Peri-implant and periodontal microbiome diversity in aggressive periodontitis patients. A pilot study.

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Abstract

Aim

To investigate the bacterial microbiome in periodontal and peri-implant biofilms deriving from aggressive periodontitis patients (AgP) in conditions of health and disease.

Material and Methods

Ninety-one plaque samples were collected from 18 patients previously diagnosed and treated for AgP. The samples were taken from (1) 24 residual periodontal pockets (TD) (*n*= 6 patients), (2) 24 healthy periodontal sites (TH) (*n*= 6 patients), (3) 24 dental sites from the same implant patients (TM) (*n*= 6 patients), (4) 5 peri-implantitis sites (II) (*n*= 2 patients), (5) 6 peri-mucositis sites (IM) (*n*= 2 patients), and (6) 8 healthy implant sites (IH) (*n*= 2 patients). All subjects underwent periodontal clinical and radiographic assessments. Bacterial DNA was extracted, PCR amplified using 16S rRNA gene V5-V7 primers (barcoded amplicons 785F;1175R), purified, pooled at equimolar concentrations and sequenced (MiSeq, Illumina) yielding 250bp paired-end reads. The 16S rRNA reads were filtered, assembled and analysed.

Results

The genera *Propionibacterium, Paludibacter, Staphylococcus, Filifactor, Mogibacterium, Bradyrhizobium* and *Acinetobacter* were unique to peri-implant sites (*p*= 0.05). In TM samples, different proportions and bacterial spp. were found when compared with the same patients' samples at implant sites. Specifically, *Actinomyces* (*p*= 0.013) and *Corynebacterium* (*p*= 0.030) genera showed to be significantly more abundant in the TM group when compared to the II. The highest phylogenetic diversity was observed in residual periodontal pocket sites (TD). Increased annual tooth loss rate and residual pocketing was related to high proportions of the genera *Actinomyces, Porphyromonas, Prevotella, Streptococcus, Sharpea, Actinomycetaceae, TM7-3, Selenomonas, and Dialister, Treponema, Parvimonas,* and *Peptostreptococcus* in the TD group.

Conclusion

Within the limitations of this pilot study, the periodontal and peri-implant microbiome presents a dissimilar taxonomic composition across different niches within AgP patients. The host response, the habitat structure and the vast coexistence of strains and species surrounding implants and teeth in health and disease are likely to be shaping the heterogeneous composition of the subgingival biofilms. The *TM7* phylum was found only in TD cases. The investigation of the impact of periodontal and peri-implant keystone species on these complex ecosystems in states of health and disease seems to be essential.

Introduction

Over the last decades peri-implant diseases (*i.e.* peri-implant mucositis and peri-implatitis) have become a burden for the clinical practice (Donos et al. 2012). Clinically, peri-implantitis is characterised by loss of supporting bone surrounding the implant fixture as well as inflammation of the supporting soft tissue (Lang & Berglundh 2011). This chronic infection represents a clinical challenge due to a) the burdensome disinfection process of the contaminated implant surface (Schwarz et al. 2012), b) the unpredictable nature of re-osseointegration on a previously infected implant surface (Renvert et al. 2009), c) the complex interplay and diversity of the microbiota colonizing the implant surface (Dabdoub et al. 2013; Kumar et al. 2012) and d) the host response (Lamont & Hajishengallis 2015, Faot et al. 2015). In previous systematic reviews, it has been indicated that the prevalence of peri-implant mucositis occurs in about 80% of the subjects and in 50% of the implants; and peri-implantitis in between 28% and ≥56% of subjects (with severe periodontal disease) and in 12% and 43% of implant sites (Zitzmann & Berglundh 2008) after a 5-10 year follow-up. In two more recent systematic reviews, the prevalence of peri-implantitis was reported to be in order of 10% of implants and 20% patients (ca. 5-10 year follow-up after implant placement) (Mombelli et al. 2012), and from 1 to 47% (EWM of 22% [95%CI: 14-30%]) (Derks & Tomasi 2015).

Notably, periodontal disease is one of the main risk factors for the development of peri-implantitis (Heitz-Mayfield 2008; Karoussis et al. 2003; Pérez-Chaparro et al. 2014; Swierkot et al. 2012). The term ‘Aggressive Periodontitis’ (AgP) defined a group of destructive periodontal diseases with a rapid progression of attachment loss (Armitage 1999). AgP is clinically presented as either localised (LAgP) or generalised (GAgP) and it has a low prevalence –*e.g.* 0.06% - 7.6% (Albandar et al. 1997; Haubek et al. 2001). The diagnosis of AgP is often difficult due to the indirect assessment of the rate of progression (Nibali et al. 2013). Furthermore, despite the fact that (for research purposes) subcategories of AgP were proposed to measure the degree of uncertainty of the diagnosis (Mombelli 2002), currently, the clinical, radiographic and historical data remain the most reliable parameters for diagnosis (Buduneli & Kinane 2011). The main features of AgP are rapid progression of attachment loss and bone destruction, systemic health and familial aggregation (Lang et al. 1999).

Other secondary features of AgP include elevated proportions of certain putative pathogens such as *Aggregatibacter actinomycetemcomitans* (and particularly the JP2 clone, especially in LAgP) and, in certain populations, of *Porphyromonas gingivalis.* Recently, other microbial profiles including *Selenomonas* genus, *Centipeda* genus, *Mitsuokella* spp., *Treponema denticola, Campylobacter, Acinetobacter baumannii, T. lecithinolyticum* and *Archaea* have been associated with AgP (da Silva-

Boghossian et al. 2011; Drescher et al. 2010; Gonçalves et al. 2012; Matarazzo et al. 2011; Riep et al. 2009) and the hypothesis of 'genetic dysbiosis' has been described as part of the onset and progression process of AgP (Nibali et al. 2014; Nibali 2015).

Similarly, multi-species bacterial biofilms are the main causal factor of peri-implant diseases (Lang & Berglundh 2011). The progression from peri-implant health to peri-implant disease is characterised by a microflora shift (Zhuang et al. 2014). Microbiologically, peri-implantitis is characterised mainly by the presence of aerobic Gram-negative bacilli (AGNB), facultative anaerobic species, black-pigmented, motile bacilli, anaerobic species (Charalampakis & Belibasakis 2015), *Staphylococcus* spp., *Candida* spp., *Tannerella forsythia, Parvimonas micra, Fusobacterium necrophorum* and *Campylobacter rectus* (Eick et al. 2015).

Our understanding on the diversity of the commensal human oral microbiome has been increased by culture-independent methods such as 16S rRNA gene analysis using massively parallel next generation (NGS) DNA sequencing and computational phylogenetics (Paster et al. 2006; Zaura et al. 2009). It is estimated that more than 19,000 species-level phylotypes contribute to the ultimate oral species diversity (Keijser et al. 2008). Interestingly, 16S rRNA sequencing analyses have indicated significant differences between healthy and chronic periodontitis groups (Li et al. 2014). There is also evidence that smoking negatively affects the subgingival microbiome, supporting the formation of pathogen-rich communities (Tsigarida et al. 2015, Mason et al. 2015). Likewise, it has been reported that smokers exhibit a significantly lower diversity than non-smokers and this community is enriched with bacterial species traditionally regarded as periodontal or systemic pathogens, such as, *Capnocytophaga, Treponema, Propionibacterium, Pseudomonas, Lactobacilli, Propionibacterium,* and *Leptotrichia* (Mason et al. 2015, Camelo-Castillo et al. 2015).

In light of the new evidence emerging with metagenomics, the aim of this pilot study was to investigate the microbial profile of the peri-implantitis microbiome in patients with a history of AgP in states of health and disease.

Materials and Methods

Study population

Informed consent

The study was conducted in line with the principles outlined in the Declaration of Helsinki (2008) on experimentation involving human subjects. Ethics approval for the conduct of the study was granted by the NRES Committee London –Queen Square (ref 13/LO/0874). Prior to initiation of the study visit, informed consent for participation was obtained.

Patient selection

This recall, longitudinal, observational study included a total of 18 patients, which were randomly selected from a patient cohort of aggressive periodontitis –AgP cases (70 patients) treated in the previous 15 years –from 1997 to 2011 (350 patients initial database) at the Eastman Dental Hospital –EDH (Table 1 and 2). Patients were included only if they met the clinical periodontal status criteria (at the time of the follow-up examination), as defined within the study groups below.
The patients were examined by 1 calibrated operator at EDH. In brief, an examiner repeatability exercise was performed to confirm adequate inter-examiner repeatability. Following, a calibration exercise was carried out on 10 subjects per examiner to confirm inter-examiner agreement. The data were analysed to confirm intra-examiner repeatability and inter-examiner calibration at a predetermined success criteria level of agreement within 2 mm for CAL in a minimum 98% of sites measured as previously reported in the literature (Suvan et al. 2015). All examiners reached levels above 98% agreement threshold for repeatability and calibration. The majority of these cases had been treated within the Hospital setting by a staff specialist periodontists and specialist trainees within the context of National Health Service delivery.

All patients had been assigned a diagnosis of AgP –either localised or generalised aggressive periodontitis (Armitage 1999; Lang et al. 1999; Mombelli 2002), at the initial examination visit to EDH as follows:

- Localised Aggressive Periodontitis (LAgP): presenting interproximal probing pocket depth (PPD) and localized clinical attachment level (LCAL) ≥5 mm and radiographic bone loss of ≥ 30% of root length on at least two permanent teeth, of which at least one was a first molar or incisor, and no more than three teeth other than first molars or incisors.
- Generalised Aggressive Periodontitis (GAgP): presenting generalized interproximal PPD and LCAL ≥5 mm and radiographic bone loss of ≥ 30% of root length affecting at least three permanent teeth other than first molars and incisors.

The AgP population was further subdivided into four groups based on the clinical periodontal status at the time of the follow-up examination (Table 1) and defined as follows:

- Active aggressive periodontitis (GAgP [TD]): this group was defined as patients presenting with a diagnosis of active GAgP, that is, residual periodontal pocket sites (*i.e.* PPD ≥5mm and BOP).
- Successfully treated aggressive periodontitis (treated GAgP [TH]): patients presenting at study visit with no sites ≥5mm and no bleeding on probing (BOP).
- Peri-implant health (implant health [IH]): patients treated for AgP and without signs of peri-implant mucositis and peri-implantitis (Lindhe & Meyle 2008; Ong et al. 2008).

- Peri-implant mucositis (implant mucositis [IM]): patients treated for AgP and presenting with peri-implant redness and swelling of the soft tissue and/or BOP (Lindhe et al. 2008) but not charactertics of peri-implantitis as below.
- Peri-implantitis (peri-implantitis [II]): patients presenting with peri-implantitis defined as PPD ≥5 mm with BOP and/or suppuration and radiographic signs of bone loss of ≥2.5mm or bone loss extending ≥ the first 3 threads (Ong et al. 2008; Albrektsson & Isidor 1994; Karoussis et al. 2003; Roos-Jansåker et al. 2006).
- Match implant (subgingival samples from implant patients [TM]): this group is comprised with periodontal samples drawn from dental sites from the same implant patients (*i.e.* groups: IH, IM, II).

Periodontal Therapy

The treatment provided prior the study visit has ranged from non-surgical therapy without adjunctive antimicrobials, to surgical periodontal therapy. Once the periodontal condition was successfully treated, all the patients were enrolled into an individually tailored supportive periodontal therapy programme (SPT) with at least 3 recall visits/year including the removal of supragingival deposits and subgingival deposits for teeth with PD>4 mm and BOP, and charting. If needed, the replacement of extracted teeth with dental implants was performed only after periodontal therapy was completed and until patients had proceeded to the maintenance phase of the therapy which included oral hygiene advice and support, debridement of supragingival deposits and removal of sub-gingival deposits for implants with PD>4 mm and BOP and charting, suprastructures were not removed at recall sessions (Table 2). After completion of treatment at EDH, patients were generally referred back to their referring general dental practitioners for the provision of periodontal maintenance care.

Baseline measurements

Periodontal charts and intra-oral radiographs were obtained from the initial periodontal examination patients' records.

Follow-up evaluation

Clinical Parameters

Calibration of the examiner

30 non-study subjects were recruited for the calibration examination of the study examiner. These subjects were selected among those with periodontal disease referred to the EDH. The examiner measured full-mouth PPD and REC (CEJ-FGM distance), using a manual, UNC-15 periodontal probe with mm markings. Six sites were measured for each natural tooth (excluding third molars).

PPD and CEJ-FGM distance measurements were rounded up to the nearest millimetre, CAL was then calculated (PPD minus CEJ-FGM). On the same day, the examiner evaluated the same subjects for a second time (minimum 30 minutes after the first exam). Upon completion of all measurements, intra-examiner repeatability for CAL measurement was assessed. Examiner's reproducibility was assessed with the Bland-Altman analysis and diagram and Lin's concordance correlation coefficient was also calculated. Paired t-test was used to assess if there was bias (systematic error).

Periodontal measurements

All measurements were taken by one calibrated examiner at 6 sites (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) for each natural tooth and recorded to the nearest millimeter (UNC-15, Hu-Friedy Mfg. B.V., Rotterdam, Netherlands). The measurements were made at the corresponding contact points or their equivalent in case of a missing tooth, and at the midpoint of buccal and lingual/palatal surfaces. The following information was collected: a full mouth dichotomous plaque score –FMPS (Guerrero et al. 2005), gingival index –GI (Loe et al. 1965), tooth mobility (Laster et al. 1975) furcation involvement (Hamp et al. 1975) probing pocket depth –PPD (single pass, whole mouth measures of the distance from the free gingival margin to the base of the sulcus), distance from the cement-enamel junction –CEJ to the free gingival margin –FGM (recession), clinical attachment loss (CAL), full mouth bleeding score (FMBS, measured as percentage of total bleeding surfaces upon probing).

Peri-implant measurements

In case of the presence of one or more implants, peri-implant clinical parameters were collected using gentle probing (UNC-15 TI, Hu-Friedy, Mfg. B.V., Rotterdam, Netherlands) as described above. The following parameters were recorded on implants: suppuration (Buser et al. 1990); mobility, radiolucency around implants and bone loss (Albrektsson et al. 1986); PPD (Mombelli & Lang 1994).

Radiographic examination

Full-mouth long cone parallel periapical radiographs using Rinn holders (Updegrave 1951) were taken to detect marginal bone levels and confirm diagnoses.

Subgingival periodontal and peri-implant plaque sampling

Subgingival periodontal and peri-implant plaque samples were obtained at a 15-year follow-up visit. The patients reported no antibiotic use in at least the past three months. All subjects refrained from eating, drinking and smoking for at least 30 minutes prior to plaque samples collection. Samples were taken from 4 sites (one in each quadrant) with sterile Gracey curettes. The distribution of the

selected sites were as follows: 16 distobuccal, 26 distobuccal, 36 distobuccal and 46 distobuccal. In case of absence of any of these teeth, the neighboring teeth (preference to the distal tooth if present) were chosen. In patients with dental implants, the disto-buccal surface of each implant (for a maximum of four was sampled. In cases of patients with more than 4 implants, 1 implant per quadrant was sampled. The samples were assigned into the previously described experimental AgP subgroups (*i.e.* TH, TD, TM, IH, IM, II) based on the recorded deepest pocket (from different quadrants). The supragingival portion of the root surface of the site was carefully cleaned with a curette. With the area isolated from saliva (gentle air spray or cotton rolls) a sterile Gracey curette was inserted to the bottom of the pocket. After a single stroke each microbiological sample was extracted from the pocket and then immediately placed into 1 mL of reduced transport fluid (RTF) and stored at -80°C for further analysis.

16S rRNA gene amplification and sequencing.

The 16S rRNA gene fragments were sequenced using by synthesis chemistry for bi-directional amplicon sequencing (MiSeq Desktop Sequencer; Reagent Kit v2; Illumina Inc., San Diego, CA, US). DNA was extracted (Griffiths et al. 2000) and then independent PCR reactions were performed for each sample to amplify the V5–V7 hypervariable region with barcoded primers 785F (F 5'-GGATTAGATACCCBRGTAGTC-3') and 1175R (R 5'-ACGTCRTCCCCDCCTTCCTC-3') (Sigma, Dorset, UK) (Bonder et al. 2012). Barcoded primers allow for multiplexing into one amplicon library (Table 1S). The PCR mixtures contained 2.5 uL of 1X Molzym PCR Buffer (Molzym), 0.5 uL dNTPs (10mM stock, Bioline, London, UK), 1 uL of each primer (10uM stock), 0.125 uL of Moltaq (0.025uM, Molzym, VH Bio Ltd., Gateshead, UK), 0.25 uL of $MgCl_2$ (50mM stock), and approximately 100 ng of DNA template in a final volume of 25 uL (PCR grade water, Bioline, London, UK). The PCR conditions were 95°C for 5 min, 30 cycles of 94°C for 30s; 55°C for 40s; and 72°C for 90s extension; followed by 72°C for 10 min. The PCR products were quantified (Qubit HS DNA kit, Life Technologies, England, UK), purified (High Stringency Agencourt AMPure XP, Beckman Coulter, UK) and combined in equimolar ratios (EB Buffer, Qiagen, Manchester, UK) to create a DNA pool for pyrosequencing on a MiSeq Sequencer (MiSeq Desktop Sequencer; Reagent Kit v2; Illumina Inc., San Diego, CA, US).

Statistical Analysis

Sample size

This study can be considered a recall longitudinal study with a convenience sample size determined by the number of patients able to or willing to attend a re-evaluation appointment and who met the inclusion criteria for the study groups.

Sequencing processing and metagenomic analysis

The raw amplicon data sets were processed using the QIIME pipeline (Quantitative Insights Into Microbial Ecology, qiime.org, version 1.8) (Caporaso et al. 2010). In brief, the multiplexed paired-end reads were filtered for quality and assigned to corresponding reference database (Greengenes, http://greengenes.lbl.gov). The reads were demultiplexed and assigned to the samples using the barcode sequence. Generation of OTU's by clustering reads based on 97% similarity (UCLUST) and chimera checking (UCHIME) were performed in order to avoid overestimates of the microbiota (Kunin et al. 2010). Thus, the taxonomic assignments (Operational Taxonomic Units –OTU's) were done in collections of sequences that were highly similar (97%) (Edgar 2010). Furthermore, a negative control sample was included in order to assess bacterial DNA contamination, which may arise from the extraction kits and laboratory reagents; this was performed following previous suggested protocols (Salter et al. 2014). The rarefaction curves and the taxonomic composition of the samples were visualized (R Development Core Team, R-project.org, 2008; qiime.org; version 1.8). Diversity within a sample (alpha-diversity –Chao1 and Shannon indexes) and diversity across samples (beta-diversity) were done. Dendrograms were used along the sides of the heatmaps to cluster groups and taxa by using the UPGMA method. The identification of the core microbiome was performed (qiime.org; version 1.8). In order to identify the most significantly different taxonomic category at a genus level and differences between groups we performed a series of statistical tests. For comparison between the five different niches ANOVA (one-way) and *post hoc* Games-Howell, which accounts for multiple comparisons and for different group size and variances (Bluman 2007) was performed. We used two-sided Welch's t-test for the comparison of two independent groups (*i.e.* teeth and implants) and the 95% confidence intervals (CI) were determined by Welch's inverthed method and multiple test correction by Storey FDR (Parks & Beiko 2010). Differences between two percentages were analysed by the two-sided Fisher's exact test and the 95%CI's were determined using the Newcombe-Wilson method and multiple test correction by Benjamin-Hochberg FDR (Tout et al. 2014, Benjamini & Hochberg 1995). The significance level was established as 0.05 to be stringent enough to avoid overcorrection. All quoted *p*-values represent that have been corrected for multiple testing, with only $p<0.05$ reported (Tout et al. 2014).

Results

Recruitment and characteristics of the population sample

A total of 91 sites (19 implants and 72 teeth sites) from 18 AgP patients were included in the present study. This study population belongs to a long-term retrospective evaluation on disease progression in aggressive periodontitis patients. The demographic and systemic clinical features of the subject groups were reported by the patients. 58.82% of the subjects were females and 41.18% males. Overall, two patients (11.76%) were current smokers, however 47% reported to have smoked before. 5.48% of the subjects were Asian, 23.52% African, and 71% Caucasian.

At study visit, patients presented with a clinical periodontal diagnosis (at a patient level) of either active aggressive periodontitis (*n*=11) (GAgP) or successfully treated aggressive periodontitis (*n*=7). Interestingly, none of the included patients presented at the recall visit with a diagnosis of LAgP. Fourty-four periodontal sites were diagnosed as active GAgP and twenty-eight as successfully treated AgP. The peri-implant diagnosis included eight peri-implant health sites (2 patients), six peri-implant mucositis sites (2 patients) and five peri-implantitis (2 patients) sites. Samples were pooled on a per patient according to their condition. Finally, twenty-four samples including 6 implants (*i.e.* IH, IM and II) and 18 teeth (*i.e.* TH, TD and TM) were processed for metagenomic analysis (Table 1).

Clinical observations

The average follow-up period since the first examination at EDI was 9.58 years (min. 5 – max. 15). The mean number of years in SPT was 6.65 years (min. 2 – max. 12 years). 47% of the patients had received surgical periodontal therapy.

The mean annual tooth loss rate in the population sample was 0.19 (*per* year). Overall, higher annual tooth loss rate (0.23 *per* year) was observed in the patients that were diagnosed with active GAgP (residual PPD) at the study visit in comparison to the successfully treated group (0.14 *per* year) (Table 2). Patients presented at the study visit with a mean FMPS of 36.18% and with a mean 22.35% (95% CI) of sites with positive BOP. Most of the patients (52.94%) presented with a plaque control of up to 20% FMPS. The mean GI was 0.87 (95% CI). Most of the implants were positioned in the premolar and molar areas (Table 2).

Taxonomy

Metagenomic analysis was used to assess variability in the biofilm community composition across five different subgingival and peri-implant niches on AgP patients in states of health and disease. The relative abundance proportions and phylogenetic distribution covering the total sequences in subgingival and peri-implant plaque samples of patients that have been treated for AgP are summarised in the supplemental material (Figures 1S and 2S).

The identified sequencing reads or reads pass filter (Q30 >70% and with chastity scores of ≥0.6) were 1.1M. Only stringent quality based reads were included for analysis (Figure 1 [a,b]). The highest number of sequences was found in the TD group (44.15%). In contrast, the lowest number of sequences was found in the IH group (1.55%). Furthermore, out of the 24 samples, one of them (TM1) was excluded from the analysis since it had fewer sequences then the requested rarefaction depth (Figure 1S). The OTU that was present in at least 50% of all the samples was represented by the OTU belonging to the genera *Streptococcus* (Figure 2).

Descriptive analysis

Overall, sequences from both teeth and implants were represented by the following major phyla: *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes,* or candidate division *TM7*. In teeth the most abundant phyla was *Actinobacteria* (52.2%), whereas in implants *Firmicutes* predominated (80.1%) (Figure 1S).

Sequences were represented by 51 different genera within teeth and implants. When sequences could not be identified at the genus level, they were classified at the next highest possible resolution level –family, order, class or phylum. From these, implants comprised 24 genera and teeth 44. Unique genera observed in the peri-implant groups (*i.e.* IH, IM, II) were *Propionibacterium, Paludibacter, Staphylococcus, Filifactor, Mogibacterium, Bradyrhizobium* and *Acinetobacter.* In teeth (*i.e.* TH, TM, TD), unique observed bacterial taxa were represented by: a) orders *Actinomycetales* and *Bacteroidales;* b) families *Actinomycetaceae, Propionibacteriaceae, Bifidobacteriaceae, Coriobacteriaceae, Porphyromonadaceae, Paraprevotellaceae, Flavobacteriaceae, Carnobacteriaceae, Lachnospiraceae, Peptostreptococcaceae, Veillonellaceae, Erysipelotrichaceae, Leptotrichiaceae, Comamonadaceae, Neisseriaceae, Campylobacteraceae, Cardiobacteriaceae,* the candidate division class *TM7-3* and *Rs-045* family; and c) genera *Granulicatella, Oribacterium, Schwartzia, Selenomonas, Eikenella,* and *Neisseria* (Table 2).

The less relative abundant genera at the different conditions were as following: 1) peri-implant health: *Acinetobacter, Veillonella* and *Acinetobacter;* 2) peri-implant mucositis: *Bradyrhizobium, Paludibacter, Mogibacterium* and *Lautropia;* 3) peri-implantitis: *Treponema* and *Bradyrhizobium.* In contrast, the less abundant genera at the different conditions were: 1) periodontal health: *Oribacterium, Prevotella, Leptotrichia, Porphyromonas, Tannerella, Parvimonas, [Propionibacteriaceae]* family, *Granulicatella, [Bacteroidales]* order, *[Mogibacteriaceae]* family, and *Treponema;* 2) periodontitis: *Peptostreptococcus, Capnocytophaga, Neisseria, [Veillonellaceae]* family, *Cardiobacterium, [Rs-045]* family, *Parvimonas, Aggregatibacter, [Lachnospiraceae]* family, *[Neisseriaceae]* family, *Prevotella, Eikenella, Lautropia, Selenomonas, [Streptococcaceae]* family, and *Campylobacter.*

Interestingly, the co-ocurrence of bacterial species seems to be more diverse in periodontal samples in comparison to peri-implant samples (Figure 3). The phylogenetic diversity of total bacterial communities of teeth and implants was calculated by rarefaction analysis (Shannon and Chao1 metric, alpha diversity) (Chao et al. 2014). It appears that the subgingival plaque presented a higher phylogenetic diversity than the peri-implant plaque (Figure 2S). Furthermore, in contrast with peri-implant sites, diseased states (*i.e.* TD) presented with the highest diversity in teeth. The shapes of the rarefaction curves indicate that overall approximately by 200 sequences per group, all of them reached the plateu, indicating that bacterial richness is complete.

Metagenomic profile differences

Shifts in the microbial community composition were observed between teeth and implants (Figure 4) and were found to be statistically significant (p<0.05) using ANOVA, Games-Howell *post hoc* test and Eta-squared (effect size). Statistically significant shifts in the relative abundance of *Streptococcus* (p=1.67e^{-3}) were observed between teeth and implants. *Streptococcus* was significantly less abundant in teeth than in implants. Significantly abundant genera found in teeth in contrast to implants included: *Actinomyces (p=1.31*e^{-3}*), Selenomonas (p=0.016), Capnocytophaga (p=0.020), Neisseria (p=0.020), Leptotrichia (p=0.022), Corynebacterium (p=0.026), Porphyromonas (p=0.032),* the *TM7-3* class (p=0.034), the order *Bacteroidales (p=0.041),* and the family *Propionibacteriaceae (p=0.049).*

The metagenomic data also revealed significant differences (p<0.05) in the microbial community composition across the peri-implant and periodontal niches (Figure 5). While *Actinomyces* and *Streptococcus* were generally the most abundant bacteria in all niches, several bacteria occurred in different frequencies across the different included niches. These included *Rothia* and *Aggregatibacter,* while *Propionibacterium, Mogibacterium, Paludibacter, Filifactor, Bradyrhizobium, Staphylococcus, Acinetobacter* were unique to the peri-implant niche.

We also observed that the periodontal and peri-implant niches differed significantly in states of health and disease. The Welch's t-test revealed that the genera *Rothia* is more abundant in the periodontal healthy niche –TH (p=0.036), conversely *Actinomyces* is the most abundant genera during the diseased states –TD (p=0.044) (Figure 6a). The genus *Streptococcus* (p=1.16e^{-3}) was significantly more abundant in IH group in comparison to the TH group (Figure 6b). Interestingly, *Rothia* (p=0.036) had a higher abundance in the groups TH in contrast to the II group (Figure 6c). *Actinomyces* (p=0.013) and *Corynebacterium* (p=0.030) genera showed to be significantly more abundant in TM group when compared with the II group (Figure 6d). Furthermore, *Staphylococcus, TG5* and *Corynebacterium* differed significantly between healthy and diseased niches in implants (Figure 7), with *Corynebacterium* being the most abundant in healthy peri-implant sites.

Comparison of the taxonomic composition between samples was done by UPGMA hierarchical clustering dendrogram of the included samples (Lozupone & Knight 2005). It seems that on the basis of the pairwise similarities between samples their taxonomic composition reflects strong support for overall distinction between implants and teeth (Figure 8).

Discussion

Differences between periodontal and peri-implant microbiome

The challenge of understanding peri-implant and periodontal microbial ecology in relation to states of health and disease has been dramatically advanced by the use of highthroughput 16S rRNA gene sequencing (NGS). This approach has increased the resolution at which microbial communities can be examined. The present recall longitudinal study focused on the investigation of the diversity of the subgingival periodontal and peri-implant microbiomes (within and between samples) from AgP patients and on the description of significantly abundant not previously identified metagenomic profiles, in states of health and disease.

One of the main risk factors for the development of peri-implant infection is periodontal disease (Heitz-Mayfield, 2008). Recent studies suggest that patients treated for aggressive periodontitis are associated with higher incidence of biological complications (*e.g.* bone loss and peri-implantitis) and lower success and survival rates (De Boever et al. 2009; Mengel et al. 2007; Mengel & Flores-de-Jacoby 2005; Swierkot et al. 2012). Specifically, a study (Swierkot et al. 2012) reported higher incidence of peri-implantitis in GAgP patients (*i.e.* 26%–39 implants, 15 patients) as compared with a non-periodontitis group (*i.e.* 10%–3 implants, 2 patients). Moreover, it has been suggested that the initial colonisation pattern of peri-implant pockets by periodontal patoghens could contribute to the development of peri-implant lesions (Leonhardt et al. 1999; Quirynen et al. 2006). Few studies have described the concept of intra-oral translocation of periodontopathogens (Quirynen et al. 2001), the association of these pathogens with peri-implant lesions (Leonhardt et al. 1999) and related patterns of bacterial colonisation (Mombelli 2002), suggesting that the microbiota harboured in peri-implantitis sites is similar to periodontal sites (Renvert et al. 2007). However, one of the main limitations of these studies is the use of low-resolution microbiological methods that partially target for the identification and quantification of selected periodontal pathogens. The present study applied a deep metagenomic sequencing analysis of the periodontal and peri-implant subgingival AgP plaque samples. Our study showed that the peri-implant microbiome in AgP patients substantially varies from the periodontal microbiome. It also demonstrates a substantial heterogeneity in the microbial community composition in states of health and disease, which is in agreement with a recent publication (Dabdoub et al. 2013). This might be one of the reasons why peri-implantitis cannot be eliminated by using therapies that have been traditionally proposed for the treatment of periodontitis. The results presented here also suggest that a complex interaction between the host and the periodontal and peri-implant niches takes place across the different microenviroments within the oral cavity; thus the translocation of periodontopathogens hypothesis needs to be revisited using larger datasets and high resolution analyses (*e.g.* NGS). These studies may benefit by taking into account the host response (Nibali 2015). It seems that the host response, the habitat structure and the vast coexistence of strains and species surrounding implants and teeth in health and disease are likely to be shaping the heterogeneous composition of the subgingival microcosms.

Specifically, by including the TM group, we were able to compare directly, at a patient level, residual periodontal pockets sites against peri-implantitis sites in the same subjects. This observation suggests that less-abundant organisms may act as keystone species (*e.g. F. alocis* or *P. gingivalis*) in complex subgingival plaque communities (Lamont & Hajishengallis 2015, Aruni et al. 2014, Wade 2002). Interestingly, the candidate phylum *TM7* was detected in periodontal plaque samples in disease conditions (*i.e.* residual periodontal pocket sites [TD]) but not during states of health (TH) or around implants (*i.e.* IH, IM and II). These results suggest its association with disease activity (TD) in treated AgP patients. These results are in agreement with a previous study, which recently reported that *TM7* is an obligate epibiont of an *Actinomyces odontolyticus* strain (XH001), and that it has a parasitic lifestyle (He et al. 2015).

From the present findings, also appears that in patients treated for AgP, increased annual tooth loss rate and residual pocketing was related to higher proportions of the genera *Actinomyces, Porphyromonas, Prevotella, Streptococcus, Sharpea, Actinomycetaceae, TM7-3, Selenomonas, and Dialister, Treponema, Parvimonas,* and *Peptostreptococcus* in teeth (*i.e.* TD). However, the conclusions in terms of causal relationships between the metagenomic composition of the subgingival (periodontal and peri-implant) biofilms and clinical characteristics (in states of health and disease) of the present study are limited by the sample size, thus generalisations could not be made.

Some technical limitations regarding the use of NGS methods are the sequence coverage and depth –specially the low sequence diversity, which we attempted to overcome by pooling the plaque samples from each individual by periodontal/peri-implant diagnosis (*i.e.* health and disease). In addition, operational taxonomic units (OTU's) were grouped at a genus-level phylotype in order to circumvent potential taxonomic misclassifications due to sequencing bias (Ching et al. 2014, Caporaso et al 2010). Higher phylogenetic resolution (i.e. species or strain level) could be approached by employing whole genome sequencing (Forster et al. 2015). Furthermore, not all the taxa could be assigned down to genus-level phylotype, instead it was assigned to the lowest common taxon.

Strenghts of the present study include the definition of the study groups, long period of time follow-up and related implant outcomes of the included subjects in AgP patients (*e.g.* 15 years), and detailed description of interventions and outcomes.

While 16S rRNA approaches have shown that the microbial community composition can shift within periodontal niches (Dabdoub et al. 2013), the diversity of the metagenomic profile and significant relative abundant taxa of the subgingival periodontal and peri-implant biofilms in states of heath and disease in AgP cases had not previously been documented. This research has unveiled for the first time in the literature the heterogenic bacterial composition of subgingival biofilms in aggressive periodontitis patients.

Recommendations for Further Research and Clinical Implications

Future Research

Currently, several tools are available for sample collection, sequencing and data analyses for metagenomics. Over the past fifteen years, the sequencing technologies platforms –including their chemistry, have improved dramatically. However, downstream analyses are still in its infancy. The field lacks general standard methods for bioinformatics pipelines to filter the 16S rRNA data, methods to estimate power and sample size (*e.g.* overcome low sequence diversity), and statistical analyses of the assembled data. These factors are of major importance for future comparison between long-term clinical longitudinal studies and their potential combination in meta-analyses.

The results from this recall longitudinal study need to be confirmed in larger metagenomic datasets looking into an AgP cohort who have received dental implants (*i.e.* health and disease/before and after treatment).

Another important issue that has to be taken into account in terms of study design is the clinical heterogeneity. Universal definitions for the experimental groups should be used, confounding factors must be controlled/adjusted, and it is advisable to include unit of analysis based at both patient and site level. Specifically, the investigation of microbial colonisation patterns, looking into keystone species' roles (such as between the *TM7* phylum and *A. odontolyticus, F. alocis* or *P. gingivalis* spp.*)* and interacions may shed light into clinical significant mechanisms and disease progression. The overall aim is to improve diagnostics, that is, presence as indication for intervention or disease activity, prevention and to develop novel therapeutics (*e.g.* targeting specific virulence factors). These studies could benefit from the integration of high-throughput multiplexed data to investigate the interactions, metabolic pathways and function prediction (Teles et al. 2013).

Conclusions

The composition of the microbial communities substantially varies within and between the subgingival periodontal and peri-implant niches in states of health and disease in AgP patients. While there were some shared communities across all metagenomes, each niche was characterised by specific traits. Specifically for AgP cases, the periodontal microbiome appears to be more diverse than the peri-implant microbiome. Likewise, in contrast to peri-implantitis sites, subgingival disease states (*i.e.* residual periodontal pockets TD) harboured the highest microbial diversity. Specifically, the *TM7* phylum was found only in TD cases. More investigations with a long-term follow-up (*i.e.* ≥10 years) in an aggressive periodontitis population with dental implants reviewing the impact of inter-taxa associations, and disease progression in the subgingival microbial ecosystem are needed. Furthermore, an approach to investigate the impact of periodontal and peri-implant keystone species on these complex ecosystems and the identification of their role and

species interactions in states of health and disease (*i.e.* AgP and chronic periodontitis) seems to be essential.

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