Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation

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ABSTRACT
The aim of this cohort study was to determine whether periodontitis and gingivitis are associated with impaired salivary antioxidant status and increased oxidative injury. One hundred and twenty-nine patients attending a routine dental check-up were recruited for the study. Periodontal disease status was characterized using the Community Periodontal Index of Treatment Needs (CPITN) system. Total salivary antioxidant capacity and salivary ascorbate, urate and albumin were determined in a sample of whole unstimulated saliva. Protein carbonyl concentrations were determined as an index of oxidative injury. Patients in the lowest tertile of CPITN score exhibited decreased salivary delivery of antioxidants and specifically urate than patients in the upper tertile. Poor periodontal health was associated with increased concentrations of protein carbonyls in saliva. Women had significantly lower total antioxidant status than men, regardless of periodontal health. Periodontal disease is associated with reduced salivary antioxidant status and increased oxidative damage within the oral cavity.

INTRODUCTION
Periodontal diseases (gingivitis and periodontitis) are among the most widespread chronic conditions affecting populations worldwide [1,2]. Gingivitis, characterized by inflammation of the gums due to the accumulation of plaque, affects some 50% of the adult population [1]. More severe destructive periodontitis associated with gum recession, loss of gingival tissue and underlying alveolar bone is also widespread, affecting 10–15% of the world population [2].

Periodontal disease is initiated by the colonization of the gums by bacterial pathogens, such as Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Bacteroides forsythus, of which P. gingivalis is of most significance [3]. This species has the capacity to activate host defence mechanisms that break down the epithelia and other structures of the gum, while at the same time inactivating repair systems [4,5]. Activated neutrophils recruited to the infected area become immobilized and produce reactive oxygen species that have the capacity to destroy the pathogen, but which also damage the host tissue in the vicinity [6,7]. Pathogens such as P. gingivalis possess their own antioxidant enzymes and can resist the killing actions of neutrophil-derived radicals [3]. This process results in host tissue damage and may thus contribute to the destructive process in severe periodontal disease.

Tissue injury due to free radical production has been suggested to be enhanced in individuals with periodontal disease due to a lack of adequate antioxidant defence [8,9]. Exaggerated neutrophil activity may be attributable to defects of the inflammatory response in some individuals [6], but low antioxidant capacity may be caused by

Key words: antioxidants, gums, oxidative injury, periodontitis, saliva, uric acid.
Abbreviations: CPITN, Community Periodontal Index of Treatment Needs; FRAP, ferric reducing ability of plasma; TAA, total antioxidant activity.
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a number of factors including smoking and poor nutritional status. In two small-scale studies, Chapple et al. have reported that patients with periodontal disease have reduced total antioxidant capacity in whole saliva [8] and lower concentrations of glutathione in serum and in gingival crevicular fluid [9]. In contrast, Moore et al. [10] reported similar salivary antioxidant profiles in periodontal patients and apparently healthy controls.

The aim of the present study was to investigate whether there is a relationship between total antioxidant status in whole saliva and periodontal disease status, using a larger and better-characterized population than in previous studies. The use of simple methods for the determination of both antioxidant status and oxidative injury may be considered appropriate for the future clinical monitoring of disease and treatment progression.

METHODS

Participants
One hundred and twenty-nine patients attending for routine dental examination at the Wilson House Dental Practice (Newport Pagnell, Bucks., U.K.) were recruited to the study. The age range of the subjects was 39–76 years (mean 58.5 years) and there were similar numbers of men (64) and women (65). All subjects were white Caucasians. Those taking nutritional supplements were excluded from the study. Also excluded were those having just received dental treatments to avoid contamination from debris or anaesthetic. All participants gave their written informed consent for inclusion in the study. Recruitment took place between September 2001 and August 2002. Saliva collections were made between 13:00 and 17:00 hours. Ethical approval was obtained from the Milton Keynes Medical Ethics Committee.

Classification of periodontal health status
An adaptation of the Community Periodontal Index of Treatment Needs (CPITN) in use at the dental practice was used to provide a detailed assessment of gum health in all subjects. The examination procedure involved the insertion of a graduated periodontal probe between teeth and gums at a standard force to measure pocket depth, combined with assessment of calculus accumulation on the sub-gingival surfaces of the teeth [11]. These assessments were made for sextants of the dentition, with the third molars only included if second molars were missing. The scoring index for this procedure ranged from 0 (no bleeding, pocketing or plaque-retentive factors) to 4 (extensive disease, pockets > 5.5 mm). Taking the sum of the sextant scores and subtracting this figure from a theoretical maximum of 24 gave the final periodontal disease score. Therefore in this study a high CPITN score represents healthy gingivae and a low score indicates disease.

Sample collection and preparation
There are numerous methods available for saliva collection, including harvesting whole saliva, individual gland saliva and stimulated or unstimulated saliva. Whole unstimulated saliva was collected in this study, as it represents the major intra-oral condition regarding saliva state and composition. It also contains some elements of gingival crevicular fluid and tissue metabolites which can be useful in the determination of tissue degradation [12,13]. In addition, stimulating saliva flow has been demonstrated to increase saliva volume and disrupt the antioxidant concentration [10]. Moreover, the mastication process employed in most stimulation techniques may cause the expulsion of relatively high quantities of gingival crevicular fluid from the periodontal pocket, artificially increasing the antioxidant capacity in saliva with plasma-derived antioxidants [8].

Saliva samples were collected after patients had received their routine check-up. With the patients seated, the saliva was collected over a 5-min period with instructions to allow saliva to pool in the bottom of the mouth and drain to a collection tube when necessary. Subjects were asked not to swallow any saliva for the duration of the collection to allow the calculation of salivary flow rates. At the end of the collection period, saliva volume was measured, the tube sealed and then frozen in dry ice until taken back to the laboratory for processing. Prior to analysis, the saliva was placed into Salivette (Sarstedt, Leicester, U.K.) tubes using a natural cotton swab insert, and centrifuged at 4000 g for 10 min at 4 °C. The supernatant fraction was then aliquotted into storage vials and kept at –80 °C until required for analysis. The use of the Salivette tubes was necessary to reduce the high viscosity of the saliva samples that would otherwise prevent accurate pipetting. Preliminary work to compare fresh saliva centrifuged in standard centrifuge tubes with saliva prepared by centrifugation in three types of Salivette (natural cotton insert, citric acid impregnated insert or plain polyester insert) demonstrated that the antioxidant profile from samples prepared using the natural cotton insert was identical to the native sample. It was inferred from this that the analytes of interest in this study were not absorbed into the inserts.

Antioxidant assays
Total antioxidant activity (TAA) was determined in whole saliva using the ferric reducing ability of plasma (FRAP) assay [14] adapted for a microplate reader. Salivary ascorbate concentrations were determined using the colorimetric method of Butts and Mulvihill [15]. Urate was assayed using an enzymic conversion method (Sigma Diagnostics kit). Salivary albumin was determined using the Bromocresol Green method [16].

Unlike other fluid compartments such as plasma, saliva is not a static fluid and its composition changes...
rapidly. Absolute antioxidant concentrations may thus be misleading and it is more important to consider the rate of delivery of antioxidant components into the oral cavity. Antioxidant flow rates (nmol · ml⁻¹ · min⁻¹) were calculated from absolute concentrations (antioxidant; µmol/litre) and the known flow of saliva (litre/min) during collection over a 5 min period using the equation:

$$\text{Antioxidant flow rate} = \frac{[\text{antioxidant}]}{\text{saliva flow}}$$

This approach is consistent with that reported by Moore et al. [10].

### Determination of oxidative injury

Protein carbonyl concentrations in whole saliva were determined as an index of oxidative injury [17]. Protein carbonyls are formed as a consequence of free radical attack upon amino-acid side chains. The carbonyl assay of Levine et al. [18] utilizes the dye 2,4-dinitrophenylhydrazine, which binds to damaged residues and has an absorbance of 370 nm. Protein carbonyl concentrations were expressed per g of total protein in saliva, as determined by the method of Bradford [19].

### Statistical analysis

Data are expressed as the means ± S.E.M. throughout the paper. Where covariates (salivary flow, sex) had a material impact upon the outcome of the analyses, data are expressed as marginal means adjusted for these variables, calculated using a univariate analysis. Means were otherwise compared using one-way ANOVA with a least-significant-difference post-hoc test, or using an independent samples t test, as appropriate. The relationship between periodontal disease status and antioxidant–oxidative balance was assessed using stepwise linear regression models using a significance level for inclusion in the model of $P < 0.05$ and a removal probability of 0.1. Binary logistic regression was applied to assess the contribution of total antioxidant capacity to risk of developing clinically significant periodontal disease (CPITN < 18). Tertiles of total antioxidant flow rate, sex and smoking habit were treated as categorical data in this analysis. The logistic regression model was generated with all three variables entered [20].

### RESULTS

Comparison of salivary antioxidant status in male and female subjects revealed a significant gender-related difference in saliva composition. TAA was significantly lower in women than in men, whether considered as an absolute concentration or as an antioxidant flow rate (Table 1). The difference in TAA flow rate was related to reduced flow of both ascorbate and urate in the women. Protein carbonyl concentrations in saliva were 2.3 times higher in women than in men, though this failed to achieve statistical significance, even after adjustment for salivary flow rate. A wide variation in protein carbonyl concentration was noted among the women with values between 0 and 177 fmol/g of protein. The women in the study tended to be younger than the men, but this difference was not statistically significant. Periodontal health and smoking habits were similar in men and women.

Following dental examination the study population included a broad range of periodontal disease states characterized on the basis of CPITN score. The patients were grouped into tertiles based upon this score, producing three groups that corresponded to severe disease (CPITN 11–14, $n = 37$) and a third group which included healthy

<table>
<thead>
<tr>
<th>Table 1 Salivary antioxidant status and periodontal disease in male and female participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects were classed as diseased if CPITN score was below 18. Subjects were grouped into tertiles on the basis of their CPITN score. The data shown are the unadjusted means ± S.E.M.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 64)</th>
<th>Women (n = 65)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.8 ± 1.0</td>
<td>57.2 ± 0.9</td>
<td>0.058</td>
</tr>
<tr>
<td>CPITN score</td>
<td>12.3 ± 0.7</td>
<td>13.6 ± 0.6</td>
<td>0.173</td>
</tr>
<tr>
<td>TAA (µmol/litre)</td>
<td>654 ± 25</td>
<td>545 ± 23</td>
<td>0.002</td>
</tr>
<tr>
<td>Protein carbonyls</td>
<td>8.46 ± 1.71</td>
<td>19.26 ± 7.09</td>
<td>0.104</td>
</tr>
<tr>
<td>TAA flow rate</td>
<td>0.31 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ascorbate flow rate</td>
<td>4.27 ± 0.44</td>
<td>2.88 ± 0.30</td>
<td>0.011</td>
</tr>
<tr>
<td>Albumin flow rate</td>
<td>5.44 ± 0.74</td>
<td>4.03 ± 0.74</td>
<td>0.180</td>
</tr>
<tr>
<td>Urate flow rate</td>
<td>108.8 ± 12.0</td>
<td>58.7 ± 6.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Salivary flow rate</td>
<td>2.58 ± 0.16</td>
<td>1.87 ± 0.11</td>
<td>0.001</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>8.9</td>
<td>9.1</td>
<td></td>
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<tr>
<td>Diseased (%)</td>
<td>81.5</td>
<td>82.8</td>
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</tbody>
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individuals and those with mild disease (CPITN > 14, n = 46). As shown in Table 2, the patients in each group were similar in age, but there were more smokers in the severely diseased group than in the moderate disease group. There were no smokers in the mild/no disease group. This group had higher salivary urate flow rates than the severe disease group (Table 2). Salivary ascorbate and albumin flow rates were similar in all groups, as was TAA. TAA flow rate did not differ significantly between the groups until a correction for sex was applied. Corrected TAA flow was significantly lower in the severe disease group than in the mild/no disease group. Similarly salivary protein carbonyl concentrations corrected for sex and salivary flow rate were significantly elevated in the severely diseased group relative to both of the other groups.

Stepwise linear regression modelling indicated that smoking had the strongest influence upon CPITN score (P = 0.003). This relationship was strengthened by the inclusion of protein carbonyl concentrations in the model (P = 0.001). Linear regression modelling also demonstrated that the primary influences upon the salivary protein carbonyl concentration (oxidative injury marker) were low CPITN score (P = 0.018) and female sex (P = 0.020). Logistic regression analysis was applied to the data to demonstrate that with the inclusion of sex and smoking as known contributors to total salivary antioxidant status and risk of disease respectively, risk of developing disease (classified as CPITN <18) was significantly related to TAA flow rate (P = 0.043). Patients were grouped into tertiles of TAA flow rate. Taking the highest TAA flow group as the baseline, the odds of disease were significantly greater with the lowest TAA flow rates (odds ratio = 4.46, 95% confidence interval 1.04–19.15).

### DISCUSSION

The main finding of this study is that subjects with the worst periodontal health status tended to have greater oxidative injury, as indicated by the presence of protein carbonyls in whole saliva. This has not been previously reported, but is wholly consistent with the hypothesis that there is enhanced reactive oxygen species-mediated damage to tissues in the most advanced states of periodontal disease [21]. A recent report has also demonstrated increased levels of protein carbonyls in the saliva of smokers relative to non-smokers [22]. We did not observe any significant increase in protein carbonyl concentrations related to smoking in the present study and when the analyses were repeated excluding smokers, a similar pattern of results was observed. Protein carbonyl concentrations in the lower, mid and upper tertiles of CPITN score, as presented in Table 2, were 26.16 ± 5.9, 8.56 ± 6.66 and 5.98 ± 5.43 fmol/g of protein respectively (P < 0.05).

It is unlikely that oxidative processes play a causal role in the aetiology of periodontitis, but they are likely to contribute to disease progression unless abated through antioxidant action. Subjects with lower CPITN scores and evidence of oxidative injury also had the lowest TAA flow rates. This could either be the reason why they had sustained more oxidative damage, or might be a consequence of antioxidant depletion due to ongoing free radical activity and destruction of scavenging antioxidant species. The latter explanation is most likely. It is remarkable that we have been able to detect differences in antioxidant status and oxidative processes at the level of whole saliva, as gingival crevicular fluid is the main site where oxidant–antioxidant interactions occur. Chapple et al. [9] have, however, demonstrated that

<table>
<thead>
<tr>
<th>Tertile of CPITN score</th>
<th>&lt; 11</th>
<th>11–14</th>
<th>&gt; 14</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>59.6 ± 1.3</td>
<td>57.4 ± 1.5</td>
<td>60.3 ± 1.3</td>
</tr>
<tr>
<td>TAA (µmol/litre)</td>
<td>593 ± 29</td>
<td>601 ± 32</td>
<td>605 ± 29</td>
</tr>
<tr>
<td>Protein carbonyls (fmol/g of protein)</td>
<td>24.77 ± 5.42</td>
<td>7.36 ± 5.97</td>
<td>6.36 ± 5.26*</td>
</tr>
<tr>
<td>TAA flow rate (µmol·ml⁻¹·min⁻¹)</td>
<td>0.21 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.27 ± 0.02*</td>
</tr>
<tr>
<td>Ascorbate flow rate (nmol·ml⁻¹·min⁻¹)</td>
<td>3.59 ± 0.44</td>
<td>3.59 ± 0.50</td>
<td>3.52 ± 0.48</td>
</tr>
<tr>
<td>Albumin flow rate (nmol·ml⁻¹·min⁻¹)</td>
<td>4.99 ± 0.84</td>
<td>4.96 ± 0.99</td>
<td>4.11 ± 0.95</td>
</tr>
<tr>
<td>Urate flow rate (nmol·ml⁻¹·min⁻¹)</td>
<td>66.9 ± 10.8</td>
<td>86.6 ± 12.5</td>
<td>101.8 ± 12.2*</td>
</tr>
<tr>
<td>Salivary flow rate (ml/min)</td>
<td>2.23 ± 0.21</td>
<td>2.21 ± 0.17</td>
<td>2.22 ± 0.15</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>16.1</td>
<td>9.7</td>
<td>0</td>
</tr>
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Table 2 Salivary antioxidant status in participants grouped on the basis of CPITN score

Subjects were grouped into tertiles on the basis of their CPITN score. The data shown for age and salivary flow are the unadjusted means ± S.E.M. Antioxidant data are adjusted for sex; protein carbonyls are adjusted for sex and salivary flow rate. *Significantly different (P < 0.05) to lowest tertile.
even plasma glutathione becomes depleted in diseased individuals.

The findings of the present study are consistent with the previous work of Chapple et al. [8,9] but, importantly, represent the outcome of a much larger trial. Unlike other reports in this area [8–10], the subjects were not just considered as disease cases or controls and we have considered periodontal health as a continuous variable (CPITN score). In other studies the control group is sometimes not subject to a dental examination [10] and a detailed definition of ‘healthy’ is generally absent from most papers in this area. The approach we have adopted reflects the fact that most people in the population will have some degree of inflammation [1].

In the present study, antioxidant status in unstimulated whole saliva was considered in terms of antioxidant flow rate. Absolute concentrations of antioxidants in this context are not meaningful as this would represent the concentration in the pool of saliva accumulated over 5 min. In normal individuals this would tend not to occur due to swallowing. When considering the exposure of the gums and teeth to antioxidant species, the rate of delivery is more important. Antioxidant flow rate was obviously affected by the amount of saliva collected during sampling. Interestingly, saliva production tended to be lower in women than in men. Saliva flow rate has been incorporated into regression analyses.

Contamination of saliva samples by bleeding from the gums is also an important issue. Patients with inflammation and pocketing in one or more sextants would be expected to bleed on probing [11]. Saliva sampling took place after dental examination. Leakage of antioxidants from the plasma component into saliva may thus have impacted upon our findings. There is, however, no evidence that this has had any significant influence on outcome. The FRAP assay is very sensitive to contamination with haemoglobin [14] and significant blood contamination would have been detected at this stage.

Urate was the major antioxidant component in saliva samples from these patients. This is consistent with the finding of Moore et al. [10] who suggested that urate contributes in excess of 70% of salivary TAA. Urate, ascorbate and albumin appear to contribute most of the antioxidant protection in whole saliva, although there is evidence of a salivary peroxidase enzyme [23]. In this respect, saliva is very different in composition from plasma and other extracellular fluids. Fluids such as gingival fluid and lung epithelial lining fluid contain a broader complement of antioxidant components, including glutathione [24]. Glutathione concentrations are high within gingival crevicular fluid and appear to be reduced in patients with periodontal disease [9]. We have not previously detected any glutathione in whole saliva from healthy individuals (D. V. Sculley and S. C. Langley-Evans, unpublished work) and have not included this measurement in the present study.

In the present study, urate was the only antioxidant component in saliva that was present in lower concentrations and delivered at a slower rate in individuals with lower CPITN scores. It is not clear whether the urate content of whole saliva directly reflects plasma concentrations or concentrations present in gingival crevicular fluid. However, interactions between scavenging antioxidants may occur [25]. Where one antioxidant is depleted another may be used to regenerate it, or to compensate for its function. Relationships between urate in whole saliva and antioxidant components of the gingival fluid, or nearby epithelial cells, will need to be evaluated in more detail.

In common with other studies, we have found that smoking is the largest single risk factor for periodontal disease [26]. After adjusting for this factor associations were noted between periodontal disease status and salivary antioxidant status. Individuals with the lowest TAA flows were nearly 4.5 times more likely to have periodontal disease, as defined by CPITN below 18, than those with higher TAA flow rates. Although it is unclear whether reduced antioxidant capacity is a cause or an effect of periodontal disease, it is certain that lower antioxidant concentrations in the gingival crevicular fluid will contribute to increased damage to the gingivae and surrounding structures by activated neutrophils. TAA flow rate as measured using FRAP in the present study may be a useful biochemical marker of disease progression and treatment. The potential use of this simple system for monitoring of treatment without dental examination may be attractive. Our finding of elevated protein carbonyl concentrations in whole saliva from patients with low CPITN scores also raises interesting possibilities. The presence of oxidized protein in saliva implicates widespread oxidative processes in the progression of more destructive periodontal disease. Protein carbonyls can be relatively easily determined [18] and may be used as a biochemical marker of disease progression or treatment.

Local decreases in antioxidant capacity in gingival crevicular fluid [9] are likely to be of greater significance in the aetiology of periodontitis and associated damage to the gums and teeth than the more systemic changes we have noted in whole saliva. The presence of antioxidants bathing the gingival crevice may be of major importance in dampening down inflammatory processes initiated by bacterial infection. Obtaining and analysing gingival fluid samples is, however, a complex process requiring a degree of specialism. The results of this study suggest that whole saliva may contain simply measured indicators of oxidative processes and may provide a tool for the development and monitoring of new treatment strategies.
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REFERENCES