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Activation of the Urokinase Plasminogen Activator/Urokinase Plasminogen Activator Receptor System in Periodontitis: A Case–Control Study

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ABSTRACT

Introduction: The plasminogen activating (PA) system has a multitude of functions such as wound healing, proteolytic activity, collagen degradation and cell growth, and the role of the urokinase plasminogen activator/urokinase plasminogen activator receptor (uPA/uPAR) system has been studied in many disease states. The aim of this study was to investigate salivary concentrations of uPA, uPAR and uPA activity in patients with periodontitis to identify biomarkers and novel pathogenic relationships.

Methods: Saliva samples were obtained from 169 participants, comprising patients with periodontitis ($n = 103$) and periodontally healthy volunteers ($n = 66$) and analysed for uPA and uPAR with a multiplex protein assay using proximity extension analysis in a subset of samples, followed by validation with ELISA. The protease activity of salivary uPA was quantified using a fluorometric assay.

Results: Patients with periodontitis had a 4.0-fold higher ($p < 0.001$) salivary uPA and a 2.5-fold higher ($p < 0.001$) salivary uPAR concentration in comparison to periodontally healthy participants. The salivary uPA activity (median [IQR]) from patients with periodontitis (123.21 [188.29] U/mL) was 1.6-fold higher ($p < 0.01$) than the salivary uPA activity from periodontally healthy participants (76.83 [98.09] U/mL). Levels of uPA and uPAR were strongly correlated with periodontal indices, whereas only weak correlations were found with BMI and age.

Conclusion: Activation of uPA/uPAR likely plays a role in the pathogenesis of periodontal diseases. uPA/uPAR may have potential utility as candidate salivary biomarkers in periodontal pathogenesis.

1 | Introduction

Urokinase plasminogen activator (uPA) and its receptor (uPAR) both belong to the plasminogen activation (PA) system. In the presence of a stimulus, whether host-derived or bacterial such as IL-1β from infiltrating neutrophils and local periodontal tissues or LPS from *Porphyromonas gingivalis*, uPA binds to its receptor uPAR, which activates and localises uPA on the cell surface [1]. Consequently, activated uPA converts plasminogen into active plasmin, thereby triggering the activities of the PA system [1].

The PA system has a multitude of functions such as wound healing, proteolytic activity, collagen degradation, cell growth, differentiation and proliferation, as well as anti-apoptotic functions [2–5]. Moreover, a recent review generally emphasised the roles of PA system proteins such as uPA, uPAR, tissue plasminogen activator (tPA) and plasminogen inhibitors (PAI-1 and PAI-2) in oral inflammatory and infectious diseases such as periodontal diseases, salivary gland diseases and oral cancer [6]. Specifically, it is thought that the balance between the plasminogen activator and inhibitors of the PA system proteins is disrupted in periodontal disease, contributing to an increase in overall inflammation [6].

Much attention has been given to the uPA/uPAR system in cancers with the potential for novel prognostic markers or drug targets [7–9]. However, as identified from review of the literature, fewer studies have investigated the uPA/uPAR system in periodontal disease. It is important therefore to study further the potential role of this important biochemical and signalling system in the pathogenesis of periodontal diseases, particularly given links with systemic health and disease.

Whilst a role for the PA system in periodontal disease pathogenesis has been recognised for some time [10, 11], only a limited number of studies have investigated its components as potential diagnostic biomarkers. In general, findings are mostly consistent, regardless of whether GCF or saliva was chosen as sample type. The majority of studies have focused on tPA, uPAR and the inhibitors PAI-1 and PAI-2, with only one study having evaluated uPA concentrations [12–19]. No study to date has determined uPA activity in periodontitis. Overall, concentrations of uPAR, uPA and tPA are increased in patients with periodontitis in comparison to periodontal health [12–18]. This relationship appears to be directly related to periodontal disease pathogenesis and was confirmed in periodontitis patients with diabetes for uPAR [12], rheumatoid arthritis for tPA [16] and cardiovascular disease for uPAR [14]. It was also confirmed for uPA and tPA in smokers with periodontal disease [15]. The present study aimed to investigate simultaneously salivary concentrations of uPAR and uPA, together with uPA activity in patients with periodontitis.

2 | Materials and Methods

2.1 | Study Population

The analysed samples were collected as previously described [20, 21] in case–control studies. Participants provided written informed consent, and were recruited from patients of the Newcastle upon Tyne Dental Hospital (2009–2012), and entered into the study at the Dental Clinical Research Facility of Newcastle Dental Hospital (Newcastle upon Tyne Hospitals NHS Foundation Trust) and Newcastle University School of Dental Sciences, following ethical approval by the National Research Ethics Service North East Newcastle and North Tyneside 1 committee (Refs: 12/NE/0396, 09/H0905/49). Inclusion criteria were males or females aged between 18 and 65, with a minimum of 20 natural teeth (excluding third molars) who were non-smokers. Exclusion criteria included evidence of infectious or systemic disease, currently undergoing treatment with antibiotics or immunosuppressants and a smoking history within the last 2 years. The criteria for allocating participants to the periodontally healthy and periodontitis groups were as follows: periodontally healthy participants had probing pocket depths (PPDs) of ≤ 3 mm at all sites (full-mouth measurements at six sites per tooth with a manual UNC-15 periodontal probe), no sites with interproximal attachment loss, modified gingival index (MGI) [22] scores of ≥ 2.0 in ≤ 10% of sites and % bleeding on probing (BOP) scores of ≤ 10%; periodontitis patients had interproximal PPDs of ≥ 5 mm at ≥ 8 teeth (following full-mouth measurements) and %BOP scores of ≥ 30%.

2.2 | Saliva Collection and Processing

Whole unstimulated saliva samples were obtained from study participants at least 1 h after their last food or drink intake, and at least 1 h after their last oral hygiene measure (tooth brushing, flossing and/or mouth rinse). The participants were seated in the dental chair, avoiding any noise or distraction. Neither stimulation nor examination of the oral tissues and mouth were carried out during sample collection. A pre-labelled, sterile, 50 mL polypropylene tube was given to each participant and the participant was instructed to drool saliva into the tube until approximately 5–10 mL were collected. The estimated collection time was 5–10 min. The samples were placed on ice and taken to the lab for processing. Saliva samples were centrifuged (at 1500 g, 15 min, 4°C), the supernatant was aliquoted, snap frozen in liquid nitrogen and then stored at −80°C until use in assays.

2.3 | Proximity Extension Assays for Salivary uPA and uPAR

A total of, 15 saliva samples from periodontally healthy individuals and 15 saliva samples from patients with periodontitis were sent to Olink Proteomics for analysis using proximity extension analysis (PEA) for a panel of 92 inflammation-related proteins, including uPA and uPAR [23–25].

PEA is a multiplex assay that utilises pairs of specific antibodies labelled with oligonucleotides. If the two antibodies for a given protein are bound within close proximity (i.e., bound to the same protein) hybridisation will occur between the corresponding oligonucleotide labels which are then extended by DNA polymerisation. This process occurs for target proteins and internal controls spiked into samples. The DNA sequences are then utilised, along with the DNA sequence generated by internal controls, in qPCR reactions to generate the relative expression of the target from C_t values, which were then normalised against internal extension controls to give ΔC_t values. These ΔC_t values were then used to generate $\Delta\Delta C_t$ from interplate controls.

The equations used were as follows:

$$\Delta C_t\text{: } C_{t_{\text{analyte}}} - C_{t_{\text{extension control}}} = \Delta C_{t_{\text{analyte}}}$$

$$\Delta\Delta C_t\text{: } \Delta C_{t_{\text{analyte}}} - \Delta C_{t_{\text{interplate control}}} = \Delta\Delta C_{t_{\text{analyte}}}$$

The $\Delta\Delta C_t$ was then converted into normalised protein expression, which is $\log_2$-transformed, using a correction factor generated using negative controls and the following equation:

$$\text{Correction factor} - \Delta\Delta C_{t_{\text{analyte}}} = \text{normalised protein expression}_{\text{analyte}}$$

2.4 | uPA And uPAR ELISAs

uPA and uPAR ELISAs (R&D Systems) were carried out according to manufacturer's instructions to validate the PEA results. uPAR was analysed in a subset of samples as there was not enough sample amount remaining for a complete re-analysis of all samples. Briefly, for the uPAR Duoset ELISA, a 7 point two-fold dilution standard curve was created in reagent diluent (RD), with the top standard of 2000 pg/mL uPAR. For the uPA Quantikine ELISA, a 7 point two-fold dilution standard curve was created in RD, with the top standard of 2000 pg/mL uPA. Samples were diluted in RD as appropriate prior to assay. All standards and samples were assayed in duplicate. Absorbance was read at 450 nm on a spectrophotometer (FL 600 Microplate Fluorescence Reader, BioTek). A reading at 550 nm was subtracted to correct for plate background. Protein concentrations of samples were calculated from the standards with the supplied software (KC4 KinetiCalc, BioTek) for the spectrophotometer using a four-parameter logistic curve fit.

2.5 | uPA Activity Assay

The uPA activity fluorometric assay kit (Sigma-Aldrich) measures urokinase activity ranging from 0.01–0.5 IU/well in a variety of samples. uPA activity in saliva was determined using the enzymatic cleavage of an AMC (amido-methyl-coumarin) based peptide substrate, which results in the generation of AMC (λ excitation = 350/λ emission = 450 nm) proportional to the enzymatic activity present, measured by fluorometric multi well microplate reader (FL 600 Microplate Fluorescence Reader). The assay was carried out according to manufacturer's instructions and saliva samples were diluted as appropriate in phosphate buffered saline. All standards and samples were assayed in duplicate. uPA activity of samples was calculated from the standards with the supplied software (KC4 KinetiCalc) for the spectrophotometer using a four-parameter logistic curve fit.

2.6 | Statistical Analysis

The null hypothesis was that there would be no difference in salivary uPA/uPAR levels between patients with periodontitis and those with periodontal health. Laboratory analyses were conducted without knowledge of the clinical status of the patients. Data were tested for normal distribution using the Shapiro–Wilk test and parametric or non-parametric tests were performed accordingly. Demographical and clinical data were analysed using the Chi square test and Mann–Whitney *U* test as appropriate. uPA and uPAR data from both the proximity extension screening assay and ELISAs were analysed using the Student's *T*-test. uPA activity data were analysed using Mann–Whitney *U* test. Correlations between parameters were performed using Spearman Rank test and corrected for multiple comparisons with Benjamini-Hochberg. All statistical analysis were performed in SPSS (version 28, IBM). A p value of < 0.05 was considered statistically significant.

3 | Results

3.1 | Demographics and Periodontal Parameters

A total of, 169 participants were recruited, comprising patients with periodontitis ($n = 103$) and periodontally healthy volunteers ($n = 66$). Patients with periodontitis presented with increased mean clinical attachment loss, PPDs and % BOP in comparison to periodontally healthy participants (Table 1). These patients also scored higher in the modified gingival index in comparison to periodontally healthy participants. The two groups were equally matched with regards to gender distribution, however patients with periodontitis overall were significantly older and had a higher BMI in comparison to the periodontally healthy participants.

3.2 | Salivary uPA and uPAR Protein Expression Is Increased in Periodontitis

Salivary uPA and uPAR normalised protein expressions (NPX) from patients with periodontitis were increased in comparison to periodontally healthy individuals in the proximity extension assay (Figure 1A,B). Patients with periodontitis had a 1.5-fold higher ($p < 0.001$) uPA (4.24 ± 1.11 NPX) (Figure 1A) and a 1.2-fold higher ($p < 0.001$) uPAR (6.7 ± 0.8 NPX) (Figure 1B) NPX in comparison to periodontally healthy participants (2.82 ± 0.99 and 5.43 ± 0.79 NPX, respectively). These differences were confirmed using ELISAs (Figure 2A,B). Patients with periodontitis had a 4.0-fold higher ($p < 0.001$) uPA

TABLE 1 | Demographics and periodontal parameters (mean ± SD for continuous variables) of study participants.

	Periodontal health ($n = 66$)	Periodontitis ($n = 103$)
Age (years)	34.91 ± 12.66	46.55 ± 8.06*
Gender (male/female)	28/38	51/52
BMI (kg/m^2)	24.79 ± 4.0	27.86 ± 5.0*
Bleeding on probing (%)	2.16 ± 2.46	54.89 ± 18.17*
Modified gingival index score	0.23 ± 0.31	2.64 ± 0.46*
Probing pocket depth (mm)	1.44 ± 0.21	3.43 ± 0.85*
Clinical attachment loss (mm)	0	4.29 ± 1.20

*$p < 0.001$ between groups using Mann–Whitney *U* test, gender was analysed using Chi Square test.

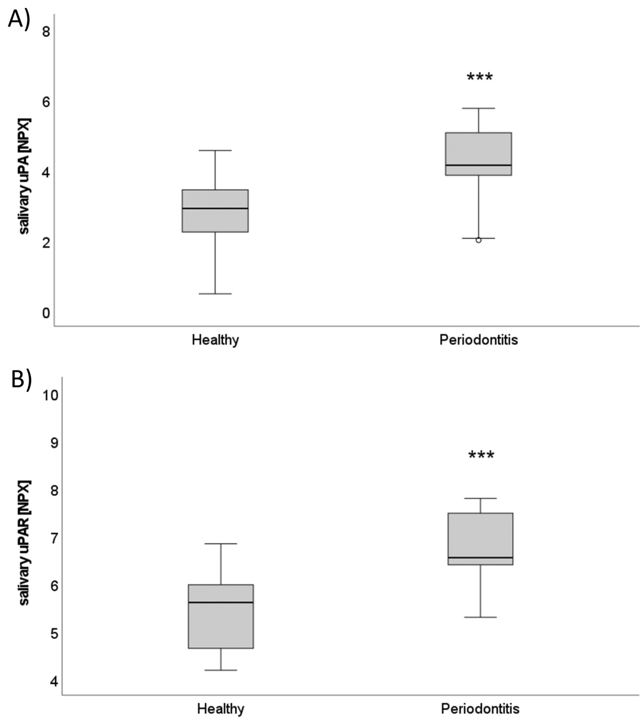

FIGURE 1 | Boxplots presenting normalised protein expression (NPX) for salivary urokinase (uPA) and receptor (uPAR). NPX expression for both salivary uPA and uPAR was identified by proximity extension screening assay in a subset of 15 patients with periodontitis and 15 periodontally healthy participants. (A) Salivary uPA expression was significantly higher in the patients as compared to the periodontally healthy participants (***$p < 0.001$, Student's *T*-test). (B) Salivary uPAR expression was also significantly higher in the patients as compared to the periodontally healthy participants (***$p < 0.001$, Student's *t*-test).

(843.43 ± 709.24 pg/mL) (Figure 2A) and a 2.5-fold higher ($p < 0.001$) uPAR (12677.03 ± 11595.63 pg/mL) protein concentration (Figure 2B) in comparison to periodontally healthy participants (208.64 ± 159.51 and 5104.44 ± 3708.11 pg/mL, respectively).

3.3 | Salivary uPA Activity Is Increased in Periodontitis

Salivary uPA activity from patients with periodontitis was increased in comparison to periodontally healthy individuals (Figure 3). The salivary uPA activity (median [IQR]) from patients with periodontitis (123.21 [188.29] U/mL) was 1.6-fold higher ($p < 0.01$) than the salivary uPA activity from periodontally healthy participants (76.83 [98.09] U/mL).

3.4 | uPA/uPAR System and Periodontal Parameters Are Correlated

Components of the uPA/uPAR system (uPA activity, uPA and uPAR) were positively correlated with each other ($p < 0.001$, Table 2). Salivary uPA, uPAR and uPA activity also showed positive correlations with periodontal indices, which can be strong at times ($\rho > 0.7$ for uPA). In contrast, correlations between uPA/uPAR system parameters and BMI or age in general were weak ($\rho < 0.3$). As expected, CAL and MGI score are strongly and significantly correlated ($p < 0.001$).

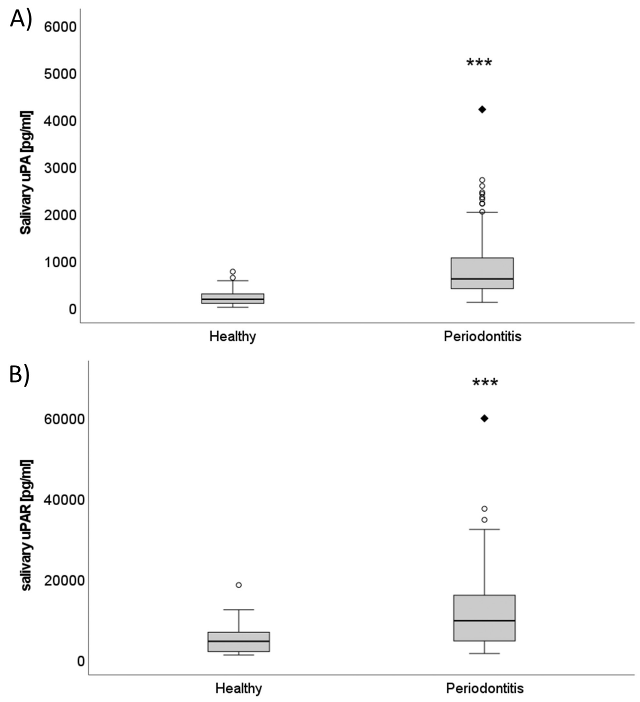

FIGURE 2 | Boxplots presenting salivary urokinase (uPA) and receptor (uPAR) levels. (A) Salivary uPA levels were measured by ELISA assay in samples obtained from 103 patients with periodontitis and 66 periodontally healthy participants, salivary uPA levels were significantly higher in the patients as compared to the periodontally healthy participants (***$p < 0.001$, Student's *T*-test). (B) Salivary uPAR levels were also measured by ELISA assay in samples obtained from 45 periodontitis patients and 37 periodontally healthy participants, in a similar manner to salivary uPA, the uPAR levels were significantly higher in the patients as compared to the periodontally healthy participants (***$p < 0.001$, Student's *T*-test). ◆ outlier more than three times the IQR from the box boundaries, ○ outlier more than 1.5 but less than three times the IQR from the box boundaries.

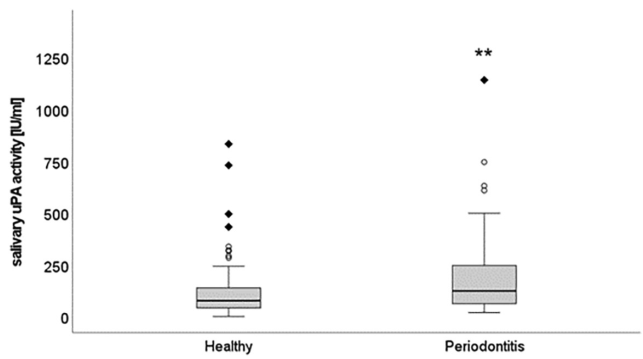

FIGURE 3 | Boxplot presenting salivary urokinase activity. Salivary uPA activity was assayed in saliva samples obtained from 103 periodontitis patients and 66 periodontally healthy participants. Salivary uPA activity was significantly higher in the patients as compared to the periodontally healthy participants (**$p < 0.01$, Mann–Whitney *U* test). ◆ outlier more than three times the IQR from the box boundaries, ○ outlier more than 1.5 but less than three times the IQR from the box boundaries.

TABLE 2 | Spearman Rank correlations between uPA/uPAR system, periodontal and general parameters.

	uPA activity (IU/mL)	uPA (pg/mL)	uPAR (pg/mL)	Age (years)	BMI (kg/m^2)	CAL (mm)	MGI score
uPA activity (IU/mL)							
uPA (pg/mL)	0.497***						
uPAR (pg/mL)	0.384***	0.595***					
Age (years)	0.291*	0.313**	0.227*				
BMI (kg/m^2)	0.257*	0.305**	0.223*	0.270*			
CAL (mm)	0.370**	0.757***	0.450***	0.402***	0.289*		
MGI score	0.239*	0.711***	0.324**	0.352**	0.284*	0.837***	

Note: ***$p<0.001$, **$p<0.01$, *$p<0.05$ after correction for multiple comparisons.

4 | Discussion

In the present study, we found a significant increase in salivary uPA and uPAR protein concentrations, as well as an increase in the salivary uPA activity in patients with periodontitis in comparison to periodontally healthy individuals, making components of the uPA/uPAR system candidate diagnostic biomarkers for periodontal disease. This is very relevant to the pathogenesis of periodontal diseases, given that periodontitis is a highly prevalent clinical condition, characterised by inflammation leading to tissue destruction that we recognise clinically as disease.

The very first mention of the plasminogen activating system in relation to saliva reported the lysis of plasminogen containing bovine-fibrin plates following the addition of stimulated mixed human saliva [26, 27]. Decades later Moody [28] demonstrated the presence of the substrate plasminogen in human saliva suggesting that it was due to the tissue plasminogen content of leukocytes and epithelial cells present in saliva as well as due to the fibrinolysis of the surrounding oral mucosa.

Increased salivary protein concentrations of uPAR in patients with periodontitis in comparison to periodontal health have been reported previously. Tasdemir et al. [13], Ali, Qasem and Baskaradoss [12] and Isola et al. [14] show a 6.5-fold, 5.2-fold and 2.2-fold increase of salivary uPAR concentrations in patients with periodontitis in comparison to periodontally healthy participants, respectively. Our own findings (a 2.5-fold increase in salivary uPAR concentrations in patients with periodontitis in comparison to periodontally healthy participants) are comparable with Isola et al. [14], however of somewhat lower magnitude than the findings of Tasdemir et al. [13] and Ali, Qasem and Baskaradoss [12]. This may be due to for example minor variations in saliva sampling and processing techniques or the different ELISA manufacturers used. uPA concentrations in periodontitis have only been investigated in GCF and a two-fold increase in comparison to periodontal health has been reported [15]. Similarly, uPA activity in saliva or GCF has only been reported once to date, in a study unrelated to periodontitis in patients with various neurological symptoms [29]. In that study, salivary uPA activity was only detected in 9 out of 34 samples, with high activity reported in a sample from a patient with hypophyseal tumour [29]. This led the authors to suggest that salivary uPA activity is inhibited by PAI-2 [29]. In contrast, we detected uPA activity in all of the analysed samples in the present study, with a significant increase in patients with periodontal disease in comparison to periodontally healthy participants.

The increased activity of uPA that was found in the present study in patients with periodontitis may simply be a reflection of the increased concentrations of uPA, however it is worth bearing in mind that only the activated uPA can catalyse plasminogen into plasmin, in turn activating the PA system. This makes the increased observed uPA activity an important indicator of the overall heightened state of the uPA/uPAR system in periodontitis and may reflect the extent of periodontal tissue destruction.

Both uPA and uPAR are produced by gingival fibroblasts in response to inflammatory and periodontal bacterial stimuli [30, 31]. uPA binds to uPAR, which will activate and localise uPA leading to the conversion of plasminogen into active plasmin, thereby initiating the process of proteolysis [1]. In the presence of inflammatory stimuli (such as IL-1β from infiltrating neutrophils and local periodontal tissues) or bacterial stimuli (such as LPS from *P. gingivalis*), the uPA/uPAR binding may be enhanced leading to more production of plasmin, in turn stimulating the proteolysis of supporting periodontal tissues associated with periodontitis. In addition to proteolysis, the active uPA/uPAR complex exerts chemotactic activity on inflammatory cells such as monocytes and neutrophils [32, 33] which in turn release cytokines, proteolytic and lysosomal enzymes which degrade the supporting periodontal tissues. Whilst synthetic uPA inhibitors are showing potential in cancer treatment [34], targeting the uPA/uPAR system as a treatment for periodontal disease remains to be investigated.

Though gender differences have been reported in the uPA/uPAR and PA system which may have impacted on our findings [35, 36], the periodontal disease and periodontal health groups in the present study were equally matched with regards to gender distribution, making a gender impact unlikely. However, the two groups were not equally matched with regards to BMI and age. Patients with periodontitis overall were significantly older and had a higher BMI in comparison to the periodontally healthy participants. A number of studies demonstrate concentration changes in the components of the uPA/uPAR system in serum according to BMI and age [37–40] and we therefore cannot discount these confounding factors could have had some influence on our findings. However, we have found evidence for strong correlations between parameters of the uPA/uPAR system and periodontal parameters contrasting with weak correlations of uPA/uPAR system parameters and BMI or age. This may further indicate that whilst there may be some confounding effect of BMI or age on the uPA/uPAR system measurements, periodontal aspects at least contribute to, if not outweigh, those effects.

In the present study, we have not investigated plasminogen inhibitors. The PA system is carefully balanced between plasminogen activators and inhibitors and it is a potential imbalance of this system which may contribute to inflammation in periodontal disease [6]. Whilst we have observed an increase on the activator side of this system, we cannot draw conclusions about a disruption of the PA system as a whole as we do not have information on whether PA inhibitors concentrations would have increased or decreased in our patient group in comparison to the periodontally healthy control participants. Future studies should consider a more holistic approach and investigate both inhibitors and activators of the PA system.

5 | Conclusion

We have demonstrated for the first time increased salivary uPA activity in patients with periodontitis in comparison to periodontally healthy individuals. This is relevant to clinical practice given that treatment strategies for periodontitis focus on inflammation reduction as an end-point of treatment (characterised by reductions in PPDs and BOP), which are achieved by implementation of improved patient selfcare in combination with professionally delivered treatments. Activation of uPA/uPAR likely plays a role in the inflammatory pathogenesis of periodontal diseases and these may be potential biomarkers in periodontal pathogenesis.

6 | Clinical Relevance

6.1 | Scientific Rationale for Study

To investigate the role of the plasminogen activator system (urokinase plasminogen activator (uPA)/urokinase plasminogen activator receptor (uPAR)) in periodontal disease, given its known role in a variety of chronic diseases and lack of knowledge of its relevance in periodontitis.

6.2 | Principal Findings

Salivary uPA/uPAR levels and salivary uPA activity are significantly higher in patients with periodontitis compared to periodontally healthy patients, and strongly correlate with clinical signs of periodontitis.

6.3 | Practical Implications

The plasminogen activator system potentially plays a role in the pathogenesis of periodontal diseases, contributing to the tissue damage that characterises the clinical signs of disease. Furthermore, uPA/uPAR may be potential biomarkers in periodontal pathogenesis.

Author Contributions

A.K., J.J.T., P.M.P., K.M.J. contributed to the conception and design of the study and data analysis. All authors contributed to data interpretation, drafting and critically revising the manuscript, and review of the final version for publication.

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Conflicts of Interest

P.M.P. reports personal fees from Springer Nature and Kenvue, and book royalties from Wiley, outside the submitted work. All other authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the authors upon reasonable request.

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