The presence of C-reactive protein, salivary agglutinin and complement protein C4 in the oral cavity

Sabrina T.G. Gunput¹*
Wijnand J. Teeuw²*
Enno C.I. Veerman¹
Patrick Rijkschroeff²
Elena A. Nicu²
Bruno G. Loos²
Antoon J.M. Ligtenberg¹

¹ Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam (ACTA), VU University and University of Amsterdam, Amsterdam, The Netherlands
² Department of Periodontology, Academic Centre for Dentistry Amsterdam (ACTA), VU University and University of Amsterdam, Amsterdam, The Netherlands

*These authors contributed equally to this work

Manuscript in preparation
Abstract

Periodontitis is an inflammatory disease of the supporting tissues of the teeth. It is caused by an excessive immune response in which the complement system plays a key role. Both C-reactive protein (CRP) and salivary agglutinin (SAG) can activate the complement system, but it is unknown if CRP and SAG play a role in the etiology of periodontitis and in which areas of the oral cavity these proteins may be found.

In a first attempt to clarify the role of CRP and SAG in the etiology of periodontitis we investigated the presence of CRP, SAG and complement protein C4 in serum and in various oral fractions, including supragingival and subgingival plaque, saliva, and salivary cell fraction of healthy subjects and periodontitis patients. In addition, serum and saliva of edentulous subjects were tested for the presence of these proteins. Samples were screened by immunoblotting. CRP concentrations in serum and saliva were measured by ELISA.

CRP was detected in supra- and subgingival plaque samples of healthy subjects and periodontitis patients. CRP was present in all salivary cell fractions. No significant differences in the serum or salivary CRP concentrations were found between the three patient groups. A positive correlation was found between CRP concentrations in serum and in saliva \( (p<0.05) \) in healthy subjects. SAG was detected in all saliva samples and salivary cell fractions, in supragingival and subgingival plaque of both periodontitis patients (7/10) and controls (7/10). C4 was found in cell fractions, plaque samples, and saliva of healthy controls (5/10) and periodontitis patients (3/10), but not in saliva of edentulous subjects.

CRP, SAG, and C4 are detectable in dental plaque and saliva, both in healthy and inflammatory conditions. This suggests that these proteins play a role in processes in the vicinity of the gingival crevice.
Introduction

Periodontitis is a complex, chronic inflammatory disease resulting in the loss of connective tissue in gingival and periodontal ligament as well as loss of alveolar bone support of the teeth (Pihlstrom et al., 2005). It is the major cause of tooth loss in adults above 40 years and affects human populations worldwide at prevalence rates up to 10–20% for the most severe forms (Eke et al., 2012; Hugoson et al., 2008). Although the relative contribution of several causal factors may vary among patients, the uncontrolled inflammatory response upon bacterial infection seems the major factor determining the onset and/or progression of the disease (Loos et al., 2015). In this perspective, the complement system, an essential initiator of the inflammatory response, plays an important role in the immune reactions in periodontitis (Hajishengallis et al., 2015).

Activation of the complement system results in direct killing of a pathogen by the membrane-attack complex, as well as the recruitment of phagocytes, and subsequent phagocytosis. The complement system can be activated via three different pathways: the classical pathway, the lectin pathway and the alternative pathway. Both the classical and lectin pathway lead to further downstream activation of the complement system via complement protein C4 (Merle et al., 2015).

An activator of the classical pathway of the complement system that has been detected in the oral cavity, i.e. in saliva and gingival crevicular fluid, is the acute phase protein C-reactive protein (CRP) (Baser et al., 2014; Kinney et al., 2014; Lu & Jin, 2010; Pradeep et al., 2010). Bacterium-bound CRP functions as opsonin, which enhances recognition of bacteria by leukocytes and activates neutrophils (Du Clos & Mold, 2004). Plasma or serum concentrations of CRP are low under healthy conditions. Upon infection or tissue injury, plasma levels of CRP can increase within minutes. CRP concentrations in serum of periodontitis patients are slightly, but significantly higher than in healthy subjects (Andrukhov et al., 2013; Bertl et al., 2013; Paraskevas et al., 2008; Teeuw et al., 2015). Furthermore, CRP levels in plasma and saliva correlate with the severity of periodontitis. After periodontal treatment, CRP levels in both plasma and crevicular fluid decreased (Bertl et al., 2013; Paraskevas et al., 2008; Teeuw et al., 2014; Torumtay et al., 2015). A potential activator of the lectin pathway of the complement system in saliva is salivary agglutinin, also known as DMBT1, gp340 or SALSA (Leito et al., 2011; Reichhardt et al., 2012).

Although complement activation plays a role in periodontal diseases, relatively little is known about the involvement of putative complement activating factors present in the oral cavity. In the present study we investigated the presence of CRP, SAG, and C4 in saliva, salivary cell fraction, and supra- and subgingival plaque.
Materials and Methods

Study population

Referred periodontitis patients \((n = 10)\) were recruited during their first visit at the Department of Periodontology of the Academic Centre Dentistry Amsterdam (ACTA). Periodontitis was defined based on the criteria for generalized severe periodontitis proposed by Tonetti and Claffey (Tonetti & Claffey, 2005): the presence of proximal attachment loss of at least 5 mm in at least 30% of teeth present. The bone loss was confirmed on recent x-rays (vertical bite-wings or periapical radiographs less than 1-year old). All subjects had not received periodontal treatment in the year preceding the study.

Dentate subjects without periodontitis (control group, \(n = 10\)) and edentulous subjects \((n = 10)\) were selected among individuals registered for restorative dental procedures or who visited ACTA for regular dental check-ups. Control subjects were included if they were not missing more than one tooth per quadrant (3rd molar excluded), had no probing pocket depth (PPD) ≥3 mm, and showed on dental bitewing radiographs ≤1-year-old on all teeth a distance of ≤3 mm between the cemento-enamel junction and the alveolar bone crest. Edentulous subjects were included when the last tooth extraction was performed more than 1 year before screening. All subjects had to be at least 36 years of age. The following exclusion criteria applied to the whole study population: any systemic disease, autoimmune disease or immunodeficiency, use of antibiotics or other immune-influencing medication in the past year, acute bacterial or viral infections, oral wounds, any chemotherapy, pregnancy and lactation currently or in the past year. The subjects, who agreed to participate in this research, signed a written informed consent. The Medical Ethics Committee of the Medical Center of the VU University (VUMC) of Amsterdam approved the study protocol.

Collection of serum, saliva and dental plaque

Blood samples were obtained by venipuncture in the antecubital fossa, without excessive venous stasis. The venous blood was collected in 9 ml vacuum tubes (Z Serum Clot Activator, Vacuette, Greiner Bio-One GmbH, Austria) and allowed to coagulate for 1 hour and centrifuged \((2,000 \text{ g}, 10 \text{ minutes})\) at room temperature. The clot was discarded and aliquots of serum samples were stored at -80 °C until use.

Saliva was collected by expectoration without stimulation for 2 minutes, homogenized on a Vortex mixer and centrifuged \((10,000 \text{ g}, 10 \text{ minutes})\). The supernatant was transferred to a new tube and defined as saliva. The pellet, consisting of dislodged epithelial cells and bacteria, was resuspended in phosphate-buffered saline (PBS), pH 7.2 to the original saliva volume. Saliva and cell fraction were stored at -80 °C.

Supragingival and subgingival plaque samples were obtained with sterile Gracey curettes. Supragingival plaque was collected, after saliva was kept from the site by using cotton rolls and air-drying. The subgingival plaque was sampled at sites with the deepest pocket, after first removing supragingival plaque. All samples were washed with 0.5 ml
PBS, resuspended in 150 µl protease inhibitor cocktail (Complete Mini, Roche diagnostics, Indianapolis, IN, USA), sonicated, and stored at -80 °C.

**SDS–PAGE and immunoblot**

Samples were diluted 1:4 in NuPAGE® LDS sample buffer (Thermo Fischer Scientific, Waltham, MA, USA) with NuPAGE® reducing agent (Thermo Fischer Scientific) and boiled for 10 minutes. As reduction destroys the epitope recognized by anti-SAG mAb 213–06, SDS-PAGE was conducted under non-reducing conditions. Hereafter, 20 µl of sample was loaded on a NuPAGE® 4–12% Bis-Tris gel (Thermo Fischer Scientific). Novex® sharp pre-stained protein standards (Thermo Fischer Scientific) were used as a reference. Proteins were transferred to nitrocellulose membranes using an iBlot® dry blotting system (Thermo Fischer Scientific). After transfer, unbound sites were blocked with PBS supplemented with 0.1% Tween-20 (PBST) and 3% bovine serum albumin (BSA) (blocking buffer) for 1 h at room temperature. Next, blots were incubated with primary antibodies in blocking buffer. Blots were washed in PBST, followed by incubation with alkaline phosphatase-linked secondary antibodies in blocking buffer. Blots were washed in PBST and finally washed in water. Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (SIGMAFAST® BCIP®/NBT, Sigma, St. Louis, MO, USA). Reactions were stopped by transfer of the nitrocellulose in distilled water. Blots were dried between filter papers and scanned.

**Antibodies and conjugates**

The following antibodies were used: biotinylated mAb anti-C4 (a kind gift of Diana Wouters and Mieke Brouwer, Sanquin, Amsterdam, The Netherlands), mAb HYB 213–06–2 anti-SAG (Bioponto, Hellerup, Denmark), alkaline phosphatase-conjugated streptavidin (Caltag laboratories, Burlingame, CA), alkaline phosphatase-conjugated rabbit-anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark), rabbit anti-human albumin (Dakopatts, Glostrup, Denmark) as a capturing antibody, horse radish peroxidase (HRP)-conjugated rabbit anti-human albumin (GeneTex, Simpson, PA, USA).

**Enzyme-linked immunosorbent assays**

CRP concentration in serum was determined using the human CRP ELISA kit (Sigma). CRP in saliva, cell fractions and plaque was determined using the salivary CRP ELISA kit (Salimetrics, Carlsbad, CA, USA) according to the manufacturer’s protocol. Salivary albumin concentrations were determined essentially as described by Prodan et al., 2015.

**Statistics**

Results were analyzed with GraphPad Prism 5.0 (San Diego, CA, USA). Statistical differences were calculated using an ANOVA, chi-square test or Kruskal-Wallis test. Values were considered significantly different when \( p < 0.05 \).
Results

Study population

In total, 30 subjects (10 periodontally healthy, 10 periodontitis, and 10 edentulous patients) were included in this study (Table 1). All subject groups consisted of 7 males and 3 females. The edentulous group (age 66.8 ± 7.8) was significantly older than the periodontitis group (age 48.2 ± 13.0) \((p = 0.005)\). More smokers were present in the periodontitis group (6) than in the edentulous (0) or control (1) group \((p = 0.030)\). The number of teeth in the periodontitis group and the control group was equal.

Table 1.
Information about age, sex, smoking status, and number of teeth for the subjects of the study population. Results are presented as mean ± SD. Statistical differences were calculated using one-way ANOVA and Bonferroni’s multiple comparison post test. N/A; not applicable.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Edentulous (n = 10)</th>
<th>Periodontitis (n = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.8 ± 12.9</td>
<td>66.8 ± 7.8</td>
<td>48.2 ± 13.0**</td>
<td>0.005</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>1.000</td>
</tr>
<tr>
<td>Smoking</td>
<td>1</td>
<td>0</td>
<td>6*</td>
<td>0.030</td>
</tr>
<tr>
<td>Teeth</td>
<td>28.1 ± 2.4</td>
<td>N/A</td>
<td>27.4 ± 2.1</td>
<td>0.496</td>
</tr>
</tbody>
</table>

Detection of CRP, SAG, and C4 in dental plaque, oral cell fractions, and CHWS

CRP was found in supra- and subgingival plaque of controls (supragingival plaque 6/10; subgingival plaque 8/10) and of periodontitis patients (supragingival plaque 7/10; subgingival plaque 9/10) (Table 2). Cell fractions of all controls, periodontitis patients, and all but one edentulous subjects contained CRP. All saliva samples contained CRP.

SAG was also found in supragingival plaque samples of most controls (9/10) and all periodontitis patients (10/10) (Table 2). Notably, SAG was also found in subgingival plaque of controls and periodontitis patients (both 7/10). SAG was detected in all saliva and cell fractions of edentulous subjects, periodontitis patients and healthy controls.

Complement component C4 was detected in supra- and subgingival plaque of controls (supragingival plaque 8/10; subgingival plaque 5/10) and periodontitis patients (supragingival plaque 6/10; subgingival plaque 5/10) (Table 2). C4 could be demonstrated in saliva of healthy controls (5/10) and of periodontitis patients (3/10), but not in saliva of edentulous subjects. The cellular fractions of controls (6/10), periodontitis patients (8/10) and also of the edentulous (7/10) subjects contained C4.
Correlations for CRP concentrations between various sample origins

No statistically significant differences in serum and salivary CRP concentrations were found between periodontitis patients, edentulous subjects and controls (Figure 1). A positive correlation between serum and salivary CRP concentrations was found for the controls ($p<0.05$) (Figure 2A), but not in the edentulous subjects ($p=0.5960$) (Figure 2B) or the periodontitis group ($p=0.8598$) (Figure 2C).

Since both CRP and C4 are blood-borne proteins, we aimed to determine if their presence in saliva is due to leakage of plasma into the oral fluid, rather than by local inflammatory processes. We therefore examined to what extent the concentrations of these proteins correlated with that of serum albumin. Periodontitis patients showed a higher concentration

### Table 2.
Presence of CRP, SAG, C4 in supragingival and subgingival plaque, salivary cell fractions and saliva of controls, edentulous subjects and periodontitis patients. Exact salivary CRP concentrations are presented in figure 1. N/A; not applicable.

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein</th>
<th>Subgingival plaque</th>
<th>Supragingival plaque</th>
<th>Salivary cell fraction</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CRP</td>
<td>8/10</td>
<td>6/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>SAG</td>
<td>7/10</td>
<td>9/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>5/10</td>
<td>8/10</td>
<td>6/10</td>
<td>5/10</td>
</tr>
<tr>
<td>Edentulous</td>
<td>CRP</td>
<td>N/A</td>
<td>N/A</td>
<td>9/10</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>SAG</td>
<td>N/A</td>
<td>N/A</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>N/A</td>
<td>N/A</td>
<td>7/10</td>
<td>0/10*</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>CRP</td>
<td>9/10</td>
<td>7/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>SAG</td>
<td>7/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>5/10</td>
<td>6/10</td>
<td>8/10</td>
<td>3/10</td>
</tr>
</tbody>
</table>

Figure 1. CRP concentration in CHWS (A) and serum (B) in controls, edentulous subjects and periodontitis patients.

CRP concentration was measured by ELISA. No statistical differences were found (Kruskal-Wallis test). Mean±SE is indicated.
Figure 2. Correlations between CRP concentrations in serum and CHWS in controls (A), edentulous subjects (B), and periodontitis patients (C).

Correlations were estimated with linear regression analysis. Positive correlations were found for controls ($p<0.05$).

($p<0.05$) (Figure 3A) and output of albumin ($p<0.01$) (Figure 3B) than edentulous subjects. No statistically significant differences in these values were found between controls and periodontitis patients or between controls and edentulous subjects. A positive correlation was found between the salivary CRP concentration and albumin concentration in the periodontitis group ($p<0.05$) (Figure 4C). This suggests that plasma e.g. via the gingival crevice may contribute to CRP concentrations in saliva of periodontitis patients.
Discussion

In this study, the presence of the acute phase protein CRP, the salivary protein SAG and the complement protein C4 were determined at different sites of the oral cavity, such as supragingival plaque, and saliva. All proteins were present at all sites of the oral cavity of controls, periodontitis patients and edentulous subjects. However, when differentiating saliva in fluid and epithelial cells/bacteria, C4 was only found in the epithelial cells and bacteria of edentulous subjects, but not in the fluid.

Interestingly, in the present study we detected the salivary protein SAG in subgingival plaque, suggesting that salivary proteins can diffuse into the gingival crevice. Although binding of salivary proteins to supragingival plaque bacteria has been demonstrated earlier (Groenink et al., 1996; Liu et al., 2002; Rudney & Chen, 2004), this is the first time that a protein secreted by salivary glands has been demonstrated in subgingival plaque. It may suggest a role of SAG in the cross-talk between innate immune systems in saliva and in plasma such as complement factors.

The presence of CRP in dental plaque of healthy individuals suggests that, even in a healthy oral cavity, CRP may play a role in antimicrobial defense. CRP is an acute phase protein of which the serum levels are increased upon inflammation. Although chronically elevated CRP levels are considered as a prognostic risk marker for atherosclerotic cardiovascular disease (Geluk et al., 2008; Ridker et al., 2010; Schenkein & Loos, 2013), in acute infection or inflammation CRP might exert a protective role (Du Clos, 2013). Other studies have shown that the plasma levels of CRP are higher in periodontitis patients than in periodontally healthy controls. In this present study, no differences in serum- or saliva levels of CRP between controls and periodontitis patients were found, possibly because of the small size of the study population. Several studies revealed that periodontal therapy reduces the plasma concentrations of CRP (Kinney et al., 2014; Paraskevas et al., 2008;
Teeuw et al., 2014). In one study the concentrations of CRP in serum were not different between severe and mild cases of periodontitis (Baser et al., 2014). In contrast, another study showed that concentrations of CRP in serum correlated with the severity of periodontitis (Pradeep et al., 2010).

Interestingly, CRP was also present in saliva of edentulous subjects. CRP is a blood-borne protein and therefore it is likely that salivary CRP originates from plasma or GCF, as demonstrated by the correlation between salivary CRP and salivary albumin concentrations in periodontitis. Since edentulous patients have no gingival crevice, and therefore hardly leakage of plasma proteins, it is unlikely that CRP in these patients was derived from plasma. This is supported by the low salivary albumin levels in these patients. The salivary

Figure 4. Correlations between albumin and CRP concentrations in saliva.
Correlations were calculated with linear regression analysis in controls (A), edentulous subjects (B), and periodontitis patients (C). A positive correlation between albumin and CRP was found for periodontitis patients ($p<0.05$).
cell fractions of virtually all subjects tested in our study were also positive for CRP. It can be speculated that, besides from plasma, CRP in saliva may originate from oral cellular sources, such as gingival epithelial cells (Lu & Jin, 2010).

As CRP activates the classical pathway and SAG activates the lectin pathway of the complement system, we also investigated complement protein C4, which is involved in both pathways. Previously C4 was found on the bacteria obtained from plaque of periodontitis patients (Chisikovsky et al., 1982), but we show that C4 may also be deposited in healthy conditions. We also assessed saliva for the presence of C4. We could detect C4 in the saliva of periodontitis patients and healthy controls, but not in saliva of edentulous subjects. Although we could not detect C4 in saliva of edentulous patients, we detected C4 in the salivary cell fraction. This suggests that the C4 present in the oral cavity binds to epithelial cells or microorganisms.

Different forms of CRP are found in the human body. Native CRP is present in the circulation as a pentamer (pCRP). When bound to a surface, for instance a pathogen, pCRP can activate the classical pathway of the complement system via C1q. The modified or monomeric form of CRP (mCRP) can also activate complement via C1q. However, mCRP inhibits further activation by binding C4b-binding protein or factor H (Holers, 2008; Mihlan et al., 2011; Mold et al., 1984; Suankratay et al., 1998). In this scenario, the complement cascade is activated to enhance the clearance of invading pathogens, whereas at the same time its deleterious effects on host tissues remains limited (Du Clos, 2013). Currently, it is unknown which molecular form of CRP (pCRP or mCRP) is present in the different oral compartments and their contribution to oral inflammatory processes. Future studies addressing the specific molecular forms of oral CRPs may increase our insight in the role of this protein in the modulation of inflammatory processes at the gingival margin.
References


