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ORIGINAL ARTICLE

IL6 −174 genotype associated with *Aggregatibacter actinomycetemcomitans* in Indians

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AIM:Genetic factors have recently been associated with presence of *Aggregatibacter actinomycetemcomitans* subgingivally in populations living in industrialized countries. The aim of this study was to analyse associations between Interleukin-6 (*IL6*) single nucleotide polymorphisms and presence and levels of *A. actinomycetemcomitans* and other subgingival microbes in a rural Indian population.

SUBJECTS AND METHODS:A total of 251 individuals from a rural village in India with a periodontal phenotype ranging from healthy to severe periodontitis were included. Checkerboard DNA-DNA analysis was performed to detect 40 periodontal taxa in subgingival plaque samples. Genomic DNA was extracted to genotype five polymorphisms in the *IL6* promoter region.

RESULTS:The *IL6* −174 GG genotype was associated with high (above median) counts of *A. actinomycetemcomitans* (both in all subjects and in periodontally healthy only) and with presence and counts of *Capnocytophaga sputigena*. Differences in detection of several other bacteria were noted between periodontitis and healthy subjects.

CONCLUSIONS:These findings support the influence of genetic factors on the subgingival microbiota.

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Keywords: periodontitis; interleukin-6; genetic; bacteria; *Aggregatibacter actinomycetemcomitans*

Introduction

The oral cavity may contain as many as 19 000 bacterial phylotypes (Keijser *et al*, 2008). We have recently introduced the concept of periodontal infectogenomics, which studies the effect host genetic factors have on the composition of the subgingival microbiota of each individual. This is based on the assumption that genetic factors in the host seem to play a major role in deciding which bacteria (commensal and pathogenic) are able to colonize the host (Nibali *et al*, 2009a).

Most studies on subgingival microbes with a putative influence on periodontally relevant immune responses have been carried out in industrialized communities with routine access to dental care (Hardie and Bowden, 1975; Papapanou *et al*, 1993; Umeda *et al*, 1998). Analysis of rural populations not subjected to dental care allows the study of possible effects of host genetic factors in the composition of the subgingival microbiota in almost pristine conditions, undisturbed by routine tooth scaling and use of antibiotics.

We have recently shown an association between interleukin-6 (*IL6*) genetic factors and clinical status in non-smokers living in a rural Indian village (Franch Chillida *et al*, 2009). The main aim of this analysis was to study the association between *IL6* genetic variants and the presence and levels (counts) of *Aggregatibacter actinomycetemcomitans* in subgingival plaque samples from the same population. Secondary aims were to explore the association between *IL6* genetic factors and the detection and counts of other bacteria, and the association between periodontal status and the detection and levels of other bacteria.

Materials and methods

Subject selection

The population reported here has also been described elsewhere (Franch Chillida *et al*, 2009). The study took place in the village of Dokur, Andhra Pradesh, where the Institute for Rural Health Studies (IRHS), a local Non-Governmental Organization (NGO), runs a rural clinic. All adult inhabitants between the ages of 18 and 70 were invited to attend a screening visit by means of local advertising within the village. A group of 500 villagers attended and were initially screened based on the following exclusion criteria: (i) presence of serious systemic conditions (malaria, tuberculosis or

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cardiovascular disease), (ii) presence of infectious or parasitic diseases, (iii) pregnancy or breastfeeding, (iv) antibiotic or anti-inflammatory medications within the last 3 months, (v) fewer than 16 standing teeth.

Two hundred and fifty-one suitable subjects (10% of the total village population) were identified and included in the study.

Ethical considerations

The study was conducted in full accordance with ethics principles, including the World Medical Association Declaration of Helsinki (version 2002). Ethical approval for the study was obtained from the Medical Board of IRHS and conformed to the Indian Council of Medical Research Guidelines on Medical Ethics 2000 (Indian Council of Medical Research, Ansari Nager, New Delhi). All subjects were native to the village and voluntary informed consent to participation in the study was obtained from each by means of a written consent form, translated into Telugu and also read aloud to each subject by a fluent Telugu speaker. Demographic data and self-reported data on education, social status, type of toothbrushing, alcohol consumption and smoking habit were recorded (Franch Chillida *et al*, 2009).

Clinical data collection

Full-mouth periodontal examination of probing pocket depth (PPD) and clinical attachment level (CAL) was carried out for each subject with an EN-15 probe on six sites per tooth. The clinical examination was performed by one single operator (IM), calibrated to an exact kappa level of 0.85 for PPD. Patients were divided into two groups (periodontitis/healthy) based on their periodontal status. Both definitions suggested by the European Workshop on Periodontitis (EWP) (Tonetti *et al*, 2005) were used:

- Presence of proximal attachment loss of ≥3 mm in ≥2 non-adjacent teeth (EWP1)
- Presence of proximal attachment loss of ≥5 mm in ≥30% of teeth present (EWP2)

Microbiological analysis

Dental plaque samples were taken from the mesiobuccal aspects of the upper right and lower left first molar teeth, unless a deeper pocket had been found at another site, in which case this site was used in preference. Subgingival plaque was removed from each site isolated with cotton rolls, following removal of supragingival plaque, using sterile 13/14 double-ended Columbia curettes. The scraped mass was transferred from the curette to an Eppendorf tube containing 150 μl of sterile TE buffer (10 mM Tris HCl, 1.0 mM EDTA, pH 7.6) and subsequently transferred to the University of Berne. The presence of 40 periodontal pathogens was evaluated using checkerboard DNA-DNA hybridization technique (Socransky *et al*, 1994).

Genetic analysis

Samples of buccal mucosal cells were taken from all subjects using wire cytology brushes (Medical Wire and Equipment Company, Wiltshire, UK) (Franch Chillida *et al*, 2009). Genomic DNA was extracted from these samples and blindly genotyped for polymorphisms at positions −174 (CCTTTAGCAT[C-G]GCAAGAC, rs 1800795), −572 (CAACAGCC[C-G]CTCACAG, rs 1800796), −1363 (CACTGTTTTATC[G-T]GATCTTG, rs 2069827) and −6106 (TCTCTACA[A-T]TAAGAAA-TAC) and −1480 (ACCGTCTCT[C-G]TGTTTAG) in the *IL6* gene by real time polymerase chain reaction (PCR) as previously described (Nibali *et al*, 2008b).

Statistical analysis

Comparisons of continuous and categorical data between groups (periodontitis and healthy) were analysed with ANOVA and Chi-square test, respectively. The alpha value was set at 0.05.

The approach to evaluate the association between genetic factors, clinical diagnosis and microbiological outcomes was performed using the SPSS 12.0 package. The main outcome was association between *IL6* −174 GG genotype and the presence (at detection threshold 1×10^4) and levels (total counts) of *A. actinomycetemcomitans*. An exploratory analysis was performed to investigate associations between all studied *IL6* genetic variables (and clinical diagnosis) and other taxa and bacterial complexes. The α value for this exploratory analysis was lowered to 0.01 to adjust for multiple testing. Microbiological data were available from two sites for all subjects. Data on detection or not of all 40 studied taxa are reported. For analysis of bacterial counts, negative (no detection) reactions were given an arbitrary value of 0.99×10^4, just below detection limit. The mean individual count for each species was obtained as the averaged between the two sites in each patient. *A priori*, median values for each bacterium were calculated and two clusters of patients for each bacterium were identified: high (above median) and low (below median) counts. Therefore, the microbiological outcomes were as follows: (i) detection of each bacterium (at 1×10^4 detection value); (ii) high or low levels (counts) of each bacterium (both categorical data) and (iii) absolute counts of each bacterium (continuous data). Subgroup analyses were performed in subjects divided by their periodontal status (healthy/periodontitis). Multiple logistic regression analysis adjusting for confounders (age, smoking, gender and type of toothbrushing) was performed to investigate these associations (categorical variables). Linear logistic regression adjusting for confounders (age, smoking, gender and type of toothbrushing) was used to detect differences in bacterial counts (continuous variables) between genotypes and by clinical diagnosis.

Results

A total of 251 subjects took part in the study. Their demographic and clinical characteristics have been reported elsewhere (Franch Chillida *et al*, 2009) (Table 1). Forty-two per cent and 19% of subjects were diagnosed with periodontitis, according to EWP1 and EWP2 definitions respectively (Tonetti *et al*, 2003).

Table 1 Demographic and clinical characteristics of the participants, as divided by their periodontal status

			EWP1		*EWP2*	
		All subjects (n = 251)	*Periodontitis (n = 105)*	*Non-periodontitis (n = 146)*	*Periodontitis (n = 47)*	*Non-periodontitis (n = 204)*
Age			45.8 ± 10.5	37.5 ± 11.1	49.6 ± 10.3	39.0 ± 10.9
Gender (female)		162 (64.5%)	62 (59.0%)	100 (68.5%)	28 (59.6%)	134 (65.7%)
Smoking	Current	44 (17.5%)	26 (24.8%)	18 (12.3%)	14 (29.8%)	30(14.7%)
	Never	207 (82.5%)	79 (75.2%)	128(87.7%)	33(70.2%)	174(85.3%)
Clinical data	Number of sites PPD 4–6 mm	16.1 ± 22.1	38.5 ± 17.4	0.1 ± 0.4	45.5 ± 15.9	8.7 ± 15.6
	Number of sites PPD >6 mm	3.3 ± 8.1	7.9 ± 11.1	0.0 ± 0.2	15.1 ± 13.1	0.6 ± 1.8
	Number of sites CAL >4 mm	16.6 ± 22.1	39.5 ± 16.3	0.1 ± 0.6	47.5 ± 15.5	9.5 ± 16.6
	Number of sites CAL >6 mm	6.1 ± 14.0	14.5 ± 18.5	0.0 ± 0.4	27.5 ± 21.1	1.1 ± 2.7

PPD, probing pocket depth; CAL, clinical attachment level.

Thirteen subjects resulted *IL6* −174 C homozygous, 46 CG and 185 GG, while 7 could not be scored. The distribution of genetic polymorphisms has been reported elsewhere and satisfied the chi-squared analysis for Hardy–Weinberg equilibrium on all subjects (Franch Chillida *et al*, 2009).

The detection of the 40 studied taxa ranged from 98% of subjects for *Prevotella intermedia* to 51% for *Capnocytophaga sputigena*. In particular, 93% of subjects were positive for *A. actinomycetemcomitans*, 93% for *Porphyromonas gingivalis*, 90% for *Tannerella forsythia*, 70% for *Treponema denticola* and 60% for *Campylobacter rectus*. The mean counts ranged from 12.7×10^5 for *N. mucosa* to 0.2×10^5 for *Propionibacterium acnes*. Average counts for *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* for all subjects were respectively 5.1×10^5, 12.0×10^5 and 6.4×10^5.

Association between IL6 genetic factors and bacterial detection

IL6 −174 GG genotype subjects exhibited high (above median) levels of *A. actinomycetemcomitans* compared with CC and CG individuals, irrespective of clinical diagnosis [adjusted $P = 0.043$, odd ratio (OR) = 1.89, 95% confidence interval (CI) = 1.02–3.50] (Figure 1). This association was confirmed among the subgroup of non-periodontitis individuals (according to EWP2) (adjusted $P = 0.037$, OR = 2.11, 95% CI = 1.0–4.2).

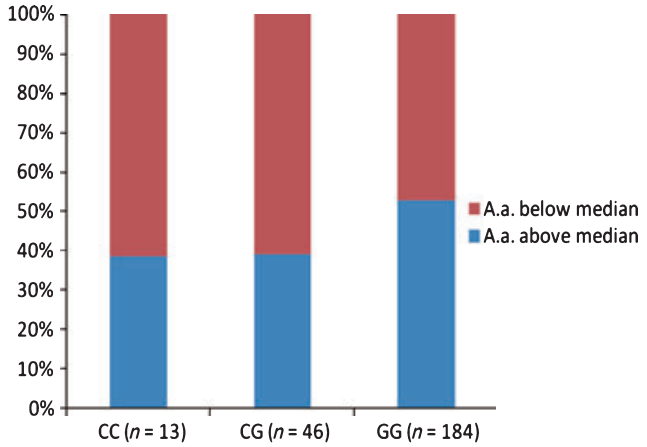

Figure 1 Representation of number of subjects with *Aggregatibacter actinomycetecomitans* above or below median according to their *IL6* −174 genotypes. GG *vs* CG/CC $P = 0.043$, odd ratio (OR) = 1.89, 95% confidence interval (CI) = 1.02–3.50

Among other bacteria, *IL6* −174 GG genotype subjects showed increased detection, and high (above median) levels of *C. sputigena* (adjusted $P = 0.003$, OR = 2.60, 95% CI = 1.38–4.90 and $P = 0.009$, OR = 2.29, 95% CI = 1.23–4.26 respectively). No statistically significant differences in percentage of bacterial complexes (Socransky *et al*, 1998) were found according to *IL6* −174 genotypes (Figure 2).

Associations between clinical diagnosis and bacterial detection

Figure 3 shows the frequency of detection of the 40 studied bacteria in periodontitis and healthy individuals according to EWP1 (Tonetti *et al*, 2003). Statistically significant associations were found between diagnosis of periodontitis (EWP1) and presence of *N. mucosa* ($P = 0.008$) and *Capnocytophaga ochracea* ($P = 0.001$), low (below-median) counts of *N. mucosa* ($P < 0.001$), and high (above median) counts of *Parvimonas micra* (previously *Peptostreptococcus micros*) ($P = 0.002$). Periodontitis patients also exhibited lower absolute counts of *N. mucosa* ($P = 0.003$).

EWP2 diagnosis of periodontitis was associated with absence (no detection) of *C. ochracea* ($P = 0.005$) and *S. noxia* ($P < 0.001$) and higher absolute counts of *P. micra* ($P = 0.025$). When bacteria were grouped in complexes, periodontal status (EWP1) was associated with percentage of bacteria undefined in complexes ('others') ($P = 0.012$).

Discussion

We have previously shown an association between *IL6* −174 genotypes and presence of periodontitis in non-smokers living in a rural village in India (Franch Chillida *et al*, 2009). The present study describes associations between *IL6* −174 GG genotype and presence of *A. actinomycetemcomitans* in periodontal pockets in these subjects (with a periodontal phenotype ranging from healthy to severe periodontitis), confirming

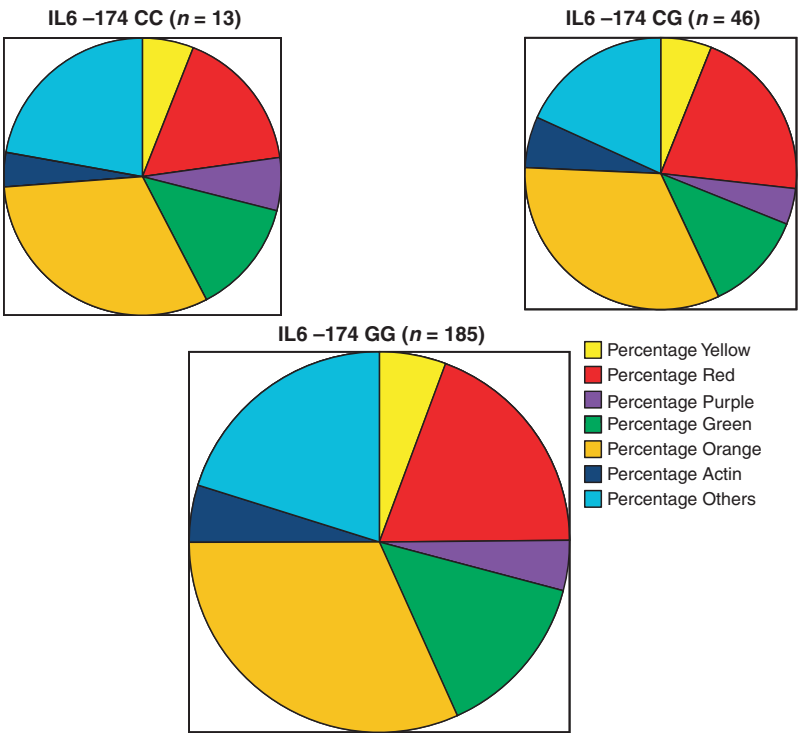

Figure 2 Pie charts with distribution of bacterial complexes between IL6 −174 GG and CG/CC individuals. % DNA probe counts for each bacterium was determined at each site and averaged per patient. The % of the DNA probe count for each species in each complex (according to Socransky *et al*, 1998) was summed and the proportions of each complex were calculated

association previously observed in subjects living in the United Kingdom (Nibali *et al*, 2007, 2008a). Among these Indian villagers living in Andra Pradesh, *A. actinomycetemcomitans* was almost ubiquitous subgingivally, as it was harboured by 93% of participating individuals. However, *IL6* −174 GG individuals had higher counts of this bacterium.

The *IL6* −174 GG has been associated with presence of periodontitis (Trevilatto *et al*, 2003, Nibali *et al*, 2009b). The study reported here confirms the previously reported association between *IL6* genotypes and *A. actinomycetemcomitans* (Nibali *et al*, 2007, 2008a), despite being conducted on a different population of different ethnicity and socio-economic status and not subjected to routine dental care. Furthermore, the microbiological analysis consisted of a checkerboard DNA-DNA hybridization analysis (Socransky *et al*, 1994), as opposed to previously reported culture (Nibali *et al*, 2007) and PCR (Nibali *et al*, 2008a) analyses. This association was seen in all subjects, independent from clinical diagnosis. In addition, to this, this paper for the first time shows the association between *IL6* genotypes and *A. actinomycetemcomitans* also in non-periodontitis individuals (according to EWP2 definition). As previously hypothesized, this association may be attributed to the increased local inflammatory response of -174 GG individuals (Fishman *et al*, 1998; Bennermo *et al*, 2004), which favours the overgrowth of components of the microbiota that grow well in inflamed areas, such as *A. actinomycetemcomitans*. This study brings preliminary evidence that other bacteria belonging to the green complex, in particular *C. sputigena*, may also be associated with increased counts in periodontal pockets of *IL6* −174 GG individuals. In other terms, the effect of genetic factors may not be limited to the presence or levels of one or two potentially pathogenic bacteria, but may extend to bacterial complexes or in general to the whole composition of the subgingival biofilm. This is in agreement with the concept of infectogenomics, suggesting that genetic factors in the host seem to play a major role in deciding the bacteria (commensal and pathogenic) that are able to colonize the host (Kellam and Weiss, 2006; Nibali *et al*, 2009a).

The microbiological analysis of the current subject group showed a population presenting with a complex oral flora, with a breakdown in bacterial complexes quite different from that observed in industrialized populations (Haffajee *et al*, 1998). Individuals diagnosed with periodontitis had a tendency towards higher percentage of green complex bacteria, and lower percentage of bacteria that were not defined in complexes ('others'). Overall, there was a predominance of bacteria belonging to the orange complex, which have been associated with established periodontitis in industrialized populations, while no significant differences in percentage of orange or red complex bacteria were noted between periodontitis and non-periodontitis individuals. This was consistent with studies in other untreated populations, which have shown ubiquitous presence of certain bacterial species and a tendency towards a stable, mixed microflora (Timmerman *et al*, 2000, Colombo *et al*, 2002; Dowsett *et al*, 2002; Papapanou *et al*, 2002). *N. mucosa*, a Gram-negative commensal bacterium, was the bacterium found in highest counts, followed by periodontopathogenic bacteria *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans*. This shows a considerable shift from the bacterial profile shown in a study with checkerboard DNA-DNA

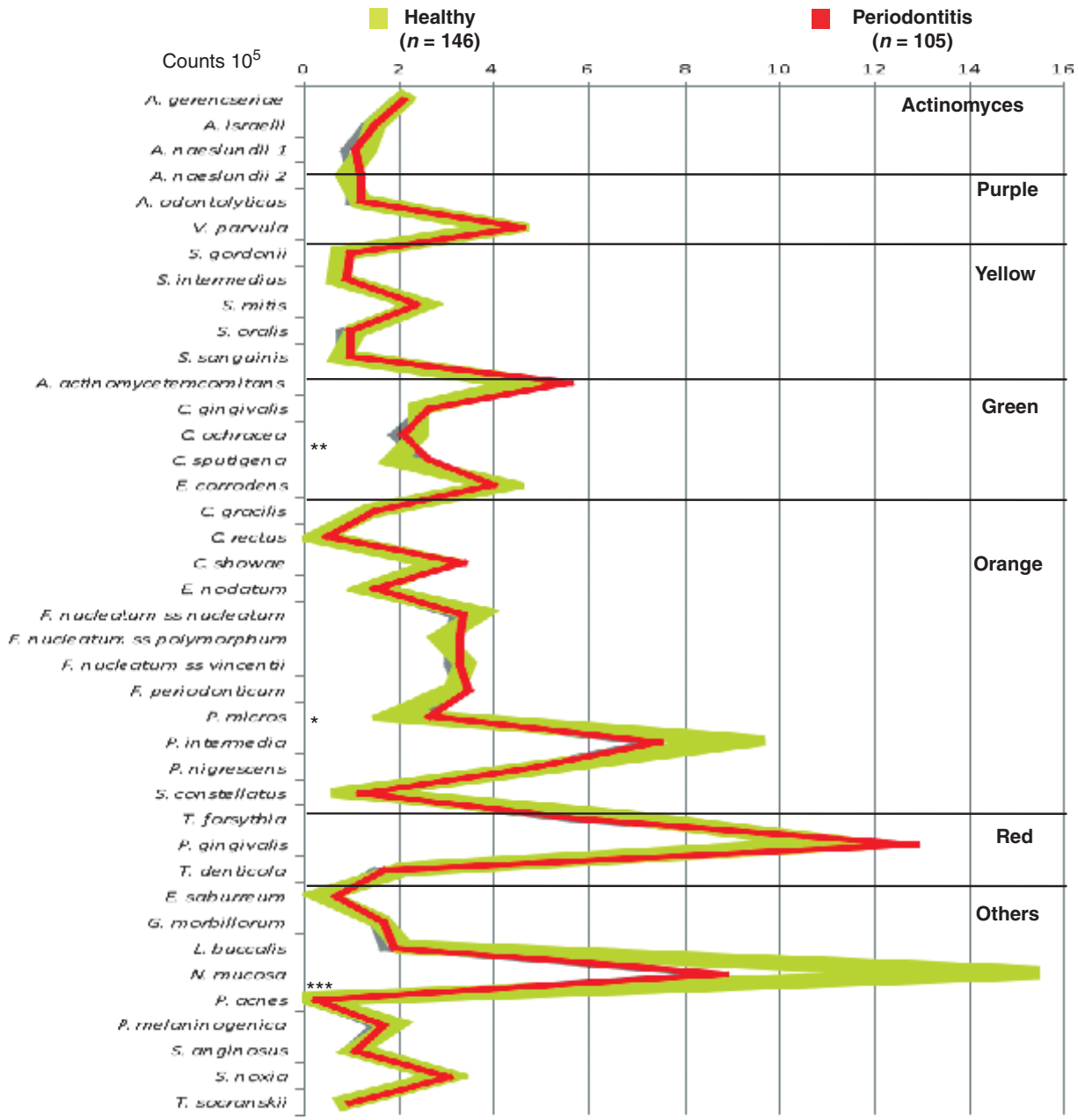

Figure 3 Counts of 40 studied taxa divided in complexes in healthy and periodontitis subjects (according to EWP1 definition). * *Parvimonas micra*: $P < 0.01$ for differences in high (above median) counts between healthy and periodontitis. ** *Capnocytophaga ochracea*: $P < 0.01$ for differences in detection and high (above median) counts between healthy and periodontitis. *** *Neisseria mucosa*: $P < 0.01$ for differences in detection, high (above median) counts and absolute counts between healthy and periodontitis

hybridization on a large United States population (Haffajee *et al*, 2006). The difference in prevalence and counts of *A. actinomycetemcomitans* between the study described here and the one by Haffajee *et al* is particularly striking. It needs to be highlighted that only two of the deepest pockets per subject were sampled in this study. Therefore, the average bacterial counts in these cases in not representative of the whole subgingival microbiota. However, even when the sampled site was used as unit for analysis, no differences were detected between bacterial complexes between deep (>6 mm) and shallow (<4 mm) pockets (data not presented).

Among the drawbacks of this study, we have to acknowledge the limitation of having only two plaque samples sites per patient, the risk for false positive results associated with DNA-DNA checkerboard analysis (vanSteenbergen *et al*, 1996) and the difficulty of defining periodontitis in this population (Franch Chillida *et al*, 2010; Preshaw, 2009). The results reported here show an association between *IL6* −174 polymorphism and *A. actinomycetemcomitans* in subjects living in a rural Indian village, bringing further evidence, consistent with the previous studies, of the importance of infectogenomics in periodontitis (Nibali *et al*, 2009a).

Further studies should analyse the possible effect of these and other genetic factors (in both periodontitis and healthy populations) on a wider range of components of the oral microbiota, on different serotypes and clones of *A. actinomycetemcomitans*, and should investigate possible functional mechanisms for the observed associations.

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