SUMMARY:
Objective: Calprotectin is a calcium binding heterocomplex protein which appear to have regulatory functions in the inflammatory process. Epithelial cells which expressing calprotectin are more resistant to bacterial invasion. In acute phase inflammatory reactions calprotectin is detectable in elevated levels.

The aim of the present study is to detect the calprotectin level in saliva in patients with periodontal disease (chronic and aggressive periodontitis and gingivitis) and to follow calprotectin level during azithromycin treatment.

Methods and materials: In nine healthy patients without systemic disease and malignancy whole unstimulated saliva was investigated. Salivary calprotectin levels were measured by enzyme-linked-immunosorbent using a commercial kit (ELISA Hycult Biotech). Azithromycin treatment was taken as 500 mg (2 x 250 mg tabs.) once daily at 10.00 am for 4 consecutive days.

Results: At baseline Day levels of calprotectin ranged between 1.45 and 2.97; median 2.19. On Day 5 (first day after azithromycin treatment) the salivary calprotectin levels decreased in 6 of the patients. The measured values at Day 10 were more similar to those at Day 5, than those at Day zero. This was confirmed by the values of the average median of calprotectin.

Conclusion: We present the first study with the application of antibiotic and measurement the calprotectin levels before, during and after azithromycin intake with no side effects reported.

Measuring calprotectin levels could benefit the monitoring of antibiotic treatment efficacy in patients with gingivitis and periodontitis

Key words: calprotectin, saliva, periodontal disease.

INTRODUCTION
Calprotectin is a calcium binding heterocomplex protein (heterodimer of MRP8 and MRP14), which belongs to the S-100 protein family and is derived predominantly from neutrophils and monocytes. This putative protective protein is distributed in myelomonocytic cells, epithelial cells, human gingival keratinocytes and in various tissues and body fluids [18]. Calprotectin and its subunits appear to have regulatory functions in the inflammatory process by regulating the adhesion of myeloid cells to endothelium and extracellular matrix and activation of effectors cells, direct antibacterial effects by zinc-capturing, induction of CD 11b [29]. Epithelial cells which expressing calprotectin are more resistant to bacterial invasion [7].

In acute phase inflammatory reactions calprotectin is detectable in elevated amounts that, in some instances correlate to elevated levels of C reactive protein (CRP) or erythrocyte sedimentation rate (ESR) [18].

More recently increased S100A8 and S100A9 levels were also detected in various human cancers presenting abundant expression in neoplastic tumor cells. The expression of S100A8 in oral tumorigenesis is helpful only in the transition from severe dysplastic tissue to cancer [13].

CALPROTECTIN IN SALIVA SAMPLES
The mRNAs of S100A8 and S100A9 are expressed at minimal levels in the submandibular and parotid glands of C3H/HeN mice [17]. The levels of calprotectin in 12 healthy adults using different collection devices – parotid saliva, stimulated whole saliva and “mucosal transudate” showed a great variation – mean concentrations of 3.2; 22.0 and 40.9 mg/l in the respective oral fluids. These results illustrate the importance of careful sampling procedures [9, 10].
CALPROTECTIN IN ORAL DISEASE
DENTAL CARIES, GINGIVITIS AND PERIODONTALITIS

Toomarian L. et al. have tested 87 children (aged 3-5 years) with multiple dental caries and cannot determine if the level of calprotectin in unstimulated whole saliva is predictive of severe early-childhood caries [31]. While in patients with chronic and aggressive periodontitis and gingivitis elevated calprotectin levels play a role as a reliable inflammatory marker in the pathogenesis of periodontal disease [1, 26].

In other study patients with cyclosporin A (CsA)-induced hyperplasias were estimated and the expression of calprotectin in gingiva was detected. The results reveal that several positive S100A8/A9 cells were seen within the connective tissue, whereas in normal gingiva very few positive S100A8/A9 cells were detected [12].

ORAL CANDIDIASIS AND HIV

The candidacidal calprotectin production, or release, or both, may be increased in subjects with candidiasis. Calprotectin is present in saliva at levels which are biologically active. Calprotectin concentration positively correlate with intensity of candidal infection [5, 6, 20]. A raised whole saliva calprotectin levels was found in patients predisposed to oral candidiasis due to HIV infection or Sjögren’s syndrome and in patients with candidiasis associated with various oral disorders (e.g. lichen planus. oral ulceration). Mean levels of calprotectin were higher in whole saliva (2 microgram/ml) than in parotid saliva (0.3 microgram/ml) [30].

On the other hand lower calprotectin levels are communicated in HIV infection [5, 6]. Those who developed oral candidiasis had significantly lower parotid calprotectin levels than those who did not (67 micrograms/l vs. 216 micrograms/l) [3, 23].

SJÖGREN SYNDROME

Salivary calprotectin levels correlated with the plasma calprotectin levels and with several ocular variables and tend to reflect the local inflammatory activity, providing a convenient and non-invasive tool for diagnosis [4, 9].

AIM

The aim of the present study is to detect the calprotectin level in saliva in patients with periodontal disease (chronic and aggressive periodontitis and gingivitis) and to follow calprotectin level during azithromycin treatment.

PATIENTS, MATERIALS AND METHODS

Oral health status

Nine general healthy patients without systemic disease and malignancy (age range 23-52, 7 females and 2 males), underwent dental examination and were screened for oral lesions and dental problems. Oral-hygiene (assessed by Debris Index) and gingival status (assessed by papilla bleeding index - PBI) were recorded before treatment with azithromycin.

We investigated Debris Index (DI) on the representative teeth (16, 11, 24, 31 – vestibular, 36, 46 - lingual) to obtain an accurate oral-hygiene status.

Papilla bleeding index of Saxer and Muhleman (PBI) was used to assess gingival inflammation [28]. The exploration was done lingual in I and III quadrant and vestibular - in II and IV quadrant. We marked the gingival status in four stages (Table 1).

Table 1. Degree of PBI (Krasteva A. et al.. 2009) [21]

<table>
<thead>
<tr>
<th>Stage</th>
<th>PBI</th>
<th>Gingival status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>until 0.5</td>
<td>no gingivitis</td>
</tr>
<tr>
<td>2</td>
<td>0.5 – 2</td>
<td>mild gingivitis</td>
</tr>
<tr>
<td>3</td>
<td>2 – 3</td>
<td>moderate gingivitis</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>severe gingivitis</td>
</tr>
</tbody>
</table>

Collection of the samples

Whole unstimulated salivary probe was collected into 15ml test tubes for 5 min between 7.30 a.m. and 8.30 a.m. Subjects were asked to refrain from eating, drinking, smoking for at least 1 hour before saliva collection. The last oral hygiene procedure had been accomplish in the previous night, according to Dawes, C and JA Weathrtrll. [11]. The specimens (approximately 5 ml) were stored at -20°C and kept until the analysis.

Saliva was collected 3 times during the study – at baseline – Day zero, Day 5 (the first day after azithromycin treatment) and at Day 10.

Azithromycin treatment

Azithromycin treatment was taken as 500 mg (2 x 250 mg tabs.) once daily at 10.00 am for 4 consecutive days.

METHOD:

Salivary calprotectin levels were measured by enzyme-linked-immunosorbent using a commercial kit (ELISA Hycult Biotech). Two parallel testes were assayed for each sample. The assays were performed according to the manufacturer’s instructions.

RESULTS

The data of oral health, saliva calprotectin levels at baseline (Day zero), at Day 5 and Day 10 after azithromycin treatment and patients self-assessment (PSA) of gingiva inflammation are presented at Table 2.
At baseline Day levels of calprotectin ranged between 1.45 and 2.97; median 2.19 (Table 3). On Day 5 (first day after azithromycin treatment) the salivary calprotectin levels decreased in 6 of the patients. Only in 2 patients calprotectin levels were elevated compared to baseline Day (patient 6 and 7) (Table 2). The measured values at Day 10 were more similar to those at Day 5, than those at Day zero. This was confirmed by the values of the average median of calprotectin (Table 3).

Table 2. DI, PBI stage, calprotectin levels of whole saliva at baseline (Day zero), at Day 5 and Day 10 and patients self-assessment (PSA) of gingiva inflammation after azithromycin treatment.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>DI</th>
<th>PBIstage</th>
<th>Day 0 calprotectin (ng/ml)</th>
<th>Day 5 calprotectin (ng/ml)</th>
<th>Day 10 calprotectin (ng/ml)</th>
<th>PSA after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>1.4</td>
<td>2</td>
<td>2.97</td>
<td>1.86</td>
<td>1.93</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>2.2</td>
<td>4</td>
<td>2.19</td>
<td>1.74</td>
<td>1.57</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>2</td>
<td>4</td>
<td>2.77</td>
<td>2.49</td>
<td>2.50</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>1.7</td>
<td>2</td>
<td>3.03</td>
<td>2.29</td>
<td>2.43</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>1.6</td>
<td>2</td>
<td>1.45</td>
<td>1.29</td>
<td>1.46</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>2</td>
<td>4</td>
<td>1.58</td>
<td>2.19</td>
<td>1.91</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>1.6</td>
<td>3</td>
<td>1.50</td>
<td>2.00</td>
<td>2.13</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>2.2</td>
<td>4</td>
<td>1.86</td>
<td>0.91</td>
<td>1.85</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>2</td>
<td>2</td>
<td>2.78</td>
<td>1.53</td>
<td>2.51</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. Salivary calprotectin levels (results are expressed by Mean and Median)

<table>
<thead>
<tr>
<th>Salivary calprotectin level (ng/ml)</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>2.19</td>
<td>1.81</td>
<td>2.03</td>
</tr>
<tr>
<td>Median</td>
<td>2.19</td>
<td>1.86</td>
<td>1.93</td>
</tr>
</tbody>
</table>

Mean and Median values of salivary calprotectin level at Day zero, Day 5 and Day 10 are presented in Table 3.

Individual Salivary calprotectin level at Day zero, Day 5 and Day 10 are present in figure 1.

Figure 1. Salivary calprotectin individual level at Day zero. Day 5 and Day 10.

DISCUSSION

Most often calprotectin level is used as a marker and is detected in domain of gastroenterology. The fecal calprotectin correlate well with the parameters of disease activity in patients with Crohn’s and ulcerative colitis and this protein serves as a good biomarker and indicator of relapse of the disease [8, 24, 27]. Also fecal calprotectin may be a useful marker for application in children during infectious diarrhea [8].

On the other hand, plasma calprotectin levels have been shown to be lower (<159 ng/mL) in nonperforated appendicitis in children compared to perforated appendicitis [19].

Last year Ortega F. et al. [25] evaluated the serum and urinary concentrations of calprotectin as markers of insulin resistance in type 2 diabetes. Circulating calprotectin was significantly increased in insulin resistance and type 2 diabetes, and positively associated with HOMA-IR, obesity measures, inflammatory markers, and parameters of glucose and lipid metabolism. Similar findings were reported for
calprotectin concentrations in urine [25]. Whereas urinary calprotectin levels in prerenal disease (28 ng/ml) are comparable with healthy controls (45 ng/ml). Intrinsic acute kidney injury leads to highly increased calprotectin concentrations (1692 ng/ml) [15].

**Laboratory method for choice - ELISA**

Different ELISA test are present: Calprotectin (PhiCal® Calprotectin) (MRP8/14) (SPU), range 3.9-250 ng/ml; Calprotectin (PhiCal® Calprotectin) (MRP 8/14) (Stool. 1h), range 13-840 ng/ml; CalproLab™ , range 7.8 – 500 ng/ml; Calprotectin R-Biopharm with low range 5.8 ng/ml.

Expecting a very low concentration of calprotectin in saliva we selected ELISA Hycult Biotech, ranging 1.6 - 100 ng/ml, already used for quantification of the individual MRP-8 (S100A8) or MRP-14 (S100A9) proteins.

**Why we used Azithromycin?**

Azithromycin and cloxacinil have salivary excretion with correlation coefficients of 0.99 between saliva and plasma concentrations. The saliva Cmax/plasma Cmax ratio for azithromycin is 16.89 and 2.61 for cloxacinil. Saliva/plasma concentrations ratio for azithromycin is 13.4, compared to cloxacinil: 1.24. These recent communication support the choice of azithromycin in our study design [16].

In another study Azithromycin 500 mg/day (per os) was applied for 3 consecutive days. The highest concentrations of azithromycin were observed 12 hours after the last dose in plasma, saliva, gingiva and bone and then declined gradually. However, consistent levels of the drug in saliva and periodontal tissues could be detected up to 6.5 days, indicating that azithromycin was retained in target tissues and fluids for a long time after the end of treatment. Azithromycin levels in pathological tissues were significantly higher than those in normal gingiva 0.5, 2.5, and 4.5 days after the last dose [2, 22]. In 6 patients we observed a decrease in calprotectin level at the Day 5 compared to Day zero, which could be a consequence of antibiotic treatment. We detected increased calprotectin level at Day 10 in 3 patients than those in Day zero. In 6 of the patients the salivary calprotectin level was decreased after azithromycin treatment and the patients noted improvement of gingival inflammation.

Gomi K. and co-workers [14] mention that clinical parameters of oral health (probing depth, gingival index, bleeding on probing and gingival crevicular fluid level) significantly improved after administration of azithromycin 500 mg once daily for 3 days. The total number of cultivated bacteria also significantly decreased by Day 4, but slightly increased after Day 7. Sustained reduction in levels of six periodontopathogenic bacteria was not apparent until Day 14. On Day 7 the azithromycin concentration in the tissues lining the periodontal pockets was 50% of that on Day 4 and on Day 14 only 20% [14].

Our patients were suggested to take azithromycin 500 mg once daily for 4 consecutive days. Patients gave self-assessment opinion concerning gingival status after azithromycin treatment. Eight of the patients mentioned improvement of gingival inflammation which indirectly confirmed the second result of clinical parameters of oral health by K. Gomi et coworkers [14].

**CONCLUSION**

Saliva calprotectin levels are detectable with the mentioned kit: Calprotectin ELISA Kit for *in vitro* determination of Calprotectin (MRP 8/14) in stool and this technique (ELISA). The chosen technique for saliva collection is suitable for testing the calprotectin levels, which is a proof of unidirectional change of salivary calprotectin and whole saliva objectively reflects the changes of this protein after antibiotic treatment. The levels in saliva reduced during treatment.

We present the first study with the application of antibiotic and measurement the calprotectin levels before, during and after azithromycin intake with no side effects reported.

Measuring calprotectin levels could benefit the monitoring of antibiotic treatment efficacy in patients with gingivitis and periodontitis.

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