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Association between oral health, apical periodontitis, CD14 polymorphisms and coronary heart disease in middle-aged adults.

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Abstract

Introduction: There is evidence to suggest an association exists between oral infections and coronary heart disease (CHD). Subjects presenting lesions of endodontic origin (LEO) or pulpal inflammation had an increased risk of developing CHD. However, findings concerning systemic manifestations of apical periodontitis (AP) remain controversial. An association between CD14 gene polymorphisms and atherosclerosis-associated diseases has been demonstrated but there are no data regarding an association between CD14 polymorphism and AP. This study evaluated associations between clinical oral health status, CD14 polymorphisms and CHD. **Methods:** A case-controlled clinical trial was designed to compare middle-aged adults with acute myocardial infarction or unstable angina (n = 51), within 12 months of the acute event defined as first manifestation, with healthy controls (n = 49). Participants were matched for age, gender and socio-economic status. Indicators of oral disease and compliance were evaluated. CD14 polymorphisms were analyzed by RFLP-PCR. **Results:** CHD subjects had higher prevalence of oral diseases and lower compliance to oral preventive strategies than healthy controls. Multivariate analysis showed a positive association between missing teeth (odds ratio [OR] = 1.37; 95% confidence interval [CI] 1.02–1.85), number of LEO (OR = 4.37; 95% CI 1.69–11.28), chronic periodontitis (OR = 5.87; 95% CI 1.17–29.4), and CHD. No statistically significant association emerged between the CD14 C(-260)T and the CD14 C(-159)T polymorphism, endodontic or periodontal disease, and CHD. **Conclusion:** Chronic oral diseases may increase the risk of CHD and may be an unconventional risk factor for CHD.

Key words cardiovascular disease, coronary heart disease, oral disease, chronic periodontitis, apical periodontitis, CD14, polymorphisms

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death and hospitalization for both genders in nearly all European countries. Among CVD, ischemic heart disease alone is the single most common cause of death in Europe (1). However, it is largely preventable through identification of high-risk individuals. Conventional risk factors for atherosclerosis and coronary heart disease (CHD), such as hypertension, diabetes, gender, socio-economic status, smoking, obesity, high low-density-lipoprotein (LDL) serum levels, and genetic disposition, have been clearly established (2).

In young patients, other risk factors, such as smoking and family history (3), appear to play significant roles in heart disease. Recently, unconventional factors, such as the presence of chronic inflammatory diseases, have also been considered as correlates of heart disease. Circulating markers of activated inflammation and hemostatic factors are closely associated with the development of fatal and non-fatal myocardial infarction (4). Thus, chronic inflammatory processes may be considered predictors for atherosclerosis. Chronic oral infections in particular have been associated with CHD (5-7).

Chronic periodontitis is associated with atherosclerosis and an increased prevalence and incidence of ischemic heart disease, independent of conventional risk factors (8). Periodontal infection may influence the development of CHD by hematogenous exposure to Gram-positive and Gram-negative bacteria through a mechanism of molecular mimicry. The binding of LPS with CD14 receptors present on the endothelial membrane is thought to cause the release of pro-inflammatory factors and exposure of adhesins involved in processes of atherogenesis and thrombogenesis (9). The presence of *Porphyromonas gingivalis, Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* DNA has also been demonstrated in atheromas (6). Chronic periodontitis induces an increase in C-reactive protein (CRP) and anti-periodontal pathogen IgA antibodies (10). Increased CRP has been associated with an increased risk of CVD in healthy individuals (11). Recent studies have detected elevated CRP levels, both in patients with coronary atherosclerosis and in those with periodontal disease (12), with the biggest increase found in patients suffering from both diseases. Chronic periodontitis has also been associated with increased levels of total cholesterol, triglycerides and LDL, and with reduced levels of high density lipoproteins (HDL). Furthermore, after periodontal treatment, an increased serum concentration of HDL cholesterol has been found, with a consequent increase in the HDL/LDL ratio, and reduced serum levels of CRP (13).

Apical periodontitis (AP), as an immune response to chronic bacterial contamination of the endodontic and peri-radicular spaces, presents significant similarities with the inflammatory response involved in chronic periodontitis (14). Endodontic pathogens, which tend to be the same as those involved in periodontal infections, may reach the atherosclerotic plaques through a mechanism of metastatic infection, or may act through the above-mentioned mechanism of molecular mimicry. An increase in pro-inflammatory cytokines has been reported in the pulp, periapical tissues and serum of patients with pulpitis and apical periodontitis (15,16). In particular, IL-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-alpha (TNF-α) promote activation of neutrophilic granulocytes, monocytes/macrophages, the alternative complement pathway and hemostasis. These cytokines also increase the binding strength of LDL to the endothelium and smooth vessel musculature. Furthermore, they increase expression of the gene for the vascular receptor of LDL, favoring atherosclerosis (14). Peri-radicular inflammatory lesions of endodontic origin (LEO) have also been associated with increased levels of serum CRP, in the same manner observed in periodontal disease (17). Findings concerning systemic manifestations of periradicular inflammatory processes remain controversial. However, some studies have shown an increased risk of developing ischemic heart disease in subjects presenting peri-radicular LEO (18) or pulpal inflammation, using root canal treatment (RCT) as a surrogate parameter (15, 19).

Genetic disposition to CHD can be assessed by examining any family history of early CHD, the genetic determinants of phenotypes involved in the pathophysiology of CHD and the analysis of gene-environment interaction, as well as the role of genetic polymorphisms (20). A large number of candidate genes have already been investigated in relation to CHD traits and to the risk of CHD itself, such as the C(-260)T single nucleotide polymorphism in the promoter region of the CD14 receptor gene and the C(-159)T in the CD14 gene. The innate immune system is the first line of defense against invading microorganisms. It is activated through the recognition of pathogen-associated molecules, such as bacterial cell wall components. CD14, a 55-kDa membrane glycoprotein expressed predominantly on the surface of monocytes/macrophages and neutrophils, plays a crucial role in the recognition of several microbial products, such as lipopolysaccharide (LPS) and peptidoglycan, which are major components of Gram-negative and Gram-positive bacterial surfaces, respectively (21). The LPS– or peptidoglycan–CD14 complex, together with other accessory proteins, interacts with the cell-surface Toll-like receptors. Activation of multiple signaling pathways leads to the up-regulation of inflammatory cytokines (22). CD14 is expressed on the cell surface via a glycosylphosphatidylinositol anchor but is also found free in plasma, being referred to as soluble CD14 (sCD14). sCD14 mediates the LPS-activation of CD14-negative cells, including endothelial and epithelial cells (23). Recent studies have demonstrated the role of CD14 C(-260)T gene as a genetic marker of susceptibility for atherosclerosis, the effect of the T allele being more evident in later stages of disease (24). Furthermore, as a higher frequency of the T allele appears to be correlated to an increased amount of sCD14 in patients, the TT genotype of CD14 C(-260)T and CD14 C(-159)T genes has been proposed as a risk factor of myocardial infarction (25). Any association between CD14 and chronic periodontitis remains controversial (26, 27) and no data exist about endodontic disease.

The aim of this study was to evaluate the association between clinical oral health status, presence of peri-radicular LEO, CD14 polymorphisms and CHD, comparing middle-aged adults affected by CHD with a healthy control group.

MATERIALS & METHODS

The study was authorized by the S.G. Battista University Hospital Ethics Committee and by the Piedmont Regional Health System Review Board. All subjects gave informed written consent for participation in the study, which was approved by the institutional ethic committee and performed according to the principles of the last update of the Helsinki Declaration.

An observational case-controlled clinical trial was designed. Dentate subjects with a minimum of five teeth, as suggested in a previous study (28), and no RCT in the last two years, were enrolled according to the following inclusion criteria: aged below 55 years, no medical history of diabetes, systemic, oncological, or immune-system diseases, no immune-suppressive or cortisone drug treatment in progress, and normal weight (i.e. body mass index [BMI] < 25 kg/m^2). The CHD group comprised the first consecutive, informed and cooperating patients with a diagnosis of acute myocardial infarction (STEMI) or unstable angina (UA/NSTEMI). Subjects were recruited within 12 months of the acute event defined as first manifestation, without known conventional risk factors for coronary disease, except for smoking and family history, and examined at the Cardiology Department of San Giovanni Battista University Hospital between October 2009 and June 2010.

Healthy controls were randomly enrolled from a general medical database from the same district as the CHD group, using a computer-generated random numbers sequence, and then matched for age (matching range ± 2 years), gender and socio-economic status with CHD group. The presence of cardiovascular disease was excluded in the control group prior to data collection through cardiologic check-up and electrocardiogram.

In both groups, familial characteristics for CHD were evaluated: medical history of cardiovascular disease among first-degree relatives (parents, siblings: aged below 55 years for male relatives and below 65 years for female relatives at time of ischemic episode). This independent risk factor, more significant in younger subjects than in the elderly, was included in the multivariate statistical analysis.

A structured patient history, including demographic and socio-economical status (age, gender, occupation, qualifications), medical and behavioral factors (smoking, compliance with preventive strategies, i.e. frequency of dental appointments and professional oral-hygiene sessions), previous dental history (prior treatment, causes of tooth-loss), was collected before dental examination. Smoking and family history were considered to be confounding factors of the association and were included in the statistical analysis.

The intra-oral examination was performed using 3.5X Galilean loupes (Orascoptic, Middleton, WI) by the same previously calibrated examiner who was blinded to participant's CHD status. For each patient, pulpal and periradicular status was assessed through vitality thermal and electric pulp tests (Diagnostic Unit, Sybron, Orange CA), palpation and percussion. Complete periodontal charting, including full-mouth plaque score (FMPS), was recorded. Full-mouth radiographic examination was performed (Planmeca Intra - Helsinki, Finland) using Rinn XCP devices (Rinn Corp., Elgin Ill.) and PSP imaging plates. The images were processed and archived by dedicated scanner and software interface (OpTime Soredex, Finland). Radiographs were analyzed by three Clinical Assistant Professors at the Endodontics Department who were blinded to the CHD status of each subject.

Examiners' performances were calibrated on the evaluation criteria, by means of a case-series presentation, until inter-examiner reliability ($K > 0.70$) could be expected; concordance between examiners was analyzed through the Fleiss' K score. In cases of non-unanimous opinion, the majority opinion was accepted. For each patient, the following indicators of dental disease were evaluated:

- number of missing teeth
- caries history (DMFT) and number of untreated dental caries
- pulpal (vital, non-vital) and periradicular status, presence of lesions of endodontic origin (LEO), RCT teeth, and LEO-associated previous RCT. Teeth were classified as having LEO by the loss

of lamina dura and periodontal ligament (PDL) enlargement of more than 2 mm as the largest diameter.

- periodontal status according to the American Academy of Periodontology classification of periodontal diseases and conditions (29).

DNA extraction and analysis of genetic polymorphisms.

The third finger tip of one hand of each subject was cleaned with antiseptic wipes and punctured with a sterile lancet. Blood samples were collected with a heparinized capillary and DNA was extracted using the Extract-N-Amp^{TM} Blood polymerase chain reaction (PCR) kit from Sigma Chemical Co. (St. Louis, MO, USA), containing all the reagents needed to rapidly extract and amplify human genomic DNA from whole blood. Briefly, 10 µl of whole blood was mixed with 20 µl of the Extraction Solution and the mixture was incubated at room temperature for 5 min. Neutralization solution (180 µl) was added and PCR was performed. An aliquot of the neutralization extract was combined with the Extract-N-Amp Blood PCR Ready Mix^{TM} (containing buffer, salts, dNTPs, Taq polymerase and JumpStart Taq antibody) and user-provided PCR primers to amplify the target DNA.

Detection of polymorphisms.

PCR primers were synthesized by Sigma-Aldrich (San Giuliano Milanese, Italy) and restriction enzymes obtained from Promega (Madison, WI, USA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). PCR-restriction fragment length polymorphism (PCR-RFLP) method was determined according to the procedure of Baldini M. et al. (30) and Ito D. et al. (31), respectively, as follows.

CD14 promoter -159 (C→T) polymorphism. Primers: A: 5'-GTGCCAACAGATGAGGTTCAC-3' and B: 5'-GCCTCGAGAGTTTATGTAATC-3' (1 μM); cycling: 96°C for 1 min, 38 cycles of 96°C for 40 sec, 56°C for 40 sec, 72°C for 40 sec, and 72°C for 10 min. PCR products were digested for 1 h at 37°C and inactivated at 65°C for 15 min with AvaII (2 units/10 μl reaction mixture).

The restriction pattern was visualized by 0.2 μg/ml ethidium bromide staining after electrophoresis of the PCR-RFLP products (10% polyacrylamide gel, 150 V, 30 min). This procedure produced two fragments (353 bp and 144 bp) in subjects homozygous for allele T (-159 T) or one fragment of 497 bp in subjects homozygous for allele C (-159 C). All three fragments were present in heterozygous subjects.

CD14 promoter -260 (C→T) polymorphism. Primers: A: 5'-CAAGGCACTGAGGATCATCC-3' and B: 5'-CATGGTCGATAAGTCTTCCG-3' (1 μM); cycling: 85°C for 15 min, 94°C for 4.5 min, 42 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 40 sec, and 72°C for 7 min. PCR products were digested for 1 h at 37°C with Hae III (2 units/10 μl reaction mixture).

The restriction pattern was visualized by 0.2 μg/ml ethidium bromide staining after electrophoresis of the PCR-RFLP products (10% polyacrylamide gel, 150 V, 30 min). This procedure produced one fragment of 418 bp in subjects homozygous for allele T (-590 T) or two fragments (263 and 155 bp) in subjects homozygous for allele C (-590 C). All three fragments were present in heterozygous subjects.

In order to check the validity of the methods, 10 samples were genotyped twice, with identical results.

Statistical analysis

The Kolmogorov-Smirnov test for normality was used to check data distribution. The difference between groups was analyzed through inferential analysis. The Student's t-test was utilized for continuous normally-distributed variables (full-mouth plaque score [FMPS], last dental appointment or professional dental hygiene session, DMFT). The non-parametric Mann-Whitney U-test was used for non-normally distributed variables (oral hygiene per day, number of missing teeth, untreated caries, LEO, RCT teeth, LEO-associated RCT teeth). The chi-square test was used for dichotomous variables (family history, smoking, prevalence of chronic apical periodontitis, chronic periodontitis, combined endo-perio diseases and healthy subjects). The level of statistical significance was set at $p < 0.05$. A multivariate logistic regression model was utilized to analyze the effects of each variable on CHD risk. Estimates were showed as odds ratio (OR) and relative 95% confidence intervals (95% CI), reciprocally adjusted for clinical factors, family history and smoking. Subjects were classified in the adjustment set up of the regression analysis as SMOKERS (current smokers + ex-smokers at any condition, duration of smoking habits and quantity) and NON-SMOKERS (never smoked). A descriptive analysis was utilized to evaluate the frequency of the CD14 polymorphisms of interest. The association with risk of CHD was evaluated through a crude estimation of OR and relative 95% CI. All statistical analyses were performed using the SPSS for Windows 17.0 software package (SPSS, Inc. Chicago, IL).

RESULTS

Kappa values estimated to evaluate inter-examiner reliability showed a high concordance between the raters: 1 vs. 2 (K = 0.73; 95% CI 0.43–1.02); 1 vs. 3 (K = 0.76; 95% CI 0.48–1.04); 2 vs. 3 (K = 0.83; 95% CI 0.60–1.06).

Fifty-one patients were enrolled in the CHD group (40 males vs. 11 female, mean age 48 ± 5.7 years) and 49 subjects were enrolled in the control group (39 males vs. 10 females, mean age 47 ± 7.1 years). In CHD group, 29.3% of patients had a family history of CHD, compared with 7.1% in control group. This difference was not statistically significant (Table 1). Enrollment rates were 80% and 65% in CHD and control groups, respectively. The distribution of occupations and education in the two groups was descriptively compared and was relatively uniform, with similar socio-economic middle-class backgrounds.

Behavioral factors: smoking, compliance, oral hygiene

Behavioral, compliance and oral-health indicators are summarized in Table 1. Smokers were significantly more prevalent in the CHD group at the time of the ischemic event compared with healthy controls (53.7% vs. 10.7%, respectively). Of current smokers in CHD group, 45.5% smoked fewer than 10 cigarettes per day, 27.3% smoked 10–20 cigarettes per day, and 27.2% smoked more than 20 cigarettes per day. Of the ex-smokers (22% of the group), 55% had stopped smoking within the last year, 22.5% had stopped smoking 1–5 years previously and 22.5% had stopped more than 20 years previously. Current smokers and ex-smokers in the CHD group were exposed to tobacco abuse for an average period of 30 ± 6 years. Only 24.3% of the subjects in CHD group had never smoked.

In the control group, one third of smokers reported smoking fewer than 10 cigarettes per day and two thirds smoked 10–20 cigarettes per day. Of the ex-smokers (17.9%), 20% had stopped within the last 10 years and 80% had stopped smoking more than 20 years previously. Current smokers and ex-smokers in control group were exposed to tobacco abuse for an average period of 23 ± 8 years. However, 71.4% of subjects in the control group had never smoked.

Participation in an oral health preventive program through routine dental appointments and professional dental hygiene sessions was assessed, as well as the oral hygiene (OH) performance of subjects in both groups. The CHD group was less compliant with preventive strategies, with significantly longer intervals between dental appointments and professional dental hygiene sessions, compared with the control group. A comparison of daily OH frequency and FMPS suggested a less consistent and less efficacious OH performance in the CHD group as compared with the control group.

Oral health status

Oral health status indicators and the statistical significance of differences between groups are summarized in Table 1.

The number of missing teeth, considered as an indicator of previous irreversible oral diseases, was significantly higher in the CHD group, as compared with the control group (5.7 vs 2.2, respectively). DMFT values were significantly higher in the CHD group (Table 1). Mean DMFT values of the CHD group (15.6 ± 5.5) were also higher than the World Health Organisation (WHO) mean range (9–13.9) for subjects in the same age group/area. Mean DMFT values of the test group (12.8 ± 5.7) were instead in line with available WHO epidemiological data (http://www.who.int/oral_health/media/en/orh_report03_en.pdf). Furthermore, CHD subjects presented more untreated caries than the control group, although the difference was not statistically significant.

The prevalence of apical periodontitis (AP) was higher in the CHD group (85.3%) than the control group (53.5%). Subjects in the CHD group also had a higher mean number of LEO per person compared with the control group (3.4 vs. 1.1, respectively).

Patients in the CHD group had received more frequent RCT than those in the control group (3.5 vs. 2.6, respectively), although no significant differences existed between the groups. However, RCTs in the CHD subjects appeared to have had a higher failure rate, more frequently being associated to a LEO compared with the control group (85.7% vs. 38.5%, respectively).

The prevalence of chronic periodontitis was significantly higher in the CHD group compared with the control group (82.9% vs. 42.8%, respectively). Furthermore, the prevalence of a combined diagnosis of LEO and lesions of periodontal origin (LPO) was significantly higher in CHD subjects than in controls (68.2 vs. 21.4%, respectively). No subject in the CHD group had total oral health, all showing at least one LEO or a diagnosis of chronic periodontitis. In comparison, 25% of subjects in the control group had complete oral health.

Multivariate analysis reciprocally adjusted for clinical variables (Table 2, column A), showed a significantly positive association between the number of missing teeth, number of LEO, diagnosis of chronic periodontitis, and CHD. The number of caries also showed a positive but not statistically significant association. Other variables (DMFT, diagnosis of chronic apical periodontitis with the presence of at least one LEO, and number of RCT teeth) did not show any positive association. Among oral health indicators, chronic periodontitis showed the strongest association with CHD (OR = 5.87). However, the amplitude of the 95% CI range indicates that the estimate is more uncertain than those of other variables. The association was still present after adjustment for family history, but not after adjustment for smoking, possibly due to the impact of smoking and family history, or to the effect of stratification on sample size. The association between the number of LEO and risk of CHD was still evident (Table 2, column B), after adjusting for all confounding factors, demonstrating a statistically significant stability of the estimates.

Polymorphism analysis

Based on the data obtained in this study, the presence of the genotypes CC, CT and TT in the analyzed Caucasian population were 18%, 42% and 40%, respectively, for the CD14 polymorphism C(-159)T and 18%, 20% and 62%, respectively, for allele CD14 C(-260)T. The frequencies of alleles C and T were 39% and 61% 61% for the CD14 polymorphism C (-159), respectively. The frequencies of alleles C and T were 28% and 72% for CD14 polymorphism C(-260)T, respectively. The CD14 C(-159)T and CD14 C(-260)T polymorphisms were evaluated in the healthy control and CHD groups (Table 3). After adjusting for the presence of endodontic and periodontal disease, only the polymorphism of CD14 C(-159)T gene seemed to evidence a positive association with CHD. This was not statistically significant (OR = 3.13; 95% CI 0.87–11.29). Furthermore the effect of T allele of the same polymorphic gene seemed to evidence an association with an increased risk of CHD (OR = 2.02; 95% CI 0.82–4.96). This was not statistically significant. No statistically significant associations were found between the studied CD14 polymorphisms and endodontic and periodontal disease.

DISCUSSION

Coronary heart disease is not common in patients younger than 55 years old. However, for those who suffer from CHD at a young age, the disease can impose a significant morbidity, psychological effect and financial constraint (32).

In this study, the test group consisted of an infrequent population of CHD patients aged below 55 years and recruited within 12 months of the first acute event, with the typical risk profile for CHD of this age group. It may be assumed that smoking, family history and unconventional risk factors, including genetic factors and chronic inflammatory status, rather than major risk factors which affect older people, could have been disease determinants in this study population.

In this study we only recruited patients who survived the CHD event. Those with fatal events could have not been included and this may represent a potential inclusion bias. Coronary interventions, fibrinolytic agents, antithrombotic therapy, and secondary prevention have reduced the overall – 1 month mortality of STEMI to 4–6% of patients treated in hospital. The timelines of care, rather than the type of reperfusion (coronary interventions vs. antithrombotic therapy), are the most important determinant of favourable outcome for patients affected by STEMI (33). Hospital mortality is higher in patients with STEMI than among those with UA/NSTEMI (7% vs. 3–5%, respectively). During the enrollment period, all patients treated for STEMI in the Coronary Unit of Turin Molinette Hospital were subjected to early reperfusion (percutaneous coronary intervention- PCI) within 90 minutes from the onset of symptoms and no fatal events occurred. The same favourable outcome occurred for patients with UA/NSTEMI during the enrollment period. However, no clinical oral health data are available concerning CHD subjects eligible for this study who eventually died out of hospital.

Patients affected by CHD had a significantly higher prevalence of chronic oral diseases and poorer compliance to oral health behaviors compared with the control group, although their socio-economic background was similar. A strong age-dependent association between poor oral health and sudden cardiac death has been demonstrated by a previous study (34). Dental pathological lesions were prevalent among victims who suddenly died out of hospital. Among men aged below 50 years, poor oral health seemed to be a significant risk factor for pre-hospital sudden cardiac death (34). A significant age-dependent association between the incidence of lesions of endodontic origin (LEO) and time to CHD diagnosis was also found by Caplan *et al.* (2006) among subjects aged below 40 years (18).

As expected, the two major risk factors for CHD in the adult population aged below 55 years, i.e. smoking and family history, were more prevalent in the CHD group. Smoking was highly prevalent in the CHD group (53.7%), even in comparison with Italian public health data (updated until May 2011: http://www.iss.it/fumo/index.php?lang=2). National monitoring for smoking, alcohol intake and drug usage reports a smoking frequency of 22.7% among adults, with a peak of 28.3% in the 25–44 year age range. Smokers made up just 10.7% of the control group in this study, confirming smoking as a predominant risk factor for CHD. Smoking increases the risk of endothelial injury to the peripheral vascular system and development of a chronic inflammatory state, both of which are risk factors for CHD and incidence of RCT. Compared with those who had never-smoked, current cigarette smokers were 1.7 times more likely to have RCT (35). Current smokers and ex-smokers (at any condition, duration of smoking habit and quantity) were classified together in the regression analysis as *smokers* and compared with *non-smokers* (never-smoked). As reported in *2012 European Society of Cardiology Guidelines (*http://www.escardio.org/guidelines-surveys/esc-guidelines/Pages/cvd-prevention.aspx*) and 2010 US Report of the Surgeon General (http://www.surgeongeneral.gov/library/reports/tobaccosmoke/index.html)*, there is a significant decrease in mortality and morbidity after only 6 months of smoking cessation. However, the cardiovascular risk significantly decreases to the lowest level after only 10–15 years of smoking cessation and never to the *never smokers*' value. As it is not possible to quantify the impact of different modalities of cessation (how many years since cessation, how many years of smoking a certain dose per day) on the deleterious effect of smoking, we considered ex-smokers together with smokers, just as "exposed subjects", in the adjustment of the regression analysis. The above-mentioned reports state that there is a clear, non-linear dose-response relationship for the risks associated with smoking. A sharp increase at a low level of exposure was reported, together with a shallower dose-response relationship as the number of cigarettes increased. However a significant risk appears to be more correlated with continuative smoking itself than differences in the number of cigarettes per day, or type of cigarettes. Also not-inhaled smoke is associated with an increased risk of CHD.

Only white-caucasian subjects presented for enrollment in the study. Race itself is not yet considered a cardiovascular risk factor so this should not be considered a limit in the external validity of the study *(*http://www.escardio.org/guidelines-surveys/esc-guidelines/Pages/cvd-prevention.aspx*)*. Genetic information are mainly represented by family history evaluation, the study of genetic determinants of phenotypes involved in the pathophysiology of CHD, gene-environment interactions and genetic polymorphisms.

In this study we included only subjects of normal weight, corresponding to BMI < 25 kg/m^2. Overweight was defined by BMI ranging between 25–29 kg/m^2 and obesity as BMI ≥30 kg/m^2 . Both conditions are significantly associated with an increased risk of CVD *(*http://www.escardio.org/guidelines-surveys/esc-guidelines/Pages/cvd-prevention.aspx*)*. This was excluded as a further confounding factor.

Among behavioral factors, CHD subjects were significantly less compliant to OH and to oral prevention strategies than control subjects. Participation in an oral health preventive programme was characterized by the interval between routine dental visits and professional dental hygiene sessions. These intervals tended to be longer in the CHD group, as compared with the control group. Furthermore, OH performances seemed to be less consistent and efficacious in the CHD group, compared with the control group, when analyzing the daily OH frequency and FMPS. A tendency towards poor oral health behaviours by CHD patients has been previously observed (36) where routine OH and regular dental care were not consistent in CHD patients. A recent survey of 11,869 subjects (37) investigated the association between poor OH and the risk of CVD, and between OH, inflammatory markers and coagulation in a sub sample of 4,830 subjects. Poor oral hygiene was associated with a higher risk of CVD and increased concentrations of CRP and fibrinogen.

Tooth loss has been implicated as a valid indicator of oral disease history and has been associated with an increased risk of CHD (38-40). An investigation involving 8,000 subjects demonstrated a weak but significant association between missing teeth and CHD, suggesting a common behavioral background of poor self care and low health prevention awareness (40). Another study demonstrated a higher incidence of CHD among men with fewer than 11 teeth and periodontal disease, compared with subjects with an intact dentition (38). It was also suggested that tooth loss may lead to unfavorable changes in diet and a further increase in CHD risk. More recently, data from the Glasgow Alumni Cohort of 12,631 subjects were utilized to investigate the association between tooth loss and CVD mortality in young adults (30 years or below). Former students with nine or more missing teeth at baseline had a 35% greater risk of CVD mortality than those with four or fewer missing teeth (39). In our study, CHD subjects had over 250% more missing teeth than the control group, illustrating the tendency to only refer to the dentist in later stages of disease for tooth extraction. The number of missing teeth was statistically significantly associated to CHD in this study.

Chronic periodontitis is associated with atherosclerosis and with an increased prevalence and incidence of CHD, independent of the presence of conventional risk factors (8). The National Report from the Italian Society of Periodontology stated that 60% of the population is affected by mild to severe (10%) periodontal disease. In this study, the prevalence of chronic periodontitis was

42.8% in the control group compared with 82.9% in the CHD group, dramatically more prevalent than in the general population. Periodontitis was strongly associated to an increased risk of CHD.

Apical periodontitis (AP) presents significant similarities with the inflammatory response involved in periodontitis (14). A recent review of epidemiological studies (41) reported a prevalence of AP ranging between 14–70% of all subjects and 0.6–8.5% of all teeth, while root-filled teeth were evident in 22–78% of subjects and 1.3–21.5% of all teeth. Furthermore, it was evidenced that AP is approximately four times as common in root-filled teeth as in non-root-filled teeth (41). This tendency was confirmed by another study (42) where the radiographic evidence of root fillings appeared to be the most important risk indicator of AP in the individual. This unfavorable outcome has been associated to poor quality endodontic therapy, which was found in 44–86% of treated teeth or roots (43-47).

In this study, CHD subjects showed a significantly higher prevalence of AP compared with the control group (85.3% vs. 53.5%, respectively) and higher than available epidemiological data for adults aged over 18 years. Data extrapolated from clinical-based prevalence studies (43,48–56) suggest a mean number of LEO per subject ranging between 1–1.9. These data were coherent with our findings where healthy controls had a mean LEO of 1.1. However, CHD subjects had a mean LEO of 3.4 per subject, which was significantly different to the control group. As expected, the majority (95%) of LEO were associated to RCT teeth. A possible, modest association between the incidence of CHD and RCT emerged in previous studies (15, 19), where RCT was considered a surrogate indicator of pulpal inflammation. However, in this study no statistically significant differences were found in the number of RCT between the groups. Subjects with recent RCT (less than 2 years) were not included in this study, as this was assumed to be the minimum time required to assess healing of an endodontic lesion. If lesion healing had still been in progress, the study could have been exposed to the risk of bias. A significant difference was seen between the number of LEO associated with RCT in the CHD group compared with the control group (85.7% vs. 38.5%, respectively). However, as no pre-treatment data were available and evaluation of the overall standards of treatment quality was not the objective of this study, these findings were not correlated to outcomes of therapy.

AP has been associated with an increased risk of CHD. However, the systemic manifestations of periapical inflammatory processes remain controversial. Several studies have demonstrated a significant association between CHD and LEO through multivariate analysis, after adjusting for confounding factors (18 28,57). This is contradicted by other studies where no significant associations were seen (36,58). In this study, the diagnosis of LEO alone did not show an evident association to an increased risk of CHD. However, the *number of LEO* was strongly associated, and the association was still evident and stable after adjusting for confounding factors such as family history and smoking. The findings demonstrate that an increase in the number of LEO may lead to an increased risk for CHD. This outcome is in concordance with the investigation by Caplan *et al.* (18) and in contrast with Frisk *et al.* (58).

Furthermore, when analyzing the combined prevalence of lesions of periodontal and endodontic origin, 68.2% of subjects in the CHD group were affected by both diseases, compared with 21.4% in the control group. Twenty-five percent of subjects in the control group were orally healthy, compared with no subjects in the CHD group. This outcome further confirms the poor oral health of CHD subjects compared with control subjects of the same age and area and with a similar socio-economic profile.

The distribution of the locus for polymorphism C(-260)T CD14 gene and its susceptibility to ischemic heart disease in different ethnic groups were evaluated by Hai-Feng Zhang *et al.* (59). A meta-analysis of 19 studies included a total of 11,813 cases and 6,196 controls. The data were divided into studies from Europe, India and East Asian countries (China and Japan). The analysis indicated that the prevalence of the genotypes CC, CT and TT in European populations are 26.8%, 49.6% and 23.18%, respectively, with a minimum variation in the percentage of these cases compared with controls. This underlines the difficulties in associating the polymorphism C (-260) T CD14 gene with an increased risk of ischemic heart disease in this population.

These studies, indicate that the frequency of T at position C(-260)T varies considerably in the control groups of the different ethnic populations studied. Values range from 48.3% in European studies, to 56.47% in Indian studies and 51.3% and 47.7% from studies in East Asia, reported in English and Chinese, respectively. Furthermore, in spite of the limitations of this review, a potential association can be observed between the T allele at position C(-260)T CD14 gene and the risk of ischemic heart disease in all populations analyzed. There is greater evidence for such an association in the Asian population.

The aim of this study was to identify a possible association between the CD14 gene promoter polymorphisms at positions -159 and -260 and the different levels of chronic inflammation, both in periodontal and endodontic disease. A population of young adults suffering from recent coronary artery disease, even in the absence of conventional risk factors, was compared with a healthy control group. It is evident that only the proportion of subjects homozygous for the T allele for the polymorphism CD14 C(-260)T is greater than that for the C allele in both the CHD and control groups. No difference was found in the frequency of C or/and T allele in CD14 C(-159)T polymorphism analysis. Although these values were not statistically significant, the frequency of allele T (calculated as TT + 1/2 CT) for both the analyzed polymorphisms, was higher in the CHD and control groups compared with the frequency of allele C. Despite these results, the polymorphism of CD14 C(-159)T gene and the effect of the T allele of the same polymorphic gene showed a positive association with CHD, even after adjusting for the presence of endodontic and periodontal disease. This association was not statistically significant. On the contrary, no associations were found between the studied CD14 polymorphisms, endodontic and periodontal disease and risk of CHD for the polymorphism of CD14 C(-260)T.

The stratification required to statistically evaluate the association of polymorphisms with different levels of endodontic and periodontal disease has been unfortunately conditioned by the low sample size. However, the data emerging from this preliminary study are sufficient to stimulate further studies with a larger population.

In conclusion, within the limits of this study, middle-aged subjects affected by CHD exhibited a poorer oral health status and a more unfavorable attitude to oral health preventive behaviors than healthy controls with similar demographic and socio-economic factors. Tooth loss, caries experience, endodontic and periodontal diseases were significantly associated with an increased risk of CHD. This study demonstrates that a strong association exists between an increased risk for CHD and the number of LEO. Furthermore, it was possible to identify a typical CHD patient profile as: smoker, low compliance to oral preventive strategies, and a higher prevalence of late stage oral diseases. This profile has much to do with life style, suggesting that chronic oral diseases may be considered among unconventional risk factors of CHD.

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Figure legends

Table 1: Descriptive statistics of variables of interest: mean values, (standard deviation) and [median]. FMPS: full mouth plaque score; OH: oral hygiene; DDS: dentist; RDH: dental hygienist; ENDO: diagnosis of at least 1 lesion of endodontic origin (LEO); RCT: root canal treated; LEO*RCT: lesions associated to RCT teeth; PERIO: prevalence of chronic periodontitis; ENDOPERIO: diagnosis of both LEO and PERIO lesions; HEALTHY: rate of orally healthy subjects.

Table 2. Multivariable logistic regression analysis of the variables of interest: odds ratio (OR) and relative 95% confidence intervals (CI). DMFT: Decayed Missing Filled Teeth value; ENDO: diagnosis of at least 1 lesion of endodontic origin; LEO: lesion of endodontic origin; PERIO: prevalence of chronic periodontitis; RCT: root canal treatment. **Bold** indicates statistical significance (p-value <0.05). A) Odds Ratios reciprocally adjusted for clinical factors; B) adjustment for clinical factors and family history; C) adjustment for clinical factors and smoking; D) adjustment for clinical factors, smoking and family history.

Table 3. Distribution of genotype (%) and allele frequencies (%) of -159 (C→T) and -260 (C→T) polymorphisms of the CD14 gene in both groups.

Table 1: Descriptive statistics of variables of interest: mean values, (standard deviation) and [median]. FMPS: full mouth plaque score; OH: oral hygiene; DDS: dentist; RDH: dental hygienist; ENDO: diagnosis of at least 1 lesion of endodontic origin (LEO); RCT: root canal treated; LEO*RCT: lesions associated to RCT teeth; PERIO: prevalence of chronic periodontitis; ENDOPERIO: diagnosis of both LEO and PERIO lesions; HEALTHY: rate of orally healthy subjects.

		CHD Patients	Healthy controls	*p* value
Family history		29.3%	7.1%	0.06
Smoking habits		53.7%	10.7%	0.001
Oral Hygiene (OH)	FMPS	73,2%	58,5%	0.007
and compliance	OH per day	1.6 (0.6) [2]	2.5 (0.7) [2]	<0.001
	last visit DDS (m)	18.7 (15.2)	9.1 (9.2)	0.001
	last visit RDH (m)	20.9 (18.8)	10.7 (12.6)	0.01
Oral health status	Missing Teeth	5.7 (5.1) [4]	2.2 (2.6) [1.5]	0.002
	DMFT	15.6 (5.5)	12.8 (5.7)	0.05
	Number of Caries	3 (2.28) [2]	2.2 (3.1) [1]	0.21
	ENDO	85.3%	53.5%	0.02
	Number of LEO	3.4 (2.7) [3]	1.1 (1.6) [1]	<0.001
	RCT teeth	3.5 (3.5) [3]	2.6 (2.6) [2]	0.24
	LEO*RCT	3 (2.8) [2]	1 (1.5) [1]	0.002
	PERIO	82.9%	42.8%	0.005
	ENDOPERIO	68.2%	21.4%	0.001
	HEALTHY	0	25%	0.08

Table 2: Multivariable logistic regression analysis of the variables of interest: odds ratio (OR) and relative 95% confidence intervals (CI). DMFT: Decayed Missing Filled Teeth value; ENDO: diagnosis of at least 1 lesion of endodontic origin; LEO: lesion of endodontic origin; PERIO: prevalence of chronic periodontitis; RCT: root canal treatment. **Bold** indicates statistical significance (p-value <0.05). A) Odds Ratios reciprocally adjusted for clinical factors; B) adjustment for clinical factors and family history; C) adjustment for clinical factors and smoking; D) adjustment for clinical factors, smoking and family history.

	A			**B**			**C**			**D**		
		95% C.I.			95% C.I.			95% C.I.			95% C.I.	
Clinical factors	OR	lower	upper	OR	lower	upper	OR	lower	upper	OR	lower	upper
MISSING TEETH	**1.37**	**1.02**	**1.85**	**1.36**	**1.02**	**1.83**	1.35	0.95	1.91	1.35	0.94	1.92
DMFT	0.81	0.62	1.06	0.81	0.62	1.07	0.85	0.64	1.12	0.87	0.65	1.15
CARIES (number)	1.28	0.95	1.73	1.30	0.96	1.77	1.18	0.81	1.74	1.21	0.81	1.81
ENDO	0.89	0.13	6.28	0.86	0.12	6.34	0.71	0.16	17.98	1.40	0.12	16.04
LEO (number)	**4.37**	**1.69**	**11.28**	**4.09**	**1.59**	**10.54**	**4.79**	**1.79**	**12.81**	**4.45**	**1.66**	**11.89**
RCT teeth (number)	0.54	0.29	1.01	0.58	0.30	1.08	0.47	0.24	0.91	0.49	0.25	0.97
PERIO	**5.87**	**1.17**	**29.4**	**5.05**	**0.99**	**25.85**	5.15	0.81	32.76	3.67	0.52	25.62
FAMILY HISTORY				2.37	0.30	18.48				4.48	0.44	45.03
SMOKING							**13.64**	**2.01**	**92.51**	**17.86**	**2.33**	**137.1**

Table 3. Distribution of genotype (%) and allele frequencies (%) of -159 (C→T) and -260 (C→T) polymorphisms of the CD14 gene.

Polymorphism of CD14 gene	**CHD patients (n = 51) %**	**Healthy subjects (n = 49) %**
-159 (C→T) CD14		
genotype:		
CC	13.6	25
CT	45.4	37.5
TT	41	37.5
allele:		
C	36	44
T	64	56
-260 (C→T) CD14		
genotype:		
CC	20	15.7
CT	30	10.6
TT	50	73.7
allele:		
C	35	21
T	65	79