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Original Contribution

Oxidative stress, chronic inflammation, and telomere length in patients with periodontitis

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ABSTRACT

The aim of this study was to determine leukocyte telomere length (LTL) in individuals with periodontitis and controls, exploring its relationship with systemic inflammation and oxidative stress. Five hundred sixty-three participants were recruited for this case–control study: 356 subjects with and 207 subjects without periodontitis. LTL was measured by a qPCR technique from leukocytes' DNA. Global measures of oxidative stress (reactive oxygen metabolites) and biological antioxidant potential in plasma were performed together with high-sensitivity assays for C-reactive protein (CRP). Leukocyte counts and lipid profiles were performed using standard biochemistry. Cases had higher levels of CRP (2.1 ± 3.7 mg/L vs 1.3 ± 5.4 mg/L, $P<0.001$) and reactive oxygen metabolites (378.1 ± 121.1 U Carr vs 277.4 ± 108.6 U Carr, $P<0.001$) compared to controls. Overall, cases had shorter LTL with respect to controls (1.23 ± 0.42 vs 1.12 ± 0.31 *T/S* ratio, $P=0.006$), independent of age, gender, ethnicity, and smoking habit. When divided by subgroup of periodontal diagnosis (chronic, $n=285$; aggressive, $n=71$), only chronic cases displayed shorter LTL ($P=0.01$). LTL was negatively correlated with age ($P=0.001$; $R=-0.2$), oxidative stress ($P=0.008$; $R=-0.2$), and severity of periodontitis ($P=0.003$; $R=-0.2$) in both the whole population and the subgroups (cases and controls). We conclude that shorter telomere lengths are associated with a diagnosis of periodontitis and their measures correlate with the oxidative stress and severity of disease.

Telomeres are long lengths of TTAGGG repeats that cap the end of a chromosome and protect it from degradation and fusion [1]. In humans, leukocyte telomere length (LTL) is heritable but modified by a host of environmental variables. Particularly, an increased oxidative stress, which is usually associated with a systemic inflammatory state, has been shown to exert a major influence on the rate of telomere shortening [2,3]. The GGG triplets of telomere sequences are highly sensitive to the hydroxyl radical damage that can cause single-stand breakage during DNA replication. Consequently, increased free radical concentrations may lead to clipping of greater stretches of telomeres with each cell replication, over and above the well-described effect on telomere shortening due to the end-replication problem [4]. This concept led to the suggestion that telomere shortening is not strictly programmed, with telomere length acting as a mere cell-division counting device, but instead that telomeres act as sentinels for cumulative oxidative stress and inflammatory exposure over the individual's life span [5]. On the other hand, because inflammation and oxidative stress are at the center of the "free radical theory of aging" [6], the unique feature of LTL as a record of the cumulative burden of inflammation and oxidative stress might explain its association with age-related diseases such as atherosclerosis and neoplasm [7]. Although several in vitro and animal experiments have confirmed the central role of oxidative stress in promoting telomere shortening, data regarding the possible role of LTL as marker of cumulative oxidative stress and inflammatory exposure in humans are still lacking.

We identified a model of systemic and chronic inflammatory exposure with a large prevalence in the general population (30%) and related to an increased risk of premature death from age-related diseases [8]. Periodontitis is a common chronic infection of the adult population affecting gingival tissues, periodontal ligament, and alveolar bone [9]. It is believed to be caused by a deregulated immunoinflammatory response [10] to a specific gram-negative microflora present on the tooth root surface organized as dental

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biofilm [11,12]. Susceptible individuals initiate and perpetuate a local inflammatory process in response to the dental biofilm, characterized by recruitment of inflammatory cell infiltrates into the connective tissue adjacent to the gingival pocket epithelium. These infiltrates consist of polymorphonuclear leukocytes, monocytes/macrophages, and T and B cells, homed to the site of infection by a multitude of cytokines and chemokines, most of them produced by the inflammatory cells themselves [10,13]. The local inflammatory process is associated with a systemic inflammatory response, possibly due to the passage of inflammatory cytokines from the inflamed gingival tissue into the peripheral circulation [14–16]. Oxidative stress, defined as an imbalance between oxidant and antioxidant protection [17,18], has been linked with both onset of periodontal tissue destruction and systemic inflammation [19,20].

The primary aim of this study was to evaluate LTL in patients affected by periodontitis compared to controls. The secondary aim was to ascertain the degree of association between chronic inflammation, oxidative stress, and LTL in the same study sample.

Materials and methods

Study groups

In a case–control design, a total of 563 participants were recruited among subjects referred to the UCL Eastman Dental Institute in London between 2002 and 2006. For all participants a complete medical history was obtained via interview (including smoking history and current or previous use of medications). Subjects with any medical disorder, including cancer, type 2 diabetes, hypertension, and major cardiovascular/endocrine diseases, were excluded from the study. All subjects currently taking any medication were also excluded. Cases consisted of 356 individuals diagnosed with severe periodontitis as previously described [21]. All cases were diagnosed as suffering from periodontitis (chronic or aggressive) according to the AAP 1999 Consensus classification [22]. Chronic periodontitis is the most common form of periodontitis in the worldwide population (estimated prevalence up to 40%), whereas the aggressive form is often diagnosed in younger populations (<40 years of age) and exhibits a faster rate of alveolar bone loss [8]. A comprehensive clinical periodontal examination was performed. Full mouth measures of probing pocket depth (PPD), recession (REC; measured as distance from the cementoenamel junction to the gingival margin), and clinical attachment levels (CAL; measured from the formula CAL = PPD + REC) were obtained at six sites per tooth in all cases. Two hundred seven individuals without clinical signs of periodontitis were also recruited among those attending the Oral Surgery or Restorative Clinic based on gender and age (within 5 years) distribution as previously described [21]. Controls were excluded if they presented at least one site with PPD and CAL ≥ 4 mm after a basic screening periodontal examination was performed or based on radiographic evidence of bone loss. All patients gave written informed consent; the study was reviewed and approved by the Eastman/UCLH joint ethics committee.

Biochemical measurements

A blood sample was collected from all subjects before the dental examination. Leukocyte counts, lipid profiles, and glucose levels were assessed by standard biochemical testing. Serum levels of C-reactive protein (CRP) were measured with an immunoturbidimetric, high-sensitivity assay (Tina-Quant CRP assay performed on a Cobas Integra analyzer; Roche Diagnostics; intra-coefficient of variation (CV) = 2.1% on 20 randomly selected samples repeated). Serum levels of interleukin-6 were measured by a high-sensitivity enzyme-linked immunosorbent assay (Quantikine HS; R&D Systems). Intra-CV for the assays ranged from 1.9 to 4.9% (each sample in duplicates) and inter-CV was 4.1% (one sample in duplicate in each plate).

Oxidative stress

Photometric quantification of both reactive oxygen metabolites (D-ROM test) and plasma antioxidant capacity (BAP test) was performed in a subgroup of samples (204 cases, 184 controls) with a multiple analyzer according to the manufacturer's instructions and as previously described [23]. The D-ROM test is used to measure hydroperoxides and expressed in relative units (one U Carr is equivalent to 0.08 mg/dl of a hydrogen peroxide water solution). Intra-CV of 3% was reported ($n = 20$ duplicate samples). The BAP test is a global measure of the biological antioxidant potential of plasma (mmol/L), including agents able to reduce the iron from its ferric (Fe^{3+}) to its ferrous form (Fe^{2+}) (intra-CV of 5%, $n = 20$ duplicate samples).

Determination of leukocyte telomere length

DNA was extracted from peripheral blood cells from a 10-ml blood sample using the Nucleons BACC2 kit (Nucleon Bioscience, Coatbridge, UK) as previously described [24]. Telomere length was measured with a validated quantitative PCR-based method [25]. Briefly, the relative telomere length resulted from the ratio of telomere repeats to single-copy gene (SCG) copies (*T*/*S* ratio). For each sample the amounts of telomere repeats and SCG copies were defined in comparison to a reference sample, in a telomere and a SCG quantitative PCR, respectively. All PCRs were performed on the Rotor-Gene 6000 (Corbett Research Ltd., Cambridge, UK) and the final raw data from each PCR were analyzed using comparative quantification analysis (Rotor-Gene 6000 software; Corbett Research Ltd.). Every sample was run in duplicate and the mean data were used for the calculations. The specificity of all amplifications was determined by melting curve analysis. The intra-CV between duplicate measurements was 5.6%. A subset of 15 randomly selected samples was run in duplicate on two different days to test the reproducibility of our method. The inter-CV was 3.15%. The comparison between the *T*/*S* ratio of our quantitative PCR assay and the standard terminal restriction fragment (TRF) analysis has been previously reported [25]. By means of the regression line resulting from this correlation, we calculated the corresponding telomere length in base pairs from the *T*/*S* ratio measured in each subject, as previously reported [25].

Statistical analysis

The SPSS statistical program (version 17, Windows) was used for all analyses. All data are presented as means and standard deviation unless differently specified. Age and gender variables were used to identify comparable controls and not to perform individual case-matching and therefore were included as covariates in the analyses. Baseline biochemical variables were log-transformed as appropriate. Differences in LTL and all other continuous variables between cases and controls were tested with an independent *t* test, and when a statistical significant association was found multiple linear regression models were fitted, including as covariates age, gender, ethnicity, lipids, and smoking differences. Fully adjusted models were used to compare slopes of *T*/*S* by age in cases and controls. Spearman correlation analyses were used to assess the associations between the LTL and the various measures of systemic inflammation, oxidative stress, and periodontitis in both the whole study population and the subgroups (cases and controls). χ^2 analyses were performed to detect differences in categorical variables. The α value for statistical significance for association was set at 0.05.

Table 1
Characteristics of the study population

Variable	Controls ($n=207$)	Cases ($n=356$)	P value
Age (years)	46.8 ± 10.2	46.9 ± 8.2	0.905
Gender (male)	88 (42.5%)	165 (47.8%)	0.410
Smoking (current)	86 (41.5%)	122 (35.4%)	0.291
Ethnicity (Caucasian)	126 (60.9%)	227 (65.8%)	0.463
WBC ($10^3/mm^3$)	6.1 ± 1.6	6.8 ± 1.8	<0.001
LDL (mmol/L)	2.8 ± 0.9	3.2 ± 0.9	<0.001
HDL (mmol/L)	1.7 ± 0.4	1.5 ± 0.4	<0.001
Total cholesterol (mmol/L)	5.0 ± 1.0	5.3 ± 1.0	0.017
Triglycerides (mmol/L)	1.3 ± 0.8	1.4 ± 1.2	0.359
CRP (mg/L)	1.3 ± 5.4	2.1 ± 3.7	<0.001
IL-6 (ng/ml)	1.3 ± 1.9	1.2 ± 3.0	0.529
D-ROM (U Carr)	277.4 ± 108.6 ($n=184$)	378.1 ± 121.1 ($n=204$)	<0.001
BAP (mmol/L)	3686.5 ± 1955.5 ($n=184$)	2798.1 ± 1445.1 ($n=204$)	<0.001
T/S ratio	1.23 ± 0.42	1.12 ± 0.31	<0.001
			0.006[a]

Data are presented as means ± SD. P values were calculated from comparisons between cases and controls with independent t test, with the exception of categorical variables compared with χ^2 test (not normally distributed variables were log-transformed before comparisons and back-transformed values are presented).
[a] P value derived from the multivariate model with LTL as dependent variable and age, gender, ethnicity, smoking, and lipids as covariates.

Results

The characteristics of the study population are summarized in Table 1. Periodontal patients had higher serum levels of CRP ($P<0.001$) and leukocyte counts ($P<0.001$) compared to controls. Further, greater total cholesterol ($P=0.003$) and LDL cholesterol ($P<0.001$) levels, as well as lower HDL cholesterol ($P<0.001$), were observed. Cases exhibited higher levels of reactive oxygen metabolites ($P<0.001$) and lower antioxidant potential ($P<0.001$) as assessed by D-ROM and BAP tests, respectively (Table 1). Periodontal patients presented shorter LTL compared to the control group (1.23 ± 0.42 vs 1.12 ± 0.31 T/S ratio; 8311 ± 2723 vs 7670 ± 2632 bp; $P<0.001$) and this difference was independent of age ($P=0.001$), gender ($P=0.288$), ethnicity ($P=0.655$), and smoking ($P=0.012$) differences. Interesting, when cases were subdivided into those with aggressive ($n=71$) or chronic ($n=285$) periodontitis, the difference in LTL was statistically significant only in the chronic group (1.12 ± 0.31 T/S ratio; 7673 ± 2632 bp; $P=0.01$ vs controls), whereas no differences were noted between aggressive cases and controls (1.15 ± 0.33 T/S ratio; 7807 ± 2632 bp; $P=0.201$) nor between aggressive and chronic cases ($P=0.571$; Fig. 1A). These differences were independent of age, ethnicity, gender, and smoking. Both periodontitis groups exhibited higher CRP serum levels ($P<0.01$, Fig. 1C) and higher D-ROM ($P<0.05$, Fig. 1E) and BAP ($P<0.05$, Fig. 1F) serum levels than controls. Furthermore, aggressive periodontitis cases were younger ($P<0.05$, Fig. 1B) than chronic cases and controls and presented with higher serum IL-6 levels ($P=0.048$, Fig. 1D) than chronic cases.

A statistically significant inverse correlation was found between LTL and age ($R=-0.2$, $P=0.001$) in the whole sample and in individual groups (cases, $R=-0.1$, $P=0.04$; controls, $R=-0.2$, $P=0.01$). Notably, LTL was inversely related to both reactive oxidative metabolites as assessed by D-ROM in the whole population ($P=0.008$, $R=-0.2$) and in cases ($R=-0.2$, $P=0.03$), but not controls ($R=0.05$, $P=0.516$) (Fig. 2A), and a measure of severity of periodontitis in the cases (clinical attachment levels in millimetres) ($P=0.003$, $R=-0.2$) (Fig. 2B). Fig. 3 shows the mean predicted LTL by age in cases versus controls deriving from the fully adjusted model (which included age, gender, ethnicity, and smoking). A stronger age slope was observed in cases ($P=0.01$) versus controls, showing a shortening of the LTL at a faster rate in individuals with periodontitis compared to controls over the age groups of the whole population studied. No statistically significant correlations were found between LTL and acute markers of inflammation, such as CRP and IL-6, nor with other biochemical parameters reported in Table 1 (whole sample correlation for CRP, $R=-0.1$, $P=0.649$; IL-6, $R=-0.07$, $P=0.149$; triglycerides, $R=-0.038$, $P=0.644$; total cholesterol, $R=-0.05$, $P=0.332$; HDL cholesterol, $R=-0.01$, $P=0.767$; LDL cholesterol, $R=-0.07$, $P=0.143$; white blood count, $R=-0.03$, $P=0.489$).

Discussion

This is the first report to describe an association between shorter LTL and periodontitis. Patients with periodontitis presented shorter LTL, higher systemic inflammation, and higher oxidative stress compared to controls. Furthermore, LTL was negatively correlated with total levels of reactive oxidative metabolites detected by D-ROM test and severity of periodontitis. These associations were independent of age, gender, ethnicity, and smoking differences. In subgroup analyses of periodontal diagnosis (chronic versus aggressive), telomeres were shorter only in chronic cases compared with controls, even after adjustment for all confounders.

In the literature there is only one previous report comparing LTL between subjects suffering from periodontitis and age-matched controls [26]. However, the authors enrolled a group of patients with the aggressive form of disease and, in agreement with our results, were unable to show a significant difference between cases and controls.

As previously reported [16,27], patients with periodontitis show higher levels of inflammation and oxidative stress compared with controls [20]. This evidence supports the hypothesis that shorter LTL detected in periodontal patients could result from a local and systemic exposure to a higher inflammatory environment. In apparent contrast with our interpretation, patients with the aggressive periodontitis tend to show higher levels of systemic inflammatory markers and longer LTL compared with chronic cases. However, we can argue that only a long-term exposure to inflammation, like that experienced by individuals with chronic disease, could affect the rate of telomere shortening. This “chronic exposure” hypothesis is supported by current understanding of the possible mechanisms involved in telomere attrition [4,28–30] and seems to be further confirmed by our results. Indeed, although biomarkers of acute inflammation (i.e., CRP and IL-6) do not correlate with LTL, surrogate measures related with a chronic inflammatory exposure, such as the lifetime periodontal clinical attachment loss, represent possible predictors of LTL. The mechanisms involved in the pathogenesis of periodontal tissue destruction at the dentogingival interface could give further support to this hypothesis. Indeed, in patients with chronic periodontitis, a typical leukocyte infiltrate into the gingival tissue is coupled with a local and systemic chronic inflammatory state, which creates a continuous stimulus to the recruitment, differentiation, and replication of new inflammatory cells [13]. This in turn could lead to a higher rate of telomere shortening in peripheral white cells as a direct

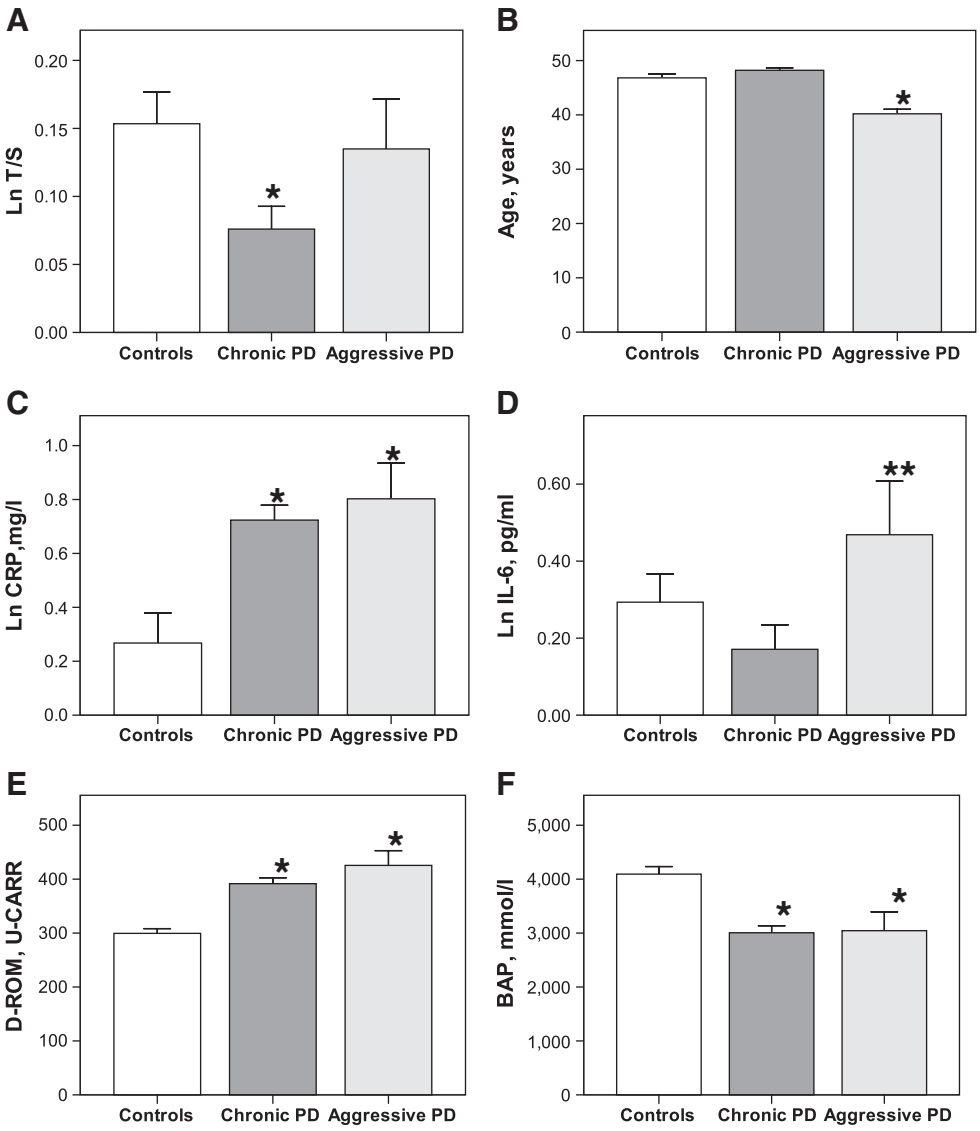

Fig. 1. Means (±standard errors) of (A) log *T/S*, (B) age, (C) log CRP, (D) log IL-6, (E) D-ROM, and (F) BAP of controls ($n=207$) and chronic ($n=285$) and aggressive ($n=71$) periodontitis (PD) cases. *Statistically significant difference ($P<0.05$) compared to controls as computed using analysis of variance. **Comparison between chronic and aggressive cases using analysis of variance.

consequence of the end-replication problem [31]. In parallel, the increased burden of oxidative stress, both systemically and at the gingival level, might cause the clipping of greater stretches of telomeres with each cell replication, exponentially increasing the rate of telomere attrition [4].

The local exposure (gingival tissue) to higher levels of oxidative stress could represent the common driver of the observed correlation between LTL and severity of periodontitis. Although in this paper we did not collect measures of local oxidative stress, a large body of evidence suggests its central role in the pathogenesis of the periodontal tissue destruction, further explaining the relationship between LTL and severity of periodontitis. An excess of reactive oxidative metabolites coupled with a depletion of the antioxidant capacity in gingival crevicular fluid [17,18] might be responsible for the chronic local activation of periodontal inflammation and lead to gingival tissue destruction.

Our data further provide a possible biological explanation for a plethora of epidemiological studies describing an association between periodontitis and higher mortality rate, mainly attributable to life-threatening diseases such as cancer, atherosclerosis, and diabetes. These diseases are typically related to shorter LTL [32] and LTL has been shown to be inversely related to the mortality rate of the patients [33]. Using the previously validated regression line relating our PCR assay with the standard TRF method for measuring telomere lengths we can estimate that the difference in LTL between cases and control corresponds to 641 bp, which would be equivalent to a biological age gap of approximately 21 years [34]. Within the limitation of our study design (not prospective), the fully adjusted models predicting LTL by age clearly showed a faster rate of telomere shortening in individuals with periodontitis.

A number of limitations should be discussed when interpreting our results. In this study the ratio of cases to controls is 1:0.6, and the inclusion of equal numbers of control subjects would improve the power. Additional screened control subjects were not available and rather than selecting or reducing the cases to an equal ratio 1:1 it was deemed more appropriate to include all recruited cases in the analyses. Similarly for the oxidative stress analyses, measures were determined only in a random subset of subjects for which there was sufficient biological material to perform the analysis. Both the D-ROM and the BAP tests give global measures of oxidative stress, and therefore they lack the specificity to determine which exact metabolites are affected by the presence of periodontitis. Moreover,

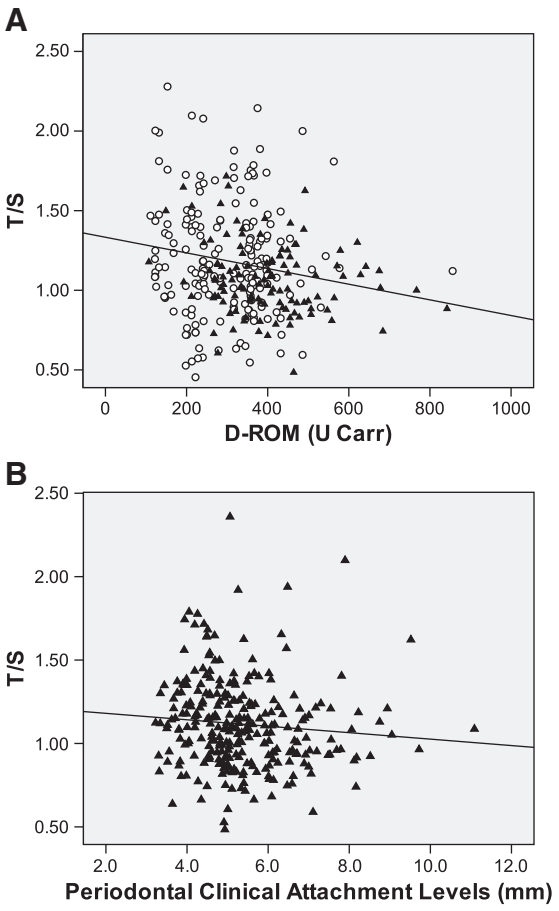

Fig. 2. Scatter plots of leukocyte *T*/*S* ratios against (A) D-ROM test serum levels ($R=-0.2$, $P=0.001$) and (B) periodontal clinical attachment levels ($R=-0.2$, $P=0.008$), by Spearman rank correlation test. Controls are drawn as open circles and cases as filled triangles.

the relationship between clinical attachment levels and LTL could reflect an early exhaustion of the regenerative ability of the local gingival tissues. This could be driven by an early senescence of the various cell lines involved in the tissue turnover. Given the lack of data regarding the changes in telomere length from normal gingival tissue we can only suggest that this relationship is representative of a concomitant and generalized aging process of all different tissues that are involved in the inflammatory response. Recent evidence, however, suggests that there could be a synchronization of telomere length along somatic cells recovered from different tissues [35,36]. Takahashi et al. did not find a significant correlation between LTL and human gingival fibroblast telomere lengths [26]. However, this relationship was explored in patients with aggressive periodontitis, which, based on our data, might not show shorter LTL. The quantitative PCR-based assay measures the average telomere length across all leukocytes in the peripheral blood. Because of the observed difference in the total leukocyte count between cases and controls in our study, we could not exclude that different leukocyte subpopulations with shorter telomeres could have been present in the cases. Nevertheless, a recent report suggested that aging processes, including oxidative stress and inflammation, could involve all different subpopulations of white cells in the peripheral blood as a reflection of their common origin from the bone marrow hematopoietic stem cells [37]. Finally, the low number of patients with a diagnosis of aggressive periodontitis could probably account for the lack of difference observed in the LTL with respect to the control group. However, it is important to emphasize that our results do not show a trend of shorter LTL in the subgroup of patients with aggressive disease and are in agreement with previously reported data [26].

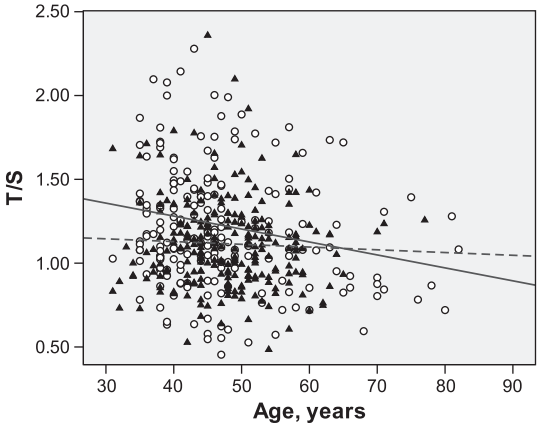

Fig. 3. Scatter plot of predicted *T*/*S* ratios based on fully adjusted models (age, gender, ethnicity, smoking, lipids) by age in cases and controls. Controls are drawn as open circles and cases as filled triangles. Solid line corresponds to the slope of *T*/*S* by age in cases and dashed line to the slope of *T*/*S* by age in controls. *P* value of the statistical difference in slope between cases (slope = 0.002) and controls (slope = 0.035) is 0.001.

Conclusions

In conclusion our data suggest that chronic inflammation could be the main driver of shorter LTL in patients with periodontitis and that the local and systemic effects of oxidative stress could be considered the main predictor of the LTL attrition. Moreover, the evidence of shorter LTL detected in individuals with chronic periodontitis could provide a possible biological explanation for the reported higher mortality rate in these patients compared to the general population. Further research and longitudinal studies, however, are needed to confirm the prognostic importance of these findings.

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