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Genetic Polymorphisms of Foxp3 and IL-17A In Inflammatory Periodontal Disease

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Abstract: *The role of newly identified two subpopulations of helper T cells and closely related - regulatory T cell [Treg; positive forkhead box P3 (Foxp3)], and Th17 cells [interleukin-17-positive (IL-17)] - specifically in periodontal disease and gingivitis has yet to be clarified. The aim of the study was to identify polymorphic variants in the promoter region of the Foxp3 gene and IL-17A their possible association with gingivitis in 141 women. Foxp3 and IL-17 polymorphisms of genes were analyzed using the method length polymorphism reaction-restriction fragment polymerase chain (FRLP) and degrees. DNA samples were obtained from peripheral blood. Comparison of the distributions of genotypes and alleles of rs3761549 and rs2275913 polymorphisms showed no statistically significant differences between groups with different degrees of gingivitis. However, there was an association between gingival index and the presence of the C allele in Foxp3 polymorphism (rs3761549). For the IL-17 polymorphism (rs2275913), no association was found with the IG and distributions of genotypes or different alleles. Foxp3 polymorphisms, especially the SNP involving the C allele, are associated to the Gingival Index.*

Keywords: *Foxp3, Gingivitis, Interleukin-17, Periodontal diseases, Polymorphism Genetic)*

I. Introduction

Periodontal disease (PD) is an inflammatory condition brought about by the interaction between microorganisms that compose supragingival and subgingival biofilms and the host inflammatory response (1).
In the last classification of periodontal diseases, gingivitis, considerably prevalent in most populations, was divided into two groups: periodontal diseases induced and not induced by the biofilm, which when enduring, represents a risk factor to the loss of the periodontal attachment and teeth (2-5).

The absence of prognostic markers indicates the need of follow-up examinations to assess the progression, recurrence and therapeutical needs. In PD the immune system is induced to arrange an inflammatory response (6). Metabolic alterations, genetic and environmental factors are related in the modulation of the gingivitis' clinical expression (7), then it is interesting to know the periodontal profile in different populations and associations to systemic parameters.
Most kinds of periodontitis result from interactions between the genome, behavior and environment throughout life. The practical utility of the genetic knowledge in it is still limited and the genes allelic variants probably influence its susceptibility (8). Due to this fact, some studies point that the single nucleotide polymorphisms (SNPs) are related to the host's response to the PD, and it may foresee susceptibility or severity (9).

Based on this assumption, increasing evidences suggest that distinct inflammatory cytokines convert Forkhead box protein P3 (Foxp3 (+) and regulatory T-cells (Tregs) into IL-17-producing cells (Th17 cells), and it has contributed to the pathogenesis of periodontal disease. However, the role of Foxp3 Th-17-mediated immune responses and its relationship in the context of periodontitis remains unclear (10).

An important study showed that the number of Th17 (IL-17A (+) (-) Foxp3) and Tregs (IL-17A (-) Foxp3 (+)) were higher in injuries derived from periodontitis than in gingivitis. The findings indicate that the conversion of Treg-Th17 may happen in injuries of periodontitis, needing more studies covering the role of the Treg conversion during inflammatory responses against periodontal bacteria (10). Another research showed that Foxp3 (+) cells are more prominent than IL-17(+) cells in periodontal disease processes, which may suggest a predominant role for Foxp3 (+) cells in periodontal disease (11).

The Tregs are important in the maintenance of the immune homeostasis, since its ability to low the immune aggression through the production of anti-inflammatory cytokines like IL-10. There are reports of problems in the Tregs' function or reduction of their numbers in autoimmune diseases (12).

Regarding the genetic factors, the genes have an important role in the immunopathology of the PD, since the cytokine genes are the main candidates to the polymorphism analysis (13).

The role of IL-17 and its polymorphism is yet to be investigated more carefully throughout further research. In fact, that polymorphism of IL-17R plays significant role in incidence of chronic periodontitis (14). However, other works suggest that the presence of IL-17A (rs2275913), an allele, is associated with the absence of periodontal disease (14, 15).

It seems reasonable to affirm that genetic mutations by themselves are not enough or needed to explain the disease's phenotype, although, in the results of periodontitis, they may contribute in a significant way to the environmental standards and lifestyle (16).

Polymorphisms in Foxp3 may, for example, influence in the cell function and in patients with psoriasis. In this case an only SNP affects the gene Foxp3 transcription because of a problem in the linkage of the factors E47 and C-myb, caused by an alteration in allele C to A -3279 C / A (rs3761548) in the promoter region of the Foxp3 gene (17). Additionally, available information about the polymorphism of Foxp3 gene and association of the periodontal disease is scarce.

The aim of the study was to identify polymorphic variants in the promoter region of the Foxp3 and *IL-17A* gene and their possible association with severity of gingivitis in Northeastern Brazil population.

II. Materials and Methods

1.1. Subjects .

A cross-sectional and analytic study conducted by collecting clinical and laboratory data on gingival conditions from a group of 141 adult women. Some participant were excluded when a) they were undergoing treatment with antibiotics; b) had fewer than 14 teeth; c) were using an orthodontic device; d) periodontitis or ed) had undergone periodontal treatment or professional oral prophylaxis within the previous six months.

2.2 Clinical assessment

A clinical assessment was conducted in a dental office, blind, by a single evaluator who had no previous knowledge of other study variables. The dentist was subjected to intra-examiner calibration, obtaining a level of Kappa agreement above 0.70 for the observed parameters.

A magnification device (Zeiss EyeMag Pro F400 Loupe, 3.5x magnification) was used to increase the accuracy of the measured data. The following clinical periodontal parameters were evaluated: Plaque Index (PI), Gingival Index (GI), and Periodontal Screening and Recording (PSR). Dental elements were examined at six sites (Disto- buccal [DBV], buccalVestibular [BV], Mesio-buccalvestibular [MBV], Mesio-lingual [ML], Lingual [L], and Disto-lingual [DL]). The PSR was obtained concurrently with the PI and GI at all sites. The PSR data were applied to a pre-analysis for inclusion in groups generally considered to have gingivitis and periodontitis. The evaluation of the PI and GI used the classifications of LÖE and SILNESS (18-20). The PI was analysed as a secondary and association variable, and the GI was used to classify the gingivitis severity. The choice of these indices was due to their wide applicability in the local setting. Based on the GI values obtained, the women were classified according to the severity of gingival inflammation into mild, moderate, or severe gingivitis (19).

2.3. DNA Isolation and Genotyping Analysis.

To the analysis of the genetic polymorphisms rs2275913 (*IL-17A*) and rs3761549 (*Foxp3*) it was performed an extraction of the genomic DNA by the salting out method, described by Miller and collaborators (21) with some modifications. The extracted DNA showed a good integrity after the electrophoresis using 3.5% agarose gel. The quantification and determination of the DNA purity (absorbance ratio A260/A280) were performed in a spectrophotometer (Nanodrop, Thermo Scientific- GE). Much of the samples presented a concentration higher than 300ng/µl and ratio A260/A280 between 1.80 and 2.00. After this the DNA was diluted in a final concentration of 100ng/µl to be used in the polymerase chain reaction (PCR).

The amplification of the genetic material was performed through PCR technique to a final volume of 25µL/well, containing: 19.125µL autoclaved Milli-Q water; 5µL of 10x Taq polymerase buffer, 1µL of DNA in 100ng/µL, 0.75µL of $MgCl_2$ in 50mM, 0.5µL of 10mM dNTP mix, 0.125µL Taq polymerase 5U / uL and 0.5µL in 20pmol of the sense (5'-GCCTGGCACTCTCAGAGCTT-3') and anti-sense primers (5'-TGCCCACGGTCCAGAAATAC-3') for the rs3761549 of Foxp3, and 0,5µL in 20pmol of the sense (5'-AGGTACATGACACCAGAAGACC-3') and anti-sense primers (5'- TGCCCACGGTCCAGAAATAC-3') for the rs2275913 of *IL-17A.* Then, plates were accurately position in the thermocycler (MyCycler Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA) under the circumstances described in Table 1. Finally, the electrophoresis was conducted in 2% agarose gel aiming to identify the presence of the amplified products.

The difference among the SNPs was performed by the cleavage of the products amplified, using restriction fragment length polymorphism (RFLP) (Table 1). Thus, the separation and disclosure of the presence of such fragments, and consequently the polymorphisms, was performed in a UV-translluminator after an electrophoresis at 2% Agarose gel colored with *SYBR® Green* (Life Technologies, USA).

2.4. Data Analysis.

The data was organised into a database in the statistical program SPSS® (Statistical Package for the Social Sciences) version 20 and subjected to statistical testing after evaluating their distributions. The association between the gingival conditions (Mild, Moderate) and the qualitative aspects was assessed applying the *Likelihood Ratio* Chi-Squared test (χ^2). To the 2x2 tables the Fischer's exact test was applied and calculated the odds ratio. The Student T-test was applied to compare the means of the Gingival Index between the two groups, as well as the One-way ANOVA to compare the means between more than two groups. Box-plot graphs were drawn to show the properties and distribution between the groups and the descriptive statistics were calculated. The significance level adopted was 5% in the statistical tests. The Hardy-Weinberg equilibrium was assessed by the Chi-Square Goodness-of-Fit Test.The experimental protocol applied was in accordance with the ethical guidelines, was submitted to the Ethics and Research Committee and approved under Opinion No. 227,271, March 22, 2013.

III. Results

The demographic characteristics of the studied population are presented in Table 3. In this regard, data from frequencies of polymorphisms IL-17A and Foxp3 were investigated in blood samples of subjects and after the genotyping it was conducted the calculation of the Hardy-Weinberg equilibrium (EHW) in both SNPs, Foxp3 2383 and IL-17, in both polymorphisms the frequency of the genotypes showed conformity with the EHW, as shown in Table 2.

A total of 141 women between 18 and 81 years old were included in this study. The age average was 33.07 years old. Gingivitis was diagnosed through the GI in all women examined, in 74 women (52.9%) it was diagnosed as mild and in 67 (47-1%) it was considered moderate. There was no record of severe gingivitis or periodontitis in any one of them.

Regarding the clinical findings, we point out that there was a significant correlation to the age, where the risk of moderate gingivitis is higher in women older than 38 years old. It also happened that the increase in the daily brush frequency lowered the risk of moderate gingivitis (ρ=0.012), as reported in Table 3.

In fact, there is no significant difference between the Foxp3 and IL-17 polymorphisms and severity of the gingivitis (data not shown). The analysis of variables: presence of the G allele, A allele and genotype distribution (GG, AA and AG) of IL-17 showed no significant correlation with the diagnosis of gingival conditions, as well as presence of the C allele, the T allele and the genotype distribution (CC, TT and CT) of Foxp3 did not show significant associations regarding the diagnosis of gingival conditions. However, there was a significant association between Gingival Index and having allele polymorphisms of Foxp3 C (rs3761549) (p=0.032). For the IL-17 (rs2275913) polymorphism, no association was found between the Gingival Index and distributions of different alleles (Table 4).

The means of the gingival index (GI), when considered as a quantitative variable, according to the genotype distributions and according to the presence of the G and A alleles in the IL_17 and there is no significant differences .

Regarding genotype distributions to the Foxp3, the means of the Gingival Index, when considered as a quantitative variable, presented no significant differences between the mild and moderate groups.

About the presence of the C allele of the Foxp3, it is possible to observe different variances, as shown in Fig. 1 and Table 4. There is a significant difference, although the qualitative test did not detected correlations (p=0.017), showing that there is no significant correlation when considered the degree of inflammation if mild or moderate.

About the presence of the T allele of the Foxp3 it happened no significant difference.

The analysis of the genotype distribution of the IL-17 and Foxp3 did not show any significant differences between the means of the groups, as shown in Table 5.

IV. Discussion

The study showed the Th17 and Foxp3 polymorphisms together in gingivitis. Th17 cells are generally considered proinflammatory, particularly through the production of the interleukin-17A cytokines (IL-17A) and IL-17F. These cells and cytokines have been associated to the pathogenesis of a long list of inflammatory diseases as, for example, periodontitis (22). Besides, Tregs cells may regulate the activation, proliferation, differentiation and effector function of immune cells (16).

The IL-17A polymorphisms may influence the functionality of the Tregs molecules and the role of the IL-17A and Foxp3 in PD is yet to be determined (11). In this study we sought to determine polymorphic variants in the promoter region of the Foxp3 and *IL-17A* gene their possible association with and severity of gingivitis in Northeastern Brazil population.

Our study point out to the influence of the age in the severity of the gingivitis. Age has been broadly researched and pointed as an important risk factor of PD, as most of the attachment insertion loss verified is due PD in individuals older than 30 years old (23). This suggests that the consequences of the development and advance of periodontitis are more prevalent in older individuals, and corroborates with of other researches (13).

The educational level has also been evaluated as a risk factor for periodontitis, and studies point that the disease was more prevalence in individuals who had not completed high school (13). In another Brazilian population sample, the risk of dental insertion loss (attachment loss and/or dental loss?) due to disease progression increased by 53% in individuals who studied four years or less, when compared to those with higher education (23). The prevalence of a PD periodontal disease in a German sample was related to the socioeconomic and educational levels, which were essential to the outcome (24). In our study the educational level had no association to the severity of the gingivitis, opposing to the findings about periodontitis in other populations.

Studies have shown that IL-17 levels are elevated in diseased human periodontal tissues and may play a destructive role in periodontal disease (16). In this study, we investigated the involvement of IL-17 and Foxp3 genes polymorphisms in gingivitis. Unlike the data found in the literature, our results have not shown association of the IL-17 polymorphism and GI. More importantly, we have shown for the first time that Foxp3 polymorphisms, especially the SNP involving the C allele, are associated to the Gingival Index. Indeed, IL-17 polymorphism have been presented to be associated with periodontal tissue damage, however, other works suggest that the presence of IL17A (rs2275913) A allele are associated to the absence of periodontal disease (14, 15). The enigma of IL-17 and its polymorphism-role in gingivitis is yet to be investigated more carefully throughout further researches, but this article demonstrates that IL-17A polymorphism plays no significant role in the gingivitis severity.

In contrast, there was a significant association between Gingival Index and having C allele in Foxp3 (rs3761549) polymorphism *(p=0,032)*. In fact, regarding Foxp3 polymorphisms there was no development to investigate the SNP -2383 in patients with periodontal diseases. There are a few reports in the literature involving polymorphism -2383 C/T in Foxp3 gene (25). Recently, our group has described that a high percentage of individuals carrying the C allele in patient kidney transplant when compared to healthy control subjects (Submission date), suggesting that the Foxp3 polymorphism may play a role in the tools that difficult the inflammatory reaction. Other recent studies show that Foxp3 (+) cells are more prominent than IL-17 (+) in processes of periodontal diseases, which may suggest a predominant role of the Foxp3 (+) cells in the periodontal diseases (11).

Since we do not have data concerning the rate of progression of gingivitis due to the study design, we cannot reject the hypothesis that the presence of Foxp3 polymorphism could control the intensity of the inflammatory process favoring slower progressions of the periodontal injury.

V. Figures and Tables

Table 1 Pcr- Rflp Characteristics

SNP	PCR Conditions	Amplified product	Restriction enzyme	Fragments produced by each genotype
IL17A rs2275913	94°C for 5min; 35 cycles: 94ºC for 45s, 60°C for 45s, 72°C for 45s; 72°C for 7 min	514pb	XagI	GG: 297pb, 217pb. GA:514pb, 297pb, 217pb. AA: 514pb.
FOXP3 rs3761549	94°C for 5min; 35 cycles: 94ºC for 1min, 60°C for 1min, 72°C for 1min; 72°C for 10min	943pb	BseNI	CC: 525pb, 213pb, 170pb and 35pb. CT: 525pb, 383pb, 213pb, 170pb and 35pb. TT: 525pb, 383pb and 35pb.

Table 2 Calculation of Hardy-Weinberg equilibrium (EHW) to the SNPs of the Foxp3 2383 and IL-17

Gene / SNP	Patients (%)	*P* value / χ^2	Controls (%)	*P* value / χ^2
IL17A - rs2275913				
Alele G	212 (85,5)	**0.576**[a] 0,289	265 (75,7)	**0,780**[b] 0,078
Alele A	36 (14,5)		85 (24,3)	
Genotype GG	89 (71,8)		101 (57,7)	
Genotype GA	33 (26,6)		63 (36,0)	
Genotype AA	2 (1,6)		11 (6,3)	
FOXP3 - rs3761549				
Alele C	232 (87,9)	**0,386**[c] 33,69	354 (88,5)	**0.000**[d] 33,69
Alele T	32 (12,1)		46 (11,5)	

Genotype CC	103 (78,0)		165 (82,5)	
Genotype CT	26 (19,7)		24 (12,0)	
Genotype TT	3 (2,3)		11 (5,5)	

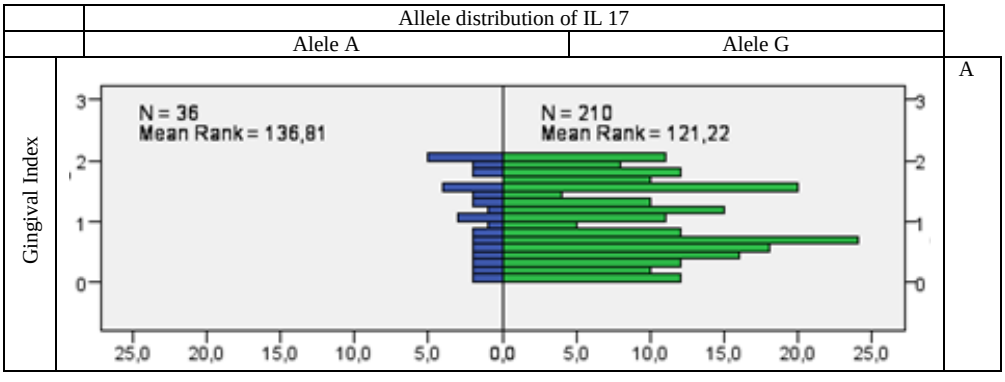
[a,b,c] $P > 0.05$ => in accordance to EHW. [d] $P < 0.05$ => in disagreement to EHW

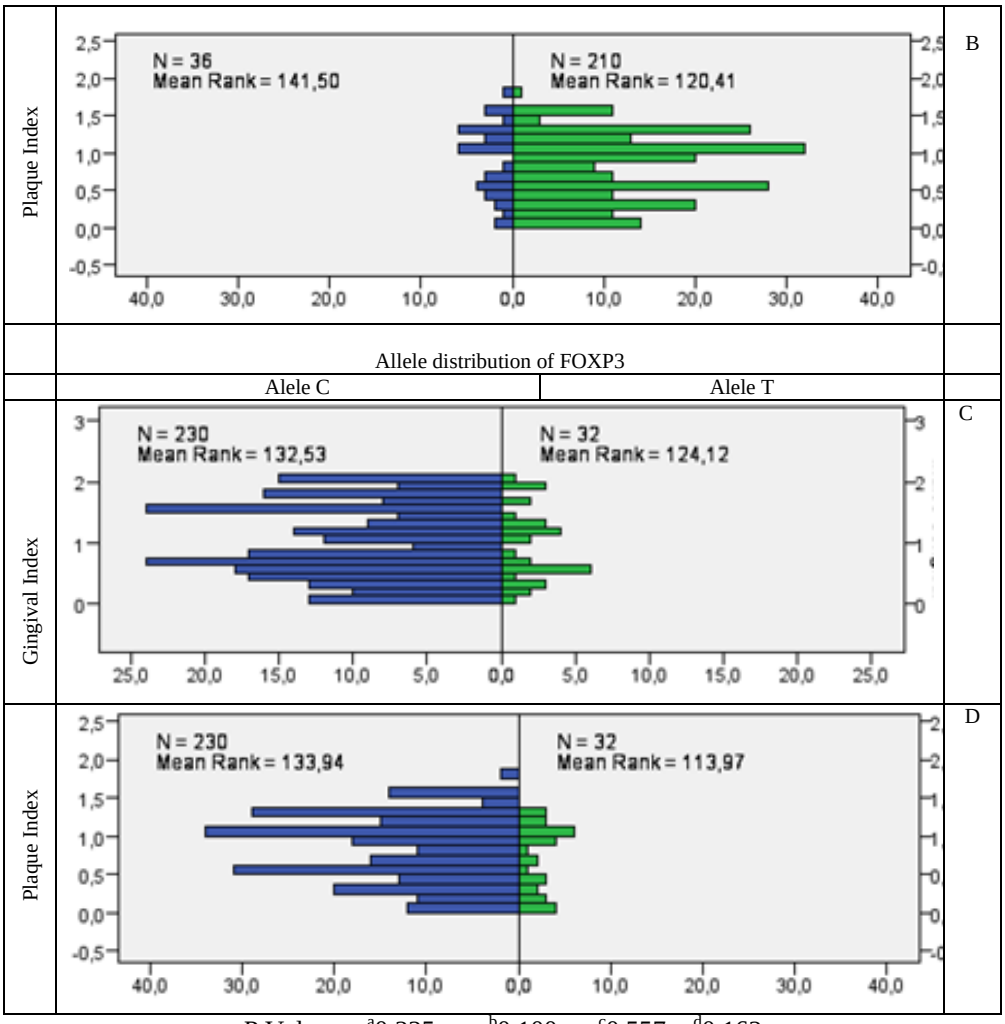
Table 3. Frequency distribution of polymorphisms in IL17A and FOXP3 in control subjects and patients with gingivitis

Gene / SNP	Patients (%)	Controls (%)	*P* value	RC (IC 95%)	patients (%)		*P* Value	RC (IC 95%)
					mild gingivitis	moderate gingivitis		
IL17A - rs2275913								
Alele G	212 (85,5)	265 (75,7)	-	1,0 (Ref.)	115 (88,5)	95 (81,9)	-	1,0 (Ref.)
Alele A	36 (14,5)	85 (24,3)	**0,004**	**1,89 (1,23-2,90)**	15 (11,5)	21 (18,1)	0,15	1,69 (0,83-3,47)
Genotype GG	89 (71,8)	101 (57,7)	-	1,0 (Ref.)	51 (78,5)	37 (63,8)	-	1,0 (Ref.)
Genotype GA	33 (26,6)	63 (36,0)	0,057	1,68 (1,01-2,79)	13 (20,0)	20 (34,5)	0,10	2,12 (0,94-4,79)
Genotype AA	2 (1,6)	11 (6,3)	0,41	4,85 (1,05-22,5)	1 (1,5)	1 (1,7)	1,00	1,38 (0,83-22,8)
GA + AA	35 (28,2)	74 (42,3)	**0,015**	**0,54 (0,33-0,88)**	14 (21,5)	21 (36,2)	0,11	0,48 (0,22-1,07)
FOXP3 - rs3761549								
Alele C	232 (87,9)	354 (88,5)	-	1,0 (Ref.)	123 (87,9)	107 (87,7)	-	1,0 (Ref.)
Alele T	32 (12,1)	46 (11,5)	0,81	0,94 (0,58-1,52)	17 (12,1)	15 (12,3)	1,00	1,01 (0,48-2,13)
Genotype CC	103 (78,0)	165 (82,5)	-	1.0 (Ref.)	55 (78,6)	47 (77,0)	-	1.0 (Ref.)
Genotype CT	26 (19,7)	24 (12,0)	0,08	0,58 (0,31-1,06)	13 (18,6)	13 (21,3)	1,00	0,83 (1,17-2,77)
Genotype TT	3 (2,3)	11 (5,5)	0,26	2,29 (0,62-8,40)	2 (2,8)	1 (1,7)	1,00	0,58 (0,51-6,66)
CT + TT	29 (22,0)	35 (17,5)	0,32	0,75 (0,44-1,31)	15 (21,4)	14 (23,0)	0,84	0,92 (0,40-2,10)

RC: odds ratio. 95% CI: 95% confidence interval. Ref .: comparison reference. P values are relative to the Fisher's exact test.

Considerations: 1st P value corresponds to comparison Allele Allele G vs A (or Allele Allele C vs T); the 2nd value of P is compared GG vs GA (or CC vs. CT); the 3rd value of P is compared GG vs AA (or CC vs. TT); the 4th value of P is compared GG vs GA + AA (or CC vs. CT + TT); I left significant value to the total homes in bold. 0.057 was borderline significance. Half the table left comparisons were made between patients and controls. The right half of the table in patients with gingivitis degrees. The n of patients with mild to moderate gingivitis has one less person in relation to the assessment of patients in general, because there was one missing value.





P Values = [a]0,225 [b]0,100; [c]0,557; [d]0,162

Figure 1 - influence of allelic distribution of SNP IL 17 and FOXP3 on dental parameters

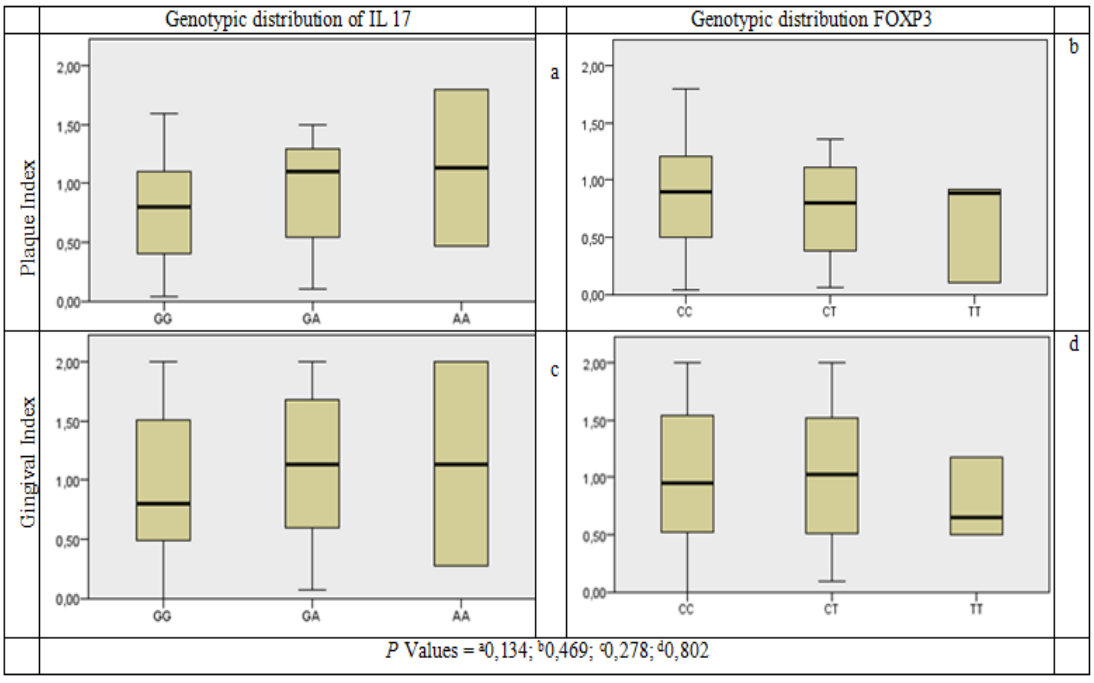


Figure 2 - influence of genotype distribution of SNP IL 17 and FOXP3 on dental parameters

VI. Conclusion

In conclusion, further studies are needed to clarify the molecular aspects of the relationship between Th17 and Foxp3 polymorphism, with potential applications for diseases prediction. The protection role of this molecule may not be excluded. Further studies are required to characterize these cells more precisely and to understand, in more details, their roles in the pathophysiology of gingivitis.

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