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CCR5Δ32 (rs333) polymorphism is associated with decreased risk of chronic and aggressive periodontitis: A case-control analysis based in disease resistance and susceptibility phenotypes

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ABSTRACT

Chronic and aggressive periodontitis are infectious diseases characterized by the irreversible destruction of periodontal tissues, which is mediated by the host inflammatory immune response triggered by periodontal infection. The chemokine receptor CCR5 play an important role in disease pathogenesis, contributing to pro-inflammatory response and osteoclastogenesis. CCR5Δ32 (rs333) is a loss-of-function mutation in the CCR5 gene, which can potentially modulate the host response and, consequently periodontitis outcome. Thus, we investigated the effect of the CCR5Δ32 mutation over the risk to suffer periodontitis in a cohort of Brazilian patients (total N = 699), representative of disease susceptibility (chronic periodontitis, N = 197; and aggressive periodontitis, N = 91) or resistance (chronic gingivitis, N = 193) phenotypes, and healthy subjects (N = 218). Additionally, we assayed the influence of CCR5Δ32 in the expression of the biomarkers TNFα, IL-1β, IL-10, IL-6, IFN-γ and T-bet, and key periodontal pathogens P. gingivalis, T. forsythia, and T. denticola. In the association analysis of resistant *versus* susceptible subjects, CCR5Δ32 mutant allele-carriers proved significantly protected against chronic (OR 0.49; 95% CI 0.29–0.83; p-value 0.01) and aggressive (OR 0.46; 95% CI 0.22–0.94; p-value 0.03) periodontitis. Further, heterozygous subjects exhibited significantly decreased expression of TNFα in periodontal tissues, pointing to a functional effect of the mutation in periodontal tissues during the progression of the disease. Conversely, no significant changes were observed in the presence or quantity of the periodontal pathogens P. gingivalis, T. forsythia, and T. denticola in the subgingival biofilm that could be attributable to the mutant genotype.

1. Introduction

Periodontitis is a chronic infectious disease characterized by the irreversible and progressive destruction of teeth-supporting structures. Periodontitis is initiated by the bacteria harbored in the teeth-attached biofilm that activate the host's inflammatory immune response, which provide protection against the infecting agents. However, host response mediators also modulates local proteolytic and osteclastogenic activities, leading to soft and mineralized tissue destruction as collateral damage [1].

In this context, destructive and protective host immune responses have been described to present specific molecular patterns in response to periodontal infection. Pro-inflammatory responses mediated by IL-1, IL-6 and TNFα, along with Th1 and/or Th17 cells, have been associated with the establishment and progression of periodontal destruction, while Th2 and Treg-associated response patterns have been described to attenuate/arrest tissue destruction [2,3]. While the exact mechanisms underlying the host response polarization in periodontium remains to be elucidated, the chemokine system have been described as an important player in the determination of the nature of the host response via the selective recruitment of immune cells subsets, which can amplify the inflammation or suppress it [4].

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The system comprised by the receptor CCR5 and its ligands CCL3, CCL4 and CCL5 has proved important in the inflammatory periodontal destruction associated with periodontitis [5,6]. CCR5 is a 352-amino acid G protein-coupled chemokine receptor which is involved in the selective recruitment of multiple leukocytes subsets, including neutrophils, monocytes, NK cells and lymphocytes [7,8]. Indeed, CCR5 mediate the recruitment of CCR5 + F4/80 + and CCR5 + CD4 + leukocytes, regarded as pre- and pro-osteoclastogenic cells involved in the bone resorption that characterizes periodontitis [9]. The activation of the transduction signals triggered by CCR5 coupling with ligands such as CCL3, CCL4 and CCL5 stimulates not only cell migration, but also influences proliferation, cytokine expression and activation of effector functions [10]. In fact, pharmacological inhibition of CCR5 using metRANTES had proved sufficient to arrest inflammatory cytokine expression, and consequently to suppress alveolar bone loss in mice [8]. Similarly, CCR5 deficient mice (CCR5KO) presents a decreased infiltration of leukocytes and decreased bone loss upon experimental periodontal infection [9]. Importantly, CCR5 + cells also contribute to the protective responses against periodontal infection [8,9].

Interestingly, the human counterpart of the CCR5KO mice strain may be represented by carriers of the CCR5Δ32 mutation. CCR5Δ32 (rs333) is a 32-base pair deletion causing a frameshift variant in the *CCR5* gene leading to a loss-of-function due by the abolition of the receptor's cell-surface expression [11,12]. Indeed, this truncated variant have been demonstrated to be clinically relevant since it has been associated with various phenotypes, including resistance to HIV infection, decreased risk for type 1 diabetes, increased risk for diabetic retinopathy in type 1 diabetes patients and increased risk of multiple sclerosis [13–15]. Thus, based in the relevance of CCR5 to experimental periodontitis pathogenesis, it is reasonable to hypothesize that CCR5Δ32 could be a relevant risk factor for human periodontitis. Two previous studies had found no association between CCR5Δ32 and chronic periodontitis, but methodological shortcomings in their designs warrant a reappraisal of the possible contribution of the mutation to periodontitis' risk profile [16,17].

Indeed, the absence of proper susceptibility and resistance phenotypes definition critically dampen such studies power and odds of identification of genetic factors potentially involved in periodontitis pathogenesis [1,18]. Chronic periodontitis is the most common form of the disease, which is characterized by destruction of teeth-supporting tissues over a long period of time, comprising progressive/active and stable/inactive periods [2,19]. A less common variant is the aggressive form of the disease, characterized by rapid progression of the periodontal destruction [20]. Conversely, chronic gingivitis is a periodontal disease characterized by widespread gingival inflammation without irreversible destruction of the teeth-supporting tissues, even over long periods of time [21]. Despite some differences in the microbial biofilm associated with the different forms of periodontal disease, such conditions are basically initiated by the same stimuli, Gram-negative periodontal bacteria [22,23]. However, the clinical course of each entity reflects inherent differences in the host's capacity to cope with the presence of periodontophatic bacteria [2,3]. Consequently, chronic and aggressive periodontitis can be considered the phenotypic expression of supposed susceptibility genotypes, while chronic gingivitis allegedly represents a resistant phenotype/genotype [18,24]. Indeed, the analysis of opposing resistant and susceptible phenotypes, determined by the observance the outcome of exposure to the microbial challenge, increases the possibilities of finding a true association between genetic markers and disease phenotype [1,18].

Therefore, it is plausible to hypothesize that CCR5Δ32 (rs333) may account for the differential expression of disease susceptibility (such as chronic and aggressive periodontitis) or resistance (such as chronic gingivitis). Also, it is possible that the hypofunctional CCR5 variant could affect the expression of host response factors in periodontal tissues, as well as influence the pattern of bacterial infection in periodontitis.

Therefore, we conducted a case-control study to investigate whether the CCR5Δ32 (rs333) is associated with periodontitis risk; performing for the first time an analysis in groups defined as susceptible and resistant, and also investigated the putative functionality of this SNP in the modulation of CCR5-related host mediators (TNFα, IL-1β, IL-6, IL-10, IFN-γ, and T-bet) *in situ*, as well as its possible impact in the composition of subgingival biofilm, in an attempt to unveil a conceivable mechanism linking the gene variant with the disease phenotype.

2. Materials and methods

2.1. Participants

The sample was recruited at the São Paulo state, south-eastern region of Brazil, from patients scheduled for treatment at the Dentistry School University of Ribeirão Preto. Patients were examined by an experienced periodontist and scored for bleeding on probing (BOP), probing depth (PD) and clinical attachment loss (CAL). Enrolled subjects provided informed consent that was approved by the Institutional Review Board. Subjects were excluded from the study if they were tobacco smokers (including former smokers), had medical history indicating evidence of known systemic modifiers of periodontal disease, and/or had received periodontal therapy in the previous 2 years. No strategy was used to identify subpopulations (population stratification) or population relatedness among the recruited subjects. After the diagnostic phase, patients were subsequently categorized into healthy (H; n = 218) [classic control], chronic gingivitis (CG; n = 193) ['resistant' phenotype control], chronic periodontitis (CP; n = 197) [susceptible subjects] and aggressive periodontitis (AP; n = 91) [highly susceptible subjects], as previously described [1,18].

The control group (H; n = 218) included subjects with clinically healthy gingival tissues scheduled to undergo dental restorative procedures. The chronic gingivitis group (CG; n = 193) corresponded to subjects with history of poor oral hygiene, BOP > 70% of periodontal sites and no CAL or radiographic evidence of alveolar bone loss. Patients in the chronic periodontitis group (CP; n = 197) included subjects diagnosed with moderate to severe periodontitis (at least one teeth per sextant with probing depth > 6 mm and clinical attachment loss > 3 mm plus radiographic evidence of extensive bone loss [> 30% alveolar bone height in at least 50% of teeth]), scheduled to receive periodontal therapy. The aggressive periodontitis group (AP; n = 91) included subject with moderate to severe periodontitis (at least one teeth per sextant with probing depth > 6 mm and clinical attachment loss > 3 mm plus radiographic evidence of extensive bone loss [> 30% alveolar bone height in at least 50% of teeth]) under the age of 35 years. The age of diagnosis was adopted as an operational criterion to define aggressive periodontitis. Table 1 summarize all relevant clinical and demographical information of each sample population.

2.2. DNA sampling and CCR5 genotyping (rs333)

Oral mucosa epithelial cells from each participant (n = 699) were obtained by scrapping the inner cheek after a mouthwash with 3% glucose. DNA was extracted from epithelial cells with sequential phenol/chloroform solution and precipitated with salt/ethanol solution. Extracted DNA was immediately used for genotyping. We genotyped CCR5Δ32 (rs333) by polymerase chain reaction (PCR) with forward primer 5′ – ATGCTGTGTTTGCTTTAAAAGCCAGG – 3′ and reverse primer 5′ – AGGACCAGCCCCAAGATGACTA – 3′ flanking the 32 nucleotide deletion in the CCR5 gene. Amplification was performed in 35 cycles as follows: denaturation step 94 °C for 30 s, annealing 56 °C for 30 s, and extension 72 °C for 30 s. The DNA amplicons were visualized under UV light on 2% agarose gel stained with ethidium bromide. The wild-type amplicon corresponded to a 220-bp band while the CCR5Δ32 allele corresponded to a 188-bp band. Negative and positive controls were included in each reaction.

Table 1
Demographical information and clinical parameters of H, CP, AP, and CG groups.

	Healthy (N = 218)	Chronic periodontitis (N = 197)	Aggressive periodontitis (n = 91)	Chronic gingivitis (n = 193)	Healthy *versus* chronic periodontitis	Healthy *versus* chronic gingivitis	Chronic gingivitis *versus* chronic periodontitis
N and gender distribution	***105 f/113 m***	***96 f/101 m***	***49 f/42 m***	***97 f/96 m***	***0.9219***	***0.8395***	***0.9583***
Age	47.48 ± 5.96	46.63 ± 7.34	26.34 ± 4.72	49.54 ± 6.47	0.19	0.0093	< 0.001
Clinical parameters	***Value ± SD***	***Value ± SD***	***Value ± SD***	***Value ± SD***			
Probing depth	2.25 ± 0.62	4.29 ± 0.75	3.75 ± 1.38	2.72 ± 0.52	< 0.001	< 0.001	< 0.001
Clinical attachment loss	0.62 ± 0.22	3.92 ± 0.64	3.88 ± 1.51	0.69 ± 0.25	< 0.001	0.0027	< 0.001
% BOP	4.86 ± 2.86	62.99 ± 8.71	51.14 ± 7.92	61.85 ± 11.49	< 0.001	< 0.001	0.2696
Plaque index	34.71 ± 8.26	51.26 ± 9.78	46.41 ± 8.51	53.47 ± 10.05	< 0.001	< 0.001	0.0284
16S DNA ($\times 10^9$)	0.09 ± 0.20	14.58 ± 34.54	11.87 ± 31.26	15.51 ± 33.18	< 0.001	< 0.001	0.7865

2.3. Gingival tissue sampling, subgingival biofilm sampling, and quantitative real-time PCR

Gingival biopsies were obtained during surgical procedures and comprised epithelium and underlying connective tissue from a fraction of the healthy (n = 63) and chronic periodontitis samples (n = 123). For the healthy sample, gingival biopsies were taken from a representative fraction of the control group (n = 63) when they underwent surgical procedures for restorative/prosthetic reasons. For the chronic periodontitis group (n = 123), gingival biopsies were taken during periodontal surgical therapy as previously described [25]. Briefly, biopsies of gingival tissue were obtained during surgical therapy of the sites that exhibited no improvement in clinical condition 4–6 weeks after the basic periodontal therapy. Aggressive periodontitis subjects were not biopsied, since the treatment protocol required a 14 days antibiotic treatment previous to the surgical therapy. Ethical considerations (lack of indication of surgical therapy) prohibited tissue sampling from the chronic gingivitis group.

Periodontal crevice/pocket biofilm samples were collected with a sterile paper point ISO #40 from the same site biopsied immediately before surgical procedure (H n = 63; CP n = 123) to quantify the bacterial load of the pathogens *P. gingivalis*, *T. forsythia* and *T. denticola*. The bacterial DNA quantification procedures has been extensively described elsewhere [1,26]. Briefly, bacterial DNA from periodontal biofilm samples was extracted by DNA Purification System (Promega) [25]. Total bacterial load was quantified using the microbial DNA qPCR assay kit (QIAgen, Hilden, Germany) for 16S rRNA gene, following the manufacturer's instructions. RT-PCR analyses were performed in a MiniOpticon system (BioRad), using SybrGreen MasterMix (Invitrogen), specific primers (*P. gingivalis*: sense TGCAACTTGCCTTACAGAGGG, antisense ACTCGTATCGCCCGTTATTC; *T. forsythia*: sense GGGTGAGTAACGCGTATGTAACCT, antisense ACCCATCCGCAACCAATAAA; *T. denticola*: sense AGAGCAAGCTCTCCCTTACCGT, antisense TAAGGGCGGCTTGAAATAATGA), and 5 ng of DNA in each reaction [27]. The standard PCR conditions were 95 °C (10 min), and then 40 cycles of 94 °C (1 min), 56 °C (1 min), and 72 °C (2 min), followed by a standard denaturation curve.

RNA was extracted from gingival biopsies using TRIzol (ThermoFisher Scientific) reagent following the manufacturer's instructions. Reverse transcription was accomplished with RevertAid First Strand cDNA synthesis kit (ThermoFisher Scientific) following the manufacturer's instructions. Quantitative RT-PCR were performed in an MiniOpticon system (BioRad, Hercules, CA, USA), using SybrGreen MasterMix (Invitrogen), specific primers (TNFα: sense AAGCCTGTAGCCCATGTTGT, antisense CAGATAGATGGGCTCATACC; IL-1β: sense GGAACCCCAGAGCGAAATACA, antisense CCTGAAGA ATGCCTCCTCACA; IL-10: sense AGATCTCCGAGATGCCTTCA, antisense CCGTGGAGCAGGTGAAGAAT; IL-6: sense AAATTCGGTACATCC TCGAC, antisense CAGGAACTGGATCAGGACTT; IFN-γ: sense ATGAAATATACAAGTTATATCATGC, antisense TGTTTCGAGGTCGAAG AGCATCCCAGTAA; T-bet: sense CCTCTTCTATCCAACCAGTAT, antisense CTCCGCTTCATAACTGTG; Beta-actin: sense ATGTTTGAGA CCTTCAACAC, antisense CACGTCADACTTCATGATGG) and 2.5 ng of cDNA in each reaction, as previously described in detail [28,29]. The standard PCR conditions were 95 °C (10 min), 40 cycles of 94 °C (1 min), 56 °C (1 min), and 72 °C (2 min), followed by a standard denaturation curve. Negative controls without cDNA and without the primer/probe sets were also performed. Calculations for determining the relative levels of gene expression were made from triplicate measurements of the target gene normalized to β-actin, using the $2^{-\Delta\Delta ct}$ method.

2.4. Statistical analysis

Differences among clinical and demographical data was tested with chi-squared test and Student's *t* test. Differences in allele frequency between the sample population and reference populations were tested by Yates's corrected chi-square test. Standard and allelic case/control association analysis was performed using chi-squared test and Fisher's exact test. Data distribution of continuous variables was tested with Shapiro-Wilk test. Differences between continuous data were tested with Student's *t*-test or Mann-Whitney *u* test. A p-value < 0.05 was considered significant. Analyses were performed in GraphPad Prism v7.02 (GraphPad software, La Jolla CA) and PLINK v1.07 [30].

The study's power was calculated *post hoc* using the Genetic Association Study (GAS) Power Calculator online tool based in the algorithm from the CaTS power calculator for two-stage association studies [31].

3. Results

3.1. Clinical characteristics, genotyping and case/control association

There were significant differences in demographics and clinical parameters among patient's groups that had been extensively described elsewhere [1,18]. As expected based on the selection criteria, the AP group presented similar periodontal destruction to the CP group (represented by attachment loss) at a significantly lower age (Table 1).

Genotype and allele frequencies of the studied polymorphism were in Hardy-Weinberg equilibrium in the H, CG, CP, and AP sample populations (p > 0.05).

The minor allele frequency in the healthy sample population was 0.076 [436/33], resembling the average allele frequency described in the latest release of the Exome Aggregation Consortium (ExAC) [12] 0.073 [112599/8813], but significantly different from the allele frequency reported for the Latino sample of ExAC 0.028 [11257/321] (Yates's corrected chi-square 96.72; p-value < 0.001) [31]. The polymorphic allele proved significantly more common in the chronic

Table 2
Genotype and allele frequencies for H, CP, CG and AP groups.

CCR5d32 (rs333)	Control (n = 218)		CP (n = 197)		CG (n = 193)		AP (n = 91)	
Genotype	N	%	N	%	N	%	N	%
WT/WT	186	85.3	175	88.8	156	80.8	81	89.0
WT/D32	31	14.2	21	10.7	31	16.1	10	11.0
D32/D32	1	0.5	1	0.5	6	3.1	0	0.0
Allele	N	%	N	%	N	%	N	%
WT	403	92.4	371	94.2	343	88.9	172	94.5
D32	33	7.6	23	5.8	43	11.1	10	5.5

gingivitis group than in the chronic periodontitis (p-value 0.01) and the aggressive periodontitis groups (p-value 0.04). All other allele frequency comparisons among groups proved non-significant (Table 2).

The association analysis demonstrated a significant protective effect of the polymorphic allele when the chronic gingivitis ['resistant' phenotype] sample was compared with both the chronic (OR 0.49; CI 0.29–0.83; p-value 0.01) and aggressive (OR 0.46; CI 0.22–0.94; p-value 0.03) periodontitis groups ['susceptible' phenotypes] (Table 3). The polymorphic allele was significantly more prevalent in unaffected/control subjects than in patients/cases. The case/control association tests using healthy patients as controls failed to prove any significant effect of rs333.

3.2. Inflammatory biomarkers

As expected, all inflammatory biomarkers where significantly up-regulated in chronic periodontitis patients irrespective of their genotype. There was a significant decrease in the expression level of TNFα in heterozygous subjects compared with ancestral homozygous in the chronic periodontitis group (p-value 0.01). There were no significant differences in IL-1β, IL-10, IL-6, T-bet, and IFN-γ mRNA expression between wild type and heterozygous subjects in the chronic periodontitis group (Fig. 1).

3.3. Subgingival biofilm profile

There were no significant differences in the bacterial load for the tested putative periodontal pathogens (*P. gingivalis, T. forsythia, and T. denticola*) between the wild type (WT) and WT/CCR5Δ32 heterozygous chronic periodontitis patients (Fig. 2).

Similarly, there were not significant differences for the frequency of detection of the three putative periodontal pathogens tested (*P. gingivalis, T. forsythia, and T. denticola*) between the WT and WT/CCR5Δ32 heterozygous chronic periodontitis patients (Table 4).

4. Discussion

Periodontitis is characterized by the inflammatory destruction of periodontal tissues concomitant to the immune response triggered by periodontal infection. The chemokine system is a critical determinant of host response by directing the selective infiltration of distinct leukocyte subsets into periodontal tissues, and consequently influences the outcome of the disease. The chemokine receptor CCR5 has a putative crucial role in periodontitis' pathogenesis, since it directs the migration of pro-inflammatory and pro-osteoclastogenic cells. Taking in consideration that CCRΔ32 (rs333) directly impacts CCR5 function, we conducted a genetic association study coupled with the analysis of secondary functional variables to address the possible influence of this genetic variant in the susceptibility to develop periodontitis.

The allele frequency for the polymorphic allele CCR5Δ32 in the control healthy population was similar to the average reported frequency in the ExAC (Exome Aggregation Consortium) [31], nevertheless it was significantly different to the frequency reported for Latino ancestry in ExAC. This is arguably due to the methodological discrepancy between our sampling and the ancestry definition strategy used by ExAC. Briefly, ExAC uses a principal component analysis to distinguish the major axes of geographic ancestry and then clusters the individuals according with their putative origin. It is possible that the Latino definition of the ExAC data base encompass a significantly broader and diverse population than the one recruited for this study, which is exclusively constituted by south-eastern Brazilians that are characterized by significant European (86.1%) and African (12.0%) heritage contributions [32,33]. Still, due to the distinctive high genetic admixture observed in Brazilian population, the ancestry cannot be inferred based on phenotypic characteristics [34–36].

The association analysis demonstrated a protective effect of the CCR5Δ32 polymorphism, which was significantly more prevalent in the resistant subjects presenting chronic gingivitis (CG) than in the two susceptible samples represented by aggressive and chronic periodontitis (Section 3.1). Interestingly, when the association analysis was performed with the traditional design used in genetic association periodontitis studies, with healthy individuals as the control population, no significant associations were observed. As previously demonstrated by our group, CG subjects are chronically exposed to the bacterial challenge to periodontal tissues (evidenced by a bacterial load similar to that of CP patients), but suffer no irreversible inflammatory destruction of the periodontium. In this sense, the CG group is a truly control population, because they share the exposure to the causal factor of the disease with the CP and AP patients. Contrariwise, the healthy sample is not equally exposed to the bacterial challenge as the diseased individuals and it is arguable composed of a heterogeneous population from the susceptibility point of view, ranging from highly resistant to highly susceptible individuals [1,18]. Even though the putative risk genetic profile for human periodontitis is extremely complex, the

Table 3
Allelic association analysis for rs333 and the phenotypes H, CG, CP and AP. OR = odds ratio. SE = standard error. 95% CI = 95% confidence interval.

	Frequency affected	Frequency unaffected	Chi square	p-value	OR	SE	95% CI
Healthy vs gingivitis	0.11	0.08	3.11	0.08	1.53	0.24	0.95–2.46
Healthy vs chronic periodontitis	0.06	0.08	0.99	0.32	0.76	0.28	0.43–1.31
Healthy vs aggressive periodontitis	0.05	0.08	0.85	0.36	0.71	0.37	0.34–1.47
Gingivitis vs chronic periodontitis	0.06	0.11	7.08	0.01	0.49	0.27	0.29–0.83
Gingivitis vs aggressive periodontitis	0.05	0.11	4.66	0.03	0.46	0.36	0.22–0.94

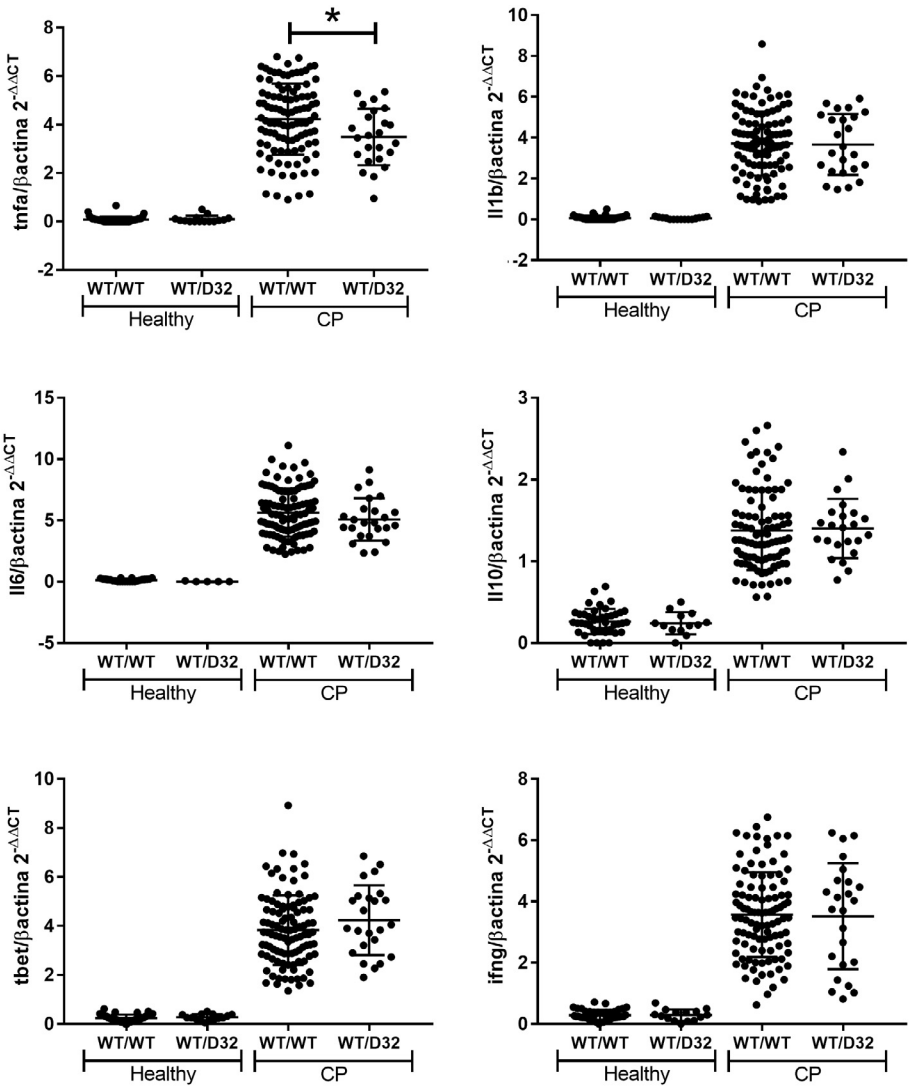

Fig. 1. Relative expression of TNFα, IL-1β, IL-6, IL-10, T-bet, and IFN-γ in H and CP subjects according to their genotype for CCR5Δ32. * = p-value < 0.05. Central line = mean; whiskers = standard deviation.

increased frequency of CCR5Δ32 in 'resistant' subjects (CG) is in accordance with our data from experimental periodontitis in CCR5KO mice, where CCR5 null mice proved resistant to periodontitis induction when compared to wild type mice [8,37].

Indeed, CCR5Δ32 analysis reinforces the mixed nature of healthy subjects, which presents an intermediary frequency of Δ32 allele/genotype, located exactly in between resistant and susceptible groups. It is noteworthy that the allele frequencies for the diseased, healthy, and resistant groups followed a gradient of enrichment of the mutant allele (0.057, 0.076, and 0.111, respectively). This finding further reinforces our proposed strategy of using the chronic gingivitis group as the true reference control for periodontitis patients.

The *post hoc* computing of the study's power resulted 0.901, using the Genetic Association Study (GAS) Power Calculator based in the CaTS power calculator for two-stage association studies [31]. The parameters for the calculation were: multiplicative (allelic) disease model, resistant *versus* susceptible populations, disease prevalence of 0.2, $\alpha = 0.05$, and a genotype relative risk of 2.04 (adjusted form the reciprocal of our computed OR = 0.49).

At this point, it is important to mention that the two previous studies, which did not find significant association between CCR5Δ32 and aggressive or chronic periodontitis [16,17] used the 'healthy' group as control, which in fact decrease the odds of finding possible associations in view of the unclear phenotypic/genotypic nature of heathy subjects.

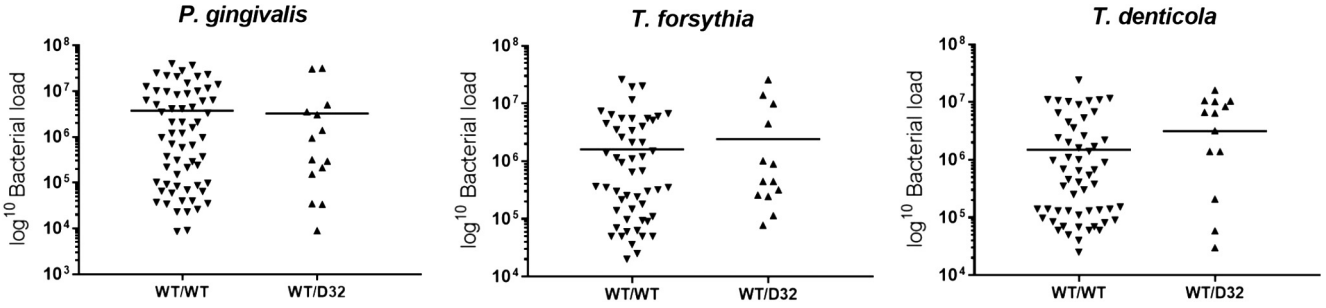

Fig. 2. Bacterial load of *P. gingivalis, T. forsythia, and T. denticola* on periodontal biofilm from chronic periodontitis patients arranged by CCR5Δ32 genotype.

Table 4
Frequency of detection of *P. gingivalis, T. forsythia, and T. denticola* on periodontal biofilm from chronic periodontitis patients.

	P. gingivalis				*T. forsythia*				*T. denticola*			
	WT/WT		WT/D32		WT/WT		WT/D32		WT/WT		WT/D32	
	N	%	N	%	N	%	N	%	N	%	N	%
Positive	64	62.1	14	58.3	61	59.2	13	54.2	61	59.2	13	54.2
Negative	39	37.9	10	41.7	42	40.8	11	45.8	42	40.8	11	45.8

In addition, the sample size of both cited studies was insufficient to perform a solid genetic association analysis, since in a complex disease such as periodontitis, the effect of any mutation is expected to be small [38]. In the first case [16], the study was most probably under powered due to the small sample recruited (total sample N = 175). This can also be said of the second study [17], which recruited only 213 subjects. In comparison, our sample consisted of 699 subjects. Moreover, the inclusion of 'resistant' (CG) and a 'highly susceptible' sample population (AP) further strengthen our results, because even with a relatively small sample size our strategy detected a significant contribution of the polymorphism in the susceptibility to the disease. This strategy has been already proposed by other groups and it has proven valuable to reveal genotype/phenotype associations without the need of recruiting extremely large sample populations [39–41]. In this sense, the careful clinical characterization of the subjects provides a homogenous sample population from the phenotypic point of view, that it is arguably also homogenous from a genetic perspective in terms of prevalence of risk mutations. Our group already demonstrated that this strategy increases the power and odds of genetic studies and that the classic healthy control used in most genetic association periodontal studies has an intermediate genotype for several polymorphisms, occupying an intermediate position between resistant and susceptible subjects [1,18].

Furthermore, the requirement for screening and exclusion of major disease co-factors during the recruitment stage is critical. Tobacco smoking is the most widely recognized behavioral risk factor for periodontitis [42], thus we explicitly excluded smokers and former smokers in the recruitment phase. This basic precaution wasn't practiced in the previous studies investigating the role of CCR5Δ32 in periodontitis [16,17]. It is of paramount importance to emphasize that tobacco smoking is the most important modifiable risk factor for periodontitis [43–46] and that failing to control for this factor completely invalidates any genetic association analysis. The confounding effect of tobacco smoking cannot be adequately corrected in small sample populations as the ones used in both cited studies [16,17], and the only sensible study design alternative is the exclusion of smokers from the sample, as we did. Further, the minor allele frequency for CCRΔ32 (0.13) reported in one of the studies [17] is more than 10-fold higher than the frequency reported in ExAC for East Asian and Southern Asian populations (0.0002 and 0.014, respectively), probably caused by a biased recruitment strategy which predisposed to a type II error due to population stratification.

Aiming to complement our association results and reveal a possible mechanism of action of CCRΔ32 in conferring resistance to periodontitis, we performed additional experiments characterizing the inflammatory expression pattern of key biomarkers and quantified the bacterial load of selected periodontal pathogens. The analysis of inflammatory biomarkers (Section 3.2) revealed a significant reduction of TNFα expression levels in heterozygous subjects. Accordingly, previous results in animal models have demonstrated that CCR5 genetic deficiency or pharmacological inhibition results in decreased TNFα levels, and more importantly, reduced periodontal destruction [9]. However, while CCR5KO mice presents a broad disruption in the cytokine production pattern during experimental periodontitis, CCR5Δ32 mutation produced no significant effect in the expression levels of IL-1β, IL-10, IL-6, T-bet, or IFN-γ. The redundancy in the functions of the chemokine receptors CCR1 and CCR5 could be partially responsible for the minor effect the mutation exerted over the expression level of inflammatory biomarkers. Our group has already demonstrated that there is a compensatory effect in the CC chemokine system, where the absence of CCR3 in an experimental periodontitis model generates no phenotypic alteration by the compensatory functions of CCR1 and CCR5 [8]. Further, we previously demonstrated that the combined pharmacological inhibition of CCR1 and CCR5 with met-RANTES has a more pronounced phenotypic effect in the progression of experimental periodontitis than the congenital absence of CCR5 receptor in a KO mice model system [9,37].

Additionally, it is important to take into consideration that the density of expression of CCR5 in the surface of leukocytes is highly variable by factors other than the underlying genotype. T cells isolated from WT homozygous individuals can express low levels of CCR5 not significantly different from those of WT/CCR5Δ32 heterozygous subjects [47]. A possible explanation for this phenomenon is the epigenetic regulation of CCR5 expression via methylation of the *cis* regulatory region of the *CCR5* gene, which can be accountable for 20-fold variations in the density of CCR5 surface expression, effectively causing WT homozygous subjects to have levels of CCR5 expression comparable to WT/CCR5Δ32 heterozygous counterparts [48].

Despite the expectation of a more prominent association between CCR5Δ32 and cytokine mRNA levels (based in our previous experimental data) the effect on TNFα expression reveals that CCR5Δ32 heterozygosity has a measurable effect in the expression pattern of effector molecules produced in response to periodontal infection, and provides support for the mutation's functionality. We must also consider that the expression levels were only compared between chronic periodontitis patients and healthy subjects, since ethical considerations impeded us to harvest gingival tissue from chronic gingivitis ('resistant') subjects, which could have provided the same resistance/susceptibility phenotypes comparison performed in our genetic association analysis. Such results also highlight the complexity of the regulatory mechanisms of inflammation during the course of periodontitis. Indeed, we encountered an equivalent result in a recently analysis of TBX21-1993T/C (rs4794067) and chronic periodontitis [1]. In the cited study, we were unable to find an upregulation of IFN-γ in subjects carrying the TBX21-1993 polymorphic allele, despite the significant effect of the polymorphism in the expression levels of the Th1-differentiation key master switch T-bet [1].

Indeed, it is important to consider that the individual contribution of single mutation to a complex disease like periodontitis is expected to be relatively small. Accordingly, previous studies demonstrated that the presence of specific pathogens, such as *P. gingivalis, T. forsythia,* and *T. denticola* have a stronger influence in the levels of pro-inflammatory cytokines than functional SNPs in their respective genes. Repeated and persistent stimulation from a constant influx of antigens derived from a pathogenic biofilm can overcome the genetic predisposition for a milder inflammatory response caused by a genetic variant [49]. In this sense, our data shows a strong and significant correlation between the bacterial load of *P. gingivalis, T. forsythia,* and *T. denticola* and the expression of the tested inflammatory biomarkers, irrespective of the genotype (data not shown).

Similarly, we did not find any effect of the CCR5Δ32 in the bacterial

load of classic putative periodontal pathogens *P. gingivalis, T. forsythia, T. denticola* (Section 3.3). Comparably, in our experiments with C-CR5KO mice, the reduced leukocyte chemoattraction to periodontal tissues did not result in increased bacterial burden and systemic biomarkers of acute response to infection remained unaltered [9,37]. However, a dose response analysis of the effects of Met-RANTES revealed a distinct scenario. While low and intermediate doses reduce inflammatory cell infiltration and bone loss and did not compromise the infection control, increased doses seemed to trespass a critical threshold, interfering with the production of the antimicrobial markers myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and IgG, resulting in increased bacterial loads [37]. In this case, the impaired protective response is possibly due the lack of the compensatory role of CCR1, also blocked by Met-RANTES [8].

In conclusion, the strategy employed in our genetic association analysis proved useful to detect the effect of CCR5Δ32 (rs333) on the risk to suffer periodontitis. Furthermore, we were able to correlate the genotype for rs333 with significant changes in the expression pattern of a key inflammatory cytokine (TNFα), providing a probable mechanistic link between the genetic background and the risk profile. These results, coupled with our previous results *in vivo* [8,37], point to a significant contribution of CCR5 in the pathogenesis of periodontitis. Thus, future studies employing a more sensitive and broad sampling strategy of inflammatory biomarkers and microbiological profile are required to unequivocally ascertain the pathway by which rs333 protects against periodontal inflammatory destruction. Also, it is possible that the single time point sampling limits the strength of the association between genotype and biomarkers microbiological data and that a time-series sampling strategy could overcome this issue. In the future, these kind of evidence will contribute to develop tailored periodontal interventions shaped to the individual risk profile of patients and will also help to direct preventive interventions at risk population before the occurrence of irreversible periodontal damage.

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