Systemic inflammatory impact of periodontitis on acute coronary syndrome

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Abstract

Aim: A causative relationship between acute coronary syndrome (ACS) and periodontitis has yet to be defined. The aim of this study was to assess ~~if there are~~ differences in levels of serum cytokines between individuals with or without ACS or periodontal comorbidity.

Material and Methods: In a case-control study, individuals with ACS (78 individuals, 10.3% females) and matching healthy controls (78 individuals, 28.2% females) were included. Medical and dental examinations were performed to diagnose ACS and periodontitis. Serum levels of cytokines were assessed using Luminex technology.

Results: A diagnosis of periodontitis in the ACS and control group was diagnosed in 52.6% and 12.8% of the individuals, respectively. The unadjusted odds-ratio that individuals with ACS also had periodontitis was 7.5 (95% CI: 3.4, 16.8, p<0.001). Independent of periodontal conditions, individuals with ACS had significantly higher serum levels of IL8 (mean: 44.3 and 40.0 pg/ml) and vascular endothelial growth factor (VEGF) (mean: 82.3 and 55.3 pg/ml) than control individuals. A diagnosis of periodontitis made no difference in serum cytokine expressions.

Conclusion: The major contributor to serum cytokine expression was associated with ~~a diagnosis of~~ ACS. Elevated serum levels of VEGF were associated with ACS. Serum cytokine expression in individuals with ACS is unrelated to periodontal conditions.

Clinical Relevance

Scientific rationale: ACS impacts coronary blood flow, causing conditions ranging from unstable angina, through to life-threatening myocardial infarctions. Limited information is available regarding the relationships between cytokine expression in individuals with acute coronary syndrome and periodontitis.

Principal findings: Independent of periodontal conditions, individuals with ACS had significantly higher serum levels of IL8 and VEGF than control individuals.

Practical implications: Although ~~Practical prediction strategies for those at greatest risk of ACS must be founded in a greater understanding of the link between circulatory and the more easily accessible inflammatory processes, such as that which sustained periodontitis~~a diagnosis of periodontitis has been associated with ACS, the inflammatory burden of periodontitis as expressed by a panel of pro-inflammatory cytokines in serum is not possible to identify at the time of the acute phase of coronary heart disease. This suggests that periodontitis is not an immediate initiating factor of ACS

Introduction

Acute coronary syndrome (ACS), has an enormous impact on mortality and morbidity worldwide (Timmis 2015). This term includes a range of conditions which precipitate the occlusion of coronary arterial blood flow, such as unstable angina through to fatal myocardial infarctions (Kaul et al. 2013). Periodontitis is a ~~relatively~~ common condition in adults ~~and children which can lead to significant oral pathology~~ (Eke et al. 2015). ~~(Keyes and Rams 2015).~~ Whilst many observational studies support a relationship between ACS and periodontitis, causation has yet to be defined (Lockhart et al. 2012). However, individuals with advanced periodontitis exhibit endothelial dysfunction along with evidence of systemic inflammation, which promotes their risk of developing cardiovascular disease (Amar et al. 2003, Holtfreter et al. 2013). Whilst studies have demonstrated that periodontal treatment improves brachial artery endothelial function (Elter et al. 2006, Seinost et al. 2005, Tonetti et al. 2007), treatment of periodontitis did not impact the incidence of cardiovascular complications (Offenbacher et al. 2009, Beck et al. 2008). In addition, survival statistics from a six-year longitudinal study also failed to show that a diagnosis of periodontitis predicted mortality in older individuals (Renvert et al. 2015). Furthermore, a systematic review of clinical trials concluded that there was insufficient evidence to support the notion that periodontal therapy can prevent the recurrence of cardiovascular disease in patients with periodontitis (Li et al. 2014). There is a need to explore the systemic impact of periodontitis in terms of inflammatory markers, which may cast light on whether periodontal inflammation actively contributes to cardiovascular complications.

Serum high sensitivity C-reactive protein (hs-CRP) has been identified as an important marker of systemic inflammation and those with elevated levels of serum CRP are at increased risk of mortality and morbidity from cardiovascular diseases (Ridker 2007).

Periodontitis is also associated with increased levels of CRP, and interleukin (IL)-18 (Buhlin et al. 2009). Some pro-inflammatory cytokines have been studied to explore the systemic inflammatory impact of periodontitis, leading to conflicting results. Elevated serum levels of IL6 and tumour necrosis factor alpha (TNF-α) have been demonstrated in individuals with periodontitis in comparison with healthy individuals (Tang et al. 2011). In contrast, a report determined TNF-α levels to be significantly lower in individuals with periodontitis than control individuals (Nakajima et al. 2010). Yet another study assessed serum TNF-α levels in individuals with or without periodontitis and failed to demonstrate a relationship between serum TNF-α levels and periodontal status (Gokul et al. 2012). Thus, the relationship between periodontitis and system expression of TNF-α is unclear.

With regard to other potential markers which may link periodontitis with systemic conditions, patients with diffuse coronary artery ectasiae have been shown to have elevated blood levels of VEGF (Savino et al. 2006). In ACS, elevated VEGF concentrations may serve as a surrogate marker of myocardial injury (Konopka et al. 2013) and indeed, serum VEGF levels increase with periodontitis severity (Pradeep et al. 2011). Hence, in patients at risk for ACS, there appears to be an important interplay between various growth factors and cytokines, which are associated with inflammatory status and platelet hyper-reactivity (Gori et al. 2009).

Although many epidemiological studies have reported on potential casual associations between oral infections and cardio-metabolic diseases it remains unclear how oral infection may have an impact on cardiovascular diseases (Janket et al. 2015). Thus, there appears to be no studies that have performed comprehensive analysis of serum cytokine levels in individuals with heart disease and periodontitis in comparison to cytokine levels in individuals without heart disease or other systemic diseases. The objectives of the present

study were to assess if there are differences in the levels of serum cytokine biomarkers between individuals who have ACS with or without periodontal comorbidity. The null-hypothesis is that there are no differences in serum cytokine expressions between individuals with or without periodontitis and/or ACS pathology.

Material and Methods

In compliance with the Declaration of Helsinki, the Regional Ethics Committee in Lund, Sweden, approved the study (Institutional Review Board approval no. LU556-00). After the details of the original study protocol had been presented (Persson et al. 2003, Renvert et al. 2010, Renvert et al. 2006) informed consent was obtained from the individuals. Briefly, consecutively surviving individuals admitted to the Kristianstad Central Hospital were enrolled if they had a diagnosis of ACS defined by chest pain associated with typical electrocardiogram (ECG) changes. The initial ECG was considered diagnostic for myocardial infarction if there was ST segment elevation of 2 mm or more in a chest lead, or ST segment elevation of 1 mm or more in a limb lead. ST depression and/or T-wave inversion changes combined with typical serial pattern of cardiac markers [i.e. creatinine kinase isoenzyme (CKMB) and troponin T (TnT)] according to local laboratory standards, were also considered diagnostic for myocardial infarction. Left bundle block (LBB) was considered diagnostic for myocardial infarction if chest pain combined with typical serial pattern of cardiac markers were present. At the time of admission, a blood sample was taken for further analysis of biomarkers of inflammation.

Approximately one month after treatment and release from hospital, all surviving ~~these~~ individuals with a diagnosis of ACS received a comprehensive periodontal examination. The periodontal examination included routine measurements of probing pocket depths, extent of

gingival inflammation and radiographic analysis of alveolar bone loss. The methods used to diagnose gingivitis and bone loss have been described in detail (Persson et al. 2003). In the present study, individuals with loss of alveolar bone, verified by a distance between cement enamel junction and the highest coronal bone level exceeding 4 mm, at ≥30 % of teeth, combined with bleeding on probing (BOP) ≥20 % and a probing pocket depth ≥5 mm at four teeth or more were considered as having periodontitis. All study individuals were examined by one and the same examiner (Susanna Persson-Sättlin, dental hygienist) (Renvert et al. 2004) . This examiner was kept unaware of the medical diagnosis by not having access to medical data. Study individuals were instructed not to provide any pertinent information in regards to cardiovascular events

Individuals matched by age, socio-economic status, and smoking habits without a preceding diagnosis of ACS, or a diagnosis of ACS within 3 years after the enrolment in the present study, were included (Persson et al. 2003, Renvert et al. 2010). The control individuals were identified among friends to those with a current ACS or from registry available to the investigators. Data based on analysis of 80 patients with ACS and 80 control individuals from a group consisting of friends of the patients (39 individuals) with ACS, and from a research registry of subjects (41 individuals) who had participated in a timely health survey (Back et al. 1999). The 41 individuals identified from the health survey were selected based on a best fit principle (age, gender, smoking status, socio-economics) in comparison to the individuals in the test group.

The control individuals also received a comprehensive cardiological medical examination at Kristianstad Central Hospital including an ECG and were cleared from evidence of ACS.

Following the medical examination, the control individuals also received a comprehensive dental examination consistent with the examinations that the individuals with ACS received. The present study design complies with the STROBE initiative.

Analysis of selected cytokines

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A broad panel including 23 pro- and anti-inflammatory cytokines was assessed using Luminex MagPix multi analyte technology (Luminex, Austin TX. USA). This panel included the following cytokines; Basic FGF , Eotaxin, GCSF (granulocyte colony-stimulating factor), IFNγ interferon gamma), Interleukin (IL): IL1β (interleukin 1 beta), IL1ra (receptor antagonist), IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12p70(active heterodimer), IL13, IL17A, IP10 (interferon-inducible protein-10), MCP1 (monocyte chemo-attractant protein-1), MIP1a (macrophage inflammatory protein 1alpha), MIP1b (macrophage inflammatory protein 1beta), PDGFBB (platelet-derived growth factor subunit B), TNFα (tumor necrosis factor alpha), and VEGF (Vascular endothelial growth factor).The cytokine kit was purchased from Bio-Rad (Sundbyberg, Sweden) and the cytokines were detected following the manufacturer's instructions. The researcher who performed the cytokine analysis was unaware of where the samples represented individuals with periodontitis or not, or any other clinical data. Briefly, samples were defrosted, incubated with antibodies, immobilized on color-coded magnetic beads, washed to remove unbound material, and then incubated with biotinylated antibodies. After further washing, a streptavidin-phycoerythrin conjugate, which binds to the biotinylated antibodies, was added before a final washing step. The Luminex analyser determined the magnitude of the phycoerythrin-derived signal. Duplicate readings were performed in a subset of samples demonstrating a high level of agreement between measurements with intra-class

correlation (ICC) varied between 0.95 and 1.0 ($p<0.001$). Serum for the analysis of cytokines were not available for all individuals, Therefore, 76 individuals in the test and control groups were included, respectively.

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Statistical analysis

The statistical package SPSS 22 for Windows was used for all analyses. Multivariate analysis with Bonferroni correction adjusted for gender was used to determine whether significant differences in cytokine levels existed between study groups. Chi-square analysis was used for dichotomized data. Independent t-tests (equal variance not assumed) were used for numerical data. Unadjusted Mantel-Haenszel common odds ratio was calculated. Due to the absence of data on serum cytokine levels from study individuals with or without heart disease and periodontitis were not available at the time of study design a sample size calculation was not possible to perform prior to the study. In previous reports on this study materiel in regards to gender, smoking status, and age we have failed to identify that these factors were confounders (Persson et al. 2003, Renvert et al. 2004).

Results

Data from 156 adult individuals including 76 individuals with a diagnosis of ACS (10.3 % females), and from 76 control individuals without clinical evidence of cardiovascular disease were included (28.2 % females). The mean age of individuals in the ACS and control groups was 59.7 years (S.D. 9.2) and 59.7 years (S.D. 9.1), respectively. Serum lipid values, white blood cell counts, HbA1c levels were within the reference range for normal conditions. Characteristic medical values are presented in Table 1.

In the ACS group as well as in the control group statistical analysis failed to demonstrate that the prevalence of periodontitis differed by gender. Statistical analysis failed to demonstrate a difference in the frequency of ACS by age (p=0.96), smoking (p=0.60) or number of remaining teeth (p=0.29). In the ACS and control groups 22.7 % and 19.2 % were smokers, respectively. A diagnosis of periodontitis in the ACS and control group was diagnosed in 52.6 % and 12.8 % of the individuals, respectively. The unadjusted Mantel-Haenszel common odds-ratio that individuals with ACS also had periodontitis was 7.5 (95 CI: 3.4, 16.8, p<0.001). The prevalence of gingivitis (bleeding on probing ≥20 % of sites, 4 per tooth) did not differ by cardiovascular status. In fact, 100 % of the individuals with ACS and 97.3 % of the control individuals presented with gingivitis. Evidence of alveolar bone loss (≥4 mm at ≥30 % of teeth) was 73.1 % in the ACS group and 23.1 % in the control group (p<0.001).

In the control group (individuals without a diagnosis of ACS) statistical analysis failed to demonstrate differences in hs-CRP levels (p=0.95) (Figure 1) between those with or without a diagnosis of periodontitis. In individuals with a diagnosis of ACS statistical analysis also did not demonstrate differences in hs-CRP levels (p=0.41) between those with or without a diagnosis of periodontitis. Serum hs-CRP values were significantly higher in individuals with a diagnosis of ACS in comparison to those individuals without a diagnosis of ACS (p<0.001). Multivariate analysis adjusting for gender failed to demonstrate differences in serum cytokine levels in both ACS and control groups.

Independent t-test (equal variance not assumed) of cytokines in serum between individuals with or without a diagnosis of ACS and periodontitis (Tables 2 and 3)

Mean values and standard deviations for 23 cytokines studied in individuals with or without ACS are presented. Independent of periodontal conditions, individuals with ACS had significantly higher serum levels of IL8 (mean value: 44.3 and 40.0, respectively, mean diff: 4.3, SE. diff: 1.6, 95 % CI: 1.2, 7.5, $p<0.01$), and VEGF (mean value: 82.3 and 55.3, respectively, mean diff: 27.0, SE. diff: 9.4, 95 % CI: 8.4, 45.4, $p<0.01$) than control individuals. Statistical analysis identified that in individuals with ACS, a diagnosis of periodontitis or not made no difference in serum cytokine expression. Individuals with ACS without periodontitis had higher serum levels of VEGF than control individuals without periodontitis (mean value: 90.0 and 55.1, respectively, mean diff: 34.9, SE. diff: 12.8, 95 % CI: 9.3, 60.4, $p<0.01$). With increase in severity of disease from periodontal and cardiovascular health to having both periodontitis and ACS serum VEGF levels increased (Figure 2).

Discussion

The present study identified a high odds ratio that individuals with ACS also had periodontitis. This finding is consistent with several other studies. The present study also demonstrated that a subset of pro-inflammatory cytokines were found at high levels in individuals without a diagnosis of periodontitis but with ACS. This suggests that periodontitis may not to any greater extent contribute to the inflammatory burden of a person at risk for or having ACS. The impact of the current ACS status may overshadow the inflammatory impact of periodontitis. A recent meta-analysis has demonstrated scientific evidence that periodontal

therapies may improve improve endothelial function and reduce biomarkers (i.e. hs-CRP, TNF-α and IL6) of atherosclerotic disease, especially in those already suffering from heart disease and/or diabetes (Teeuw et al. 2014).

The present study failed to demonstrate that a diagnosis of periodontitis enhanced the expression of serum cytokine levels in individuals with ACS. We did show that elevated levels of IL8 and VEGF were associated with a diagnosis of ACS. Consistent with other studies, however, serum hs-CRP levels were significantly higher in individuals with ACS and periodontitis than in healthy control individuals (no clinical evidence of ACS or periodontitis). Gingival bleeding and probing pocket depth did not impact serum hs-CRP or serum cytokine levels. Consistently, the present study also failed to demonstrate a difference in serum hs-CRP levels in individuals with or without periodontitis and in the absence of a diagnosis of ACS. This is in broad agreement with the current literature in this area (Baser et al. 2014, Renvert et al. 2013). The identified lack of impact on serum cytokine levels when gingival bleeding was included may be explained by the high prevalence of gingival inflammation in all study individuals. The decision to use serum samples to assess the presence of pro and anti-inflammatory cytokines was based on the concept that systemic hyper-inflammation may, in part be related to the pathology of periodontitis. To the best of our knowledge there are limited data on the impact of periodontitis on the levels of a broader panel of cytokines in serum from individuals with or without ACS.

VEGF is a cytokine that is known to be involved in angiogenesis. It has been shown that VEGF levels in myocardial infarction may reflect the progressive stages of angiogenesis activity in the ischemic-necrotic myocardium (Lee et al. 2004). Levels of VEGF in serum correlate with clinical parameters of periodontal disease and serum VEGF levels increase progressively with the severity of periodontitis (Pradeep et al. 2011), contributing to its

pathogenesis (Artese et al. 2010, Prapulla et al. 2007). Our findings are consistent with these observations. Studies of gingival biopsies at different stages of inflammation have shown that levels of VEGF are related to endothelial proliferation in gingival tissues collected from individuals with chronic periodontitis (Kasprzak et al. 2012). Elevated VEGF levels in individuals with chronic periodontitis are linked with VEGF and β-defensin-1 gene polymorphisms (Tian et al. 2013). The present study identified that serum concentrations of VEGF were associated with ACS but not to periodontitis. Data have shown that in ACS serum VEGF concentrations are elevated and can serve as a surrogate marker of myocardial injury (Konopka et al. 2013). Data have also suggested that VEGF induces IP10 expression, which is a pro-inflammatory marker which is associated with the developing and chronic pathology of ACS process (Boulday et al. 2006, Frangogiannis 2004, Wilsgaard et al. 2015).

Chronic periodontitis presents with phases of disease activity and quiescence. Although the routine criteria (extent of bone loss, pocket depth ≥ 5 mm, gingival bleeding ≥ 30 %) are well established, such criteria cannot identify active periodontitis. Most likely, analysis of serum or gingival crevicular fluid levels of pro- and anti-inflammatory cytokines may distinguish between chronic and acute periodontitis. There is a need to further explore serum cytokine threshold levels that indicate periodontal inflammation. It is possible that additional studies of the infectious bacterial aetiology in periodontitis in relation to clinical and cytokine data can cast light on periodontal disease activity and its impact on cardiovascular disease. Whilst the present report revealed that elevated cytokine levels were associated with periodontitis and coronary disease, it is recognised that there are practical limitations on the interpretation of the expression of pro- and anti-inflammatory cytokines, in terms of time- and event dependency, which make their clinical predictivities potentially problematic.

One limitation of the present study is that the subgroup analyses included relatively few cases. The present study is based on a cohort of individuals who either were admitted to emergency care with a diagnosis of ACS, or who belonged to a sample of the community without a confirmed diagnosis of heart disease. Nevertheless, the study design represents case selection based on consecutive cases with ACS and is therefore not likely to be influenced by periodontal status. In addition, the individuals that had received treatment for ACS had most likely been prescribed medications which could have impacted periodontal status during the time of dental examination. Logistically, it was not possible to perform the dental examination at the time of admittance to the hospital for ACS. The investigators have no information about past diagnosis and treatment of periodontitis, on the progression of periodontitis, or if these study individuals with or without periodontitis were in a current or recent phase of active periodontitis or not and that could have had an impact on pro-inflammatory cytokine levels. Information on Body Mass Index (BMI) was not collected. Therefore, no adjustment for this factor could be made. Data analysis on the impact of age, gender, and smoking status in previous reports (Persson et al. 2003, Renvert et al. 2004) have failed to identify that these factors were confounders to the outcome. The explanations to this can be explained by the study design to match individuals in test and control groups, and to the fact that few individuals were smokers, and that the the age range was narrow.

In spite of the fact that few individuals were smokers, all individuals had poor oral hygiene reflected by the presence of gingival inflammation approaching 100 %. Thus, the periodontal diagnosis, including data on gingival inflammation, pocket depths, and bone loss was predominantly defined by data based on alveolar bone loss evaluations. The analysis of serum from the individuals with ACS was performed on blood samples collected at the time of admission and before any medical intervention or medication. Thus, the cytokine levels in

serum represent a 'snapshot' of cytokine expression at that particular time point of admission. Likewise, the blood samples from the control individuals were collected from individuals who were not taking medication or had had their medication changed within the preceding three months.

In conclusion, a diagnosis of ACS had a major impact on serum cytokine expression and elevated serum levels of VEGF were also associated with ACS. However, we found serum cytokine expression in individuals with ACS to be unrelated to periodontal conditions.

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Figure Legends

Figure 1. Box-plot diagram illustrating differences in hs-CRP levels by cardiovascular and periodontal status (° = outlier).

Figure 2. Box-plot diagram illustrating differences in VEGF levels by cardiovascular and periodontal status (° = outlier).

Tables

Table 1. Mean levels and standard deviations of medical values in control and ACS individuals.

Variables	**Control (n=78)**		**ACS (n=78)**		**Sign**
	Mean	**S.D.**	**Mean**	**S.D.**	
Cholesterol (mmol/l)	5.6	0.9	5.0	1.2	p=0.001
Triglycerides (mmol/l)	1.8	1.7	1.6	0.8	NS
High density lipids (mmol/l)	1.4	0.4	1.2	0.3	p=0.000
Low density lipids (mmol/l)	3.4	0.9	3.0	1.2	p<0.05
HbA1c (mmol/mol)	28.3	13.7	31.7	12.8	NS
WBC ($\times 10^9$/l)	6.4	1.8	8.6	2.9	p=0.000

Table 2. Mean levels and standard deviations of serum cytokines in control individuals and individuals with a diagnosis of ACS. (* = significant differences between groups, $p<0.01$)

Cytokine	**Control (n=78)**		**ACS (n=78)**	
	Mean	**S.D.**	**Mean**	**S.D.**
BasicFGF	75.6	54.1	87.1	68.6
Eotaxin	127.2	167.3	110.1	65.5
GCSF	124.6	65.5	137.0	55.0
IFNγ	95.8	138.7	104.9	114.0
IL1β	5.8	8.2	5.3	3.3
IL1ra	592.4	2088.8	308.4	537.9
IL4	4.5	2.1	4.9	1.9
IL5	10.9	4.8	11.8	3.6
IL6	20.4	54.6	16.1	11.4
IL7	17.5	13.5	16.1	4.8
IL8*	40.0	9.3	44.3	10.3
IL9	28.7	72.1	23.3	17.1
IL10	36.2	121.3	29.3	60.8
IL12p70	71.7	118.5	74.2	63.4
IL13	9.5	11.5	8.6	3.5
IL17A	132.9	93.8	163.8	133.7
IP10	707.9	495.4	824.7	749.0
MCP1	60.8	41.0	65.9	35.1
MIP1a	11.6	6.1	12.2	8.8
MIP1b	156.6	57.9	171.7	56.1

PDGFBB	3919.0	1344.7	4026.7	1358.8
TNFα	85.3	185.5	72.1	66.1
VEGF*	55.3	53.1	82.3	63.6

Table 3. Levels of cytokines in serum from control individuals without periodontitis (n=68) or with periodontitis (n=10) and individuals with a diagnosis of ACS without periodontitis (n=37) or with periodontitis (n=41). Data are presented for mean values, mean differences, S.E. mean diff, 95 % CI and significance when adjusted for smoking history.

Cytokine	**Control**	**ACS**	**Mean diff**	**S.E. diff**	**95 % CI**	**Sign**
IL8	40.0	44.3	4.3	1.6	1.2, 7.5	p<0.01
VEGF	55.3	82.3	27.0	9.4	8.4, 45.4	p<0.01
	Control	**ACS**				
Cytokine	**Periodontitis negative**	**Periodontitis negative**	**Mean diff**	**S.E. diff**	**95 % CI**	**Sign**
VEGF	55.1	90.0	34.9	12.8	9.3, 60.4	p<0.01
	ACS	**ACS**				
Cytokine	**Periodontitis negative**	**Periodontitis positive**	**Mean diff**	**S.E. diff**	**95 % CI**	**Sign**
Data analysis failed to show differences by periodontal status in individuals with ACS.						NS