The –308 polymorphism in the promoter region of the tumor necrosis factor-alpha (TNF-α) gene and *ex vivo* lipopolysaccharide-induced TNF-α expression in patients with aggressive periodontitis and/or type 1 diabetes mellitus

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Accepted for publication September 21, 2004

ABSTRACT. Several single-nucleotide polymorphisms (SNPs) have been identified in the TNF-α gene promoter. The transition G→A at position –308 generates the TNF-α1 (G/G) and TNF-α2 (G/A or A/A) alleles, where the polymorphic TNF-α2 allele is associated with a high, *in vitro* TNF-α expression and an increased susceptibility to diverse illnesses. Here we study the association of the –308 TNF-α SNP with the susceptibility for developing aggressive periodontitis (AP), AP combined with type 1 diabetes mellitus (DM) and DM. We also explore the TNF-α capability expression and the presence of the –308 polymorphism. For this purpose we recruited 27 individuals with AP (AP+ group), 27 individuals with AP combined with DM (AP+/DM+ group), and 27 individuals with DM without signs of periodontitis upon clinical examination (DM+ group). The control group was comprised of 30 subjects. Genotyping for TNF-α promoter was performed by PCR-RFLP analysis. For TNF-α expression we used a blood culture system.

Keywords: TNF-α gene promoter, polymorphism, periodontitis, diabetes, tumor necrosis factor, promoter, cytokine, type 1 diabetes me llitus

The homozygous TNF-α1 allele was present in 81.5% of the AP+ patients, 70.4% of the AP+/DM+ patients, 89.0% of the DM+ patients, and 83.3% of controls, while the heterozygous TNF-α2 allele was present in 18.5% of the AP+ patients, 29.6% of the AP+/DM+, 11.0% of the DM+, and 16.7% of controls ($p > 0.80$; $p > 0.24$; $p > 0.55$, respectively). With respect to the *ex vivo* TNF expression, we found that the spontaneous TNF-α concentration did not differ significantly between AP+, AP+/DM+, DM+ patients and healthy controls ($p > 0.10$). However, the LPS-induced TNF-α concentration in AP+ patients was significantly higher than in AP+/DM+ and DM+ patients ($p = 0.00024$ and $p = 0.00001$, respectively) and controls ($p = 0.000001$). Although not significant, the LPS-induced TNF-α concentrations in AP+/DM+ and DM+ patients were higher than those obtained in controls ($p > 0.13$ and $p > 0.43$, respectively). The comparison of TNF-α production by –308 TNF-α genotype within the study groups showed no significant differences. The means of serum TNF-α concentrations in all patient groups were three times higher than those found in controls.
We conclude that no associations were found between the –308 TNF-α SNP, and the serum and *ex vivo* TNF-α levels in Chilean patients with AP, AP combined with DM, and DM.

INTRODUCTION

Periodontal diseases are specific, Gram-negative, anaerobic bacterial infections that lead first to the destruction of connective tissue and then the underlying alveolar bone that supports the teeth. Epidemiological studies have shown that diabetes increases the risk of both severe periodontitis and the incidence of periodontal disease progres-

sion, by approximately two to three fold [1, 2]. Additionally, periodontitis is a frequent complication in diabetic patients and may have significant consequences at functional and metabolic levels [3].

Periodontal diseases include a broad spectrum of inflammatory and destructive responses to oral microbe infections mediated by cytokines that include TNF-α and IL-1 [4]. Progression to severe periodontitis with loss of supporting structures is influenced by several factors, including genetic predisposition. In the search for genetic markers of periodontitis, cytokine gene polymorphism has emerged as an important factor [5, 6].

A single nucleotide polymorphism (SNP) in the promoter region of the TNF-α locus has been identified at position –308, involving the replacement of guanine (G) by adenine (A) [7]. This polymorphism, designated the TNF-α2 allele, leads to a higher rate of TNF-α gene transcription than that found for the wild-type TNF-α1 allele in *in vitro* expression studies [8, 9]. The presence of the TNF-α2 allele has been linked with increased susceptibility or severity to a variety of illnesses, such as cerebral malaria [10], Crohn's disease [11], systemic lupus erythematosus [12], ankylosing spondylitis [13], and rheumatoid arthritis [14]. However, the associations between –308 TNF-α polymorphism and periodontitis are still controversial [15-17].

In this study, the frequencies of the –308 TNF-α SNP in patients with aggressive periodontitis (AP), AP combined with type 1 diabetes mellitus (DM), and DM were compared to those obtained in healthy control subjects. We also analyzed a possible association of this polymorphism with the serum TNF-α concentrations, and the lipopolysaccharide (LPS)-induced TNF-α levels in all groups.

PATIENTS AND METHODS

Patients and controls

Within a protocol approved by an institutional review board, subjects signed an informed consent form after being advised of the nature of the study. Patients were enrolled from the Periodontic Clinic at the Faculty of Odontology of the University of Chile and from the Endocrinology Section of the Department of Medicine of the University of Chile Clinical Hospital. Controls were healthy students at the University of Chile Faculty of Medicine. Subjects completed personal and family medical and dental history questionnaires and were excluded for usage of antibiotics, corticosteroids, or non-steroidal anti-inflammatory drugs during the previous three weeks. Smokers and patients with an infectious episode were also excluded. We selected 27 individuals with AP (AP+ group, 26.6 ± 6.2 -mean age ± SD- years), 27 individuals with AP combined with DM (AP+/DM+ group, 24.7 ± 3.9 years), and 27 diabetics without periodontal disease upon clinical examination (DM+ group, 21.6 ± 3.3 years). Thirty healthy individuals formed the control group (C group, 25.1 ± 4.3 years). All patients, controls, and their two previous generations were born in Chile. In all groups, HIV (ELISA, Abbott, South Pasadena, CA, USA) analysis was performed. Probing depth (PD), clinical attachment loss (CAL), microbial plaque and bleeding on probing were evaluated clinically with a calibrated examiner. The inclusion criteria for AP were PD > 5 mm in at least one site and CAL > 6 mm in two or more teeth. For dental health criteria, PD < 3 mm and no sites with clinical bone loss, were used [18]. Diabetic patients met the American Diabetes Association and the World Health Organization criteria for diagnosis of type 1 diabetes mellitus [19].

Genotyping of –308 TNF-α promoter polymorphism

The genotype was analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) according to Wilson *et al.* [7]. The –308 TNF-α1 allele (G/G) is digested by NcoI, resulting in a fragment of 87 and 20 bp for an individual homozygous for this allele. In the case of an individual homozygous for the –308 TNF-α2 allele, which is not digested by NcoI, only the full-length PCR product of 107 bp is present. A heterozygous individual displays all three fragments of 107, 87, and 20 bp length.

Whole blood culture system (WBCS) for TNF-α expression

Briefly, blood samples were diluted five times in RPMI-1640 medium, supplemented with L-glutamine, penicillin and streptomycin. After 4 h of incubation, gel filtration chromatography-purified LPS (*Escherichia coli*, serotype 026:B6 -SIGMA, Chemical Co, USA) was added to each culture well to a final concentration of 10 μg/mL. After an additional incubation of 12 h, the supernatants of each culture well were centrifuged, and TNF-α was measured by immunoradiometric assay (IRMA) [20].

Immunoradiometric assay for TNF-α

This assay was performed using an anti-human TNF-α monoclonal antibody for TNF-α capturing, and an anti-human TNF-α polyclonal antibody for detection; both reagents were generated in our laboratory [21]. This inexpensive but less sensitive assay was used in order to analyze those samples with higher TNF concentrations.

Detection of TNF-α in serum

An ultra-sensitive ELISA kit (Biosource International Inc., Camarillo, CA, USA, quantification limit 0.5 pg/mL) was used according to the manufacturer's instructions.

Statistical analysis

The test on the proportions between groups was performed using the χ^2 or Fisher's exact test. For comparisons between groups, we first used a one-way analysis variance by ranks (Kruskal-Wallis test). When the previous test was significant, the Kruskal-Wallis's non-parametric test for multiple comparisons between groups was used. A p value < 0.05 was considered statistically significant. Odds ratios (OR) as an estimation of the relative risk were calculated with 95% confidence intervals (CI). The STATA 7.0 software was used for the analysis of relationships or associations between the variables [22].

RESULTS

–308 TNF-α promoter polymorphism in patients with AP, AP combined with DM (AP+/DM+), DM, and healthy control individuals.

Table 1
Allelic distribution of the –308 TNF-α promoter polymorphism in patients with aggressive periodontitis (AP), AP combined with type 1 diabetes mellitus, type 1 diabetes mellitus, and in healthy controls

TNF-α –308 genotypes	Patients with			Controls n (%)
	Aggressive periodontitis n (%)	Aggressive periodontitis and diabetes n (%)	Diabetes n (%)	
TNF-α1 (G/G)	22 (81.5)	19 (70.4)	24 (89.0)	25 (83.3)
TNF-α2 (G/A)	5 (18.5)	8 (29.6)	3 (11.0)	5 (16.7)
TNF-α2 (A/A)	–	–	–	–

Table 2
Differences in spontaneous and LPS-induced TNF-α concentrations in patients with aggressive periodontitis (AP), AP combined with type 1 diabetes mellitus, type 1 diabetes mellitus, and in healthy controls

TNF-α (pg/mL)*	Patients with			Controls n = 30
	Aggressive periodontitis n = 27	Aggressive periodontitis and diabetes n = 27	Diabetes n = 27	
Spontaneous	977 (63-2715)	1245 (105-4860)	713 (263-1351)	1212 (171-4586)
LPS-induced	8115 (977-12925)	5389 (678-10018)	4800 (1510-7718)	4863 (2488-8483)

*Values are expressed as means; range is given in parentheses.

Table 1 shows the frequencies of –308 TNF-α promoter polymorphism in AP+, AP+/DM+, DM+ patients, and in healthy controls. The homozygous TNF-α1 allele was present in 81.5% of the AP+ patients, 70.4% of the AP+/DM+ patients, 89.0% of the DM+ patients, and 83.3% of controls, while the heterozygous TNF-α2 allele was present in 18.5% of the AP+ patients, 29.6% of the AP+/DM+, 11.0% of the DM+, and 16.7% of controls (OR = 1.19, $p > 0.80$; OR = 2.11, $p > 0.24$; OR = 0.62, $p > 0.55$, respectively). The homozygous TNF-α2 allele was not detected in any group.

Spontaneous and LPS-stimulated TNF-α expression and circulating TNF-α concentrations in AP+, AP+/DM+, DM+ patients, and controls

A wide range of inter-individual variability in spontaneous and LPS-induced TNF-α concentrations in patient groups and controls was found *(table 2)*. As shown in *figure 1*, the spontaneous TNF-α concentration did not differ significantly between AP+, AP+/DM+ , DM+ patients, and healthy controls ($p > 0.10$). However, the median of LPS-induced TNF-α concentration varied throughout the

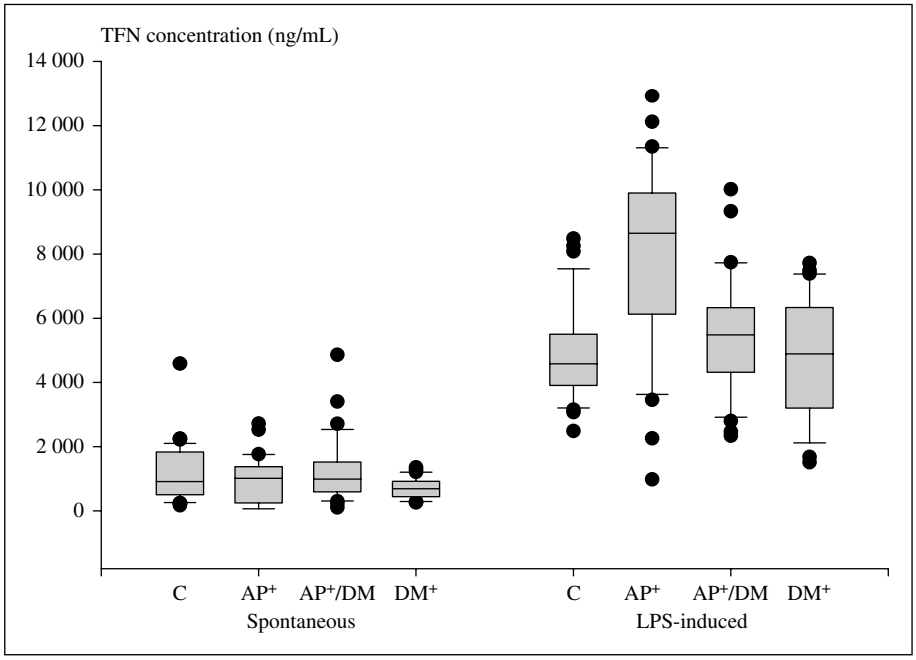

Figure 1
Spontaneous and lipopolysaccharide (LPS)-induced TNF-α expression in patients with aggressive periodontitis (AP+), aggressive periodontitis combined with type 1 diabetes mellitus (AP+/DM+), type 1 diabetes mellitus (DM+), and in healthy controls (C). TNF-α concentrations, measured by immunoradiometric assay, were detected in supernatants obtained from LPS-stimulated and non-stimulated whole blood cultures. Boxes represent median values for the TNF-α concentration of each group and quartiles 1 and 3.

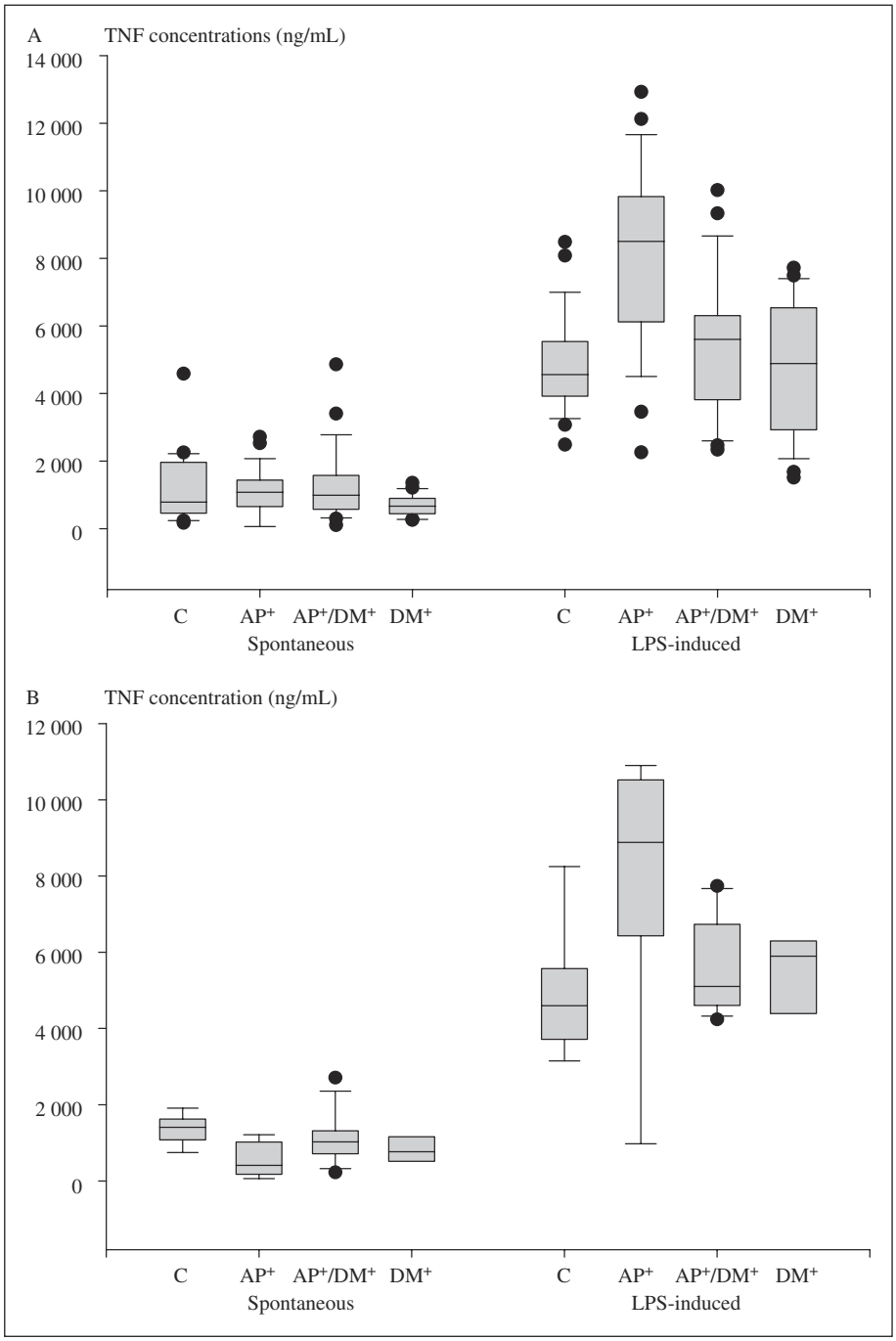

Figure 2

Spontaneous and lipopolysaccharide (LPS)-induced TNF-α expression by genotypes of the –308 TNF-α gene promoter polymorphism (**A**: G/G and **B**: G/A individuals) in patients with aggressive periodontitis (AP+), aggressive periodontitis combined with type 1 diabetes mellitus (AP+/DM+), type 1 diabetes mellitus (DM+), and in healthy controls (C). TNF-α concentrations, measured by immunoradiometric assay, were detected in supernatants obtained from LPS-stimulated and non-stimulated whole blood cultures. Boxes correspond to the median concentration of each group and quartiles 1 and 3.

groups. Thus LPS-induced TNF-α concentration in AP+ patients was significantly higher than in AP+/DM+ and DM+ patients ($p = 0.00024$ and $p = 0.00001$, respectively) and controls ($p = 0.000001$). Although not significant, the LPS-induced TNF-α concentrations in AP+/DM+ and DM+ patients were higher than those obtained in controls ($p > 0.13$ and $p > 0.43$, respectively), while the LPS-induced TNF-α concentration in AP+/DM+ patients was slightly higher than in DM+ patients ($p > 0.18$).

When all groups were divided into sub-groups by –308 TNF-α promoter genotypes (G/G and G/A), a broad inter-individual variability in the spontaneous and induced-TNF-α expression capabilities in all patient groups and controls was detected *(figure 2)*. Patient groups with the same TNF-α promoter genotype *(figure 2)* showed similar significant differences in the LPS induced-TNF-α concentration as described for all patients *(figure 1)*. However, the comparison of spontaneous and LPS induced-TNF-α pro-

duction within each individual group (controls, AP+, AP+/DM+ and DM+), according to the –308 TNF-α gene promoter genotype (G/G *versus* G/A), was not significant ($p > 0.54$, 0.77, 0.83 and 0.56, respectively) (*figure 2A* and *2B*).

Furthermore, high, inter-individual variations in the serum TNF-α concentrations were observed in all patient groups and controls. The means of serum TNF-α concentrations in AP+ (6.0 ± 1.0 pg/mL), AP+/DM+ (6.1 ± 2.1 pg/mL) and DM+ (5.5 ± 1.4 pg/mL) patient groups were at least three times higher than that obtained for controls (1.6 ± 1.2 pg/mL) ($p = 0.0001$ in all comparisons). In all patient groups, no differences were found between serum TNF-α concentration and –308 TNF-α promoter genotypes ($p > 0.05$).

On the other hand, with respect to the clinical estimation of bone loss, we did not detect any significant differences in inter-periodontitis patient groups, or when they were distinguished by –308 TNF-α promoter genotypes (result not shown).

DISCUSSION

Although periodontitis is an infection produced by specific, anaerobic Gram-negative bacteria, several factors are involved in its acquisition and progression. Thus, DM has been associated with a higher susceptibility in the development of oral pathologies including AP [23]. As reviewed by Hassel and Harris, numerous studies to date have suggested a genetic predisposition for AP [5].

HLA-DR3 and HLA-DR4 haplotypes have been associated with the presence of DM [24-26]. More specifically, an association of DM with the HLA-DR3 and TNF-α haplotype has been reported as consequence of the genetic linkage desequilibrium between the genes involved [27]. Likewise, specific HLA haplotypes have been linked to susceptibility or resistance to the expression of different forms of periodontitis (aggressive or chronic periodontitis) [28-30].

Cytokine imbalance, including an increase in pro-inflammatory TNF-α and IL-1β, is a common mechanism for diabetes and periodontitis [31, 32]. Moreover, there is an accumulation of evidence indicating associations between specific gene polymorphisms of TNF-α and IL-1β, and diverse inflammatory pathologies such as periodontitis [16, 33-35]. Of these polymorphisms, our research focused on the –308 TNF-α SNP.

Our results demonstrated that there were no significant differences between the presence of periodontitis and the increased frequency of the heterozygous TNF-α2 allele in diabetic (AP+/DM+) and non-diabetic patients (AP+), when both groups were compared with periodontologically healthy diabetic individuals (DM+) and healthy controls (C). As shown in *table 1*, the distribution of the –308 TNF-α frequencies did not reveal any significant differences between the groups. However, AP+/DM+ patients showed the highest frequency for the TNF-α2 allele (OR = 2.11), suggesting that this group may have an increased risk.

Surprisingly, the frequency of the TNF-α2 allele in DM+ patients was lower than expected and lower than that described by other authors [27, 36]. Due to the genetic linkage disequilibrium between the HLA-DR3 haplotype and TNF-α alleles in DM, it is likely that the lower frequency detected here could be explained by the presence of a high proportion of non-DR3 individuals in the DM+ group [36].

When we consider only the AP patients with respect to the control individuals, the distribution of the heterozygous TNF-α2 allele was 18.5% and 16.7%, respectively (OR = 1.19). The non-significant difference observed agrees with results obtained in Caucasian [16] and in Japanese patients [37], both with AP. The same lack of association with the –308 TNF-α promoter polymorphism has been described for patients with chronic periodontitis [17, 38, 39]. In contrast, Kornman and di Giovine reported a higher TNF-α2 allelic frequency in Caucasian patients with chronic periodontitis than in healthy individuals. This difference was correlated with the severity of the disease [15, 33]. Similarly, Galbraith *et al.* demonstrated that the TNF-α2 allele is a risk factor for the severity of adult chronic periodontitis [34], as its presence is detected in the most severe cases of the disease [40]. More recently, Lin *et al.*, demonstrated an increased frequency for the TNF-α2 allele in Chinese patients with chronic periodontitis [41]. Although we did not evaluate the severity of periodontitis, the possible participation of the TNF-α2 allele in the magnitude of the periodontal damage is the subject of current research.

One aspect to be considered in the interpretation of these studies is the way the –308 TNF-α polymorphism frequencies are distributed in different ethnic groups. As we have previously reported [42], significant differences in the –308 TNF-α genotype frequencies of the Chilean population were detected when compared to Caucasian –308 TNF-α genotypes. The frequency for TNF-α2 alleles in Caucasians is approximately 43%, while in Chileans is 16% [42]. Thus, in the sample studied here, the homozygous TNF-α2 allele was not detected. Although the –308 TNF-α frequencies in Chileans are similar to those found in the Chinese population, contrary to our findings, Lin *et al.* reported a positive association with chronic periodontitis [41, 42].

The search for a functional role of the –308 polymorphism in the TNF-α expression has produced controversial results [42-46]. Our approach for measuring the production of TNF-α was based on detecting its *ex vivo* overexpression by LPS-stimulated peripheral mononuclear cells in whole blood culture [20, 21]. Whole blood culture system (WBCS) is a useful *ex vivo* technique used to study cytokine production because it maintains the microenvironment of the blood and avoids the extraction procedure associated with modifying cell ratios and activation. It represents the technique of choice for exploring inter-individual variations in TNF-α production, and their relationship to genetic background [47].

In agreement with previous reports [21, 48], we found a wide inter-individual variability in the TNF-α expression capabilities both in controls and in all patient groups *(table 2)*. This behavior was also observed for the serum TNF-α concentrations. No significant differences were detected in either patient groups or controls when the medians of spontaneous TNF-α concentration were compared *(figure 1)*. However, the medians of LPS-induced TNF-α concentration differed significantly between groups *(figure 1)*. Our results disagree with those reported by Fokkema *et al.* [49] for LPS-induced TNF-α in patients

with periodontitis, who found no differences in the cytokine expression compared with healthy individuals. We demonstrated that patients with AP and patients with AP combined with DM (AP+/DM+) express higher LPS-induced TNF-α concentrations than healthy individuals *(figure 1)*.

Unlike the findings reported by Salvi *et al.* for IL-1β [50], indicating that diabetics had a significantly higher monocytic IL-1β production in response to LPS as compared to non-diabetic patients with adult periodontitis, we demonstrated, for TNF-α, that diabetic individuals (DM+) displayed a lower capacity for LPS-induced TNF-α expression than AP+ and AP+/DM+ patients *(figure 1)*. The contrast observed with the Salvi *et al.* report may be due to the difference in the ages within the patient groups at the time of study and at the time of onset of the diabetes. In our study, the mean age of the AP+/DM+ patients was 24.7 ± 3.9 years, while the onset of the disease was no more than five years earlier. In Salvi's study, patients had a mean age of approximately 50 years and had had the disease for a longer period of time. This implies that the development of the hyper-secretor monocytic phenotype may be more related to the time of onset and length of diabetes along with the severity of the periodontitis, than with an increased predisposition to periodontitis itself [51, 52].

A similar result was obtained when inter- and intra-group comparisons were performed for –308 TNF promoter genotype *(figure 2)*. Our results are consistent with recent studies using transcriptional activity assays in cells from rheumatoid arthritis patients describing no functional differences for the allele forms of the –308 TNF-α polymorphism [46, 53]. However, studies done *in vitro* using cells bearing the TNF-α allele showed a correlation with increased cytokine expression [37, 47]. Since chromosome 6 is highly polymorphic, and characterized by extensive linkage disequilibrium, it is possible that SNPs are haplotype markers rather than actually being responsible themselves for the disease association [53].

Multiple studies have shown that patients with periodontitis present higher TNF-α levels in both serum and gingival fluid than those measured in healthy individuals [32, 54]. A similar situation has been described in the serum of type 1 diabetic patients [55]. As expected, all patient groups showed an increased serum TNF-α concentration (approximately three times higher than that of the controls). Increases in serum IL-1β and TNF-α have been shown to produce alterations in lipid metabolism leading to hyperlipidemia. Within this context, periodontitis may contribute to elevated pro-inflammatory cytokines, increased serum lipids and potentially to systemic disease resulting from chronic hyperlipidemia and/or increased inflammatory mediators. These cytokines can produce an insulin-resistance syndrome similar to that observed in type 2 diabetes, and initiate the destruction of pancreatic beta cells leading to the development of type 1 diabetes. Thus, there is potential for periodontitis to exacerbate diabetes-induced hyperlipidemia, immune cell alterations, and diminished tissue repair capacity. It may also be possible for chronic periodontitis to contribute to triggering diabetes in susceptible subjects [32].

In summary, the –308 TNF-α promoter polymorphism may not be associated with the presence of AP, AP combined with DM, and DM, and the increase in patients' circulating TNF-α, or the capacity to produce TNF-α in the whole blood *ex vivo* culture system. Other factors may therefore be important in determining the circulating levels of TNF-α in AP, AP combined with DM and DM patients.

Acknowledgements. *This research was supported by Fondecyt-Chile grant 1040860 and DID-University of Chile grant ENL-02/13. We thank Ms. Juana Orellana and Nancy Fabres for their excellent technical help.*

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