Ex vivo lipopolysaccharide (LPS)-induced TNF-α, IL-1β, IL-6 and PGE_2 secretion in whole blood from Type 1 diabetes mellitus patients with or without aggressive periodontitis

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ABSTRACT. Several studies have demonstrated that diabetes is a risk factor for developing periodontal disease, increasing its prevalence and severity. Furthermore, periodontitis may impair the metabolic control and adequate treatment of diabetic patients. LPS from Gram-negative bacteria penetrates the periodontal tissues and subsequently recruits and activates immune cells. Progression to severe periodontitis with loss of supporting structures is mediated by several factors, including secretion of a broad spectrum of inflammatory and destructive mediators such as cytokines (TNF-α, IL-1β and IL-6), chemokines (IL-8) and prostaglandin E_2 (PGE_2).

The aim of this work is to investigate differences in the TNF-α, IL-1β and IL-6 expression and prostaglandin E_2 (PGE_2) release in blood from diabetic patients with and without aggressive periodontitis (AP) stimulated with lipopolysaccharide (LPS). For this purpose we recruited 29 Type 1 diabetes mellitus (DM) patients, 14 with AP and 15 without AP. Fourteen healthy individuals formed the control group. For cytokine expression and PGE_2 secretion, an ex vivo whole blood culture system was used . Cytokines and PGE_2 were detected by commercial immunometric assays. A wide range of inter-individual variability in spontaneous and LPS-induced TNF-α, IL-1β and IL-6 levels in patient groups and controls was found. The mean of spontaneous and LPS-induced TNF-α and IL-1β levels did not differ significantly ($p > 0.5$) when patients were compared to control individuals. Although not significant, the spontaneous TNF-α, IL-1β and IL-6 levels in the group of Type 1 DM with AP were higher than in controls, while in diabetic patients without AP, these values were depressed in comparison with controls. In both groups of patients, the means of LPS-induced IL-6 levels were higher than the controls but the differences observed were not significant ($p = 0.07$).

However, the LPS-induced PGE_2 levels varied significantly when all groups were compared ($p = 0.007$). The means of LPS-induced PGE_2 levels for Type 1 diabetic patients with AP ($p = 0.0009$) and without AP ($p = 0.024$) were significantly higher than the levels observed for healthy controls. Finally, we conclude that Type 1 diabetic patients with or without AP did not express higher LPS-induced TNF-α, IL-1β and IL-6 levels than controls. However, the PGE_2 levels released were significantly higher than those detected in controls.

Keywords: TNF-α, IL-1β, IL-6, PGE_2, diabetes

INTRODUCTION

Diverse studies have demonstrated that diabetes is a risk factor for developing periodontal disease, increasing its prevalence and severity [1-5]. According to previous reports, the prevalence of periodontitis in Type 1 diabetic patients between the ages of 13 and 18 is approximately 9.8% [6], while in the 20-29-year-old age group, the rate rises to 40.0% [2, 7].

Since Type 1 diabetes mellitus (Type 1 DM) generally develops at a very early stage of life, there may be a high probability of a severe, early onset periodontitis, resulting in tooth loss if not detected sufficiently quickly.

Furthermore, periodontitis may impair the metabolic control and adequate treatment of diabetic patients, being associated with a reduction in the glycosylated haemoglobin levels. There may also be an additional potential risk of bacteraemia leading to systemic inflammatory response syndrome [8, 9].

It has been demonstrated that LPS from Gram-negative bacteria penetrates the periodontal tissues and subsequently recruits and activates immune cells [10]. Progression to severe periodontitis with loss of supporting structures is mediated by several factors, including secretion of a broad spectrum of inflammatory and destructive mediators such as cytokines (TNF-α, IL-1β and IL-6), chemokines (IL-8) and prostaglandin E_2 (PGE_2) [11-13]. Moreover, increases in serum IL-1β and TNF-α have been shown to produce alterations in lipid metabolism, leading to hyper-lipidaemia. Periodontitis may contribute to elevated pro-inflammatory cytokines, increased serum lipids with potentially atherosclerotic vascular risk, and/or increased inflammatory mediators [14].

Within this context, TNF-α can produce insulin resistance by direct action on the insulin signaling pathway, and it could play a role in the pathogenesis of Type 2 diabetes related to obesity [15-18]. Additionally, TNF-α can initiate the destruction of pancreatic beta cells, leading to the development of Type 1 DM [14].

The aim of this study is to define possible differences in cytokines expression and PGE_2 release when whole blood from Type 1 diabetic patients with (DM+/AP+) and without aggressive periodontitis (AP)(DM+/AP-) is stimulated with LPS in an *ex vivo* culture system.

METHODS

Patients. Within a protocol approved by an institutional review board, subjects signed an informed consent form after being advised of the nature of the study. Subjects completed personal and family medical and dental history questionnaires, and were excluded for usage of antibiotics, corticosteroids, and non-steroidal anti-inflammatory drugs, during the previous 3 weeks. Patients with any infection and who smoked were also excluded. We selected 29 Type 1 DM individuals of both sexes between 18 and 30 years old, 14 with AP (DM+/AP+) and 15 patients without AP upon clinical examination (DM+/AP-). Fourteen healthy individuals, matched by age and sex, formed the control group (controls). All patients and controls, as well as their two previous generations were born in Chile. HIV analysis (ELISA, ABBOTT, South Pasadena, Ca, USA) was performed in all groups. AP individuals were included only if they had at least 10 functional teeth.

Probing depth (PD), clinical attachment loss (CAL), microbial plaque and bleeding on probing were clinically evaluated. The inclusion criteria for AP were PD > 5 mm at least in 1 site and clinical bone loss > 6 mm in 2 or more teeth. For dental health criteria, PD < 3 mm and no sites with clinical bone loss, were used [19]. Diabetic patients fulfilled World Health Organization criteria for diagnosis of Type 1 DM [20]. Table 1 shows the demographic characteristics of patients and controls.

Ten ml of venous blood were obtained by trained medical personnel from each individual at 3:00 pm.

Whole blood culture system (WBCS) for TNF-α, IL-1β, IL-6 expression. Blood samples were diluted 5 times in RPMI-1640 medium, supplemented with L-glutamine, penicillin and streptomycin. After 4 h of incubation, LPS (*Escherichia coli*, serotype 026:B6 -SIGMA, Chemical Co, USA) was added to each culture well to a final concentration of 10 μg/ml. Blood and culture medium incubated in the absence of LPS were used as controls. The supernatants of each culture well were obtained by centrifugation after incubating for 12 h. Cytokines and PGE2 were measured by immunometric assays [21, 22].

Table 1
Demographic characteristics of Type 1 diabetes mellitus patients (Type 1 DM) with aggressive periodontitis (AP) (DM+/AP+), Type 1 DM patients without AP (DM+/AP–), and healthy individuals (Controls)

	DM+/AP+	DM+/AP-	Controls
No of subjects men/women	4/10	8/7	5/9
Age (years)	23.9 ± 4.4	22.2 ± 3.1	25.1 ± 3.7
BMI (Kg/m^2)*	24.8 ± 3.5	23.9 ± 2.8	23.9 ± 3.6
Duration of Type 1 DM (years)	10.8 ± 7.7	12.0 ± 5.9	-

Values are expressed as means with their specific standard deviations.
*Body Mass Index.

Detection of TNF-α, IL-1β, IL-6 and PGE_2. Ultrasensitive immunometric assays based on chemiluminiscence for Immulite analyser (Diagnostic Products Corporation, Los Angeles, Ca, USA) were used for detecting serum TNF-α (quantification limit: 0.1 pg/ml), serum and ex vivo IL-1β and IL-6 (quantification limits: 1.5 and 5.0 pg/ml, respectively). PGE_2 was determined by ELISA kit (R,D System, Minneapolis, USA). All procedures were performed according to the manufacturer's instructions.

Immunoradiometric assay (IRMA) for detection of TNF-α in supernatants of WBCS. Polyvinyl chloride (PVC) plates were coated with 50 μl of the anti-human recombinant TNF-α (hrTNF-α) monoclonal antibody at 3 μg/ml. The plates were then washed and blocked with 1% w/v bovine serum albumin (BSA) in phosphate buffered saline (PBS), for 2 h at 4°C. After washing, 50 μl of the WBCS supernatants were added per well in triplicate and incubated for 2 hours at 4°C. The plates were washed and 50 μl/well of the anti-hrTNF-α polyclonal antibody were added, in a 1/1000 dilution and incubated for 2 h at 4°C. After washing, 25 μl/well (100,000 cpm) of a ^{125}Iodo-labelled affinity purified IgG goat anti-rabbit IgG were added, followed by incubation for 2 h at room temperature. Finally, the radioactivity was quantified in cpm. Each measurement was interpolated into a standard curve built with hrTNF-α dilutions (7-8000 pg/ml). Both anti-hrTNF-α monoclonal and polyclonal antibodies were generated in our laboratory [23].

Detection of glycosylated haemoglobin (HbA_{1c}). This was performed using the DCA 2000 technique (Bayer, Germany).

Statistical analysis. Results are expressed as means, and standard deviations were calculated. When comparing groups, we used one-way and multiple comparisons analysis of variance via Kruskal-Wallis's non-parametric test. To evaluate the relationships between variables, the Spearman's Rho correlation coefficient was used. A p value < 0.05 was considered statistically significant. For comparing median LPS-induced IL-6 levels between diabetic patients (with and without AP) and controls, the range test of Wilcoxon for independent-samples was used. The STATA 5.0 software was used for the analysis of relationships or associations between the variables [24].

Table 2
Glycosylated haemoglobin (HbA_{1c}) and serum TNF-α, IL-1β and, IL-6 concentrations in Type 1 diabetes mellitus patients (Type 1 DM) with aggressive periodontitis (AP) (DM+/AP+), Type 1 DM patients without AP (DM+/AP-), and healthy individuals (Controls)

	DM+/AP+	DM+/AP-	Controls	p-value
HbA_{1c} (%)	9.5 ± 2.2	8.7 ± 1.6	–	> 0.05
TNF-α (pg/ml)	5.1 ± 1.4	5.7 ± 1.5	1.3 ± 0.2	0.001
IL-1b	u	u	u	–
IL-6	u	u	u	–

Values are expressed as means with their specific standard deviations.
u: undetectable.

RESULTS

As shown in Table 1, the Type 1 DM associated with AP (DM+/AP+), the Type 1 DM without AP (DM+/AP-), and the control individual groups were composed of 4 men and 10 women, 8 men and 7 women, and 5 men and 9 women, respectively. When all groups were compared, there were no significant differences for age or BMI. In all diabetic patients, (DM+/AP+ and DM+/AP-) no differences were observed as regards time of onset of diabetes.

As summarised in Table 2, although not statistically significant, the mean HbA_{1c} percentage in diabetic patients with AP was slightly higher than in those without AP ($p > 0.05$). In addition, high inter-individual variations in the serum TNF-α concentrations were observed in all patient groups and controls. The means of serum TNF-α concentration in diabetic patients with and without AP were at least three-fold higher than that obtained for controls ($p = 0.001$ in all comparisons). However, in all individuals, no serum IL-1β or IL-6 were detected by the immunoassay used. In the group with AP, high levels of HbA_{1c} were correlated with serum TNF-α concentration ($r = 0.6$, $p = 0.02$). This observation was not detected in diabetic patients without AP.

On the other hand, a wide range of inter-individual variability in spontaneous and LPS-induced TNF-α, IL-1β and IL-6 levels was found in patient groups and controls. As shown in Figure 1, the mean of spontaneous and LPS-induced TNF-α and IL-1β levels did not differ significantly ($p > 0.5$, respectively) when patients with Type 1 DM or diabetes associated with AP were compared to control individuals. Although not significant, the spontaneous TNF-α, IL-1β and IL-6 levels in the group of Type 1 DM with AP were higher than in controls, while in diabetic patients without AP these values were depressed in comparison with controls. In both groups of patients, the means of LPS-induced IL-6 levels were higher than the controls but the differences observed were not significant ($p = 0.07$). Additionally, when LPS-induced IL-6 levels in diabetic patients (with or without AP) and controls were compared, the first group showed a significantly higher production ($p < 0.03$).

Unlike the observations made for the cytokines studied, LPS-induced PGE_2 release varied significantly ($p = 0.007$) when all groups were compared by one-way analysis of variance (Figure 2). When multiple comparisons between groups were performed, the means of LPS-induced PGE_2 levels both for Type 1 diabetic patients with AP ($p = 0.0009$) and without AP ($p = 0.024$) were significantly higher (four-fold and two-fold, respectively) than those obtained for healthy controls. However, although the

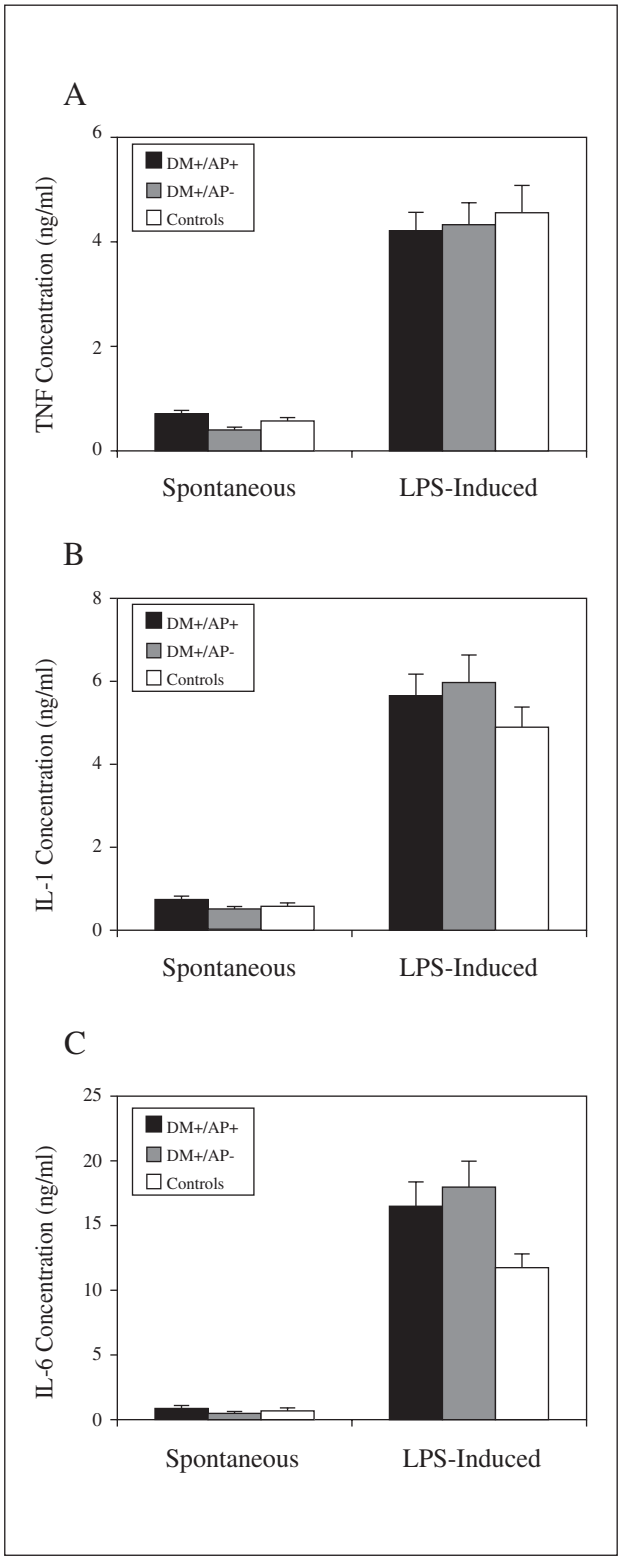

Figure 1
Spontaneous and lipopolysaccharide (LPS)-induced TNF-α (A), IL-1β (B) and IL-6 (C) expression in patients with Type 1 diabetes mellitus (Type 1 DM) associated with aggressive periodontitis (AP) (DM+/AP+), Type 1 DM without AP (DM+/AP-), and in healthy controls. TNF-α, IL-1β and IL-6 levels, measured by a chemiluminiscent ELISA, were detected in supernatants obtained from LPS-stimulated and non-stimulated whole blood cultures. The bars represent mean values ± SD of the three groups.

level of PGE_2 released by Type 1 diabetic patients with AP was higher that that released by those without AP, the difference was not significant ($p = 0.116$) (Figure 2). All the spontaneous PGE_2 levels were undetectable by the immunoassay used.

DISCUSSION

It has been established that diabetic subjects have both an increased prevalence and severity of periodontal disease when compared to non-diabetic individuals [5]. Moreover, the presence of Type 1 or Type 2 DM may predict the development of periodontal disease, and it should be considered as an additional chronic complication of diabetes [25]. For Type 1 diabetic patients, Karjalainen *et al.*, have reported that the presence of chronic complications are associated with an increased severity of the periodontal disease [26].

In diabetic patients, an early loss of periodontal tissue adherence and alveolar bone has been observed. Numerous factors are responsible of this massive destruction, including defects in the chemotactic functions, impairment of the macrophages' adherence and phagocytosis [27], collagen metabolism alterations leading to an excessive breakdown [28], and the presence of hyper-secretory monocytes responsible for releasing pro-inflammatory mediators such as TNF-α, IL-1β, IL-6 and PGE2, in response to a bacterial endotoxin stimulus [13, 29].

Several studies have described how patients with periodontitis, express higher TNF-α levels in both serum and gingival fluid than those measured in healthy individuals [14, 30]. A similar situation has been described for Type 1 diabetic patients [31]. As expected, our study found that

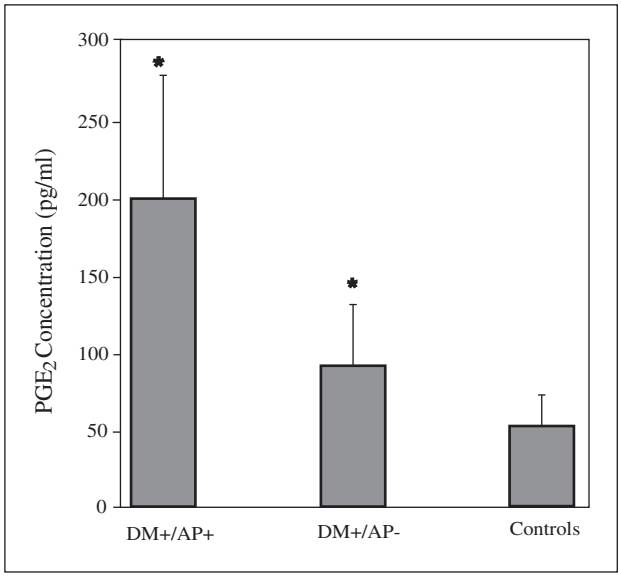

Figure 2
Lipopolysaccharide (LPS)-induced prostaglandin E_2 (PGE_2) release in patients with Type 1 diabetes mellitus (Type 1 DM) associated with aggressive periodontitis (AP) (DM+/AP+), Type 1 DM without AP (DM+/AP-), and in healthy controls. PGE_2 levels, measured by ELISA, were detected in supernatants obtained from LPS-stimulated and non-stimulated whole blood cultures. The bars represent mean values ± SD of the three groups. Asterisks indicate significant differences between DM+/AP+ patients ($p = 0.0009$) and DM+/AP- patients ($p = 0.024$) compared with controls.

both patient groups showed an increased serum TNF-α concentration (approximately 3 times higher than controls). Unfortunately, we were unable to define differences in serum IL-1β and IL-6 concentrations; probably the immunoassay used was not sensitive enough. It has been shown that serum TNF-α levels are correlated with metabolic control in Type 1 DM (31). Nevertheless, we found a positive correlation with the HbA_{1c} levels only in the AP group. It is likely that these patients displayed worse metabolic control than patients without AP.

Increases in serum IL-1β and TNF-α have been demonstrated to produce alterations in lipid peroxidation leading to endothelial damage. Furthermore, the interaction of TNF-α in the insulin-signalling cascade may lead to insulin resistance. In Type 1 DM, these cytokines are involved in the process leading to the destruction of pancreatic beta cells. Periodontitis therefore has the potential of exacerbating diabetes-induced hyper-lipidemia, immune cell alterations, and diminished tissue repair capacity, and it may also contribute to atherosclerotic disease, one of the life-threatening complications in diabetic patients. It may also be possible for chronic periodontitis to contribute in triggering Type 1 or Type 2 diabetes in susceptible subjects [14].

Our approach for measuring the cytokine production was based on detecting its *ex vivo* over-expression by LPS-stimulated peripheral mononuclear cells present in whole blood culture [21]. The whole blood culture system (WBCS) is an elegant 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 a technique of choice for exploring inter-individual variations in TNF-α production [21, 32]. Furthermore, the reflected performance of monocytes in LPS-induced WBCS was found to have relatively low levels of LPS, as the cytokine production by neutrophils requires much higher amounts of LPS [33].

In agreement with previous reports [23, 34], we found a wide inter-individual variability in the cytokine expression capabilities in both controls and patient groups. This behaviour was also observed for the serum TNF-α concentrations. As depicted in Figure 1, our results do not demonstrate significant differences in any of the spontaneous or the LPS-induced cytokine levels when all groups were compared. The depressed spontaneous IL-6 levels detected in diabetic patients without AP are in agreement with those observed by Pickup *et al.*, however the differences detected by us were not significant [35]. Interestingly, diabetic patients with AP showed a tendency towards a higher spontaneous production than controls for all cytokines (Figure 1). LPS-induced IL-6 levels were in fact higher both in diabetic patients with AP (DM+/AP+) and without AP (DM+/AP-) compared with the control group. Although not statistically significant, this observation does not concur with that of Pickup *et al.* who found that the IL-6 production was slightly lower in type 2 diabetic patients than in controls after the LPS stimulation [35]. Interestingly, when we compared the LPS-induced IL-6 levels between diabetic patients (with or without AP) and controls, we found that diabetic individuals produced significantly higher LPS-induced IL-6 levels than healthy subjects. It is likely that the increased tendency for IL-6 expression observed in diabetic patients may be ex-

plained in part by the basal autoimmune process proper of Type 1 DM more than the periodontal process itself (Figure 1).

Our results are concordant with those obtained by Fokkema *et al.* [36], for LPS-induced TNF-α and IL-6 levels in patients with AP where they did not find significant differences in the expression of these cytokines compared to the healthy individuals. However, we disagreed with respect to their findings of lower IL-1β expression in AP patients than in controls. In our study no significant differences were detected in any of the patient groups compared to healthy individuals.

The levels of PGE_2 were four- and two-fold higher in WBCS from Type 1 DM patients with and without AP, respectively, than from controls. Although we did not detect a significant difference, diabetic patients with AP released twice as much PGE_2 in supernatants in comparison with patients without AP. The high PGE_2 response observed in patients with diabetes associated with AP with respect to patients without AP and controls, may imply a preponderant role for PGE_2 in the pathogenesis of AP. Additionally, we can speculate about the clinical implications of this finding, in which the monocytes/macrophage phenotypic trait (hyper-responsive with respect to LPS challenge) in patients with AP might serve as a useful screening tool for Type 1 diabetic (of a younger age group) who might not have clinical expression of AP and who may go on to develop the disease in due course. Since the size of groups studied was rather small we were unable to determine a possible association between the PGE_2 release and the severity of the AP.

In agreement with the results reported by Fokkema *et al.* [36], for LPS-induced PGE_2 levels in patients with AP, where they demonstrated that AP patients produced PGE_2 concentrations twice as high as those in healthy individuals, our findings support the opinion that PGE_2 selectively inhibits IFN-γ production and favours the development of Th2 cells [37]. Furthermore, it has been proposed that the ratio of IL-12 to PGE_2 produced by antigen-presenting cells (APC) during T cell activation is highly predictive of the high level of IFN-γ production by Th cells [38]. Since the present study showed higher PGE_2 levels in blood cultures from diabetic patients with AP, these results may support the hypothesis that postulates a Type 2-promoting phenotype of APC from AP patients. IL-12 and IFN-γ determinations could have provided useful information for defining a Type 2 APC phenotype in patients with Type 1 DM associated with AP.

Finally, we have demonstrated that there are no significant differences in the TNF-α, IL-1β and IL-6 production in whole blood cultures stimulated with LPS, either in Type 1 DM patients with or without AP when compared with healthy individuals. However, LPS-induced PGE_2 levels were shown to be higher in both Type 1 DM patients with and without AP, when were compared to the control group. The determination of LPS-induced PGE_2 in an *ex vivo* WBCS could represent a new tool in patients with high risk of periodontal disease such as Type 1 diabetic patients.

ACKNOWLEDGEMENTS. This research was supported by FONDECYT-CHILE grant 1990936, DID-University of Chile grant ENL-02/13 and Clinical Project from Faculty of Medicine, University of Chile. We thank Ms. Juana Orellana, Egardo Caamano and Ruth Mora for their excellent technical help.

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