

Plasma levels of tumour necrosis factor- α in patients with chronic periodontitis and type 2 diabetes

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Abstract

Objectives: Studies suggest that elevated circulating tumour necrosis factor- α (TNF- α) may contribute to insulin resistance in patients with type 2 diabetes. The source of plasma TNF has been thought to be adipocytes associated with obesity, but inflammation and infection result in TNF- α production as well.

Methods: We studied 46 patients with type 2 diabetes and chronic periodontitis to determine the relationship between plasma TNF- α levels and clinical measures of periodontitis, gingival crevicular fluid (GCF) interleukin-1 β (IL-1 β), plasma endotoxin, serum glucose, and glycated haemoglobin (HbA1c). TNF- α levels were measured using a high sensitivity enzyme-linked immunosorbent assay.

Results: TNF- α showed a significant positive correlation with attachment loss ($r = 0.40$, $p = 0.009$), plasma endotoxin ($r = 0.33$, $p = 0.03$), and GCF IL-1 β ($r = 0.33$, $p = 0.035$), but not probing depth ($r = 0.28$, $p = 0.07$), bleeding on probing ($r = 0.30$, $p = 0.053$), plaque index ($r = 0.22$, $p = 0.17$), serum glucose, HbA1c ($r = 0.10$, $p = 0.50$), or body mass index ($r = 0.077$, $p = 0.62$). A dose–response relationship was observed between periodontitis severity and TNF- α ($p = 0.012$).

Conclusion: The finding that chronic periodontitis is associated with plasma TNF- α levels in subjects with type 2 diabetes supports the hypothesis that periodontal infection and inflammation may contribute to insulin resistance.

Key words: chronic periodontitis; endotoxin; gingival crevicular fluid; human; interleukin-1 β ; plasma; tumor necrosis factor- α ; type 2 diabetes

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Type 2 diabetes is associated with systemic inflammation (Schmidt et al. 1999), insulin resistance, and chronic periodontitis (Taylor 2001). Increasing evidence suggests that severe chronic periodontitis represents a sub-clinical septicaemic state (Loesche & Lopatin 1998). Gram-negative species commonly inhabit the periodontal biofilm and enter the circulation during dental manipulations (Everett & Hirschmann 1977; Hockett et al. 1977). Geerts et al. (2002) have demonstrated that plasma endotoxin levels become elevated following mastication. Consistent with a release of endotoxin into the circulation, Ide et al. reported a transient increase in circulating tumour necrosis factor

(TNF) following scaling and root planing (Chen et al. 2004). Recently, Bretz et al. (2005) showed in a large geriatric cohort that circulating TNF is associated with more severe periodontitis. Thus, it appears likely that periodontitis may influence circulating TNF levels.

Tumour necrosis factor (TNF- α) has been reported to play a key role in the pathogenesis of type 2 diabetes (Argiles et al. 1994). Several lines of evidence support this concept. In animal (Lang et al. 1992; Hotamisligil et al. 1996; Kroder et al. 1996) and in vitro human cell models, TNF- α inhibits insulin action (Hotamisligil & Spiegelman 1994; Bruun et al. 2003; Lang

et al. 1992). In human cross-sectional studies, elevated circulating TNF levels are associated with insulin resistance (Mishima et al. 2001) and type 2 diabetes (Fernandez-Real et al. 2002). Longitudinal human studies show elevated plasma TNF to be concomitant with worsening of glycaemic control (Lechleitner et al. 2002). In experimental human studies, systemic administration of TNF causes a sustained elevation of circulating insulin (Tracey et al. 1986) and glucose (Van der Poll et al. 1991). Therefore, while TNF is associated with insulin resistance, the aetiology of elevated circulating TNF in diabetic individuals is not fully understood.

Adipose tissue may be a major source of TNF- α secreting cells in type 2 diabetes (Hotamisligil & Spiegelman 1994; Bertin et al. 2000), as studies of obese subjects show declining TNF levels following a weight-loss regimen (Katsuki et al. 1998; Bruun et al. 2003). However, elevated TNF- α is also observed in non-obese diabetes patients (Bruun et al. 2003), and not all studies have shown TNF to be associated with obesity (Mishima et al. 2001; Bruun et al. 2003) or insulin resistance (Blüher et al. 2001). Additionally, recent evidence suggests that adipose tissue is characterized by a monocytic cellular infiltrate (Weisberg et al. 2003) pointing to inflammation rather than obesity per se as an influence on circulating TNF.

Subjects with periodontitis appear to have an exuberant TNF response as lipopolysaccharide (LPS)-challenged macrophages derived from periodontitis subjects release greater quantities of TNF compared with non-periodontitis controls (McFarlane et al. 1990). Monocytes from subjects with both diabetes and periodontitis appear to release even greater quantities of TNF (Salvi et al. 1998). Taken together, these findings support the hypothesis that periodontitis may contribute to the heightened state of inflammation that is observed in diabetic subjects (reviewed by Iacopino 2001; Nishimura et al. 2003).

It remains unknown to what extent periodontitis influences circulating TNF levels in diabetic individuals. We hypothesize that TNF levels in the circulation of diabetic subjects are influenced by periodontal infection and inflammation. As circulating TNF may play an important role in insulin resistance, we measured the plasma levels of TNF- α in subjects with type 2 diabetes and chronic periodontitis.

Materials and Methods

Subjects

This cross-sectional study was approved by the Institutional Review Board of Columbia University Medical Center. Written informed consent was obtained from each subject. We evaluated 46 patients with periodontitis and type 2 diabetes. The data reported here are from the baseline examination of a clinical trial for periodontal therapy (Engelbreton et al. 2004). Age, gender, serum glucose level, HbA1c level, and duration of diabetes mellitus were recorded (Table 1). Patients recruited

Table 1. Clinical characteristics of the study subjects

Variable	Mean or %	N	SD	IQR	Range
Age (years)	54.0	46	9.9	18.0	37.0
% Female	55	46			
% Smoker	17	35			
Glucose (mg/dl)	190.3	44	97.0	124.0	362.0
% HbA1c	7.8	46	2.0	3.1	7.3
Body mass index	33.5	35	7.6	11.4	30.5
AL (mm)	4.0	46	1.3	1.7	5.1
PD (mm)	3.3	46	0.8	1.2	3.0
% BOP	56	46	27	43	87
% plaque	77	46	23	36	85
GCF IL-1 β (pg/ml)	77.9	44	54.0	74.0	222.7
Plasma TNF- α (pg/ml)	2.6	44	3.5	2.2	18.5
Plasma endotoxin (EU/ml)	0.09	44	0.1	0.1	0.7

AL, attachment loss; PD, probing depth; BOP, bleeding on probing; GCF, gingival crevicular fluid; IL, interleukin; TNF, tumour necrosis factor.

for this study were maintenance patients from an out-patient diabetes care centre (The Naomi Berrie Diabetes Center at Columbia University Medical Center). All subjects were taking prescribed hypoglycaemic agents and/or insulin for the treatment of their diabetes. Subjects were eligible for the study and referred for periodontal treatment if, during their routine medical examination, signs of tooth loss or tooth mobility were noted, and no recent history of periodontal treatment was evident.

A diagnosis of type 2 diabetes was made according to World Health Organization (WHO) criteria. These are positive findings from any two of the following tests on different days: symptoms of diabetes mellitus plus casual plasma glucose concentration ≥ 200 mg/dl (11.1 mmol/l) or fasting plasma glucose (FPG) ≥ 126 mg/dl (7.0 mmol/l) or 2-hour post-prandial plasma glucose (2 hr PPG) ≥ 200 mg/dl (11.1 mmol/l) after a 75-g glucose load. Vital signs and demographic data were determined from the medical record. The body mass index (BMI) was estimated by dividing the body weight (in kilograms) by the square of the height (in metres). Smoking status was assessed and defined as never smoker or ever smoker. Additional exclusion criteria were pregnancy or lactation, HIV infection, bleeding disorders and immunosuppressive chemotherapy.

Chronic periodontitis was diagnosed according to the criteria of the American Academy of Periodontology using probing depths, bleeding on probing (BOP), loss of clinical attachment, and radiographs. Subjects with periodontal treatment within the last six months were excluded.

Study protocol and methods

Clinical Measures

Venous blood samples were drawn from each subject before the periodontal examination. gingival crevicular fluid (GCF) was collected before measurement of periodontal clinical parameters. Probing depth (PD), attachment loss (AL), BOP, and plaque index (PI) were collected at six sites per tooth. PD was defined as the distance in millimetres from the coronal-most margin of the free gingiva to the most apical penetration of the North Carolina probe. AL was defined as the distance from the cemento-enamel junction to the most apical penetration of the probe. The presence of supragingival plaque was recorded dichotomously during PD measurements. BOP within 20 s was recorded dichotomously.

TNF- α

Venous blood samples were obtained before the periodontal examination using pyrogen-free heparinized collection tubes (EndoTube, Greiner, Vienna, Austria). Tubes were centrifuged according to the manufacturer's guidelines, then stored at -80°C till analysed. Plasma TNF- α was measured using a commercially available sandwich immunoassay kit (Quantikine HS Human TNF- α immunoassay kit, R&D systems, Minneapolis, MN) following the manufacturer's instructions. Briefly, 200 μl of standard or plasma samples were added to microtitre plate wells coated with a monoclonal antibody specific for TNF- α , followed by incubation at 4°C for 16 h. The wells were then washed four times with a buffered

surfactant solution, and thereafter, 200 μ l of anti-TNF- α polyclonal antibody conjugated to alkaline phosphatase was added to each well and incubated for 3 h at room temperature. After appropriate washing, 50 μ l of substrate solution (NADPH) was added to each well and incubated again for 60 min. at room temperature. After this, 50 μ l of amplifier enzyme solution was added to the well, followed by incubation for 30 min. at room temperature. The reaction was then stopped by the addition of 2N sulphuric acid to the wells, and absorbance was measured at 490 nm with corrections set at 650 nm using a microplate reader. The values of plasma TNF- α levels were extrapolated from a curve drawn using TNF- α standard. The minimum detectable concentration of this assay is 0.01 pmol/l, and the intra- and inter-assay coefficients of variation of the assay were 5.6% and 7.5%, respectively.

Serum glucose

Glucose levels were measured by an automated enzymatic method. The HbA1c was measured using an affinity chromatography method (Micromat II, Bio-Rad Laboratories, Hercules, CA, USA).

GCF collection

Each clinical evaluation was preceded by collection of GCF as previously described (Lamster et al. 1991) from the mesiolingual and mesiobuccal surfaces of all molar teeth. Briefly, teeth were air-dried and isolated with cotton rolls, supragingival plaque was gently removed, and GCF was sampled with pre-cut methylcellulose filter paper strips for 30 s. Strips were measured for fluid volume with a calibrated Periotron 6000 (Interstate Drug Exchange, Amityville, NY, USA), and then removed to separate microcentrifuge tubes containing 50 μ l phosphate-buffered saline-Tween 20. The tubes were stored at -20°C until eluted (maximum 48 h). Following elution, each GCF sample was analysed separately. Values were then pooled to give a single mean value for each patient, and where appropriate, within each probing depth category.

Analysis of interleukin-1 β (IL-1 β) in GCF

GCF samples were analysed for IL-1 β as previously described (Engebretson et al. 2004) using a commercially available enzyme-linked immunosorbent assay

(Multikine Kit, Cistron Biotechnology, Pine Brook, NJ, USA). This assay is a sandwich ELISA and was performed according to the manufacturer's instructions using human recombinant standards. Results are reported as total amount of IL-1 β (in pg) per sample, or as concentration by converting Periotron units to microlitres using calibration curves as described previously (Lamster et al. 1988) and expressed as pg/ μ l.

Limulus amoebocyte lysate (LAL) assay

LAL test kits (QCL-1000, Cambrex Corporation, East Rutherford, NJ, USA) were used to detect plasma endotoxin according to the manufacturer's protocol. This is a chromogenic endpoint assay. Briefly, plasma samples were diluted 1:10 in pyrogen-free water (Cambrex Corporation) using pyrogen-free pipette tips (BioRad Laboratories) and then heat treated for 10 min. at 70° in pyrogen-free glass tubes (Cambrex Corporation). Samples were thoroughly mixed and then added to LAL reagent and incubated for 30 min. at 37° in pyrogen-free 96-well plates (BioRad Laboratories). Chromogenic substrate was added for 6 min. The reaction was stopped with 20% acetic acid. The absorbance of the samples was determined spectrophotometrically at 405 nm. Control standard endotoxin was used to set up standard curves. Values were recorded in endotoxin units (EU), with 1 EU equal to about 0.1 ng purified *Escherichia coli* LPS. The maximum sensitivity of this assay is 0.01 EU/ml. Biologically relevant plasma endotoxin levels are between 0.1 and 0.4 EU/ml.

Statistical methods

Data are expressed as the mean \pm standard deviation (SD). Parametric and non-parametric tests were used to compare variables. Comparisons between groups were made for non-normally distributed variables using the Mann-Whitney *U* and Kruskal-Wallis tests. Correlations were calculated using Spearman's rank correlation. Multivariate regression models were developed that included variables known to be associated with plasma TNF. BMI and plasma endotoxin were chosen a priori to be independent variables. Mean attachment loss was selected among periodontal clinical parameters by a forward stepwise procedure as an independent variable. Adjustments were made for age and sex in each

model. A level of $p < 0.05$ was accepted as being statistically significant. STATA/SE version 8.2 for Macintosh (STATA Corporation, College Station, TX, USA) was used for the analysis.

Results

Study Subjects

The subjects were approximately evenly divided between male and female, with a mean age of 54. The mean number of years since diagnosis of diabetes was 9.1 years. Other demographic and clinical parameters of the individuals in this study (mean \pm SD) are provided in Table 1.

Periodontal clinical measures

Among all subjects, the mean attachment loss was 4.05 ± 1.27 mm (SD), with a range of 2.48–7.62. The mean probing depths were 3.35 ± 0.79 mm, range 2.27–5.31. The mean percent of sites per subject with BOP was 56 ± 27 , range 13–100%, and plaque $77 \pm 23\%$, range 15–100%. Smokers tended to have a greater degree of attachment loss, but this was not statistically significant (not shown).

GCF

A total of 168 GCF samples were collected. IL-1 β was detected in all samples. Samples were analysed individually, and the data were pooled for each subject. The mean subject GCF IL-1 β levels were 77.94 pg/sample ± 54.01 , range 10.70–233.38. As has been shown previously in non-diabetic subjects (Engebretson et al. 2002), GCF IL-1 β was associated with increased attachment loss (Fig. 1). The mean GCF IL-1 β levels were also significantly correlated with plasma TNF ($r = 0.33$, $p = 0.04$).

Plasma Endotoxin

Endotoxin was detected in 36 of 46 plasma samples. The mean endotoxin levels were 0.09 EU/ml ± 0.11 , ranging from 0.00 to 0.66. Plasma endotoxin showed a significant positive association with plasma TNF ($r = 0.51$, $p = 0.0005$), but not with periodontal clinical parameters or GCF IL-1 β (not shown).

Plasma TNF- α was detected in 45 of 46 subjects, mean 2.61 pg/ml ± 3.50 (SD), range 0–18.51 pg/ml. TNF- α showed a significant positive correlation with attachment loss (Fig. 2) ($r = 0.395$,

$p < 0.009$) but not probing depth ($r = 0.276$, $p = 0.07$), BOP ($r = 0.296$, $p = 0.053$), or PI ($r = 0.216$, $p = 0.173$). We tested whether plasma TNF was associated with markers of metabolic control. Neither serum glucose ($r = 0.08$, $p = 0.63$) nor HbA1c ($r = 0.103$,

$p = 0.51$) were associated with plasma TNF. BMI ($r = 0.077$, $p = 0.62$) likewise did not show an association with plasma TNF. Subjects with severe periodontitis [mean whole mouth attachment loss (AL) > 4 mm] had significantly higher plasma concentrations of TNF- α

than those with AL < 3 mm ($p < 0.03$, Kruskal–Wallis; Fig. 3).

In a multivariate model, using greater than median plasma TNF (TNF50) as the dependant variable, severe attachment loss (mean-whole mouth AL > 4 mm) was a significant predictor variable for elevated plasma TNF adjusting for age and sex (odds ratio 5.4; 95% confidence interval (CI), 1.2–24.0) (Table 2). After adjusting for BMI, the association between attachment loss and plasma TNF increased to an odds of 24.3 (95% CI, 2.0–300). We also included plasma endotoxin in a third statistical model. The presence of greater than median plasma endotoxin conferred a 4.3 odds of having elevated plasma TNF (95% CI, 1.1–17.7).

Discussion

The present study demonstrated that chronic periodontitis severity is significantly associated with plasma TNF- α in a small cohort of urban-dwelling adult subjects with long-standing type 2 diabetes. To our knowledge, this is the first report that TNF- α is associated with periodontitis in a diabetic cohort. Circulating levels of TNF- α have been linked to insulin resistance (Mishima et al. 2001),

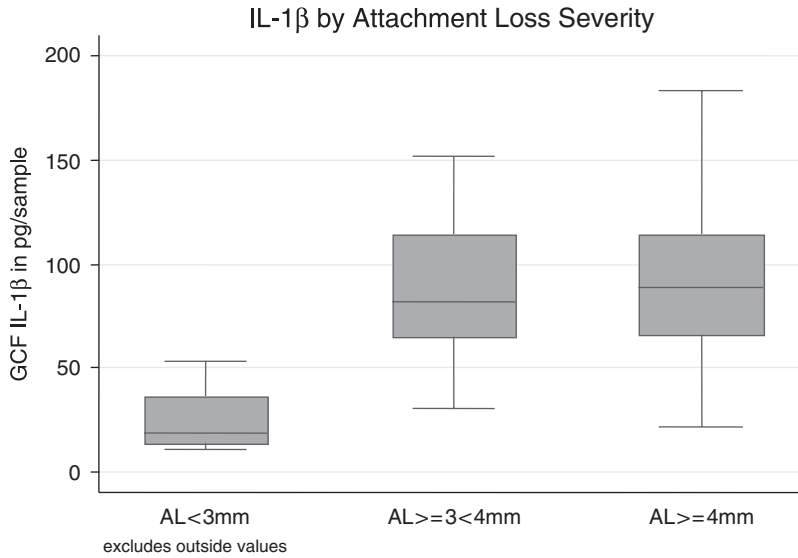


Fig. 1. Gingival crevicular fluid (GCF) interleukin-1 β (IL-1 β) levels by severity of attachment loss among subjects with periodontitis and type 2 diabetes. Subjects with severe attachment loss (AL ≥ 4 mm) had significantly greater levels of GCF IL-1 β than those with AL < 3 mm ($p = 0.0002$ Kruskal–Wallis).

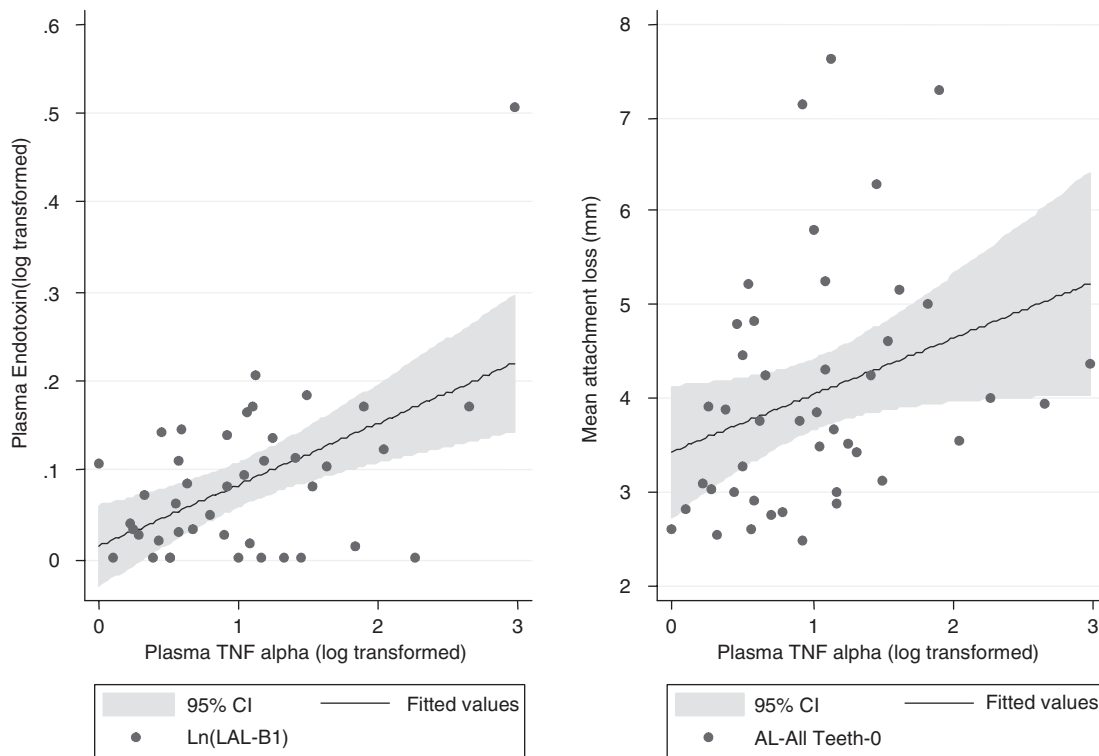


Fig. 2. A scatter plot of adult subjects with untreated periodontitis and type 2 diabetes shows a positive relation between attachment loss and plasma TNF- α (log transformed) ($r = 0.395$, $p < 0.009$). There was also a significant positive correlation between plasma levels of TNF- α and endotoxin ($r = 0.51$, $p = 0.0005$).

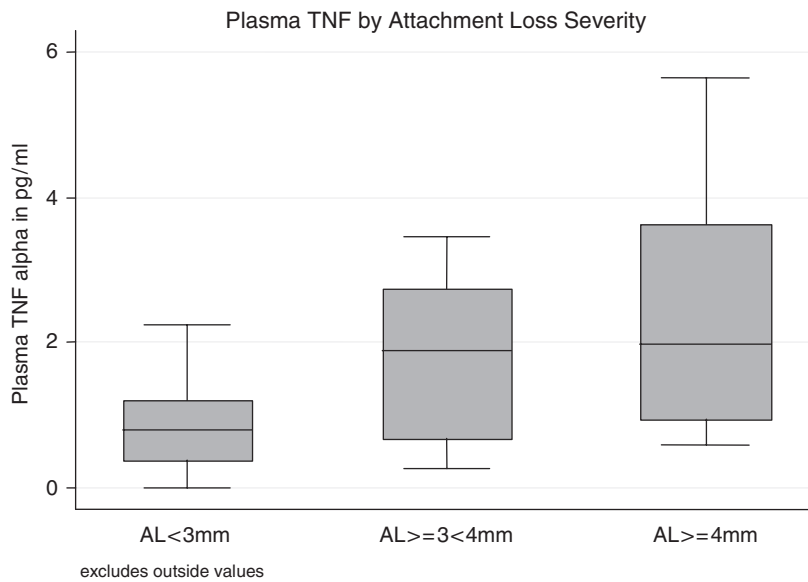


Fig. 3. Plasma TNF levels by severity of attachment loss. Subjects with severe periodontitis (mean whole mouth attachment loss (AL) ≥ 4 mm) had significantly higher plasma concentrations of TNF- α than those with AL < 3 mm ($p < 0.03$, Kruskal–Wallis).

Table 2. Logistic regression model 1 includes the covariates age, sex, and attachment loss category > 4 mm), model 2 adjusts for BMI, and model 3 adjusts for endotoxin

	Dependent variable: TNF (> 50th percentile)		
	model 1	model 2	model 3
Independent variables			
Age	0.938* (0.035)	0.930 (0.044)	0.946 (0.040)
Sex	0.388 (0.27)	0.253 (0.25)	0.459 (0.34)
AL > 4 mm	5.356** (4.10)	24.30** (31.2)	4.908* (4.07)
BMI		1.120 (0.087)	
Endotoxin (> 50th percentile)			4.304** (3.11)
Constant	28.74* (58.5)	0.706 (2.56)	8.121 (18.5)
Observations	43	33	41
R ²	0.14	0.26	0.19

Note that body mass index (BMI) data were missing for 10 subjects.

Standard errors in parentheses.

*** $p < 0.01$,

** $p < 0.05$,

* $p < 0.1$.

diabetes (Lechleitner et al. 2002), incident diabetes (Spranger et al. 2003), and visceral fat (Bertin et al. 2000). Recently, Bretz et al. (2005) demonstrated that circulating TNF- α is associated with clinical measures of periodontitis in a large geriatric cohort after adjusting for diabetes status and BMI. Another recent study by Genco et al. (2005) reported an analysis of NHANES III data and also analysis of 176 archived blood samples from subjects of known BMI and periodontitis status. That study reported an association between obesity and periodontitis, and obesity and TNF- α , but found no association between TNF- α and perio-

odontitis. The Genco study is notable for inferring that insulin resistance may mediate the effect of obesity on periodontitis, possibly through TNF. Our data tend to support the findings of both Bretz et al. (2005), and Genco et al. (2005).

The present findings may have important ramifications for interpreting studies of TNF and type 2 diabetes as periodontal parameters generally have not been measured. In previous studies, BMI was associated with circulating TNF (Bertin et al. 2000). The association between periodontitis with circulating TNF in diabetic subjects that we observed was independent of BMI as

statistical adjustment did not diminish the influence of periodontal attachment loss on circulating TNF. In fact, adjustments for BMI only strengthened the association observed between periodontal attachment loss and plasma TNF. Periodontitis therefore may be a significant confounder of the association between obesity and inflammation. A limitation of the present study is that BMI data were missing for some subjects. Also, waist–hip ratio is probably a better predictor of diabetes morbidity than BMI (Wang et al. 2005).

The association between attachment loss and plasma TNF- α that we observed appeared to be dependent on periodontitis severity. Periodontitis may conceivably contribute to circulating TNF in at least three ways. First, enhanced in vitro production of TNF- α by monocytes from diabetic subjects (Salvi et al. 1998) with periodontitis may indicate an innate (Salvi et al. 2005) hyper-responsiveness of diabetic monocytes to bacterial challenge. Salvi et al. recently corroborated previous work with an experimental gingivitis study in diabetic humans. That study found an earlier and more pronounced inflammatory response in diabetic versus non-diabetic subjects (Salvi et al. 2005) as a result of increasing periodontal plaque accumulation. Second, the translocation of Gram-negative species from the periodontal biofilm into the circulation may cause TNF levels to become elevated. Geerts et al. (2002) demonstrated that plasma endotoxin levels increased following mastication in subject with periodontitis. As systemically administered endotoxin is known to cause TNF levels to become elevated (van Deventer et al. 1990, 1993; Van der Poll et al. 1991), it is plausible that endotoxin entering the circulation from the periodontal biofilm may account for elevated TNF. It is of interest that plasma endotoxin was positively correlated with TNF in our study. Both attachment loss and plasma endotoxin were significant independent predictors of elevated plasma TNF in our adjusted statistical model. It is unknown to what degree circulating endotoxin was influenced by the periodontal biofilm in our study as there were no apparent associations between plasma endotoxin and periodontal parameters. A third possible mechanism may result from a direct cytokinaemia from the GCF i.e., a translocation of cytokines from the periodontal space into the circulation. GCF

levels of IL-1 β are known to be high in subjects with periodontitis (Engebretson et al. 2002), and even more so in subjects with diabetes (Salvi et al. 1998). Scaling and root planing result in a short-term increase in circulating TNF in non-diabetic subjects (Ide et al. 2004) that may be due to bacteraemia, or, conceivably, cytokinaemia. Attachment loss, GCF IL-1 β , and plasma endotoxin were each associated with plasma TNF in this cohort. There appeared to be a dose-response effect of attachment loss on plasma TNF in our study. Subjects with mild disease had significantly lower TNF levels than those with moderate or severe periodontitis. Our results suggest that periodontitis may influence plasma TNF- α levels by any of these three mechanisms.

Our findings do not support a role for hyperglycaemia as an influence on plasma levels of TNF- α ; however, a positive correlation between plasma TNF- α and glucose control has been reported by others (Katsuki et al. 1998; Lechleitner et al. 2002). Other studies suggest that high glucose can stimulate TNF production by monocytes in culture (Morohoshi et al. 1996). We found no correlation between serum glucose or HbA1c and plasma TNF. It is possible that the chronic periodontitis of the present study subjects influenced TNF levels to such a degree that glucose measures were no longer correlated with TNF.

While we report a cross-sectional association between circulating TNF and periodontal disease severity in subjects with type 2 diabetes, it remains unclear whether TNF levels influence diabetes severity, or indeed whether circulating TNF influences periodontitis severity. Two human studies involving diabetic subjects have measured plasma TNF levels following treatment. One small Japanese study suggests that periodontal treatment may influence circulating TNF levels (Iwamoto et al. 2001), while Talbert et al. (2006) reported that TNF levels are not reduced following periodontal treatment. Clearly, additional studies are needed to define whether periodontitis plays a causal role in systemic inflammation associated with diabetes.

Duncan et al. (1999) have demonstrated in a large prospective study that inflammation precedes diabetes onset. As periodontitis may influence systemic inflammation, it is worth considering whether periodontitis itself may influ-

ence the onset of diabetes. It is unknown whether circulating TNF precedes chronic periodontitis, or whether the reverse is true. These issues need to be resolved in clinical trials. Certainly, periodontal intervention studies are warranted to investigate the contribution of periodontal infection to the metabolic status of subjects with type 2 diabetes. This association warrants much further study. Definitive clinical trials are needed to determine the clinical significance of the relationship between periodontal inflammation and glycaemic control. In the present study, we found that the plasma levels of TNF- α are correlated with chronic periodontitis in type 2 diabetes patients.

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Clinical Relevance

Scientific rationale for study: TNF in the plasma is related to obesity and diabetes, but TNF may also be related to periodontal infection. TNF interferes with insulin function.

Principal findings: TNF in diabetic subjects is significantly higher

in subjects with more severe attachment loss. Plasma endotoxin and gingival inflammation are also related to plasma TNF.

Practical implications: Our study provides evidence that periodontitis may influence circulating TNF in diabetic subjects. As TNF interferes

with insulin function, this study may offer a new insight into the relationship between periodontitis and diabetes.