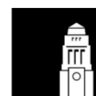

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Dysbiosis in the Oral Microbiomes of anti-CCP Positive Individuals at Risk of Developing Rheumatoid Arthritis

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ABSTRACT

Objectives

An increased prevalence of periodontitis and perturbation of the oral microbiome has been identified in rheumatoid arthritis (RA) patients. The periodontal pathogen *Porphyromonas gingivalis* may cause local citrullination of proteins, potentially triggering anti-citrullinated protein antibody production. However, it is not known if oral dysbiosis precedes the onset of clinical arthritis. This study comprehensively characterised the oral microbiome in anti-cyclic citrullinated peptide (anti-CCP) positive at-risk individuals without clinical synovitis (CCP+ at-risk).

Methods

Subgingival plaque was collected from periodontally healthy and diseased sites in 48 CCP+ at-risk, 26 early RA and 32 asymptomatic healthy control (HC) individuals. DNA libraries were sequenced on the Illumina Hiseq 3000 platform. Taxonomic profile and functional capability of the subgingival microbiome were compared between groups.

Results

At periodontally healthy sites, CCP+ at-risk individuals had significantly lower microbial richness compared with HC and early RA groups (P=0.004 and 0.021). Microbial community alterations were found at phylum, genus and species levels. A large proportion of the community differed significantly in membership (523 species; 35.6%) and structure (575 species; 39.1%) comparing CCP+ at-risk and HC groups. Certain core species, including *P. gingivalis*, had higher relative abundance in the CCP+ at-risk group. Seventeen COG functional units were significantly over-represented in the CCP+ at-risk group compared with HC (adjusted P value <0.05).

Conclusions

Anti-CCP positive at-risk individuals have dysbiotic subgingival microbiomes and increased abundance of *P. gingivalis* compared with controls. This supports the hypothesis that the oral microbiome and specifically *P. gingivalis* are important in RA initiation.

(246 words)

Keywords

Rheumatoid arthritis; oral microbiome; dysbiosis, periodontitis; *Porphyromonas gingivalis*

71 **INTRODUCTION**

72 Individuals at-risk of rheumatoid arthritis (RA) often have anti-citrullinated protein antibodies
73 (ACPA) well before the development of joint inflammation.[1, 2] Where the initiation of RA-
74 autoimmunity occurs is a critical question with significant implications for future preventative
75 strategies. Recent data have implicated mucosal sites and the local microbiome and there has been
76 considerable focus on the role of the oral mucosa and periodontium.[3, 4]

77 There is an increased prevalence of periodontitis in patients with both early and established RA.[5]
78 The subgingival microbiota in periodontitis, in particular the periodontal pathogens *Porphyromonas*
79 *gingivalis* and *Aggregatibacter actinomycetemcomitans*, may play a critical role in RA pathogenesis;
80 *P. gingivalis* by contributing to ACPA production through citrullination of proteins via its
81 peptidylarginine deiminase enzyme (PAD), and *A. actinomycetemcomitans* by inducing leukotoxic
82 hypercitrullination.[6-8]. We recently reported increased prevalence of periodontal inflammation and
83 *P. gingivalis* in anti-cyclic citrullinated peptide (anti-CCP) positive at-risk individuals without
84 arthritis (CCP+ at-risk), supporting the concept that periodontal inflammation and *P. gingivalis*
85 precede joint inflammation, as important risk factors in RA initiation.[9] *A. actinomycetemcomitans*
86 did not emerge as similarly significantly associated with at risk individuals; *A.*
87 *actinomycetemcomitans* is particularly important in severe generalised periodontitis,[10] which we did
88 not see in our cohort.

89 Periodontitis is a complex disease, mediated by consortia of co-operating bacteria and the host
90 responses to them. While *P. gingivalis* is a keystone pathogen that increases the risk of periodontitis,
91 it depends upon the activities of other members of the subgingival microbiome to establish within the
92 community and express full virulence. Thus, to fully understand the role of periodontitis in RA
93 pathogenesis, it is important to study the entire bacterial community. Although certain taxa, and
94 compositional and functional alterations were identified in RA-associated oral microbiomes,[11-13] it
95 is difficult to clarify the cause and effect of these findings once clinical arthritis has developed.
96 Furthermore, RA treatment is also likely to influence the oral microbiome.[12]

97 We therefore sought to comprehensively characterise the oral microbiome in CCP+ at-risk individuals
98 without clinical arthritis; we aimed to report differences in the metagenomes, characterised by a
99 shotgun metagenomic approach, sampled from periodontally healthy and diseased subgingival sites of
100 CCP+ at-risk individuals, early RA patients and healthy controls.

101

102 **MATERIALS AND METHODS**

103 Healthy controls (HC), CCP+ at-risk individuals with musculoskeletal symptoms but no clinical
104 synovitis and anti-CCP positive early RA patients (within the first 3 months of disease-modifying
105 anti-rheumatic drug, DMARD, therapy) were recruited. The three groups were balanced for age, sex,
106 and smoking status (Table S1).[9] Periodontal assessments and subgingival plaque sampling were
107 performed by three experienced dentists.[9] According to the latest Classification of Periodontal
108 Diseases and Conditions, periodontally healthy sites were defined as sites with ≤3 mm probing depth
109 and no bleeding on probing.[14] Diseased sites were those with ≥ 4 mm probing depth and ≥ 2 mm
110 clinical attachment loss (CAL).[15] Subgingival plaque samples from a maximum of three healthy
111 and three diseased sites were analysed for each participant using shotgun metagenomics sequencing
112 (Illumina Hiseq-3000). Microbial diversity and community composition were compared between
113 three groups. Periodontitis is a dysbiotic disease, with significant differences comparing microbiomes
114 from healthy and diseased subgingival sites. The term dysbiosis is also used here to describe
115 microbiomes from healthy sites that are distinct in composition from those of healthy sites from the
116 HC group. Further details are given in the online supplementary material.
117

118 **RESULTS**

119 **Microbial diversity**

120 Within periodontally healthy sites, the CCP+ at-risk group showed a significantly lower Abundance
121 Coverage Estimator (ACE) value compared with the HC group (*P*=0.004) and the early RA group
122 (*P*=0.021), indicating decreased estimated microbial richness of the subgingival microbiome (Figure
123 1).

124 **Bacterial community composition**

125 Overall, 28 bacterial phyla, 593 genera and 1472 species were identified. Significantly altered
126 community composition was found in the CCP+ at-risk group at different taxonomic levels. In
127 periodontally heathy sites, phylum *Synergistetes* was found with significantly higher relative
128 abundance in the CCP+ at-risk group compared with other groups (online supplementary Figure S1a).

129 Among the top 20 most predominant genera in periodontally heathy sites (Figure 2a), *Bifidobacterium*
130 and *Porphyromonas* were present with significantly increased relative abundance in the CCP+ at-risk
131 group (*P* = 0.027, 0.033). In pairwise comparison, 523 species (35.6% of the community) differed
132 significantly in membership and 575 species (39.1%) differed significantly in structure, comparing the
133 CCP+ at-risk and HC groups. Less difference was found in the community membership (62 species,
134 4.2%) and structure (42 species, 2.9%) comparing the early RA and HC groups (Figure 3a). Certain
135 significant differences were also found between groups in periodontally diseased sites, e.g. the
136 abundance of phylum *Chlorobi* was increased in the HC group compared with other groups (online

supplementary Figure 1b) (corrected $P < 0.05$). The genus *Porphyromonas* was significantly higher in the CCP+ at-risk group compared with other groups ($P = 0.015$), and *Capnocytophaga, Cardiobacterium, Neisseria* and *Streptococcus* were significantly more abundant in the early RA group (P= 0.009, 0.003, 0.024, 0.003) (Figure 2b). At species level, only 1.4% and 5.7% of the microbial community differed significantly in membership and structure between the CCP+ at-risk and HC groups (Figure 3b).

Core microbiome

The core microbiome, of which the species were present in at least 80% of the samples in each group, was used to compare stable associations between groups. Within periodontally healthy sites (Figure 4a), 81 species were identified in the core microbiome of all study participants. The core microbiome from the CCP+ at-risk group was much less diverse than that of the HC or early RA group. There was no core species exclusively belonging to the CCP+ group, unlike the HC and early RA groups which had 35 and 79 exclusive core species, respectively. In the periodontally diseased sites (Figure 4b), 42 species were found in the core microbiome of all groups. Importantly, 6, 2 and 190 species were identified as uniquely belonging to the HC, CCP+ at-risk and early RA core microbiomes, respectively (online supplementary Table S2-S3). Certain species were significantly more abundant in each group compared with the other groups within periodontally healthy or diseased sites (online supplementary Table S4). In particular, within both periodontally healthy and diseased sites, *Arthrobacter chlorophenolicus* and *P. gingivalis* were significantly more abundant in CCP + at-risk individuals.

Bacterial co-occurrence networks in subgingival microbiomes

In periodontally healthy sites, Spearman's correlation analysis identified 347, 83 and 1024 edges as strong (q < -0.7 or > 0.7) and significant (corrected $P < 0.01$) pairwise correlations between nodes (species) in each the HC, CCP + at-risk and early RA groups, respectively (online supplementary Figure S2). In periodontally diseased sites, there were 49, 139 and 365 edges identified in HC, CCP + at-risk and early RA groups, respectively (online supplementary Figure S3). The edge/node ratio (density) of the network represents the number of co-occurrence instances in a microbial community; in the early RA group this was higher than that of other groups in both periodontally healthy and diseased sites, reflecting a dysbiosis of the subgingival microbiome in early RA patients (online supplementary Table S5).

To gain deeper insights into the differences between groups, the hubs in each network were identified by ranking the top 20 nodes with the MCC algorithm. In the periodontally heathy sites (Figure 5a), the cluster of *Neisseria* spp. by which the network of HC group was dominated, was not found in the hubs of other groups. Species including *Filifactor alocis*, *Campylobacter rectus*, *Porphyromonas*

172 *endodontalis* and *Treponema vincentii* formed the network hubs for both HC and CCP + at-risk
173 groups, while the early RA group showed entirely different network hubs. Within the periodontally
174 diseased sites (Figure 5b), *Actinomyces viscosus* and *Actinomyces urogenitalis* were identified in the
175 network hubs of all groups indicating an implication in the development of periodontal disease
176 irrespective of RA status. Intriguingly, the periodontal pathogen *A. actinomycetemcomitans,* which
177 may also initiate protein citrullination in RA, was one of the hubs of the early RA group.

178 **Functional capabilities of subgingival plaque microbiomes**

179 Abundances of 3034 clusters of orthologous genes (COGs) functional units were normalized and
180 compared between groups. Within periodontally healthy sites, 17 functional units were significantly
181 over-represented in the CCP+ at-risk group compared with the HC group and 5 functional units were
182 significantly over-represented in the early RA group compared with the HC group (online
183 supplementary Table S6) (corrected $P < 0.05$). In periodontally diseased sites, significant differences
184 were found comparing the early RA group with the HC and CCP+ at-risk groups (online
185 supplementary Table S7). The functional unit of "PAD and related enzymes" were detected in 65.6%,
186 68.8% and 69.2% of samples in the HC, CCP+ at-risk and early RA groups from periodontally
187 healthy sites and in 55.6%, 69.2% and 56.3% of each group from diseased sites. No significant
188 difference was found in the normalized counts between groups either in periodontally healthy or
189 diseased sites (Figure 6).
190

191 **DISCUSSION**

192 Although intensively studied, the mechanisms of disease initiation and development of autoimmunity
193 in RA are still unclear.[16] ACPA are highly specific for RA and can be detected years before joint
194 inflammation, suggesting a preclinical phase of RA, which could be a window of opportunity for
195 disease prevention.[17] We previously showed that periodontitis and *P. gingivalis* were increased
196 before clinical or subclinical joint inflammation in individuals at risk of RA.[9]. Other studies have
197 identified increased periodontitis in the first-degree relatives of RA patients.[18, 19] Compared with
198 healthy controls, the alterations in the subgingival microbial community of RA patients has been
199 reported in different studies,[11-13] suggesting a potential role of oral microbial dysbiosis in RA
200 development. However, it is unknown if subgingival microbial dysbiosis precedes the onset of RA.
201 The present study, to our knowledge, is the first comprehensive characterisation of the subgingival
202 microbiome from both periodontally healthy and diseased sites in at-risk individuals. To preclude the
203 effect of established periodontitis on the subgingival microbiome, analysis was performed on the
204 samples from shallow gingival sulci (3 mm depth or less) with no bleeding on probing. This study
205 comprised a relatively small sample size but participant groups were well balanced for age, sex and
206 smoking status. Other variables currently being investigated for possible associations with
207 periodontal disease (e.g. BMI, race, alcohol, education level) may also influence the subgingival

208 microbiome. Larger samples size will be needed to more completely define the role of the
209 subgingival microbiome in the development and progression of RA.

210 In CCP+ at-risk individuals, significant alterations were found in the composition of the periodontally
211 healthy subgingival microbiome at different levels, which distinguished this group from matched
212 controls and early RA patients. In agreement with present study, compositional change of salivary
213 microbiota and decreased microbial diversity were found in individuals at high-risk for RA in a recent
214 study.[20]

215 Most previous studies utilized 16S rRNA gene sequencing to analyse the oral microbiome of RA
216 patients.[11, 13, 20] However, a major limitation of this method is that only a single region of the
217 bacterial genome can be sequenced and it is difficult to distinguish the species when their 16S rRNA
218 gene sequences display high similarities.[21] The present study utilized shotgun metagenomics, which
219 has several advantages including more confident identification of bacterial species, increased
220 detection of diversity and prediction of genes.[22]

221 *P. gingivalis* may contribute to RA aetiology via the citrullination of local antigens by its PAD.[7, 23]
222 While some previous studies have examined the association between *P. gingivalis*, and established
223 RA, few have looked at *P. gingivalis* in individuals at risk of RA. Studies determining levels of
224 antibodies against *P. gingivalis*, or its virulence determinants, in HC, at-risk or established RA groups
225 have been equivocal, possibly due to methodological and sampling differences.[7, 24-28] A recent
226 study demonstrated decreased levels of *P. gingival*is in the saliva of high-risk individuals compared
227 with healthy controls using 16S rRNA gene sequencing.[20] Analysis of the microbiome of saliva and
228 supra-gingival dental plaque using shotgun sequencing revealed *P. gingivalis* to be enriched in
229 healthy controls rather than RA patients.[12] In another study, periodontitis, but not the subgingival
230 presence of *P. gingivalis*, was more prevalent in patients who later progressed to classifiable RA.[29]
231 De Smit *et al* concluded that, while there was evidence that periodontitis may precede symptomatic
232 RA, there was insufficient evidence to confirm a role specifically for *P. gingivalis* in disease
233 progression.[30] Thus, while the link between periodontitis and RA is established, the specific roles of
234 *P. gingivalis* or its PAD have been less clear. Our data indicate anti-CCP positive at-risk individuals
235 have increased abundance of *P. gingivalis* compared with healthy controls.

236 A lower abundance of *P. gingivalis* as well as alterations in microbial composition and functional
237 capability were found in the early RA group, which may be related to the inflammatory burden of RA.
238 Lopez-Oliva *et al*. proposed RA may act as a condition shaping the subgingival microbiome,
239 particularly promoting the growth of certain organisms.[13] Moreover, these patients were receiving
240 DMARDs, although for less than three months. It is likely that RA therapy, particularly drugs with
241 additional antibacterial properties,[31, 32] can influence the subgingival microbiome. RA regimes

242 with immunomodulatory effects may influence both the development of the subgingival microbiome
243 and progression of periodontitis.[33, 34] A recent shotgun sequencing study identified alterations in
244 the oral microbiome in RA patients, which were partially restored by DMARD treatment.[12]

245 The presence and abundance of PAD and related enzymes (the COG functional unit representing a
246 family of orthologous protein-coding genes) were similar between groups. This is interesting given
247 the differences that were observed between the groups in *P. gingivalis* abundance. Although *P.*
248 *gingivalis* was once considered unique among prokaryotes in producing a PAD, PAD homologues
249 were recently found in other *Porphyromonas* species.[35] Thus, the PAD in the subgingival
250 microbiomes may arise from a range of species, not all of which may express PAD at the levels and
251 with similar activity to the *P. gingivalis* PAD. A recent study also reported variations in the active
252 site of PAD detected in clinical isolates of *P. gingivalis*, one of which was associated with increased
253 *in vitro* activity.[36] Our data cannot reveal differences in the expression or activity of PADs, or *P.*
254 *gingivalis* PAD specifically. Detailed comparison of the active *P. gingivalis* PAD site and potential
255 enzyme activity in different groups related to RA status would be an important area for future work.

256 Other periodontal pathogens may also contribute to protein citrullination via routes different from *P.*
257 *gingivalis*. The leukotoxin-A (LtxA) produced by *A. actinomycetemcomitans* has been implicated in
258 inducing leukotoxic hypercitrullination, and exposure to *A. actinomycetemcomitans* was associated
259 with ACPA.[6] This species was not dominant in the present study; considerable variations in
260 isolation rates of *A. actinomycetemcomitans* have been reported in the literature, which may be the
261 consequence of geographical differences in prevalence and methodological differences.[37] *P.*
262 *intermedia* was recently reported to be associated with antibody responses to a novel citrullinated
263 peptide related to RA,[38] but abundance of this organism did not emerge in our analyses as different
264 in the groups sampled. It is clear that the microbiome of these patients was highly perturbed
265 compared with both healthy controls and CCP+ at-risk individuals and the influence of DMARDs and
266 duration of therapy requires further consideration. Intriguingly, there were some species that have not
267 previously been reported as abundant in the subgingival plaque of early RA patients, e.g *Neisseria*
268 *gonorrhoeae* (online supplementary Table S4). This pathogen of the urogenital tract can adapt to
269 display asymptomatic survival in the human nasopharynx and oropharynx, providing a potential
270 reservoir for their further spread. {Marangoni, 2020 #60}{Quillin, 2018 #61} There is evidence of
271 widespread horizontal gene transfer in the genus *Neisseria* [Maiden, 2008] and of commensal species
272 sharing many gene sequences with closely related pathogenic species [Marri et al, 2010] and this may
273 have impacted on our findings regarding the relative abundance of individual *Neisseria* species. *In*
274 *vitro* culture and more in-depth analysis are necessary to clarify the presence of *N. gonorrhoea* and its
275 potential contribution to oral microbial dysbiosis.

276

277 Several species were identified as hubs of the co-occurrence networks; these in the CCP + at-risk
278 group may be indirectly involved in the pathogenesis of RA via the interplay with *P. gingivalis* and
279 possibly by supporting communities that promote citrullination by multiple routes. Among these hub
280 species, *Streptococcus* spp. are considered the principle early colonizers in dental plaque, and their
281 colonisation influences the composition of maturing plaque.[39] *F. nucleatum*, which was
282 demonstrated to accelerate collagen induced arthritis in mice, functions in a bridging complex
283 between early and late colonizers such as *P. gingivalis*.[40] A strong synergy was also observed
284 between *T. denticola* and *P. gingivalis* in biofilm formation.[41] Therefore, it is logical to consider the
285 overall capacity of the microbial community in future work.

286 In conclusion, this study has demonstrated dysbiosis in the subgingival microbiome alongside the
287 specific increase of *P. gingivalis* in individuals at-risk of RA. We propose these may play an
288 important role in the initiation of RA and that periodontitis and the observed oral dysbiosis may be
289 attractive targets for future preventative interventions, such as periodontal therapy, in individuals at
290 risk of RA.
291
292 *2847 words*
293
294
295 **Key messages:** (*up to 5 bullet points*)
296 **What is already known about this subject?**
297 • Rheumatoid arthritis (RA) patients have increased periodontal disease and a perturbed oral
298 microbiome. The periodontal pathogen *P. gingivalis* is able to citrullinate proteins via its
299 peptidylarginine deiminase enzyme (PAD) and can generate citrullinated antigens that may drive the
300 autoimmune response in RA.
301 • Periodontitis and *P. gingivalis* were increased before joint inflammation in individuals at risk
302 of RA, supporting the concept of periodontal inflammation and *P. gingivalis* as important risk factors
303 in RA initiation.
304
305 **What does this study add?**
306 • This is the first study to demonstrate dysbiosis, including an increase of *P. gingivalis*, in the
307 periodontally healthy microbiome (and altered diseased subgingival microbiomes) of individuals at
308 risk of developing RA compared with healthy controls.
309
310 **How might this impact on clinical practice or future developments?**
311 • Our results indicate that dysbiosis in the subgingival microbiome precedes the onset of joint
312 inflammation in at-risk individuals. This dysbiosis, together with the increase of *P. gingivalis*, may

313 play an important role in the initiation of RA.

314 • Taken together with our previous findings, periodontal disease and the observed oral
315 dysbiosis could be targets for future preventive interventions in individuals at risk of RA.
316 Investigation of the overall metabolic capability of the subgingival microbiome may provide novel
317 insights into the pathogenesis of RA.

318

319

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324

325 **Competing interests**

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340

341 **Author contribution**

342 ZC: Conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing,
343 visualisation

344 TD: Conceptualisation, methodology, validation, formal analysis, data curation, writing, supervision.

345 KM: Conceptualisation, methodology, validation, data curation, writing

346 JM: Conceptualisation, methodology, validation, writing, supervision

347 LH: Conceptualisation, methodology, investigation, writing

348 VC: Conceptualisation, methodology, investigation, writing

349 AS: Conceptualisation, methodology, investigation, writing

350 AT: Conceptualisation, methodology, investigation, writing
351 PE: Conceptualisation, writing, supervision, administration, funding
352 DD: Conceptualisation, methodology, validation, writing, supervision, administration, funding
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477 **Figure 1. Comparison of α-diversity in healthy control (HC), CCP+ at-risk and early RA groups using**
478 **samples from periodontally healthy sites and diseased sites.** Abundance Coverage Estimator (ACE)

479 index was significantly decreased in the CCP+ at-risk group compared with the HC group in
480 periodontally healthy sites (Kruskal-Wallis test).

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483 **Figure 2. Taxonomic profiles for the 20 most abundant genera in subgingival plaque from**
484 **periodontally healthy and diseased sites in healthy control (HC), CCP+ at-risk and early RA groups.**
485 Relative abundance of the 20 most abundant genera within (a) periodontally healthy sites and (b)
486 diseased sites was plotted for each group. The permutation test (one-sided signassoc function,
487 indicspecies R-package) was used to find the genera with significantly different relative abundances
488 between groups. *: corrected $P < 0.05$ (Sidak's correction).

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491 **Figure 3. Phylogenetic tree representing normalized mean relative abundance of species (stacked**
492 **bar chart) in the subgingival microbiome of (a) periodontally heathy and (b) periodontally diseased**
493 **sites (phylogenetic tree constructed using the webserver iTOL.embl.de).**

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496 **Figure 4. Overlap analysis of the group specific and shared core species.** Core species in each group
497 of periodontally healthy and diseased site samples were identified, respectively (> 80% prevalence).
498 Number of group-specific and shared core species were visualized for (a) healthy sites and (b)
499 diseased sites.

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505 **Figure 5. Identification in plaque from periodontally healthy and diseased sites of hubs in the**

506 **networks of healthy control (HC), CCP + at-risk and early RA groups.** The top 20 nodes (species)
507 ranked by Maximal Clique Centrality were displayed in circular layout for each group from (a)
508 periodontally healthy and (b) diseased site samples. Nodes are coloured based on rank; dark colour
509 denotes high ranks. Green dashed line: HC, orange: CCP+ at risk, blue: early RA.

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513 **Figure 6. Normalized count of peptidylarginine deiminase enzyme (PAD) and related enzymes in**
514 **healthy control (HC), CCP+ at-risk and early RA groups using samples from periodontally healthy**
515 **sites and diseased sites.** Abundance of PAD and related enzymes was normalized by sequencing
516 depth and compared between groups using the Waldtest in *DESeq2* R package. No significant
517 difference was found between groups either in (a) periodontally healthy or (b) diseased sites
518 (corrected $P > 0.05$).

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