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Investigation and quantification of key periodontal pathogens in patients with type 2 diabetes

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Background and Objective: Diabetes is a recognized risk factor for periodontitis. There are conflicting data regarding whether healthy diabetic patients or diabetic patients with chronic periodontitis have an altered subgingival microbiota compared with nondiabetic individuals. The aim of the present study was to detect quantitative differences in selected periodontopathogens in the subgingival plaque of diabetic patients using TaqMan quantitative PCR.

Material and Methods: Type 2 diabetes mellitus patients with ($n = 9$) or without chronic periodontal disease ($n = 15$) were recruited and matched to nondiabetic control subjects ($n = 12$ periodontally healthy, $n = 12$ chronic periodontitis). Subgingival plaque samples were collected from deep (> 4 mm probing depth) and shallow sites (≤ 3 mm probing depth) using paper points, and *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* were quantified.

Results: Forty-eight subjects (69 samples) were recruited. Marked differences were seen in the levels of all three bacterial species, relative to the total bacterial population, according to periodontal health status. Using real-time quantitative PCR, bacterial counts for *P. gingivalis* were significantly higher in deep pockets of diabetic and nondiabetic subjects compared with periodontally healthy subjects ($p < 0.05$) but did not differ significantly between diabetics and nondiabetics. *A. actinomycetemcomitans* was detected in all groups in low quantities, and counts did not differ significantly between groups ($p > 0.05$). *F. nucleatum* was abundant in all groups, with no clear significant differences between groups. *P. gingivalis* was found in higher quantities in periodontitis than in periodontally healthy subjects ($p < 0.05$). Statistically significant positive correlations were identified between pocket depth and counts for all three species tested ($p < 0.05$).

Conclusion: A. actinomycetemcomitans, *F. nucleatum* and *P. gingivalis* were present in significantly different quantities and proportions in subgingival plaque, according to periodontal disease status. No significant differences were identified between the subgingival microbiota of type 2 diabetes mellitus patients compared with nondiabetic subjects.

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Periodontitis is a common chronic inflammatory disease, and approximately 10–15% of the UK adult population is affected by advanced disease that threatens tooth retention (1). Diabetes is also a common chronic inflammatory disease and is a massive burden to public health across the world. Global estimates suggest that the prevalence will continue to increase significantly over the next 25–30 years. Diabetes currently affects 246 million people worldwide and is expected to affect 380 million by 2025 (2,3). In the UK, over 2.5 million people are known to be affected by type 2 diabetes mellitus (T2DM), which increases the risk of cardiovascular disease and is associated with a decreased lifespan (2).

The implications for oral health and the provision of dental care for people with diabetes are also significant, because numerous cross-sectional studies have clearly demonstrated an association between diabetes and periodontitis (4–10). Furthermore, this association is particularly pronounced in populations with high prevalence of diabetes (5,8) and also for those patients with poor or unstable glycemic control (6). The infectious and inflammatory burden of chronic periodontitis is now thought to have an important systemic impact (11), and diabetic patients with periodontal disease have an increased risk of severe systemic disease compared with those without periodontal disease (12,13).

Periodontal health is dependent on a balance between the bacterial challenge presented by dental plaque and the host response to this challenge. If this balance is altered, by alteration of either the microbial challenge or the magnitude and nature of the host response, then disease may occur, as outlined in the ecological plaque hypothesis (14,15). Environmental changes may modify the bacterial challenge or host immune response; for example, onset of a systemic disease, such as diabetes, may be responsible for such imbalance. While the microbiology of periodontitis in type 1 diabetes has been investigated in several studies, investigations of the subgingival microbiotia in T2DM are less numerous (16–19). It has been considered that in the subgingival area in patients with T2DM there is a more anaerobic environment, with increased gingival crevicular fluid glucose levels that may encourage a more pathogenic flora (20). Previous studies have identified periodontopathogens at sites of periodontal disease in patients with T2DM, using culture techniques (20,21); however, culture techniques have serious limitations when used for quantification of bacteria in natural samples. A major difficulty is that of maintaining the viability of bacteria from collection, through transportation, to growth. In addition, it is estimated that 50% of oral bacteria have not yet been grown in culture (22). This may be due to lack of knowledge of an essential nutrient or growth factor, or because the microorganism has evolved to grow as part of a community, such as in a biofilm, rather than as an isolate (15).

The aetiology of periodontitis is highly complex, and the simple detection of bacterial species without quantification may not be particularly informative. Enumeration of the bacteria can help to build a more detailed knowledge of the aetiology of periodontal disease. Technological advances have resulted in the development of novel approaches for detection and enumeration of bacteria in dental plaque, including, for example, real-time TaqMan PCR (qPCR). Quantitative PCR is highly sensitive (detection limits of the order of 10–100 cells), highly specific, and has the capability to quantify bacterial cell numbers accurately. It is a technique that has been successfully applied for the enumeration of specific periodontal pathogens in dental plaque (23,24).

While much research has focused on altered host immune-mediated inflammatory responses to plaque in diabetic patients, the role of diabetes in modulating the periodontal microbiota remains unclear. The few publications to address this issue to date have almost exclusively relied on qualitative or indirect methods for detecting bacteria or on culture techniques (25). Using PCR to detect the presence or absence of periodontal pathogens, *Porphyromonas gingivalis* was found to be more prevalent in type 2 diabetes patients than in control subjects (4). Unfortunately, in that study no account was taken of the pocket depth from which DNA samples were collected. In fact, the recruited type 2 diabetes patients had more extensive periodontal disease than the nondiabetic control subjects, and it is likely, therefore, that the increased levels of *P. gingivalis* were due to increased periodontitis rather than the presence of type 2 diabetes *per se*. A study on 63 subjects reported significant differences in some periodontopathogens, including *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, between diabetes patients and nondiabetic individuals (26); however, this was undertaken in a population with an unusually high incidence of type 2 diabetes and may not be truly representative of the average population.

By accurately quantifying three species of bacteria, two of which were identified in the above study as being present in significantly different quantities between diabetic patients and nondiabetic subjects (despite previous data showing no differences), we have attempted to resolve the conflicting data regarding shifts in the periodontopathogen population associated with T2DM. In addition to *P. gingivalis* and *A. actinomycetemcomitans*, *Fusobacterium nucleatum* was also quantified, because it is highly abundant in subgingival plaque, and small changes in the levels of this organism could have major consequences for the pathogenic potential of the biofilm.

Material and methods

Study design

This was a cross-sectional pilot study of the periodontal subgingival plaque of a population of patients with a diagnosis of type 2 diabetes, with and without periodontal disease, compared with a matched local, control population who did not have a diagnosis of type 2 diabetes. Ethical approval was granted by Sunderland Research Ethics Committee.

Study populations

Subjects recruited to this study included patients with and without a

confirmed diagnosis of T2DM, and with and without periodontitis. Patients were seen at a unique diabetes/periodontitis clinic at Newcastle Dental Hospital. Age, sex and smoking status-matched control subjects were recruited from the population of patients also attending Newcastle Dental Hospital. Initial contact with subjects of both groups was through a written or verbal invitation to participate in the study to attend a single appointment, at which written informed consent to participate was obtained.

Inclusion criteria were patients with or without T2DM, over 18 years of age with a minimum of 20 natural teeth, who conformed with the periodontal diagnostic criteria indicated below. Subjects were excluded from the study if they were pregnant, had a diagnosis of aggressive periodontitis, if they had drug-induced gingival overgrowth, if they were immunosuppressed, had a bleeding disorder, a medical history negating safe participation, had taken antibiotics in the last 3 mo or had nonsurgical management for periodontal disease in the previous 6 wk.

Control patients

Patients with T2DM were recruited who were currently deemed stable with regard to their diabetes symptoms by their general medical practitioner. Nondiabetic subjects were selected on the basis that they had not previously exhibited symptoms of T2DM nor had a diagnosis of diabetes from a medical practitioner. Glycated haemoglobin was not recorded as part of this pilot study.

Demographic data

The following data were recorded: age, sex, ethnicity, smoking status and comprehensive medical history.

Periodontal status

A comprehensive periodontal examination was undertaken using a UNC 15 (Hu-Friedy) probe. Full mouth-probing depths were recorded at six sites per tooth, excluding third molars, and were measured as the distance from the free gingival margin to the apical extent of the probe tip, rounded up to the nearest millimetre. Gingival recession was measured as the distance between the cemento-enamel junction and the free gingival margin. Clinical attachment levels were calculated as the sum of the probing depth + gingival recession measurements. Bleeding on probing was assessed following the probing depth and gingival recession measurements. Periodontal status was classified as one of the following two categories: periodontal health (no probing depths > 3 mm and bleeding on probing was $< 15\%$) and active chronic periodontitis (six or more sites with probing depth of ≥ 5 mm and bleeding on probing $> 15\%$). Periodontitis was confirmed by bone loss evident on radiographic examination. The diagnoses were assigned following consideration of diagnostic criteria for periodontal disease that were proposed by the European Workshop of Periodontology 2005 and the Centers for Disease Control & Prevention – American Academy of Periodontology collaboration 2007 (27,28).

Sample collection

Shallow sites were defined as those with probing depths ≤ 3 mm and no attachment loss, whereas deep sites were those with probing depths ≥ 5 mm, with attachment loss and confirmed radiographically. Three shallow sites were sampled and pooled for each periodontally healthy patient. For those patients designated as having chronic periodontitis, three shallow sites were sampled and pooled, and the three deepest sites present in the mouth (all with pockets ≥ 5 mm) were sampled and pooled. Fifteen T2DM patients and 12 nondiabetic control subjects who were periodontally healthy were recruited, along with nine T2DM and 12 nondiabetic patients with chronic periodontitis. Subgingival plaque samples were taken from sites by carefully removing supragingival plaque with curettes and cotton pellets before inserting three parallel, size 60, sterile endodontic paper points and leaving them *in situ* for 10 s. The points were immediately transferred to sterile, dry microcentrifuge tubes. To avoid PCR inhibition by components present in blood (29), excessively blood-soaked points were discarded and new samples taken from a less haemorrhagic site. Points were transferred for storage at −80°C within 5 min of sample collection and stored frozen until processing for qPCR.

Cultivation

Laboratory strains used in this study were *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* 9710, *Fusobacterium nucleatum* 25586 and *Porphyromonas gingivalis* W50. *A. actinomycetemcomitans* and *F. nucleatum* were cultured in anaerobic conditions or with elevated CO_2 (GasPak Plus system; Becton Dickson & Co., Franklin Lakes, NJ, USA) at 37°C in Todd Hewitt yeast extract medium containing (per litre): 36.4 g Todd Hewitt broth (Difco, Becton Dickson & Co.) and 5 g yeast extract. *P. gingivalis* was cultivated anaerobically in fastidious anaerobe broth (Lab M, Bury, UK) at 37°C.

Generation and quantification of control DNA for qPCR

Routine genetic manipulations were performed according to the methods of Sambrook (30). To prepare genomic DNA from laboratory strains, *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis* were cultured to stationary phase. Cells (10 mL) were harvested (centrifugation at 3500*g*, 20°C, 10 min) and resuspended in 0.15 mL spheroplasting buffer (20 mM Tris–HCl, 10 mM $MgCl_2$ and 26% (w/v) raffinose, pH 6.8) containing 75 units mutanolysin (Sigma, St Louis, MO, USA) and 250 μg lysozyme per millilitre. Cells were incubated at 37°C for 30 min. Lysis buffer (0.15 mL of 2× T&C lysis buffer; Epicentre Biotechnologies, Madison WI, USA) was added, and chromosomal DNA was extracted using the Masterpure DNA Purification kit (Epicentre Biotechnologies) in accordance with the manufacturer's instructions. Fragments of DNA containing template sequences

Table 1. Oligonucleotide primers used to amplify DNA fragments

Name	Oligonucleotide sequence(5′–3′)	Target region/species	References
PCR primers			
E8F	AGAGTTTGATCCTGGCTCAG	16S rRNA gene/all bacteria	(1)
E1115R	AGGGTTGCGCTCGTTG	16S rRNA gene/all bacteria	(1)
Aa_hgpAF1	GCACTTTGCAATAGAAAGAA	*hgpA*/*A. actinomycetemcomitans*	This study
Aa_hgpAR1	CAAGATCCTTATTGGGTAAT	*hgpA*/*A. actinomycetemcomitans*	This study
PgF1	ACCTTACCCGGGATTGAAATG	16S rRNA gene/*P. gingivalis*	(2)
PgR1	CAACCATGCAGCACCTACATAGAA	16S rRNA gene/*P. gingivalis*	(2)
FnF1	GCGGAACTACAAGTGTAGAGGTG	16S rRNA gene/*F. nucleatum*	(3)
FnR1	GTTCGACCCCCAACACCTAGTA	16S rRNA gene/*F. nucleatum*	(3)
AaF1	CGGTTACCGTTATGACCGTGTGA	*hgpA*/*A. actinomycetemcomitans*	(3)
AaR1	GCCCGGAATGCTTTGCTATATTTC	*hgpA*/*A. actinomycetemcomitans*	(3)
UnivF1	TCCTACGGGAGGCAGCAGT	16S rRNA gene/all bacteria	(4)
UnivR1	GGACTACCAGGGTATCTAATCCTGTT	16S rRNA gene/all bacteria	(4)
Taqman probes			
Pg_probe	ATGACTGATGGTGAAAACCGTCTTCCCTTC	16S rRNA gene/*P. gingivalis*	(2)
Fn_probe	AATGCCGATGGGGAAGCCAGCTTA	16S rRNA gene/*F. nucleatum*	(3)
Aa_probe	AGGCAAGACGGGAAGCTAACGCAAA	*hgpA*/*A. actinomycetemcomitans*	(3)
Univ_probe	CGTATTACCGCGGCTGCTGGCAC	16S rRNA gene/all bacteria	(4)

for qPCR were amplified from genomic DNA as follows. Oligonucleotide primers E8F and E1115R (Table 1; Baker *et al.*) (31) were employed to amplify fragments (approximately 1.1 kbp) of the 16S rRNA genes from *P. gingivalis* and *F. nucleatum*. The PCR reactions contained each primer at 250 nM and template DNA (2 nM) in Reddymix (Thermo Fisher, Fisher Scientific UK Ltd, Loughborough, UK) reaction mixture. The PCR amplifications were performed as follows: DNA strands were separated at 94°C for 2 min, followed by 35 cycles of 94°C for 10 s, 52°C for 30 s and 68°C for 1 min 20 s, and a further incubation at 68°C for 7 min.

The same reaction conditions were employed to amplify a 472 bp region of the *A. actinomycetemcomitans hgpA* gene, encoding haemoglobin binding protein A, with primers Aa_hgpAF1/Aa_hgpAR1 (Table 1). The three amplified fragments were each cloned in plasmid vector pCR2.1-TOPO (Invitrogen, Life Technologies Ltd, Paisley, UK) to generate plasmids pCR-Pg (5037 bp), pCR-Fn (5003 bp) and pCR-Aa (4403 bp), respectively. *Escheichia coli* DH5α was transformed with each plasmid, and transformants were selected on LB agar (Difco) supplemented with 100 μg ampicillin/mL. Plasmids were purified from *E. coli* using the Qiaprep Spin Miniprep kit (UK-Qiagen Ltd, West Sussex, UK). To ensure that plasmids contained the correct insert DNA, the appropriate portion of the plasmid was sequenced using primers M13F and M13R (supplied with the TOPO cloning kit; Invitrogen). The concentration of each plasmid was accurately quantified using Quant-iT PicoGreen DNA Assay kit (Invitrogen).

Isolation of material from paper points

Plaque samples were retrieved from the paper points by adding 200 μL phosphate-buffered saline, vortexing vigorously and centrifuging, as previously described (32) Plaque material was pelleted by centrifugation at 10,000*g* for 5 min, and the supernatant was discarded. Pellets of plaque material were resuspended in 100 μL spheroplasting buffer (20 mM Tris–HCl, pH 6.8, 10 mM $MgCl_2$, 26% raffinose) and 5 μL mutanolysin (10,000 U/mL stock) was added to each tube, followed by addition of lysozyme (Sigma) to 250 μg/mL (from a freshly prepared stock of 25 mg/mL). Tubes were vortexed to mix and incubated at 37°C for 30 min. To each tube 50 μL TE buffer and 150 μL of 2× T&C Lysis Solution (Epicentre Biotechnologies) were added. The DNA was extracted using the Masterpure DNA Purification kit (Epicentre Biotechnologies) and stored at −20°C.

Real-time qPCR

Sequences of primers and TaqMan probes are listed in Table 1. Quantitative PCR reactions contained the following reagents: forward primer (2.5 μM) 1.2 μL, reverse primer (2.5 μM) 1.2 μL, probe (2.5 μM) 0.6 μL, 2× Sensimix 7.5 μL (Quantace, London, UK), H_2O 3.3 μL and template DNA 1.0 μL. The DNA extracted from subgingival plaque was added without dilution in the first instance. In a small number of samples, the concentration of DNA was too high for accurate quantification, and qPCR was repeated using 100-fold dilutions of DNA. Reactions were performed using an Opticon 2 DNA Engine (Bio-Rad Laboratories Ltd., Hertfordshire, UK) with the following PCR programme: 95°C for 10 min, 95°C for 15 s, 60°C for 1 min, plate read and cycle repeated 40 times.

The quantity of template DNA in each sample well was calculated by reference to wells containing serial 10-fold dilutions of standards of known DNA concentration. The logarithm of the DNA concentration of the standard was plotted against the cycle threshold (C_t). This yielded a linear relationship over at least six orders of magnitude. The lowest concentration of standard for each probe was equivalent to approximately 10^3 cells. Where the 16S rRNA gene was used as a

target, corrections were made for gene copy number. Representative genome sequences of *F. nucleatum* and *P. gingivalis* have been determined, and these bacteria contain five copies and four copies, respectively, of the 16S rRNA gene per genome. For the universal bacterial primers/probe, the average 16S rRNA copy number per bacterial genome was estimated to be approximately 3.7, based on the analysis of approximately 950 complete bacterial genome sequences available in the online Ribosomal RNA database (http://rrndb.mmg.msu.edu/index.php, accessed on 30th July, 2009; 33).

Statistical analysis

A conventional power calculation was not possible owing to the lack of published studies that have investigated this area and/or that have used similar molecular techniques. Statistical analyses were performed using SPSS version 17 (SPSS, IBM, Hampshire, UK). Frequency distributions were determined and descriptive statistics calculated as means, standard deviations and ranges. Given the large variability in bacterial counts, counts were log transformed (following addition of one as a constant) to correct skewed distributions and to permit parametric analyses where appropriate. All variables were assessed for normality using the Kolmogorov–Smirnov test, supplemented with histograms. Where there was no evidence to reject normality, means and standard deviations of these parametric variables were calculated. Where the assumption of normality was rejected, medians and interquartile ranges of these nonparametric variables were calculated. For all continuous variables, box plots were constructed and one-way ANOVA or the Kruskal–Wallis test was employed to test for significant differences between groups for parametric and nonparametric variables, respectively. In the case of the parametric analyses, Bonferroni *post hoc* tests were used to identify the precise locations of the significant differences between groups, and for the nonparametric analyses, *post hoc* Mann–Whitney *U*-tests were used. Significance of all tests was assessed at the 5% level, except in the case of the *post hoc* Mann–Whitney *U*-tests, in which case the critical value of p was adjusted according to the number of *post hoc* tests required. Correlations between variables were investigated by calculation of Spearman's rho. Chi-squared analysis was used to identify whether sex, smoking and ethnicity distributions varied significantly between groups.

Results

In total, 48 patients were recruited into the study. Significant differences in age were found between nondiabetic periodontitis patients vs. T2DM patients and nondiabetic subjects with periodontal health, and these differences were compensated for during data analysis. Chi-squared analysis showed that no significant differences existed in the distribution of sex, smoking and ethnicity between groups, and the results are illustrated in Table 2.

Log bacterial counts and the proportions represented as a percentage of the total bacteria are presented in Table 3. Total bacteria numbers were quantified with a universal probe. Significant differences were identified in the bacterial counts of *P. gingivalis* in the shallow pockets of nondiabetic patients with chronic periodontitis and T2DM/nondiabetic periodontally healthy subjects. Significant differences were also identified between the shallow pockets of periodontally healthy T2DM patients and deep pockets of nondiabetic periodontitis patients. Also, significant differences existed in nondiabetic patients, between the shallow pockets in periodontally healthy subjects and the deep pockets of periodontitis patients ($p < 0.05$).

There were no significant differences between log bacterial counts of *A. actinomycetemcomitans* in any of the pocket categories. The log bacterial counts of *F. nucleatum* were found to be significantly different between the shallow and deep pockets of nondiabetic periodontitis patients, the shallow pockets of T2DM patients with chronic periodontitis and nondiabetic periodontally healthy subjects. Also, significant differences were identified between the shallow pockets of T2DM periodontally healthy patients and nondiabetic periodontitis patients ($p < 0.05$).

Expressed as a proportion of the total population, there was significantly more *P. gingivalis* present in deep pockets of nondiabetic periodontitis patients compared with shallow pockets of T2DM and nondiabetic

Table 2. Demographic characteristics of study population

	Patients with type 2 diabetes mellitus		Nondiabetic subjects	
Characteristic	Periodontal disease ($n = 9$)	Periodontal health ($n = 15$)	Periodontal disease ($n = 12$)	Periodontal health ($n = 12$)
Age (years)				
Mean (SD)	53.33 (5.43)	48.53 (5.60)[a]	55.33 (5.23)[a,b]	47.92 (6.53)[b]
Range	46.00–60.00	35.00–56.00	48.00–64.00	33.00–60.00
Sex, *n* (%)				
Male	7 (77.8)	7 (46.7)	9 (75.0)	6 (50.0)
Female	2 (22.2)	8 (53.3)	3 (25.0)	6 (50.0)
Race/ethnicity, *n* (%)				
White Caucasian	8 (88.9)	13 (86.7)	12 (100.0)	12 (100.0)
Black	0 (0.0)	1 (6.7)	0 (0.0)	0 (0.0)
Asian	1 (11.1)	1 (6.7)	0 (0.0)	0 (0.0)
Tobacco use, *n* (%)				
Ex	4 (44.4)	2 (13.3)	6 (50.0)	0 (0.0)
Current	1 (11.1)	1 (6.7)	1 (8.3)	0 (0.0)
Non	4 (44.4)	12 (80.0)	5 (41.7)	12 (100.0)

Within rows, numbers with matching superscript letters are significantly different from each other ($p < 0.05$).

Table 3. Bacterial counts and proportions according to periodontal status and diabetes status

	Patients with type 2 diabetes mellitus			Nondiabetic subjects		
	Periodontitis Shallow sites	Periodontitis Deep sites	Periodontal health Shallow sites	Periodontitis Shallow sites	Periodontitis Deep sites	Periodontal health Shallow sites
Mean (SD) probing depth (mm)	2.22 (0.44)	5.77 (1.30)	2.09 (0.20)	2.28 (0.34)	6.22 (0.97)	2.17 (0.33)
Log total bacteria	6.63 (0.75)^{a,d}	6.93 (0.94)^{b,e}	8.05 (0.54)^{a,b,c}	6.74 (0.71)^{c,f}	8.11 (0.78)^{e,f}	7.56 (0.36)^{d}
Log *P. gingivalis*	2.85 (0.00–4.80)	4.17 (0.00–5.81)	0.00^{a,b} (0.00–0.00)	4.46^{a,c} (0.68–5.66)	6.58^{b,d} (1.26–7.93)	0.00^{c,d} (0.00–0.00)
Log *A. actinomycetemcomitans*	3.85 (0.00–4.43)	3.39 (0.00–4.93)	3.78 (0.00–5.17)	0.00 0.00–3.63)	4.88 (0.00–5.63)	0.00 (0.00–3.89)
Log *F. nucleatum*	5.33 (0.59)^{b}	5.85 (1.26)	6.48 (0.72)^{a}	5.32 (0.96)^{a,d}	6.86 (0.85)^{b,c,d,e}	5.60 (0.95)^{c,e}
P. gingivalis (%)	0.03 (0.00–4.68)	0.23 (0.00–9.12)	0.00^{b} (0.00–0.00)	0.64 (0.00–5.32)	6.54^{a,b} (0.16–15.74)	0.00^{a} (0.00–0.00)
A. actinomycetemcomitans (%)	0.18 (0.00–0.64)	0.09 (0.00–1.04)	0.00 (0.00–0.11)	0.00 (0.00–0.10)	0.04 (0.00–0.20)	0.00 (0.00–0.03)
F. nucleatum (%)	4.82 (3.56–6.47)	7.27^{a,b} (3.31–24.32)	3.38^{a} (0.57–8.72)	3.81 (1.83–10.42)	5.08 (2.61–12.69)	1.94^{b} (0.27–4.70)

Data presented as means (SD) for parametric data and medians (interquartile range) for nonparametric data. For the bacterial counts, within rows, numbers with matching superscript letters are significantly different from each other ($p < 0.05$ or critical value of p adjusted for multiple testing). For the probing depth data, the probing depths at periodontitis deep sites were significantly greater than the probing depths at all other sites.

periodontally healthy subjects, whereas there was more *F. nucleatum* in the deep pocket sites of T2DM periodontitis patients compared with shallow pockets of periodontally healthy T2DM and nondiabetic subjects ($p < 0.05$).

Data from the samples from the shallow and deep sites were then pooled for each of the periodontitis patients and analysed in four categories: nondiabetic periodontitis (NDP), T2DM periodontitis (DP), nondiabetic periodontally healthy (NDH) and T2DM periodontally healthy (DH). Bacterial counts for *P. gingivalis* were significantly higher in NDP pockets compared with NDH and DH ($p < 0.05$). There was also significantly more *P. gingivalis* present in DP pockets compared with DH pockets ($p < 0.05$). There were no significant differences between the log bacterial counts of *A. actinomycetemcomitans* and *F. nucleatum* in any of the groups, as outlined in Table 4. As a proportion of the total microbiota, there was significantly more *P. gingivalis* present in NDP pockets compared with NDH and DH shallow pockets ($p < 0.05$). This was also the case for DP pockets compared with DH pockets ($p < 0.05$). There was a strong trend for a greater proportion of *P. gingivalis* to be present in DP pockets compared with NDH pockets ($p = 0.071$), which was a trend mirrored in the log bacterial counts for *P. gingivalis*.

For all three species investigated, marked differences were seen in the proportions present, relative to the total amount of bacteria, according to periodontal status. That is, they were seen in increased proportions in patients with chronic periodontitis, when compared with periodontally healthy subjects.

As a general finding, *P. gingivalis* was found in much higher quantities in periodontitis patients rather than healthy subjects. There were no statistically significant differences in the proportion of *P. gingivalis* found in the periodontal pockets of T2DM patients with periodontitis when compared with nondiabetic patients with periodontitis.

Pocket depth and clinical attachment loss were significantly greater in

Table 4. Bacterial counts and proportions according to pooled periodontal status and diabetes status

	Patients with type 2 diabetes mellitus		Nondiabetic subjects	
	Periodontitis	Periodontal health	Periodontitis	Periodontal health
Log total bacteria	6.48 (0.84)	8.05 (0.54)	7.13 (1.01)	7.56 (0.36)
Log total *P. gingivalis*	2.73 (0.00–4.90)[c]	0.00 (0.00–0.00)[b,c]	4.93 (0.61–6.92)[a,b]	0.00 (0.00–0.00)[a]
Log total *A. actinomycetemcomitans*	3.50 (0.00–4.45)	3.78 (0.00–5.17)	2.46 (0.00–4.73)	0.00 (0.00–3.89)
Log total *F. nucleatum*	5.29 (0.99)	6.48 (0.72)	5.79 (1.18)	5.60 (0.95)
P. gingivalis (%)	0.13 (0.00–3.03)[c]	0.00 (0.00–0.00)[b,c]	1.35 (0.00–12.18)[a,b]	0.00 (0.00–0.00)[a]
A. actinomycetemcomitans (%)	0.10 (0.00–0.61)	0.00 (0.00–0.11)	0.01 (0.00–0.13)	0.00 (0.00–0.03)
F. nucleatum (%)	4.93 (3.47–12.97)[a]	4.25 (0.72–10.98)	4.11 (2.38–10.78)	1.94 (0.27–4.70)[a]

Data are presented as means (SD) for parametric data and medians (interquartile range) for nonparametric data. Within rows, numbers with matching superscript letters are significantly different from each other ($p < 0.05$ or critical value of p adjusted for multiple testing).

deep sites in periodontitis patients compared with the other groups. There were no significant differences between the shallow pockets in the periodontitis patients and periodontally healthy individuals.

Finally, correlations between bacterial counts and clinical parameters were investigated. Statistically significant positive correlations were identified between pocket depth at the sampled sites and quantities of *A. actinomycetemcomitans* ($R^2 = 0.064$), *F. nucleatum* ($R^2 = 0.135$) and *P. gingivalis* ($R^2 = 0.255$). That is, with increase in pocket depth, there was a corresponding increase in quantities of all three micro-organisms.

Discussion

Individual susceptibility to periodontitis is determined by a range of genetic and environmental factors and is influenced by the presence of systemic disease, such as diabetes. It is clearly established that individuals with diabetes (if poorly controlled) are at increased risk for periodontitis, and much research has focused on investigating aspects of the host response to explain the link between the two diseases (19). Research into the role played by subgingival plaque in patients with diabetes has been rather more limited, however. Therefore, this pilot study aimed to investigate the microbiota in patients with diabetes in comparison with nondiabetic individuals, including patients with periodontitis and those who are periodontally healthy. Modern molecular techniques were employed to accurately quantify three bacterial species in subgingival plaque samples, these species having been implicated as key periodontal pathogens.

The species investigated all showed significant increases in quantity in deeper pockets encountered in periodontal disease. This is in line with studies on periodontal therapy, which show reductions in proportions of *A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis* with decreasing pocket depth (34,35). *P. gingivalis* was encountered in higher numbers and formed an increased proportion of the total species present in periodontally diseased pockets, as was *F. nucleatum*. Over the last 20 years, studies have consistently identified these species and *A. actinomycetemcomitans* to be prevalent in periodontally diseased sites (36,37). This was supported by further work by Socransky *et al*. (14), who in 1998, using modern molecular techniques, confirmed a strong association between *P. gingivalis* (described as a red complex micro-organism) and deeper pocket depths and increased levels of bleeding on probing in periodontal disease. *F. nucleatum* (a gram-negative, anaerobic rod and an orange complex pathogen) was seen to be closely associated with *P. gingivalis* in deep pockets. This may be because *F. nucleatum* is well suited structurally to co-aggregate with many species in the biofilm and can co-aggreate well with *P. gingivalis*, which usually adheres to tissue before *F. nucleatum* colonizes. The microenvironment in diseased pockets encourages further co-aggreagation between *F. nucleatum* and late colonizers, and it acts as a 'bridge' between early and late species (38–40).

A. actinomycetemcomitans has been positively associated with localized aggressive periodontitis through several studies, including its ability to induce disease in germ-free animals (41–44). It was not present in significantly different numbers between the groups in our study, and this may have been because aggressive periodontitis patients were excluded from the study. The relatively uniform presence of *A. actinomycetemcomitans* may be attributed to its versatility, as a facultative anaerobe, in colonizing both shallow and deep pockets.

Whilst the microbiology of periodontitis in type 1 diabetes has been investigated in several studies, investigations of the subgingival microbiotia in T2DM are less numerous (16–19). Although significant differences were seen between periodontally diseased and healthy sites, no significant differences were shown to exist between T2DM patients and nondiabetic subjects. This finding is supported by several studies using different detection and enumeration techniques that have also failed to identify clear differences between the microbiota in diabetic and nondiabetic subjects. This finding may be surprising, as it has been reported that in the subgingival area in T2DM, there may be a more anaerobic environment, with increased gingival crevicular fluid glucose levels that may encourage a more pathogenic flora (20).

Previous studies investigating this concept identified periodontopathogens at sites of periodontal disease in patients with T2DM, using culture

techniques (20,21). More recent studies that have used molecular techniques for identification of specific micro-organisms within this population also have failed to identify differences in the subgingival microbiota between subjects with and without T2DM, all of whom also had periodontal disease (45,46). This outcome conflicts with another study which used DNA checkerboard hybridization, which although quantitative, is less sensitive than qPCR. That study concluded that significant differences existed in the subgingival microbiota between T2DM and nondiabetic subjectts, identifying more *P. gingivalis* and *A. actinomycetemcomitans* present in sites of periodontal disease (26). Our investigation does not provide evidence for differences in quantities of *A. actinomycetemcomitans* and *P. gingivalis* between subjects with and without type 2 diabetes and therefore somewhat contradicts the above study. The differences may be due to the different sample populations, with the cohort used in this study being more representative of a population with a level of T2DM observed in the general UK population. Observed differences may have been due to technical limitations of the methods used in each study. Ebersole *et al*. (26) did not quantify levels of different bacteria, but simply noted presence or absence using a threshold of 10^5 cells. In our study, we employed qPCR to quantify bacteria above 10^3 cells per sample. Theoretically, qPCR can detect levels of bacteria as low as 10–100 cells. We did not attempt to detect such low numbers of cells, because it is unlikely that very low levels of pathogens would be responsible for disease.

Whilst *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* are generally present in higher levels in patients with periodontitis, they were virtually absent from some patients with deep pockets. Therefore, other micro-organisms must also be involved. It has been shown that that in approximately 10% of periodontitis patients, very low levels of proteolytic bacteria are observed, indicating that organisms such as *P. gingivalis* are not always abundant in periodontally diseased sites (47). It would be of interest to investigate what alternative pathogens have colonized the periodontal pockets of these subjects.

It has been postulated that increased disease severity may be due to alterations in the pathogenicity of the bacteria in the plaque biofilm or a modification in host response (26). Future research using optimized animal models, sensitive microbial detection techniques, novel technologies and clinical observations needs to consider characteristics of the bacterial initiators combined together with host response to the challenge. This requires a quantitative and time-dependent approach with tissue- and site-specific analysis, in order for conclusions to be extrapolated into the broader clinical picture (48). It would be useful to investigate whether there are subtle differences in the severity of periodontal disease between periodontitis patients with and without diabetes. This could be quantified, for example, using the recently introduced measurement, 'periodontal inflamed surface area', which is a sensitive measure of periodontitis severity that takes into account clinical attachment levels, recessions and bleeding on probing (49).

More studies with increased numbers of patients are required, using molecular techniques to ascertain whether any significant differences truly exist in the subgingival microbiota of T2DM patients. This would be more valuable if carried out with consideration of the host response (48). It is important also to consider the host response and the effect this may have on the gene expression of bacteria when looking to develop novel therapeutic strategies. Increased knowledge of pathogens and the genetic diversity between species, coupled with a fuller understanding of host genetic and environmental factors gained through further studies is likely to give a better understanding of the aetiology of destructive periodontitis (50). The improved understanding of the relationship between diabetes, altered host response and behaviour of periodontal bacteria is a prerequisite for the development of new therapeutic strategies to combat periodontitis in both diabetic and nondiabetic individuals.

Conclusion

In conclusion, *A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis* were detectable in significantly different quantities and proportions according to periodontal disease. *P. gingivalis* and *F. nucleatum* were detectable in increased numbers in deep pockets in periodontal disease compared with periodontal health. No significant differences were identified between the subgingival microbiota of T2DM patients compared with nondiabetic subjects in this study, consistent with previous research that has investigated this area. Further studies are required with increased numbers of patients, using qPCR to elicit any potential differences in the microbiota of these populations.

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