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Oral microbiota in patients with atherosclerosis

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ABSTRACT

Background and aims: Recent evidence suggests that the microbiota may be considered as an environmental factor that contributes to the development of atherosclerosis. Periodontal disease has been associated with cardio- and cerebrovascular events, and inflammation in the periodontium is suggested to increase the systemic inflammatory level of the host, which may in turn influence plaque composition and rupture. We previously showed that bacteria from the oral cavity and the gut could be found in atherosclerotic plaques.
Methods: To elucidate whether the oral microbiota composition differed between patients with asymptomatic and symptomatic atherosclerosis we performed pyrosequencing of the oral microbiota of 92 individuals including patients with asymptomatic and symptomatic atherosclerosis and control individuals without carotid plaques or previous stroke or myocardial infarction.
Results: The overall microbial structure was similar in controls and atherosclerosis patients, but patients with symptomatic atherosclerosis had higher relative abundance of *Anaeroglobus* (mean 0.040% (SD 0.049)) than the control group (0.010% (SD 0.028)) ($P = 0.03$). Using linear regression analysis, we found that *Parvimonas* associated positively with uCRP and *Capnocytophaga*, *Catonella* and *Lactobacillus* associated with blood lipid markers. In conclusion, abundance of *Anaeroglobus* in the oral cavity could be associated with symptomatic atherosclerosis.

1. Introduction

Atherosclerotic vascular disease is determined by genetic and environmental factors such as diet and lifestyle, and recent evidence suggests that the host microbiota may be considered an environmental factor that contributes to the stability of atherosclerotic plaques [1–5]. Periodontal disease has been associated with cardio- and cerebrovascular events, and inflammation in the periodontium is suggested to increase the systemic inflammatory level of the host, which may influence plaque composition and rupture [6–8]. A mechanism involving oral bacteria translocating into the blood stream through the inflamed gums has been proposed to affect atherogenesis [9]. Interestingly, improvement in periodontal status has been shown to slow progression of increased intima-media thickness in the common carotid artery in a 3-year longitudinal study [10]. We previously showed that bacteria from the oral cavity and the gut can be recovered from the atherosclerotic plaque [11] and Hyvärinen et al. (2012) showed that the periodontal pathogen *Aggregatibacter actinomycetemcomitans* was increased in the saliva of patients with both symptomatic and asymptomatic coronary disease as compared to healthy individuals [6]. *A. actinomycetemcomitans* has been shown to affect progression of plaques in mouse models [12]. However, as only 11% of the patients were positive for the bacterium [6], other pathogens or mechanisms also deserve further investigation.

To elucidate whether the oral microbiota composition was altered in patients with asymptomatic and symptomatic atherosclerosis, in the present study we analyzed by pyrosequencing of the 16S rRNA gene the composition of the oral microbiota of 92 subjects, including patients with asymptomatic and symptomatic atherosclerosis, as well as from control individuals without carotid plaques and no previous stroke or myocardial infarction.

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2. Materials and methods

2.1. Mouth swab collection

The patient samples were obtained from the Western Region INitiative to Gather information on Atherosclerosis (WINGA) study which recruits patients referred for ultrasound examination of atherosclerosis in their carotid arteries at the vascular diagnostic unit at Sahlgrenska University hospital. Control subjects were recruited from the general population. Patients were consecutively recruited from the WINGA database with the inclusion criteria of having at least one carotid artery plaque $\geq$15 mm^2. No exclusion criteria were applied. Based on medical history, patients were divided in symptomatic (having had previous myocardial infarction or stroke) and asymptomatic subjects. The control group was also asymptomatic and was recruited from the population register and initially matched to the patient groups by age and sex. For the current study the matching was not used and recruitment was done consecutively of subjects fulfilling the above criteria. Patients were asked for informed consent to participate in a more in-depth examination including ultrasound examination of the carotid arteries of all groups, blood sampling, detailed questionnaire and mouth swabs. Regular cotton swabs were autoclaved and applied in a circular movement to the hard palate, both the inner cheeks and the upper and lower gum from: *i*) patients with either symptomatic atherosclerotic disease (defined as patients with atherosclerosis in their carotid arteries ($\geq$15 mm^2) and having had prior myocardial infarction or stroke), *ii*) patients with asymptomatic atherosclerotic disease (defined as patients with atherosclerosis in their carotid arteries ($\geq$15 mm^2) and no prior myocardial infarction or stroke) or *iii*) controls (recruited from general population, no atherosclerosis (see definition below) and no prior myocardial infarction or stroke). Atherosclerosis of the carotid artery was defined according to the Manheim consensus [13]. After collection, oral swabs were placed at −80 °C until further analysis. At time of DNA extraction, the cotton swab tip was cut and placed in lysis buffer, after which DNA was extracted as described previously [14].

2.2. PCR amplification and pyrosequencing of bacterial 16S rRNA genes

16S rRNA genes were amplified and sequenced as described previously [15,16]. Additional information can be found in the supplementary material.

2.3. Statistical analysis

The Quantitative Insights Into Microbial Ecology (QIIME) software package [17] was used to analyse the composition of the oral microbiota and identify microbial taxa (operational taxonomic units, OTU:s) which relative abundance was different between the groups (more details about the analysis are presented in the supplementary material). OTU:s (at six taxonomic levels, from phylum to genus level) differing between groups were identified in QIIME with the non-parametric Kruskal–Wallis test. To determine which of the three groups differed (which was not reported in QIIME), the OTU:s that had a *P*-value < 0.1 in the QIIME analysis (n = 57) were thereafter divided into phylum and genus level, after which each OTU was analyzed separately with the non-parametric Kruskal–Wallis test together with Dunn's multiple comparison's correction using Graphpad prism version 6 (Graphpad Software Inc.). In order to correct for the variation in sequencing depth among the samples, all microbial communities were randomly subsampled at a depth of 1467 sequences per sample, which excluded two samples from the analysis. These subsampled datasets were then used to calculate weighted and unweighted UniFrac [18] and to find associations between metadata and bacterial genera. Linear regression analysis was calculated using Graphpad prism. Linear discriminant analysis effect size (LEfSE, www.huttenhower.sph.harvard.edu/galaxy/) was applied on the OTU table according to the method of Segata et al., 2011 [19], to identify potential bacterial taxa that could be associated with symptomatic atherosclerosis. In addition, LEfSE was also applied on the aggregated group of controls without atherosclerosis and asymptomatic atherosclerosis subjects, which was compared with the symptomatic atherosclerosis group.

3. Results

A total of 103 mouth swabs were collected. We excluded 11 patients from the analysis due to either missing data on atherosclerosis (n = 4) or technical failures in DNA extraction/analysis (n = 7) leaving a total of 92 patients for the analysis (symptomatic atherosclerosis, n = 27; asymptomatic atherosclerosis, n = 35 and

Table 1
Characteristics of the study population.

Variable	Symptomatic with atherosclerosis	Asymptomatic with atherosclerosis	Control group	*P*-value
N	27	35	30	–
Sex, female (n, %)	15 (50)	20 (57)	14 (52)	0.67[b]
Age (median, IQR)	69 (64–78)	69 (61–72)	64 (62–71)	0.06[a]
BMI (kg/m^2)	26.6 (24.3–30.2)	24.6 (22.2–23.3)	27.4 (24.0–29.4)	0.10[a]
Smoker (n, %)	5 (23)	3 (9)	5 (21)	0.46[b]
SBP (mmHg)	140 (120–150)	130 (124–140)	130 (126–140)	0.63[a]
DBP (mmHg)	70 (65–80)[c]	74 (68–80)[c,d]	80 (75–80)[d]	0.022[a]
Previous stroke (n, %)	13 (48)	0	0	<0.001[b]
Previous MI (n, %)	15 (56)	0	0	<0.001[b]
Diabetes (n, %)	7 (26)	2 (6)	2 (7)	0.029[b]
Treated hyperlipidemia (n, %)	18 (67)	7 (20)	6 (20)	<0.001[b]
Treated hypertension (n, %)	21 (77)	17 (49)	9 (30)	0.0014[b]
s-Cholesterol (mmol/L)	4.6 (3.9–5,2)[c]	5.9 (4.9–6.3)[d]	5.8 (5.0–6.5)[d]	0.0015[a]
s-HDL (mmol/L)	1.3 (0.9–1.7)	1.7 (1.3–2.2)	1.6 (1.2–2.1)	0.08[a]
s-LDL (mmol/L)	1.3 (0.9–1.7)[c]	3.2 (2.6–4.2)[d]	3.5 (3.0–4.3)[d]	0.0020[a]
hsC-reactive protein (mg/L)	3.4 (1.2–6.4)	1.6 (0.8–2.6)	1.4 (0.8–3.1)	0.064[a]

BMI, body mass index; DBP, Diastolic blood pressure; SBP, Systolic blood pressure; hs, high-sensitive. Median and inter-quartile range where applicable. Significant differences between groups were analyzed with the:
[a] Kruskal–Wallis test with Dunn's multiple comparison correction (values with differing superscripts (c, d) are significantly different) or with
[b] Chi-square test.