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ORIGINAL ARTICLE

Tumour necrosis factor-alpha polymorphism -308 G/a and its protein in subjects with gingivitis

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ABSTRACT

Objective: This study examined the association between tumour necrosis factor-alpha (*TNF- α)* (–308 G/A) polymorphism and gingivitis, and serum and salivary TNF- α levels, in a Mexican population.

Material and methods: This study enrolled 171 subjects, divided into two groups: healthy subjects and gingivitis patients. *TNF- α* (–308 G/A) gene polymorphism was analyzed by PCR-RFLP assay. Salivary and serum samples were used to measure cytokine levels through the ELISA technique.

Results: *TNF- α* (–308 G/A) polymorphism was shown to have a protective effect in carriers of the A/A genotype and allele A. The G/A genotype is associated with an increase in high-density lipoprotein cholesterol (HDL-C) levels in the gingivitis group. Healthy individuals had higher levels of salivary TNF-α and HDL-C, and increased salivary flow. Triglycerides, low-density lipoprotein cholesterol, and very low-density lipoprotein cholesterol levels were increased in the gingivitis group. No statistical differences were found in serum TNF- α levels.

Conclusion: Our data demonstrate that the *TNF- α* −308 A/A genotype exerts a protective effect against gingivitis. Moreover, oral conditions are associated with some biochemical parameters.

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Introduction

Periodontal disease (PD) is a condition affecting the tissues that support the teeth, such as the gingiva, periodontal ligament, and alveolar bone [1]. In this work, gingivitis refers to plaque-induced gingivitis, a reversible form involving an inflammatory lesion caused by the host's immune-inflammatory response and dental plaque interactions, confined to the gingiva, without spreading to the periodontal insertion [2]. Gingivitis is considered the precursor of periodontitis, which confers clinical importance [3].

Gingivitis affects up to 90% of the world's population and represents a public health problem. In Mexico, according to the Epidemiological Surveillance System for Oral Pathologies 2018 (SIVEPAB 2018), 57.9% of the population between 20 and 99 years of age presented some periodontal damage [4]. In addition, altered glucose levels, as well as dyslipidemias, have been described as risk indicators of PD [5,6].

Gingivitis is caused by bacterial components such as lipopolysaccharides, which induce an inflammatory response in the host [7]. The immune response to oral microorganisms induces an inflammatory cascade that promotes the release of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumour necrosis factor-alpha (TNF- α), in gingival tissue. Uncontrolled inflammation in the gingiva can cause the aggravation and destruction of the periodontal tissues, and the attachment loss may depend on the host's responsiveness to inflammatory aggression at the gingival sites [8,9]. TNF-α is a cytokine with multiple functions that can induce tissue destruction and alveolar bone resorption [10]. The production and biological activity of TNF-α depends on genetically controlled mechanisms. Single-nucleotide polymorphisms (SNPs) have been identified in the TNF-α gene promoter region, and the transition from G to A at position −308 has been shown to increase cytokine production levels [11].

Research has shown the association of this polymorphism with PD in some populations [12,13]. Nevertheless, other investigations show contradictory results [14,15]. The salivary and circulatory levels of TNF-α have been previously studied among people with and without PD, with inconsistent results [16–19]. To our knowledge, the literature does not contain information regarding the possible association between *TNF- α* (–308) polymorphism and serum and salivary levels of its cytokine in people with gingivitis. Therefore, this study aimed to analyze *TNF- α* (–308) polymorphism in healthy subjects and those with gingivitis. We also investigated

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whether *TNF-* α polymorphism (–308) is associated with serum and salivary TNF- α levels in a Mexican population. Additionally, we investigated whether some biochemical parameters are associated with gingivitis.

Material and methods

Study group

A cross-sectional study was conducted in full accordance with the general health law (NOM-012-SSA3-1012) and Helsinki declaration. The subjects of this study were recruited at the Centre for Comprehensive Medical Care, which belongs to the Centro Universitario de Los Altos of the Universidad de Guadalajara. A consecutive non-probabilistic sampling method was used to obtain the study population. The participants were informed about the study protocol, and all of them signed informed consent. The local ethics committee approved the research protocol under the official number 6/2017-2018.

This study enrolled 171 Mexican subjects of both genders, from 18 to 69 years. The subjects were submitted to anamnesis and to clinical and periodontal examination. The diagnosis of gingivitis was performed by calibrated dentists, considering bleeding on probing (BOP), with a cut-off point of 10% of the complete oral probing; probing pocket depth, considering the absence of depths greater than or equal to 4 mm; clinical attachment lost; gingival index; plaque index, and gingival bleeding index, using an oral mirror and a periodontal probe (model UNC 15 from American Eagle). The subjects were divided into two groups: healthy subjects ($n = 28$) and gingivitis patients ($n = 143$). Subjects with autoimmune diseases, undergoing orthodontic treatment, on medication for any systemic disease, on antibiotics, or who were pregnant women were excluded from the study.

Sample collection

Two blood samples were taken from the subjects with a venous puncture. One sample was collected in a sterile tube containing potassium EDTA solution, and the other in a dry tube. Both tubes were later centrifuged at 1646 relative centrifugal force (RCF) for 20 min to separate the leukocyte ring and the serum. The genomic DNA extraction was performed using Purelink™ Genomic DNA (mini Kit cat no. K182002) from Invitrogen™, following the manufacturer's instructions. Serum levels of glucose, triglycerides (TGs), total cholesterol (CHOL), and high-density lipoprotein cholesterol (HDL-C) were measured using an Abbott Aeroset autoanalyzer. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald equation. For very-low-density lipoprotein cholesterol (VLDL-C) levels, the TG values (mg/dL) were divided by five.

Unstimulated saliva was collected in 15 mL Eppendorf tubes using the spitting method. Subjects were asked to avoid eating, drinking, brushing, or using dental floss for 90 min before sampling. All participants were asked to deposit saliva in the tube for 5 min. The amount of deposited saliva was then recorded. The salivary flow was calculated by dividing the saliva volume by the collection time (5 min). The samples inside the tubes were then homogenized and clarified *via* centrifugation at 2374 RCF for 20 min at 4 °C. The aliquots of clarified supernatant were stored at −80 °C for future use in cytokine measurements. The quantification of serum and salivary TNF-a was determined using a commercially available ELISA kit (R&D Systems Inc, USA. Cat. DY008). The reagents and standards were prepared according to the manufacturer's instructions. Absorbance was measured at 450 nm and 570 nm using a microplate reader (Multiskan GO, Thermo Scientific, Finland). The results were presented in picograms per millilitre (pg/mL). For quality control, the samples were randomly analyzed between healthy subjects and gingivitis subjects, and the analyst did not know which group the samples belonged to.

Genotyping analysis

TNF-a (-308) gene polymorphism (rs1800629) was analyzed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay, according to a previous study [20]. The PCR amplified the (–308 G/A) polymorphism of the *TNF-a* gene with a set of primers, 5'AGG CAA TAG GTT TTG AGG GCC AT3' and 5'TCC TCC CTG CTC CGA TTC CG3', and digested them with *NcoI* (Invitrogen™ Anza™ 2 NcoI. COD 15481923), generating a 107 bp PCR product. In the digested fragments, the homozygous G/G genotype appeared at 86 bp and 21 bp, and the homozygous A/A genotype appeared at 107 bp. The heterozygotes exhibited all the bands: 107 bp, 86 bp, and 21 bp. The PCR products and the digested products were visualized on a 12-well 6% polyacrylamide gel, including a 50 bp DNA ladder, and stained with silver nitrate.

The samples were randomly analyzed as they were collected; the analyst did not know which group the samples belonged to. When there was doubt regarding the genotype, the sample was processed again until conclusive results were obtained.

Statistical analysis

The characteristics of the subjects were described using simple frequency distribution and percentage. The mean and standard deviation were used for the scale variables. An analysis of the association between study variables and oral conditions was performed using the chi-square test, Mann–Whitney *U* test, or students' *t*-test, after the corresponding normality tests. Disease risk was estimated using the odds ratio, with a 95% confidence interval. A p value $<.05$ was considered statistically significant.

Results

After the dental check-up, the 171 subjects were distributed into one of the two study groups: healthy ($n = 28$, 16.3%) or gingivitis ($n = 143$, 83.6%). Interestingly, gingivitis had a high prevalence, although most of the subjects mentioned

Table 1. General characteristics of the study subjects according to oral health.

Characteristic	Total $n = 171$(%)	Healthy $n = 28$ (%)	Gingivitis $n = 143$ (%)	p value
Gender				.802
Female	134 (78.4)	23 (82.1)	111 (77.6)	
Male	37 (21.6)	5 (17.9)	32 (22.4)	
Education				.001
Primary school	4 (2.3)	0 (0)	4 (2.8)	
Junior High school	12 (7.0)	0 (0)	12 (8.4)	
High school	30 (17.5)	1 (3.6)	29 (20.3)	
Bachelor's degree	110 (64.3)	17 (60.7)	93 (65.0)	
Post grad.	15 (8.8)	10 (35.7)	5 (3.5)	
Brushing				.327
1 a day	8 (4.7)	0 (0)	8 (5.6)	
2 a day	71 (41.5)	14 (50.0)	57 (39.9)	
3 a day	92 (53.8)	14 (50.0)	78 (54.5)	
Nourishment				.517
Bad	8 (4.7)	2 (7.1)	6 (4.2)	
Regular	106 (62.0)	19 (67.9)	87 (60.8)	
Good	57 (33.3)	7 (25.0)	50 (35.0)	
Smoke				.399
Yes	28 (16.5)	6 (22.2)	22 (15.4)	
No	142 (83.5)	21 (77.8)	121 (84.6)	

Table 2. Frequencies of (–308) TNF- α genotypes and alleles in healthy and gingivitis groups.

Genotype	Total $N = 171$ (%)	Healthy $N = 28$ (%)	Gingivitis $N = 143$ (%)	p value	OR (95% CI)
Genotypes					
GG	80 (46.8)	10 (35.7)	70 (49.0)	reference	reference
GA	80 (46.8)	12 (42.9)	68 (47.6)	.914	.080 (.32–1.99)
AA	11 (6.4)	6 (21.4)	5 (3.5)	.002	.119 (.03–.46)
Allele					
G	240 (70.2)	32 (57.2)	208 (72.7)	reference	reference
A	102 (29.8)	24 (42.8)	78 (27.3)	.020	.500 (.27–.90)

Table 3. Biochemical parameters and levels of TNF- α according to oral conditions.

Characteristic	Healthy (mean ± SD)	Gingivitis (mean ± SD)	p value
Age (years)	23.93 ± 3.96	26.81 ± 10.68	.402
Salivary flow (mL/min)	0.81 ± 0.30	0.60 ± 0.37	.009
Glucose (mg/dL)	89.31 ± 12.11	96.38 ± 27.31	.254
TGs (mg/dL)	87.06 ± 61.31	124.69 ± 75.01	.003
CHOL (mg/dL)	145.26 ± 43.8	174.80 ± 79.75	.053
HDL-C (mg/dL)	75.43 ± 48.33	53.87 ± 33.60	.032
LDL-C (mg/dL)	66.89 ± 84.08	103.32 ± 82.94	.048
VLDL-C (mg/dL)	17.41 ± 12.26	24.89 ± 15.20	.005
Salivary TNF-a (pg/mL)	5.57 ± 1.47	4.16 ± 1.11	.001
Serum TNF-a (pg/mL)	3.49 ± 1.57	5.52 ± 4.75	.643

CHOL: total cholesterol; TGs: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very-low-density lipoprotein cholesterol.

brushing their teeth three times a day. When we analyzed oral health with regard to general characteristics, a significant statistical difference was observed in relation to the educational background; however, we found no statistical differences related to other variables, as seen in Table 1.

The distributions of genotypes for *TNF-* α (–308) in relation to oral conditions showed significant differences. Carriers of the homozygous A/A genotype were more frequent in the healthy group ($p = .002$) than in the gingivitis group. We observed that allele A is more prevalent in the healthy group ($p = .020$). Therefore, our data show that both the A/A genotype and the A allele have a protective effect against gingivitis. These data are shown in Table 2. The genotypic distributions of the polymorphism were consistent with the Hardy–Weinberg equilibrium ($p > .05$).

The salivary flow was increased in healthy subjects compared to those with gingivitis ($p = .009$). Some of the biochemical parameters also presented significant differences. The levels of TGs, LDL-C, and VLDL-C were higher in the group with gingivitis ($p = .003$, .048, and .005, respectively). In contrast, the prevalence of HDL-C was higher in the healthy group ($p = .032$), as was that of salivary TNF- α ($p = .001$). No statistical differences were found for serum TNF- α levels (Table 3).

Finally, we analyzed whether the genotypes of *TNF-* α (–308 G/A) polymorphism have affected the biochemical parameters of the gingivitis group. We found that the HDL-C levels were increased in subjects carrying the G/A genotype ($p < .05$), while the group with the A/A genotype showed a trend of increased salivary TNF- α levels ($p = .059$), as shown in Table 4.

Discussion

TNF-α is a pro-inflammatory cytokine that plays an essential role in PD pathogenesis, and *TNF-* α gene polymorphisms can influence its production. The change from the common allele (G) to the rare allele (A) at position −308 has been described to induce the binding of nuclear factors, which causes an increase in the level of TNF- α transcription [11]. As gingivitis is considered to be the precursor of periodontitis, which involves an inflammatory lesion, we analysed the association between *TNF-* α −308 G/A (rs1800629) polymorphism and gingivitis, as well as the association of this polymorphism with serum and salivary TNF- α levels. We conducted this study for the first time in a Mexican population.

To date, there is no evidence of an association between TNF- α (–308 G/A) polymorphism and gingivitis. Interestingly, we found an increased frequency of the homozygous A/A genotype among the group with good oral health. Our data also show that the A/A genotype has a protective effect against gingivitis. This suggests that *TNF-* α (–308 G/A) polymorphisms play different roles depending on ethnicity,

Table 4. TNF- α genotypes in relation to biochemical parameters in patients with gingivitis.

	TNF- α (–308) Genotype			
Variable	GG ($N = 70$)	GA ($N = 68$)	AA ($N = 5$)	p value
Salivary flow (min/mL)	0.61 ± 0.36	0.57 ± 0.37	0.82 ± 0.41	.445
Glucose (mg/dL)	96.85 ± 34.30	96.26 ± 18.89	91.43 ± 13.11	.510
TGs (mg/dL)	112.72 ± 60.68	139.99 ± 87.10	82.69 ± 24.53	.238
Cholesterol (mg/dL)	179.92 ± 97.94	170.82 ± 60.08	160.53 ± 53.47	.862
HDL-C (mg/dL)	44.72 ± 25.80	62.89 ± 38.27*	41.01 ± 5.67	.007
LDL-C (mg/dL)	114.57 ± 102.09	90.89 ± 60.31	127.63 ± 27.99	.347
VLDL-C (mg/dL)	22.26 ± 12.38	27.99 ± 17.42	16.53 ± 4.90	.203
Salivary TNF-a (pg/mL)	3.67 ± 0.55	4.18 ± 0.81	6.34 ± 2.72	.059
Serum TNF-a (pg/mL)	5.65 ± 4.93	5.44 ± 4.72	5.09 ± 4.35	.968

The data are shown as mean ± standard deviation. CHOL: total cholesterol; TGs: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very-low-density lipoprotein cholesterol. *$p < .05$ GA *vs* GG.

which could be helpful in determining genetic variations, since other investigations have shown a lack of this association in Italian [13] and Caucasian [12] populations.

We observed that allele A was more frequent in the healthy group, a result that is consistent with a meta-analysis [21]. Furthermore, it is essential to note that differences in allele A frequencies between ethnic groups were observed. Asian controls had a lower A allele prevalence, while Brazilians had the highest in their control group. In our study, allele A exerted a significantly protective effect against gingivitis.

According to *TNF-* α (–308 G/A) polymorphism and TNF-a levels, our study showed a trend towards higher salivary levels of TNF-a in the carriers of A/A genotypes. However, it was impossible to compare this result with other reports because, to date, an association between *TNF-* α (–308 G/A) polymorphism, gingivitis, and salivary and serum cytokine levels has not been reported. Ozer Yucel *et al.* [22] investigated the association of this polymorphism with gingival crevicular fluid (GCF) TNF- α levels in PD in the Turkish population and found no significant differences. On the other hand, Escalona *et al.* [23] showed that higher GCF TNF- α concentrations were significantly associated with PD severity. However, they only measured cytokine concentrations, without analysing the polymorphism. Interestingly, an investigation carried out in a Mexican population [24] showed elevated GCF TNF-a levels in the group with bone loss. Nevertheless, it is not possible to correlate these results, because we did not measure the cytokine levels in GCF, and those studies did not analyse the *TNF-* α (–308 G/A) polymorphism.

No statistical difference concerning the relation of *TNF-* α −308 G/A polymorphism and serum levels was found in our study. A similar study conducted by Passoja *et al.* [25] showed higher serum TNF- α levels in PD, but without an association with *TNF-* α (–308 G/A) polymorphism, with only one individual presenting the A/A genotype. Importantly, lacking signs of both inflammation and gingivitis can be understood as an indicator of an underlying protective mechanism against bone loss. Additionally, exposure triggers the inflammatory process, and we are therefore unable to determine whether or not these subjects would develop inflammation once exposed to a dysbiotic biofilm, because this was a cross-sectional study.

The salivary TNF- α levels were increased in our healthy group; however, Ulker *et al.* [16] showed higher salivary TNF- α levels in subjects with gingivitis. On the other hand, Afacan *et al.* [17] found increased biomarker concentrations of GCF TNF- α in a healthy group, despite no differences arising in salivary TNF- α levels between gingivitis and control groups. In another study, serum and total TNF- α were both increased in subjects with gingivitis compared to the control group [18]. It is known that the oral epithelial barrier plays an essential role in maintaining oral health, as it keeps out harmful microbes through an immune response, which helps prevent inflammation [26]. Ketarinocytes induce the expression of cytokines, such as IL-1 and TNF- α, to attract dendritic cells, neutrophils, and macrophages, which execute a rapid response against bacteria [1], thus contributing to oral health. Therefore, the imbalanced host response, which determines the inflammatory microenvironment, is crucial for the transition from health to disease. We found no statistical difference in serum TNF- α levels. This finding suggests that cytokine concentrations might vary according to the sample. Therefore, a better understanding of the roles and properties of these fluids in maintaining tissue integrity and in the defense against pathogens should be sought [27].

The results regarding salivary flow show an increase in saliva secretion in subjects with good oral health. Other researchers have reported similar results; participants with a lower flow rate exhibited a higher odds ratio for PD than those with a higher flow rate, meaning that increased salivary flow and its components help kill some bacteria [19]. This finding suggests that saliva may contain molecules that provide protection against oral diseases, and acts as a key to the host defense against oral infections [27].

Our gingivitis group showed significantly increased TGs, LDL-C, and VLDL-C, and decreased HDL-C levels. This can be explained by the fact that the infection caused by pathogenic bacteria can increase the serum levels of free fatty acids and TGs [28]; furthermore, one study [29] showed that bacteria in PD play an essential role in the accumulation of lipids in the arteries. In another study, Cury *et al.* [30] showed that obesity and PD are individually or cooperatively associated with lipid profile abnormalities. The age of the subjects in our study was 18–69 years old, but it has been reported that age could alter biochemical parameters [31].

Additionally, in this research, *TNF-* α (–308 G/A) polymorphism analysis showed that carriers of the G/A genotype

presented increased levels of HDL-C. Other studies on this issue show contradictory results [32,33]. Although Pyrzak *et al.* [33] showed that boys carrying allele A had lower concentrations of HDL-C, it is thought that hormones play an essential role here. Furthermore, it has been described that a diet high in carbohydrates induces the production of enzymes involved in the formation of TGs, and serum lipid levels change in response to diet [34], so we must consider the effects of environmental factors, such as diet, in each population. On the other hand, the A/A carriers tend to display higher salivary TNF-a levels, although without becoming significant, possibly due to their low number.

Together, all these findings could imply that TNF-a levels vary between populations due to multiple factors and the type of sample analysed. The limitation of our study is that we did not measure the levels of the cytokine in GCF. Therefore, we consider that it is necessary to integrate the analysis of sera with those of salivary and GCF levels of TNF-α, which will further elucidate the behaviour of the cytokine and allow us to better understand the role of TNF- α in PD in the Mexican population. Another limitation is that we did not determine whether the study subjects had been exposed to bacteria that trigger the inflammatory process.

Conclusions

Our data demonstrated that the *TNF*- α (–308) A/A genotype exert a protective effect against gingivitis. Healthy subjects had higher levels of salivary TNF- α and a greater salivary flow. Gingivitis causes an alteration in lipid levels.

Disclosure statement

The authors declare that they have no conflict of interest.

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