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Relationship between Periodontal disease, Porphyromonas gingivalis, peripheral vascular resistance markers and Coronary Artery Disease in Asian Indians

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ABSTRACT

Introduction: A close association exists between oral health and cardiovascular disease. Periodontal disease induces early vascular changes while oral pathogens have been detected in sub gingival and atheromatous plaques. We examined the interrelationship between Periodontal disease, oral bacteria, surrogate sub-clinical markers and coronary artery disease (CAD) in a representative Asian Indian cohort.
Materials and Methods: 532 Gingivitis cases and 282 Periodontitis cases were assessed for early peripheral vascular changes, namely pulse wave velocity (PWV), arterial stiffness index (ASI) and ankle brachial index (ABI) using computerized oscillometry method. Relative quantitation (RQ) of Porphyromonas gingivalis (Pg) was estimated in saliva samples of 54 Periodontitis, 25 Gingivitis and 51 CAD cases (38 also had oral disease) by Taqman assay by amplifying pathogen-specific gene targets, 16srRNA and IktA, respectively, and 16s universal bacterial rRNA as endogenous control.
Results: PWV and ASI were elevated in Periodontitis compared to Gingivitis cases ($p < 0.0001$) and in those with diabetes and hypertension. Cases with Periodontitis showed higher mean expression of Pg than Gingivitis (0.37 ± 0.05 versus 0.15 ± 0.04, $p < 0.0001$), while CAD patients with oral disease ($N = 38$) showed lower mean Pg expression than those without oral disease ($N = 13$) (0.712 ± 0.119 versus 1.526 ± 0.257, $p = 0.008$). Higher Pg expression was recorded in subjects with diabetes and hypertension.
Conclusion: Oral disease induces early changes in the peripheral blood vessels. Further, common presence of Pg in subjects with oral disease, in those with established cardiovascular risk factors and in patients with symptomatic CAD reflects the importance of oral hygiene in the development of Coronary Artery Disease in Asian Indians.
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Introduction

Over the last two decades, there has been a growing interest on the possible link between periodontal disease and cardiovascular disease [1,2]. Our knowledge on this association has been gained through clinico-epidemiological and animal studies and has been extensively reviewed [3–7]. Endothelial dysfunction is an important early event in the pathogenesis of atherosclerosis, the underlying disease process in CAD [8]. Studies have shown that chronic Periodontitis causes impairment in vascular function which improves following periodontal therapy [9,10]. There are several methods available to non-invasively assess sub clinical atherosclerotic changes [11]. Among them, use of computerized oscillometry for direct measurement of arterial stiffness is a simple, reproducible technique that is gaining popularity for its predictive value in cardiovascular risk stratification [12–16]. While the causal role of different bacterial pathogens in Periodontitis has been well established, three specific bacteria, Porphyromonas gingivalis (Pg), Aggregatibacter actinomycetemcomitans (Aa) and Tanerella forsynthia (Tf) were officially designated as the etiological agents for Periodontitis in 1996 (American Academy of Periodontology,1996. Consensus report). Identification of these periodontal pathogens in atheromatous plaques [17,18] and detection of antibodies against these pathogens in serum of CAD patients [19–21], establish the necessary link between oral diseases and CAD. In fact, serological evidence of high antibody levels against Pg was shown to be an independent

Abbreviations: Apoe(shl), Apolipoprotein-E-deficient spontaneously hyperlipidemic mice; CAD, coronary artery disease; ICMR, Indian Council of Medical Research; OMP, outer membrane protein; RQ, Relative Quantitation; Tf, Tanerella forsynthia.

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predictor of myocardial infarction [22]. The plausible underlying mechanism appears to be endothelial injury, which is induced by toxins released by the oral bacterial and the consequent systemic inflammatory response [23,24]. In the present study, we have explored the association between Periodontal disease and early vascular changes in peripheral arteries and the putative association of two oral pathogens, Pg and Aa with oral disease, classical cardiovascular risk factors and CAD in a representative cohort of Asian Indians.

Materials and Methods

Study Population

A total of 969 individuals, clinically asymptomatic for CAD, were initially recruited from among those attending the dental out-patient department at Jindal rural charitable hospital, Bangalore India. Presence of oral disease was diagnosed based on standard clinical criteria such as bad breath, swollen or bleeding gums and gum recession for Gingivitis and tooth mobility, tooth decay, bone loss or tooth loss for Periodontitis. Electrocardiography (ECG) and Periscope test was performed on all participants. Of the 969 initial recruits, 66 who showed abnormal ECG and 85 subjects with missing Periscope data or with outliers were excluded from analysis. Finally, 814 individuals that included 532 Gingivitis cases and 282 Periodontitis cases remained in the study.

A total of 140 subjects were selected for the Periodontal pathogen study. Ten of these subjects showed abnormal ECG and were excluded from further analysis. The 130 remaining subjects included 51 angiographically proven CAD patients (38 with oral disease, 13 without oral disease), 25 cases with Gingivitis and 54 with Periodontitis, respectively. All CAD patients and 8 subjects with oral disease were enrolled from Narayana Hrudayalaya, a multi-specialty hospital in Bangalore. The CAD patients were a part of the Indian Atherosclerosis Research Study (IARS). The design of the IARS has been previously published [25]. Briefly, the IARS is an epidemiological study that investigates the classical and emerging risk factors that contribute to the development of CAD in the Indian population. Participants were recruited based on well-defined inclusion/exclusion criteria. All participants were of Indian origin and did not suffer from any other major illness at the time of recruitment. Presence of clinical CAD was confirmed on the basis of any of the following features - angiogram report showing >70% stenosis in any one of the major epicardial arteries or >50% in two or more arteries, percutaneous intervention, coronary artery bypass grafting procedure or history of myocardial infarction, verified as per the hospital records. All participants underwent dental examination at the dental outpatient unit at both the hospitals. In addition, five healthy subjects without oral disease or CAD, having normal ECG were treated as controls.

Detailed demographics, anthropometrics, medical history, medication and pedigree information were recorded for all participants through personal interview. Prevalence of type 2 diabetes and hypertension was ascertained based on self-report of physician's diagnosis and/or use of prescription medications along with perusal of their medical records. The IARS has been approved by the ethics committee of the Thrombosis Research Institute and designed according to the guidelines of the Indian Council of Medical Research (ICMR) [26].

Periscope Assessment

Periscope (M/S Genesis Medical Systems, India), an oscillometry based blood pressure monitoring and PC-based acquisition and analysis system was used to measure in real time, the following parameters - right brachial PWV (R bra PWV), left brachial PWV (L bra PWV), carotid-femoral PWV (C-F PVW), right brachial ASI (R bra ASI), left brachial ASI (L bra ASI), right ankle ASI (R ank ASI), left ankle ASI (L ank ASI) and ankle brachial index (ABI). All pressure recordings were done for about 10s. The report contained 8-second traces of Lead I and II ECGs and all pressure pulse waveforms.

Laboratory Methodology

After rinsing the mouth with water, about 2-3 ml saliva was collected in a sterile vial containing 1 ml of Phosphate buffered saline (PBS) from those subjects who were included in the oral pathogen study. Saliva samples were stored at 4 °C until DNA extraction. DNA was extracted by a modified salting out method [27]. Briefly, the saliva samples were re-suspended in lysis buffer (30 mM Tris, pH7.5, 12.5 mM Mgcl2 and 2.5% Triton-X-100) and incubated for 15 min at 37 °C. Following centrifugation, the pellet was re-suspended in a mixture containing 1% SDS and 4 μl Proteinase K (10 mg/ml), and incubated at 37 °C in a water bath for 30 minutes. Next, 6 M NaCl was added and the sample was mixed vigorously. Following centrifugation, 3 volumes of chilled 100% ethanol was added to the supernatant and incubated for further 30 minutes at 4 °C. The DNA precipitate was collected by centrifugation, washed with 70% ethanol, air dried at room temperature and finally re-suspended in 100 μL TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0). The purity and concentration of DNA was determined using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Detection of Relative Quantity of Oral Pathogens

Quantitative polymerase chain reaction was carried out to detect the presence of Pg and Aa on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA), using pathogen specific gene targets, 16srRNA and IktA, respectively, and 16s universal bacterial rRNA serving as the endogenous control. The list of probe-primers used for the Taqman assay were commercially synthesized by Applied Biosystems, USA and has been previously published (Table 1) [28]. 1 μl of DNA at a concentration of 50 ng/μl was mixed with 9 μl of Universal Taqman PCR mix and pathogen specific primer-probe mix (Applied Biosystems, CA, USA). All reactions were carried out in duplicates. Salivary DNA obtained from 5 healthy individuals was pooled in equimolar concentrations, used as positive control and was run with every experiment. PCR conditions included an initial denaturation of 95 °C for 10 min, followed by 40 cycles of denaturation step at 95 °C for 15 sec, annealing and extension step at 60 °C for 1 min. Relative quantitation (RQ) was calculated by $2^{-\Delta\Delta}$CT method using the CT values to analyze the relative changes in gene expression [29].

Measurement of Plasma Lipids

Venous EDTA blood sample was collected in evacuated tubes after overnight fast of 12 to 14 hours (Vacuette®, Greiner Bio-One GmbH, Vienna, Austria) for all participants. Plasma aliquots were separated and stored at -80 °C until further analysis. Pooled EDTA plasma samples prepared from blood collected from 30 healthy volunteers served as in-house lab control.

Table 1
Primer details of the three pathogen-specific gene targets.

Designation	Probe sequence (5'-3')	Amplicon size (bp)	Target
Primers			
Pg1198-F	TACCCATCGTCGCCTTGGT	126	16S rRNA
Pg1323-R	CGGACTAAAACCGCATACACTTG		
Aa1956-F	CAGCATCTGCGATCCCTGTA	147	IktA
Aa2102-R	TCAGCCCTTTGTCTTTCCTAGGT		
Uni152-F	CGCTAGTAATCGTGGATCAGAATG	69	16S rRNA
Uni220-R	TGTGACGGGCGGTGTGTA		
Fluorescent Probes			
Pg1238T	FAM- CTAATGGGACGCATGCCTATCTTACAGCT- TAMRA		
Aa2034T	FAM-TCGAGTATTCCTCAAGCATTCTCGCACG-TAMRA		
Uni177T	FAM-CACGGTGAATACGTTCCCGGGC-TAMRA		

Abbreviations: Aa, Aggregatibacter actinomycetemcomitans; Pg, Porphyromonas gingivalis; Uni, universal.

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