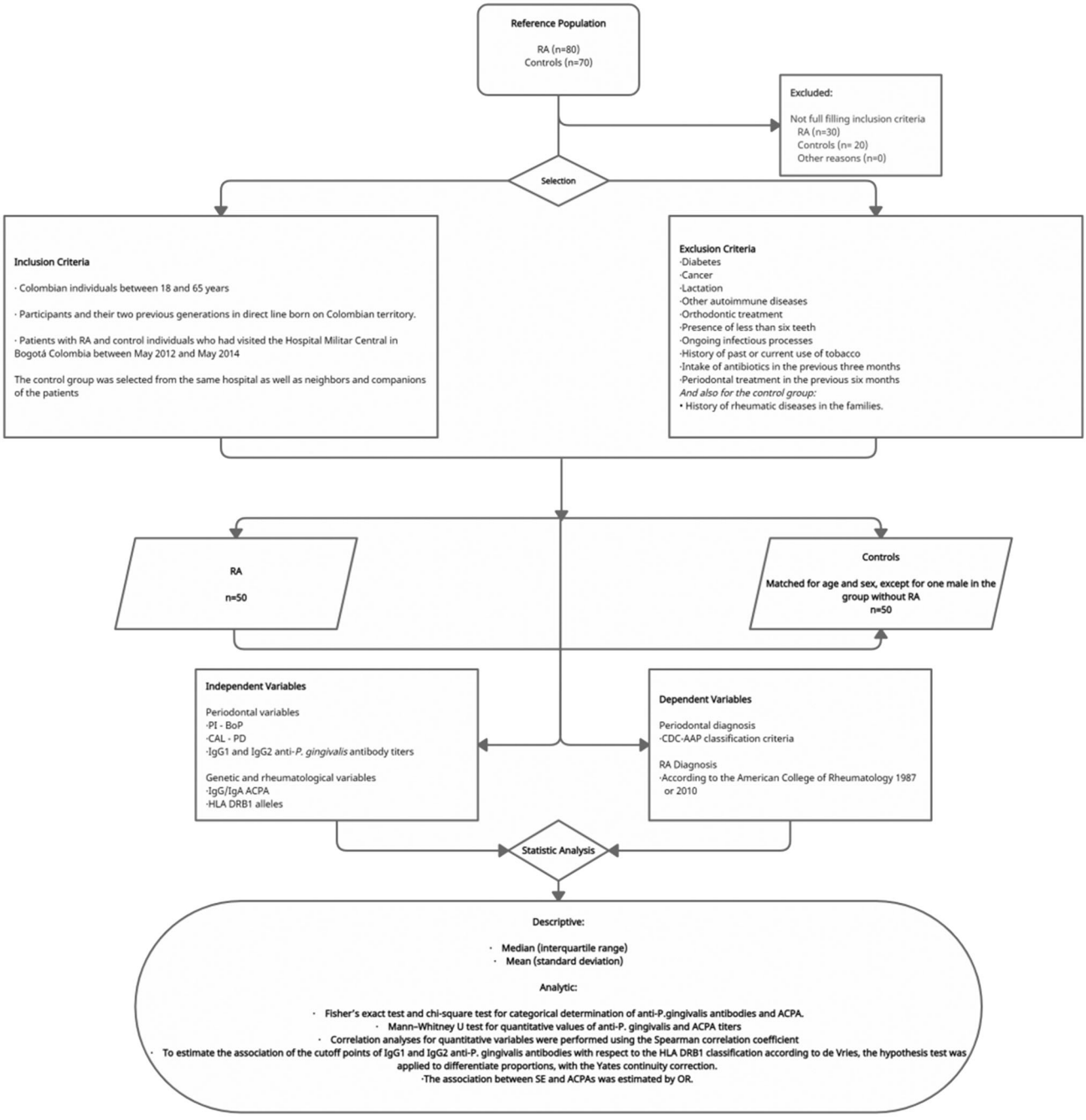

Figure 1. Flowchart of the study population selection.

the same buffer solution. Next, 150 µL horseradish peroxidase streptavidin§§§ (diluted 1:1,500) was added to each well using O-phenylenediamine|||||| diluted in 1x peroxide buffer¶¶¶ as the substrate, and incubated for 1 h at 37 °C. The reaction was stopped with 2 M H_2SO_4.### The optical density was measured at 492 and 640 nm using a microplate reader.**** The positive and negative controls were represented by pooled *P. gingivalis*-positive (quantitative PCR) serum samples from individuals with PD and pooled serum samples from edentulous individuals with lower antibody titres (1:100), respectively. The titre for anti-*P. gingivalis* antibodies in each of the samples was measured starting at 1:50 until final dilution. In addition, for further analyses three cut-off points were determined: 1:100, 1:200, and 1:400. An amount below 1:100 was considered to be negative.

†† Greiner Bio-One (cat. no. 655061), Kremsmünster, Austria.
‡‡ Ref. 1011, Immuno Concepts, Sacramento, CA.
§§ Avidin (cat. no. SP-2001), Vector Laboratories, Burlingame, CA.
|||| Cat. no. 820451, Probumin diagnostic grade, Merck Millipore, Darmstadt, Germany.
¶¶ StabilCoat (SC01-2000), SurModics, Eden Prairie, MN.
Cat. no. SP-2001, Vector Laboratories.

*** Tween 20 (cat. no. P1379), Sigma-Aldrich, St. Louis, MO.
††† Cat. no. A10650, Invitrogen, Thermo Fisher Scientific, Waltham, MA.
‡‡‡ Cat. no. B3398, Sigma-Aldrich, Gillingham, UK.
§§§ Cat. no. SNN1004, Invitrogen, Thermo Fisher Scientific.
|||||| Cat. no. 34005, John B. Pierce Laboratory, New Haven, CT.
¶¶¶ Cat. no. 34062, Thermo Fisher Scientific, Waltham, MA.
Cat. no. 6057, Baker Analysed, Centre Valley, PA.
**** Sunrise, Tecan, Männedorf, Switzerland.
†††† Cat. no. 447070, Immage 800, Beckman Coulter, Brea, CA.
‡‡‡‡ Cat. no. LKCRP, Immulite 1000, Siemens, Erlangen, Germany.

HLA DRB1 allele characterisation

DNA extraction was performed using the salting-out method by CorpoGen®. The classification of HLA DRB1 alleles was performed by the Luminex 100 system and using the INMUCOR, Lifecodes HLA-DRB1 SSO typing kit (628923, Stamford, CT, USA) as per the manufacturer's instructions. Data were analysed using LifeMatch DNA Software.

Statistical analysis

SPSS version 20.0 was used for statistical analysis. Fisher's exact test and chi-square test were used for qualitative variables (categorical determination of the presence or absence of anti-*P. gingivalis* antibodies and ACPA). The Kolmogorov–Smirnov normality test was used for quantitative values (anti-*P. gingivalis* and ACPA titres). None of the variables expressed normality in this study; hence, the non-parametric Mann–Whitney U test was used. Correlation analyses for quantitative variables were performed using the Spearman correlation coefficient. Software R version 3.22 was used to estimate the association between IgG1 and IgG2 anti-*P. gingivalis* antibody cut-off points with respect to the HLA DRB1 classification according to de Vries among control individuals and patients with RA, applying the hypothesis test to differentiate proportions, with de Yates' continuity correction. The odds ratio (OR) was estimated to determine the risk between the presence of the SE and the ACPAs. For all analyses, p values <0.05 were considered statistically significant.

Results

The study population consisted of 100 non-smoking Colombian men and women between 18 and 65 years old; 50 patients with a diagnosis of RA and 50 control individuals. In the RA group, patients who were prescribed some type of medication for their disease were not excluded (data not shown). The presence of ACPA was exclusive to patients with RA ($p = .001$), with 76% positivity. The distribution of the disease with respect to sex reflected the higher occurrence of RA in women, with 84%, being female (Table 1).

Table 1. General characteristics and genetic and rheumatologic variables of patients with RA and controls.

	RA *n*(%) 50(100.00%)	Control *n* (%) 50 (100.00%)	*p* Value
Age: Mean ± SD	51.16 ± 11.65	49.76 ± 10.33	.52
Women	42 (84.00%)	41 (82.00%)	
ACPA%	38 (76.00%)	0	**.001***
SE HLA DRB1	***n* = 50 patients with RA**	***n* = 47 control individuals**	
≥1 SE+	29 (58.00%)	15 (32.00%)	**.006***

RA: Rheumatoid Arthritis; ACPA: anti-citrullinated protein antibodies; SE: Shared epitope.

HLA DRB1 alleles in control individuals and patients with RA

According to a preliminary study [13], the HLA DRB1 allele classification systems developed by Gregersen and de Vries are appropriate for the differentiation between non-smoking Colombian control individuals and non-smoking Colombian patients with RA ($p = .006$, Table 1). We obtained information from 50 patients with RA and 47 control individuals for the Gregersen classification and 50 patients with RA and 45 control individuals for de Vries classification, which provided data of alleles with no ambiguities (data not shown).

Hardy–weinberg equilibrium (HWE) determination

According to the allele frequencies of SE, the population under study reached HWE by the chi-square test, p value = 0.87 (with a degree of freedom, $p < 0.05$ was not consistent with HWE).

Periodontal parameters

The number of teeth and the periodontal clinical indices were similar between patients with RA and control individuals. PD diagnosis was more frequent in patients with RA (80%) than in control individuals (76%). Moderate PD was the most prevalent severity level in both groups. Patients with RA showed a greater prevalence of severe PD (22.50% vs 15.78%). There was no statistically significant difference between the two groups regarding PD (Table 2).

IgG1 and IgG2 anti-P. gingivalis antibodies

The median values and the presence of IgG1 anti-*P. gingivalis* antibody titres were higher in control individuals. In contrast, the median values of IgG2 anti-*P. gingivalis* antibody titres were higher in patients with RA. However, these values were not statistically significant (Table 2).

There were no statistically significant differences between the cut-off points for IgG1 anti-*P. gingivalis* and IgG2 anti-*P. gingivalis* between the groups (data not shown).

Table 2. Comparison of periodontal parameters between the RA and control groups.

	RA *n*(%) 50 (100.00%)	Control *n* (%) 50 (100.00%)	*p* Value
Anti-*P. gingivalis* antibody titres			
IgG1 anti-*P. gingivalis*	26 (52.00%)	32 (64.00%)	.22
IgG2 anti-*P. gingivalis*	36 (64.00%)	36 (64.00%)	
IgG1 Anti-*P. gingivalis:* Median (Interquartile range)	100 (350)	200 (350)	.21
IgG2 Anti-*P. gingivalis:* Median (Interquartile range)	200 (350)	150 (350)	.45
Clinical parameters			
Teeth count: Mean ± SD	21.84 ± 6.97	21.69 ± 7.87	.92
PI: Mean ± SD	0.60 ± 0.25	0.61 ± 0.27	.82
BoP : Mean ± SD	0.45 ± 0.28	0.47 ± 0.26	.71
PD total mouth: Mean ± SD	2.10 ± 0.44	2.25 ± 0.54	.12
Interproximal PD: Mean ± SD	3.41 ± 2.08	3.35 ± 1.83	.88
CAL total mouth: Mean ± SD	1.80 ± 1.29	1.92 ± 1.38	.63
Interproximal CAL: Mean ± SD	2.58 ± 0.93	2.78 ± 0.95	.29
Periodontitis (positive)(CDC/AAP): *n*(%)	**40 (80.00%)**	**38 (76.00%)**	
Severe Periodontitis: *n*(%)	9 (22.50%)	6 (15.78%)	
Moderate Periodontitis: *n*(%)	24 (60.00%)	24 (63.15%)	.83
Mild Periodontitis: *n*(%)	7 (17.50%)	8 (21.05%)	

RA: Rheumatoid Arthritis; PI: Plaque index; BoP: Bleeding on probing; PD: pocket depth; CAL: clinical attachment loss; CDC/AAP: Centre for Disease Control and Prevention/American Academy of Periodontology.

Table 3. Proportion analyses of IgG1 anti-*P. gingivalis antibody* titres in patients with RA and control individuals regarding protective, neutral, and susceptibility alleles, according to the HLA DRB1 de Vries' classification.

IgG1 anti-*P. gingivalis*	Patients with RA *N* = 50	Control individuals *N* = 45	Alleles' risk group according to the de Vries' classification	*p* Value
Cut-off point 1:100	0.5333	0.7741	Protective	.907
1:200	0.4666	0.6774	Protective	.853
1:400	0.4	0.4516	Protective	.505
1:100	0.58	0.5555	Neutral	.486
1:200	0.44	0.4222	Neutral	.500
1:400	0.36	0.1777	Neutral	**.039** *
1:100	0.4285	0.6875	Susceptibility	.921
1:200	0.3142	0.625	Susceptibility	.962
1:400	0.2285	0.4375	Susceptibility	.882

RA: Rheumatoid Arthritis.

Association of IgG1 and IgG2 anti-P. gingivalis antibodies with SE in control individuals and patients with RA according to HLA DRβ1 gregersen and de vries classifications

Comparisons were made between IgG1 and IgG2 anti-*P. gingivalis* antibody titres with the presence of SE according to Gregersen and de Vries' classification methods. No statistically significant associations were observed; IgG1 and SE ($p = .46$)/IgG2 and SE ($p = .45$).

When analysing the alleles using de Vries' classification, a statistically significant correlation between the presence of neutral alleles and RA in the presence of high titres of IgG1 and IgG2 anti-*P. gingivalis* antibodies was observed. With regards to IgG1 anti-*P. gingivalis* antibodies, a higher proportion of patients with RA was observed when compared with control population carrying neutral alleles with titres against bacteria $\geq$ 1:400 ($p = .039$; Table 3). In the genotype analysis, the (N/N) group with high titres of IgG2 anti-*P. gingivalis antibody* was characterised with RA, IgG2 anti-*P. gingivalis antibody* titres $\geq$1:400 and the N/N genotype were exclusive for RA ($p = .008$), and anti-*P. gingivalis* antibody titres $\geq$1:200 with the N/N genotype were more common in patients with RA than in control individuals ($p = .016$). A trend towards statistical significance was observed for patients with RA carrying the P/N genotype with high titres of IgG2 anti-*P. gingivalis* antibody ($\geq$400; $p = .053$) (Table 4).

ACPA and SE

None of the control individuals were positive for ACPA. A high frequency of SE + individuals (SE+/SE + and SE+/SE−) was observed in the RA ACPA + group (63.15% of ACPA + individuals). In the ACPA- group there was a higher frequency of SE- individuals. (58.33%). The differences between ACPA + and ACPA − groups with respect to the presence of SE were not statistically significant [OR 2.4, 95% confidence interval (95% CI) 0.63–9.01; $p = .19$; Table 5].

ACPA and anti-P. gingivalis antibody

Comparisons were performed to observe a possible correlation between ACPA and IgG1 and IgG2 anti-*P. gingivalis* antibody titres. No correlations were found among these parameters [IgG1 and ACPA ($R = 0.016$; $p = .315$) and IgG2 and ACPA ($R = 0.039$; $p = .483$)].

Table 4. Proportions analyses of IgG2 anti P. gingivalis antibody titres between patients with RA and control individuals regarding P/P, P/N, P/S, N/N, N/S, and S/S genotypes, according to the HLA DRB1 de Vries' classification.

IgG2 anti-*P. gingivalis*	Patients with RA $n = 50$	Control individuals $n = 45$	Genotype	*p* Value
Cut-off point 1:100	1	1	P/P	NA
1:200	1	0.3333	P/P	.500
1:400	1	0.3333	P/P	.500
1:100	0.8888	0.75	P/N	.377
1:200	0.6666	0.5	P/N	.349
1:400	0.6666	0.25	P/N	.053
1:100	0.5	0.6666	P/S	.500
1:200	0.5	0.5555	P/S	.500
1:400	0	0.3333	P/S	.726
1:100	0.7272	0.4545	N/N	.192
1:200	0.7272	0.1818	N/N	**.016***
1:400	0.5454	0	N/N	**.008***
1:100	0.6315	0.8	N/S	.570
1:200	0.4736	0.8	N/S	.787
1:400	0.3157	0.4	N/S	.500
1:100	0.8333	1	S/S	.500
1:200	0.8333	1	S/S	.500
1:400	0.5	1	S/S	.500

RA: Rheumatoid Arthritis; Genotypes related to RA according to de Vries' classification: P/P: Protective/Protective; P/N: Protective/Neutral; P/S: Protective/Susceptibility; N/N: Neutral/Neutral; N/S: Neutral/Susceptibility; and S/S: Susceptibility/Susceptibility.

Table 5. ACPA and SE in patients with RA.

Presence of SE	ACPA + *n*(%) 38(76%)	ACPA-*n*(%) 12(24%)	OR (95% CI)	*p* Value
SE + (%)	24 (63.15%)	5 (41.66%)	2.4 (0.63–9.01)	.19
SE- (%)	14 (36.84%)	7 (58.33%)		

RA: Rheumatoid Arthritis; ACPA: anti-citrullinated protein antibodies; SE: Shared epitope (SE+ =SE+/SE + and SE+/SE−); SE−(SE−/SE−). Data available for 50 patients with RA.

Discussion

The frequency of PD in both groups corresponded to that reported in the 4th oral health study conducted in Colombia (ENSAB IV) [23]. We found a greater percentage of individuals with severe PD among the RA population, a condition reported by Mercado et al. [24]. Similarly, studies performed in Colombia by Bello-Gualtero et al. [22] and Unriza S. et al. [25] on RA groups, groups with early RA (eRA), groups at risk of having RA (pre-RA), and healthy individuals, found a higher frequency of severe cases in the eRA groups, with statistically significant differences in pre-RA individuals. The statistical significance in the reported results of the pre-RA group can be explained by the absence of the effects of disease-modifying anti-rheumatic drugs (DMARDs), which might mask PD and reduce the associated clinical variables and their progression [26].

This study did not demonstrate statistically significant differences in anti-*P. gingivalis* antibody titres between patients with RA and control individuals; moreover, no evidence of differences was found in similar studies on the Colombian population [22,25]. Contrary to our findings, other studies, such as those of Mikuls et al. [27] and Arvikar *et al.*, [28] noted a higher IgG1 anti-*P. gingivalis* antibody titres in patients with RA; however, these studies classified control individuals using self-reporting parameters of absence of PD; a greater correlation of IgG anti-*P. gingivalis* antibody titres in individuals with PD has been reported in the literature, [29,30] which could be the reason for such results. Kharlamova et al. [31] found greater antibody titres against virulence factor arginine gingipain B (Anti-RgpB) in patients with RA. Quirke et al. [10] found high levels of antibodies against PPAD in individuals with RA as well as those with PD in comparison to control individuals. These contradicting results might be attributed to the targeting of different antigens from *P. gingivalis*, with anti-RgpB and anti-PPAD being involved in the citrullination process in bacteria [10,31] as one of its potential actions in RA. In addition, differences in the inclusion criteria associated with periodontal parameters in the study subjects can contribute to the discrepancies in these results.

Antibody subclasses have different biological properties; protein antigens normally stimulate IgG1 and IgG3, whereas lipopolysaccharides (LPS) and their antigens tend to stimulate IgG2 [32]. In our study, we observed higher titres of IgG2 anti-*P. gingivalis* in patients with RA than in control individuals. This finding was also reported by Hitchon et al. [33], who demonstrated that anti-LPS *P. gingivalis* antibody titres, representative of IgG2 anti-*P. gingivalis* antibodies, were higher in individuals with RA than in their relatives and two other control groups. Increased IgG2 anti-*P. gingivalis antibody* titres have been associated with non-treated PD and alveolar bone loss even in subjects who are undergoing periodontal treatments, suggesting that the subclass IgG2 has an insufficient protective ability against *P. gingivalis* infection [32]. Further studies are warranted to confirm a greater proportion of severe PD in patients with RA and a higher median value of IgG2 anti-*P. gingivalis* antibody titres, with a potential deleterious action of *P. gingivalis* in the development of pathology in RA through pathways in addition to citrullination.

P. gingivalis has been associated with citrullination mechanisms due to the presence of PAD activity [34], with antibodies against citrullinated peptides as a specific response of RA [35]. In this study, we did not observe any correlation between anti-*P. gingivalis* antibodies and ACPA titres. A study conducted by Seror et al. [30] on a French cohort with eRA also did not find an association among titres of both antibodies. Other studies have indicated a correlation among these antibodies titres; [22,27,28] however, our study is the only one that excluded individuals who smoked. The association between anti-*P. gingivalis* antibody and ACPA titres in individuals who smoke is affected by citrullination generated due to an altered humoral immune response against *P. gingivalis* secondary to smoking and, due to a decrease in oxygen in the subgingival area secondary to increase in anaerobic flora [36]. Zeller et al. [37] and Arvikar et al. [28] indicated that the total IgG anti-*P. gingivalis antibody* titres decreased in individuals who smoke, which negatively affects their antigenic presentation, promoting bacterial survival and

contributing to an increase in virulence mechanisms related to the microorganism [37].

In this study, we found 58% of the RA population with at least one SE + allele, a result similar to that found in another study in a Colombian population (51%) [38]. Balandraud et al. [39] found however, this value increased in population of the southeast and east of France (70%).

No relation was found between IgG1 and IgG2 anti-*P. gingivalis antibody* titres and the alleles group that carry SE (according to Gregersen's classification) or the susceptibility alleles (according to de Vries' classification). Hitchon et al. [33] also did not find an association between anti-*P. gingivalis antibody* titres and SE in their study on American subjects with RA and their relatives. This lack of association is also supported by the results of the study conducted by Seror et al. 2015 [30] on an extensive cohort of French individuals with eRA. Kharlamova et al. [31] performed an interesting and one of the greatest investigations in the field of RA, they observed interactions between high levels of IgG anti-RgpB *P. gingivalis* antibodies and SE in individuals positive for ACPA. Their observations supported the biological plausibility, according to Bradford Hill's criteria, of a connection between *P. gingivalis* and SE with RA, due to the association of its pathology with citrullinated peptides [34]. We can attribute the discrepancies between different results to the multiple variables that influence the oral microbiota: geographical area, race, diet, developmental development, and lifestyle [40], as well as differences in studies' methodologies, including smoking and biological factors used for detecting anti-*P. gingivalis* antibodies.

This study showed a relevant percentage of RA ACPA + individuals with negative SE (36.84%), similar to the study conducted by Balsa et al. [41] on Spanish population where they found a similar value (32%). Regarding a research on the possible associations between *P. gingivalis* and other genome areas outside the SE, which have been associated with anti-citrulline autoimmunity, Kharmalova et al. [31] found no association between the polymorphism rs2476601_A of the PTPN22 gene and IgG anti-RgpB of *P. gingivalis*; HLA DQ alleles have also been significantly identified in the presentation of citrullinated peptides [42,43], this being a plausible factor for future bacterial analyses.

In particular, in the current research performed in a non-smoking Colombian population, we found a correlation between high titres of IgG1 and IgG2 anti-*P. gingivalis* antibodies and the presence of neutral alleles, according to de Vries' classification, in the RA population without a relationship of the bacteria's antibodies with the antibodies against citrullinated peptides. Although an association between *P. gingivalis* and RA has been attributed to the property of bacteria in modifying arginine peptides into citrulline and its own auto-citrullination mechanisms, the bacterial processes in RA pathogenesis has been recognised with both pathogenic and non-pathogenic bacteria involved in promoting autoimmune diseases through chronic activation of innate and acquired response [44]. The other contributing effects of *P. gingivalis* in the development of RA pathology can be associated with the induction of IL-1 and IL-6, which causes a Th17 pathogenic response [45]. The association of *P. gingivalis* with mechanisms other than citrullination has been confirmed as an association between IgG anti-RgpB and *P. gingivalis antibody* with RA in individuals negative for ACPA [31], furthermore, red complex periodontal pathogens (gram-negative anaerobic bacteria) have been related to some RA markers [46]. Our findings promote the study of associations between genetic mechanisms related to RA with other species related to PD.

According to de Vries' classification, neutral alleles were classified as alleles that were not specific for patients with RA or control individuals, as observed in this group [13,15]. The results of an association between IgG1 and IgG2 anti-*P. gingivalis* antibodies and neutral alleles were represented by allelic and genotypic effects and were significantly related to pathology, with the presence of high IgG2 anti-*P. gingivalis antibody* titres with the N/N genotype only observed in patients with RA. An important association was observed with the P/N genotype, having a possible homozygosity loss of the protective alleles with risky effects against neutral alleles and their interaction with *P. gingivalis*. De Vries described a homozygosity protective effect of the P/P genotypes having a central role in the regions 67–74 of the HLA-DRB1 molecule against arthritogenic peptides [15]. Currently, the biological characteristics of the group of neutral alleles in HLA-DRB1 have not been described, other than their epidemiological grouping; our findings promote further studies on this group of alleles in which possible common sequences or linkage disequilibrium relations with other DNA areas can be observed.

Our study has strengths and weaknesses. One of its weaknesses is the small sample size, which can be attributed to the need to obtain strict compliance with exclusion criteria during the period of time available to collect the data, another weakness was the lack of evaluation of *P. gingivalis* specific determinants related to RA such as anti-RgpB or anti-PPAD. However, the results provide relevant information on the relationship studied; to our knowledge, the present investigation is the first to analyse the relationship of multiple RA-related HLA DRB1 alleles with anti- *P. gingivalis* antibodies, where two classifications previously associated with RA in a mestizo Latin American population were taken into account, while ensuring to exclude important environmental factors related to citrullination, such as smoking.

Conclusions

In conclusion, based on our findings, no association was found between anti-*P. gingivalis antibody* and SE. We found a relation between high titres of IgG1 and IgG2 anti-*P. gingivalis* antibodies with neutral alleles according to the Vries' classification for RA in non-smoking population, with no association between the bacterial antibodies and antibodies against citrullinated peptides. This study focuses on other possible pathways of association—not previously described—between *P. gingivalis* and RA, plausible from the diverse virulence factors inherent in the bacteria. We recommend additional studies on neutral alleles, according to de

Vries' classification along with investigations regarding the relation between RA with other periodontopathic bacteria.

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Disclosure statement

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