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Synergistetes cluster A in saliva is associated with periodontitis

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Background and Objectives: Synergistetes is a novel bacterial phylum consisting of gram-negative anaerobes. Increasing lines of evidence demonstrate that this phylum is associated with periodontal diseases. This study aimed to compare the presence and levels of *Synergistetes* clusters A and B, in saliva of patients with chronic periodontitis (CP), generalized aggressive periodontitis (G-AgP) and non-periodontitis subjects, and investigate their correlation with clinical parameters.

Material and Methods: Saliva was collected from patients with CP (n = 20), G-AgP (n = 21) and non-periodontitis subjects (n = 18). Full mouth clinical periodontal measurements were recorded. The numbers of *Synergistetes* cluster A and cluster B or the associated species *Jonquetella anthropi* were quantified by fluorescent *in situ* hybridization and microscopy.

Results: Synergistetes cluster A bacteria were detected more frequently, and at higher numbers and proportions in the two periodontitis groups, than the non-periodontitis control group. The prevalence was 27.7% in the control group, 85% in CP and 86% in G-AgP. Compared to the control group, the numbers were significantly higher by 12.5-fold in CP and 26.5-fold in G-AgP, whereas the difference between the two forms of periodontitis was not statistically significant. Within the total bacterial population, the proportion of this cluster was increased in CP and G-AgP compared to the control group, with the difference between the two forms of periodontitis being also significant. There was a positive correlation between the levels of *Synergistetes* cluster A in saliva and all full mouth clinical periodontal parameters. Nevertheless, *Synergistetes* cluster B bacteria and *J. anthropi* species were detected infrequently and at low levels in all the three subject groups.

Conclusion: Synergistetes cluster A, but not cluster B, bacteria are found at higher prevalence, numbers and proportions in saliva from patients with periodontitis, than non-periodontitis subjects. These findings support the association of this cluster with periodontitis.

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Synergistetes is a recently identified bacterial phylum consisting of gram-negative anaerobes (1). Morphologically they have been identified by fluorescent *in situ* hybridization (FISH) as long curved bacilli (2,3). They are distributed in multiple anaerobic environments (4), also found as part of the human microbiota in health and disease (5–7). *Synergistetes* comprises one of the 13 different phyla identified in the Human Oral Microbiome

Database among the oral microflora (8). The human-originated oral *Synergistetes* are divided principally into cluster A and cluster B (2). These consist of numerous non-cultivated phylotypes and three so-far isolated species. The first cultivated oral *Synergistetes* cluster A species is *Fretibacterium fastidiosum* (9), whereas the two cultivated oral *Synergistetes* cluster B species are *Pyramidobacter piscolens* (10) and *Jonquetella anthropi* (2). *Synergistetes* have been detected in high numbers in subgingival plaques from patients with chronic periodontitis (CP) and from root canals of endodontically affected teeth (2,11–13). They are more frequently detected in patients with periodontitis than healthy subjects, and more abundantly present in subgingival plaque from diseased than healthy sites (2). Moreover, subgingival plaque from periodontitis sites foster approximately five-fold higher clonal abundance and 3.5-fold higher clonal diversity of *Synergistetes* cluster A clones, compared to healthy sites (14). Accordingly, dental plaque from patients with necrotizing ulcerative gingivitis (NUG) fosters 9.4-fold higher numbers and 2.5-fold higher proportions of *Synergistetes* cluster A bacteria, compared to plaque-induced gingivitis (3). However, as only a handful of oral *Synergistetes* strains have so far been isolated and successfully cultivated, and no virulence factors yet identified, the potential pathogenicity of these taxa is to date unclear.

Saliva is a suitable biological analyte for detecting molecular biomarkers and periodontal pathogens associated with the progression or stability of periodontal disease (15), as it contains a mixture of oral microbiota from various sites of the oral cavity, at the time of collection. The presence of *Synergistetes* has been confirmed in saliva of patients with periodontitis (2,16).

Although there is evidence of the higher prevalence and diversity of *Synergistetes* in periodontal disease, there is lack of quantitative data on the levels of *Synergiststes* clusters A and B in larger cohorts of patients with CP and generalized aggressive periodontitis (G-AgP), particularly in saliva. Therefore, the aim of this study was to evaluate the prevalence, numbers and proportions of oral *Synergistetes* clusters A and B, as well as *J. anthropi*, in the saliva of CP, G-AgP and non-periodontitis subjects.

Material and methods

Study population and clinical examination

A total of 59 unrelated Caucasians of Turkish descent residing in the same geographic region and of similar socio-economic level were included in this study, recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir, Turkey, from November 2011 until March 2012. The use of human subjects satisfied the requirements of Ege University Institutional Review Board (ethics approval number: 11-12.1/11) and was conducted in accordance with World Medical Association Declaration of Helsinki guidelines. Complete medical and dental histories, including previous orthodontic therapy, were taken from all subjects. Systemic exclusion criteria were the presence of cardiovascular and respiratory diseases, systemic inflammatory conditions, such as diabetes mellitus, or non-plaque induced oral inflammatory conditions, HIV infection or immunosuppressive chemotherapy, and current pregnancy or lactation. The patients did not receive any medication that could affect their periodontal conditions, such as antibiotics or anti-inflammatory drugs, for at least 6 mo before enrolment in the study. One week after pre-screening, patients eligible for participation in the study returned for further clinical screening. Participants smoking more than five cigarettes per day were registered as active smokers. Before enrolment in the study, written and informed consent was obtained from all participants. Patient selection was based on clinical and radiographic criteria proposed by the 1999 International World Workshop for Classification of Periodontal Disease and Conditions (17).

The periodontal status of each patient was assessed by a single calibrated examiner having experience in clinical trials (O.V-O). Full mouth clinical periodontal examination included measurement of probing pocket depth (PPD), clinical attachment loss (CAL) at six sites around each tooth with a manual probe, full mouth dichotomous presence of bleeding on probing (BOP) and plaque index (PI) (18). The control group, referred to as the non-periodontitis group, consisted of five women and 13 men (35.94 ± 12.45 years of age) with varying levels of gingival inflammation as indicated by BOP scores, and no radiographic evidence of alveolar bone loss (i.e. distance between the cemento-enamel junction and bone crest $\leq$ 3 mm at $> 95\%$ of the proximal tooth sites). They exhibited PPD $\leq$ 3 mm and CAL $\leq$ 2 mm at $\geq$ 90% of the measured tooth sites. When subjects in this group exhibited sites with CAL > 2, this was in $< 10\%$ of the sites, with most of the attachment loss confined to the facial surfaces of the teeth. This was due to traumatic tooth brushing, or orthodontic reasons, as judged by the examining clinician. CAL due to traumatic tooth brushing was denoted in the presence of abrasion of the facial surfaces of the teeth, whereas previous orthodontic tooth movement was revealed through dental history. In either case, the respective sites were free of inflammation. Five subjects in this control group had a history of orthodontic therapy. The CP group included 12 women and eight men (44.05 ± 7.81 years of age). They had at least four non-adjacent teeth with sites exhibiting CAL $\geq$ 5 mm, commensurate with the amount of plaque accumulation, PPD $\geq$ 6 mm, BOP $\geq$ 50%, and radiographic evidence of bone loss. The G-AgP group consisted of 12 women and nine men (34.19 ± 5.3 years of age). These patients demonstrated a generalized pattern of severe periodontal destruction, CAL $\geq$ 5 mm, and PPD $\geq$ 6 mm on eight or more teeth, at least three of these were other than central incisors or first molars, and radiographic evidence of bone loss. All subjects had at least 20 teeth in their mouth. Smokers were identified as four of 18 (22.2%) in the control group, five of 20 (25%) in the CP group and seven of 21 (33.3%) in the G-AgP group.

Collection and processing of saliva

All saliva samples were collected in the morning between 8 a.m. and 11 a.m. The participants were asked to avoid oral hygiene measures (i.e. flossing, brushing and mouth rinses), eating, drinking for 2 h before collection. During the collection process, the subjects were asked to rinse their mouth with tap water, and then expectorate whole saliva into sterile collection tube over a period of 5 min. The saliva samples were then centrifuged at 10,000 ***g*** for 15 min, at 4°C, and the resulting pellet was suspended in 0.9% NaCl containing Protect RNase inhibitor (Sigma Aldrich, Buchs, Switzerland) at 1 : 500 dilution, and stored at – 80°C.

Analysis of saliva samples by fluorescent *in situ* hybridization

The number of various bacterial taxa in the saliva samples was quantified by epifluorescence microscopy, following staining by FISH. Briefly, after the samples were defrosted and vortexed for 1 min, they were diluted in coating buffer (0.9% NaCl, 0.02% NaN_3, 2.5×10^{-4}% hexadecyltrimethylammonium bromide). Ten microliters of these suspensions were then spotted on individual 18- or 24-well epoxy coated ADCELL multiwell slides of a 4 mm well-diameter (CelLine, Erie Scientific Company, Portsmouth, NH, USA). After air-drying, the slides were fixed by a 20 min incubation, at 4°C in 4% paraformaldehyde, and thereafter processed for FISH, as described previously (3). Four specific oligonucleotide rRNA probes were used for *Synergistetes* cluster A, *Synergistetes* cluster B and *Jonquetella anthropi* (3), streptococci (19), and one universal probe, which identifies most eubacteria (20). The oligonucleotide sequences of the rRNA probes labelled with Cy3 or 6-FAM (Microsynth, Balgach, Switzerland), and their targeted taxa are listed in Table 1. The cluster classification of oral *Synergistetes* into clusters A and B was based on Vartoukian *et al.* (2). The final probe concentrations used for FISH were 5 ng/μL for Cy3 conjugates and 20 ng/μL for FAM conjugates, in the presence of 40% or 45% formamide (Table 1). The FISH-stained samples were visualized under an Olympus BX60 fluorescence microscope (Olympus Optical AG, Volketswil, Switzerland). The quantitative evaluation of the stained bacterial taxa was performed as previously described (3,21). Briefly, for the estimation of bacterial counts, a minimum of 10 viewing fields per well were counted at 100× magnification. The total number of positively stained bacteria was then calculated against the volume of saliva per sample.

Table 1. rRNA-targeted probe sequences, target taxa and formamide concentrations

Probe	Sequence (5′–3′)	Target taxa	Formamide (%)
EUB338-Cy3	GCTGCCTCCCGTAGGAGT	Most eubacteria	45
STR405-FAM	TAGCCGTCCCTTTCTGGT	All streptococci	45
SYN-A1409-FAM	ACACCCGGCTCGGGTGGT	*Synergistetes* cluster A	40
SYN-B1149-Cy3	TCGATGGCAGTCTCGCCG	*Synergistetes* cluster B	40
Jon219-Cy3	CACAAGCTCCTCCATCAG	*Jonquetella anthropi*	45

Statistical analysis

The differences in full mouth clinical periodontal parameters and bacterial numbers between all groups were analysed by the Kruskal–Wallis test, using Dunn's multiple comparison test to investigate differences between groups. The differences in bacterial proportions between all groups was analysed by one-way analysis of variance, using the Bonferroni test to investigate the differences between groups. The association between bacterial numbers in saliva and full mouth clinical periodontal measurements was investigated by Spearman's rank correlation analysis. Differences were considered statistically significant at $p < 0.05$. Values are expressed as mean ± standard deviations (SD).

Results

Demographics and full mouth clinical measurements

The full mouth clinical findings of the studied groups are provided in Table 2. Mean PPD, CAL, PI and BOP scores were significantly higher in the CP and G-AgP groups, than in the control group ($p < 0.0001$). No significant differences were detected in these clinical measurements between the CP and G-AgP groups.

Estimation of total bacteria and streptococci levels

The total number of eubacteria and streptococci were initially evaluated in the collected saliva samples. Total bacterial number was used as a reference to calculate the proportions of *Synergistetes* within the bacterial population. The rationale for deciding to evaluate the total number of streptococci is because these taxa are well

Table 2. Demographic information and full mouth clinical periodontal measurements

	Control (n = 18)	CP (n = 20)	G-AgP (n = 21)
PPD (mm)	1.85 ± 0.23	4.93 ± 0.37*	5.32 ± 0.77*
CAL (mm)	1.88 ± 0.25	5.57 ± 0.69*	6.02 ± 1.07*
PI	0.81 ± 0.4	2.35 ± 0.50*	2.09 ± 0.44*
BOP (%)	26 ± 15	75 ± 43*	66 ± 21*

*Statistically significant differences to control group ($p < 0.01$).
BOP, bleeding on probing score; CAL, clinical attachment loss; CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis; PI, plaque index; PPD, probing pocket depth.

characterized and highly prevalent in saliva. Therefore, the presence and levels of streptococci are used as a measure of relative comparison to the less studied *Synergistetes*.

Saliva samples from patients with CP yielded significantly higher total bacterial numbers than G-AgP patients or the control group (Fig. 1A). The number of streptococci was significantly higher in CP compared to G-AgP, but there was no difference between either form of periodontitis and the control group (Fig. 1B). When the percentage of streptococci was calculated, this was lower in the CP and G-AgP groups, compared to the control group. This difference proved to be significant only in the case of the G-AgP group (Table 3).

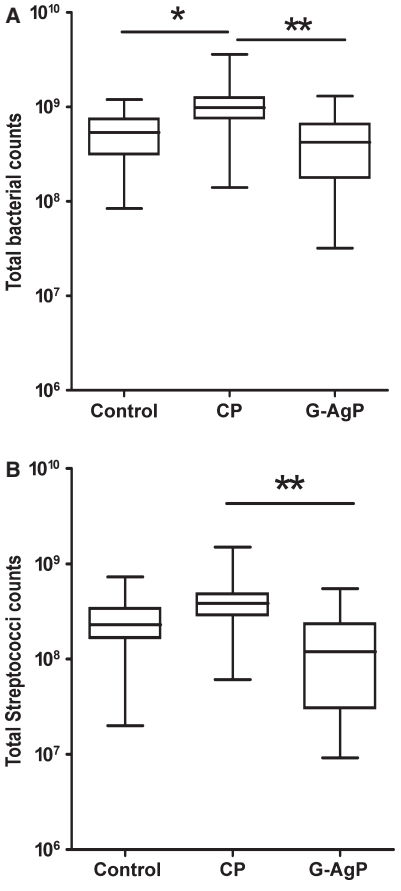

Fig. 1. Total bacterial levels (A) and total streptococci levels in the saliva of control subjects ($n = 18$) and patients with CP ($n = 20$) and G-AgP ($n = 21$). Cell numbers represent positive bacterial counts, as stained by fluorescent *in situ* hybridization, per mL of saliva. Horizontal lines in box plots represent median values. The asterisks indicate statistically significant difference between the indicated groups (*$p < 0.01$, **$p < 0.001$). CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis.

Detection of *Synergistetes* cluster A bacteria

The presence of *Synergistetes* cluster A in saliva was further considered. This cluster was detected in five of 18 non-periodontitis subjects, but its frequency of detection was increased to 17 of 20 patients in CP, and 18 of 21 patients in G-AgP (Table 4). This was also reflected by higher detection levels. In particular, compared to the control group, *Synergistetes* cluster A bacteria were found at significantly higher numbers in CP (12.5-fold) and G-AgP (26.5-fold). Nevertheless, there was no statistically significant difference between the two forms of periodontitis (Fig. 2).

The percentage of *Synergistetes* cluster A within the total bacterial population was also calculated, and was found to be considerably lower than that of streptococci. However, the percentage of *Synergistetes* cluster A in G-AgP was 6.2-fold higher than in CP, and 91.6-fold higher than in the control group, a difference that was statistically significant in both cases. Nevertheless, the difference between CP and the non-periodontitis control group did not prove to be statistically significant (Table 3).

Detection of Synergistetes cluster B bacteria and *Jonquetella anthropi*

The presence of *Synergistetes* cluster B and *J. anthropi* species in saliva was also considered. Their frequency of detection is provided in Table 4. Collectively, *Synergistetes* cluster B bacteria were detected in five of 21 samples of the G-AgP group ($1.8 \pm 1.7 \times 10^4$ bacteria/mL), but not at all in the CP or control groups. *J. anthropi* was detected in four of 18 non-periodontitis ($8.1 \pm 1.1 \times 10^2$ bacteria/mL), two of 20 CP samples ($2.1 \pm 0.7 \times 10^3$ bacteria/mL) and five of 21 G-AgP samples ($2.8 \pm 1.6 \times 10^3$ bacteria/mL). As *Synergistetes* cluster B, and *J. anthropi* in particular, were detected at low frequencies and numbers close to detection limits, no further analyses were performed on this taxa, as it would not be statistically meaningful.

Correlation of clinical periodontal parameters with numbers of total bacteria, streptococci and *Synergistetes* cluster A

Finally, correlation analyses were performed between full mouth clinical periodontal parameters and numbers of total bacteria, streptococci or *Synergistetes* cluster A in saliva (Table 5). Spearman correlation analysis revealed that the total bacterial numbers correlated only with the PI, whereas the number of streptococci did not correlate with any of the clinical mea-

Table 3. Percentage of streptococci and *Synergistetes* bacteria among the subject groups

	Control (%)	CP (%)	G-AgP (%)
Streptococci	46.7 ± 2.3	40.7 ± 3.9	28.3 ± 12.3**
Synergistetes cluster A	$2.4 \pm 4.5 \times 10^{-4}$	$3.5 \pm 3.8 \times 10^{-3}$	$2.2 \pm 4.0 \times 10^{-2}$*

Asterisks indicate statistically significant difference to the control group *$p < 0.05$ **$p < 0.001$.
CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis.

Table 4. Prevalence of *Synergistetes* bacteria among the subject groups

	Control (%)	CP (%)	G-AgP (%)
Synergistetes cluster A	5/18 (27.7)	17/20 (85)	18/21 (86)
Synergistetes cluster B	0/18 (0)	0/20 (0)	5/21 (23.8)
Jonquetella anthropi	4/18 (22.2)	2/20 (10)	2/21 (9.5)

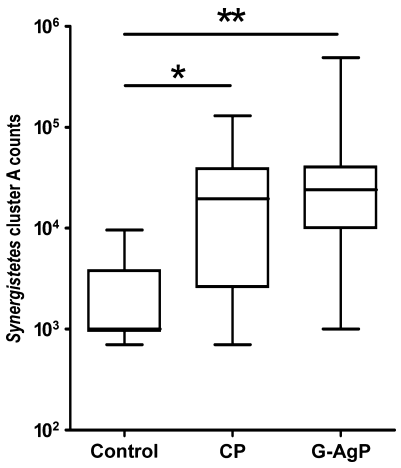

Fig. 2. Levels of *Synergistetes* cluster A bacteria in saliva of control subjects ($n = 18$) and patients with CP ($n = 20$) and G-AgP ($n = 21$). Cell numbers represent positive bacterial counts, as stained by fluorescent *in situ* hybridization, per mL of saliva. Horizontal lines in box plots represent median values. Undetectable values were ascribed the lowest detection limit value of the assay, to allow for log transformation.The asterisks indicate statistically significant difference between the indicated groups (*$p < 0.01$, **$p < 0.001$). CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis.

surements. On the contrary, *Synergistetes* cluster A numbers correlated positively with all full mouth clinical parameters, which included PPD, CAL, PI and BOP.

Discussion

The present study investigated the presence and levels of bacteria of the phylum *Synergistetes* in the saliva of a cohort of patients with CP, G-AgP and non-periodontitis subjects. The designated clusters A and B of this phylum, as well as the species *J. anhtropi*, were specifically considered in the study. It was found that the prevalence of *Synergistetes* cluster A bacteria was similar in CP and G-AgP, and approximately three-fold higher than in the control group. Moreover, bacteria belonging to this cluster were measured at 12.5-fold or 26.5-fold higher numbers in CP and G-AgP, respectively, compared to non-periodontitis, although there was no significant difference between the two periodontitis groups. These findings are in line with an earlier study demonstrating higher prevalence and diversity of *Synergistetes* clones in the saliva and subgingival plaque of patients with periodontitis than healthy subjects, also providing the first hints that cluster A is the most well represented in the disease (2). Accordingly, a recent study demonstrated that subgingival plaque from patients with periodontitis exhibits higher numbers and more diverse operational taxonomic units of *Synergistetes* cluster A, compared to periodontitis-free subjects (14). Another recent study using FISH for bacterial quantification found that *Synergistetes* cluster A bacteria were present at higher numbers in NUG than in plaque-induced gingivitis (3), indicating that the presence of this taxa could increase by the severity of the disease.

The presence of *Synergistetes* cluster B and the associated species *J. anthropi* were also investigated in the present study, but were detected in low frequencies and numbers. Accordingly, *Synergistetes* cluster B were barely detected in subgingival plaque samples of patients with periodontitis (2,14), whereas there were no differences in their proportions between plaque-induced gingivitis and NUG (3). Hence, the low prevalence and levels of *Synergistetes* cluster B, including *J. antrhopi*, indicates that these taxa may not have a crucial etiological role in periodontal diseases, hinting that further focus should be placed in studying cluster A.

The proportion (percentages) of *Synergistetes* cluster A in saliva was calculated against total bacterial numbers. This was elevated in both CP and G-AgP, compared to the non-periodontitis group, but the difference proved to be significant only in the case of G-AgP. Interestingly, the percentage of *Synergistetes* cluster A in G-AgP was significantly higher than in CP, and this is worthwhile investigating further, as it could give a clue for a differential microbial diagnosis between the two forms of periodontitis. Accordingly, the proportion of *Synergistetes* cluster A was recently found to be higher in dental plaque of patients with NUG, which compared plaque-induced gingivitis (3). Of note, earlier studies reported that *Synergistetes* constitute 0.1% of the dental plaque flora in health or shallow pockets, but this proportion can rise above 1% in deep pockets (22,23). Numerically, these proportions are considerably lower than the ones reported in the present study by two to three log scales, although the trend of differences is similar (i.e. increase in periodontitis). This may be a result of the different detection methods, or the different biological analyte used, which in the present case was saliva. Indeed, the aerobic conditions in saliva may not be favourable for the long-term survival of the strictly anaerobic *Synergistetes*. To this extent, streptococci were the predominant taxa detected in this cohort, whereas in relation to them, *Synergistetes* cluster A represented only a small fraction of the salivary flora. Still, the dynamic shift in numbers and proportions of this bacterial phylum in periodontitis may denote a strong association with the disease. However, these findings do not necessarily confer a cause–effect rela-

Table 5. Correlation analysis between microbiological findings and clinical parameters

	Total bacteria	Streptococci	Synergistetes cluster A
PPD	0.181 ($p = 0.170$)	− 0.029 ($p = 0.829$)	0.563 ($p < 0.001$)*
CAL	0.201 ($p = 0.126$)	− 0.018 ($p = 0.892$)	0.497 ($p < 0.001$)*
PI	0.318 ($p < 0,05$)*	0.153 ($p = 0.246$)	0.456 ($p < 0.001$)*
BOP	0.110 ($p = 0.408$)	0.010 ($p = 0.939$)	0.540 ($p < 0.001$)*

Spearman's rank correlation analysis was performed (r coefficient is provided).
*Statistically significant correlation.
PPD, Probing pocket depth; CAL, clinical attachment loss; PI, plaque index; BOP, bleeding on probing score.

tionship between *Synergistetes* cluster A and periodontitis. Their increased presence and levels in the disease could well be a secondary phenomenon arising from the altered microenvironmental conditions of the affected sites.

To investigate this association further, correlation analyses were performed between full mouth clinical periodontal parameters and numbers of *Synergistetes* cluster A present in saliva. A positive correlation was revealed between the levels of *Synergistetes* cluster A and all clinical measurements, including PPD, CAL, PI and BOP scores. On the contrary, the levels of streptococci did not correlate with any of the clinical parameters. These findings are in line with the demonstration that *Synergistetes* are found at low levels in shallow periodontal pockets, but their numbers and proportions can increase as the pockets become deeper (22,23), or by the extent of gingival tissue destruction (3). Hence, the findings of this study support that the presence and levels of *Synergistetes* cluster A are associated with the severity of periodontitis, which could well be related to the microecological changes, such as increased pocket depth, inflammation and anaerobiosis.

In conclusion, cluster A of the phylum *Synergistetes*, but not cluster B, is found at higher prevalence, numbers and proportions in saliva from patients with periodontitis than non-periodontitis subjects, and is associated with the severity of the disease. Although there are no differences in prevalence and numbers between CP and G-AgP, the proportion of this cluster tended to be higher in the latter form of the disease. Hence, cluster A of the phylum *Synergistetes* is associated with periodontitis.

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