

# Relationship between the salivary concentrations of proteinase-3 and interleukin-8 and severe early childhood caries

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## Conflict of interest

None declared

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## Abstract

**Background.** Severe early childhood caries (S-ECC) is a multifactorial transmissible infectious disease continuing to affect infants and toddlers worldwide. Saliva plays a modulatory role in the pathogenesis of dental caries.

**Objectives.** The present study aimed to assess the salivary levels of proteinase-3 (PR3) and interleukin-8 (IL-8) as pro-inflammatory cytokines related to the function of neutrophils in association with S-ECC and its treatment.

**Material and methods.** Fifty children aged 36–60 months were recruited (25 caries-free controls and 25 S-ECC patients). Saliva sampling was performed in all participants. In the S-ECC group, sampling was repeated 6–8 weeks after restorative treatment. The salivary concentrations of PR3 and IL-8 were determined using the enzyme-linked immunosorbent assay (ELISA). The  $\chi^2$  test, Fisher's exact test, the independent  $t$  test, and the paired  $t$  test were applied at  $p < 0.05$ .

**Results.** The baseline salivary concentrations of PR3 and IL-8 in the S-ECC group were significantly higher than in the caries-free group ( $p < 0.001$ ). A significant reduction occurred in the levels of these cytokines following restorative treatment in the S-ECC group ( $p < 0.001$ ), although they were still significantly higher than in the caries-free group ( $p < 0.05$ ).

**Conclusions.** The salivary levels of PR3 and IL-8 were significantly affected by the presence of dental caries in children, implying their potential efficiency as non-invasive indicators in the determination of the caries risk and treatment effectiveness.

**Keywords:** cytokines, dental caries, saliva, biomarker, interleukin-8

## Cite as

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## Introduction

Early childhood caries (ECC) is a major public health problem worldwide, particularly in developing countries.<sup>1</sup> It is defined as the presence of one or more decayed (cavitated or non-cavitated), missed (due to caries) or filled surfaces (dmfs) in any primary tooth of children aged  $\leq 72$  months. Severe ECC (S-ECC) in 3–5-year-old children is defined as the presence of one or more decayed, missing (due to caries) or filled smooth surfaces in primary maxillary anterior teeth, or a dmfs score  $\geq 4$  at the age of 3 years,  $\geq 5$  at the age of 4, or  $\geq 6$  at the age of 5.<sup>2</sup> Early childhood caries negatively affects the oral health-related quality of life (OHRQoL) of children and is associated with several complications, such as toothache, dental abscesses, the loss of appetite, malnutrition, and an increased risk of caries in primary and permanent dentition. Moreover, it imposes a high economic burden on the family as well as the public health system.<sup>3,4</sup> Therefore, the determination of its pathogenesis can influence caries preventive strategies.

Saliva plays a significant regulatory role in the pathogenesis of dental caries. The properties of saliva, such as its viscosity, buffering capacity, remineralization potential, antimicrobial activity, and the presence of immunological factors in its composition, affect the development of caries.<sup>5</sup> Saliva sampling is a simple, economical and non-invasive method for oral health examinations, with a minimum risk of sample contamination. The analysis of salivary biomarkers is an appropriate method for the early diagnosis of oral diseases, as well as for the determination of prognosis and treatment success.<sup>6</sup>

The first line of body defense and the immune response to dental caries consists in the activity of neutrophils present in saliva, which control bacterial infections through chemotaxis, opsonization, endothelial cell migration, and phagocytosis.<sup>7</sup> Neutrophils are also considered one of the primary sources of lysozyme, the salivary enzyme responsible for the direct antibacterial action of saliva.<sup>8</sup>

Interleukin-8 (IL-8) is a neutrophil chemotactic factor secreted by various cells, such as macrophages, T cells, fibroblasts, neutrophils, and vascular endothelial cells. It encourages the accumulation of neutrophils and the subsequent increase in the secretion of lysozyme against bacteria.<sup>9</sup>

Neutrophil defensins (human neutrophil peptides (HNPs) 1–4) also play a critical role in the defense mechanisms of saliva and microbial homeostasis. They act by opsonizing bacteria, causing their non-oxidative death and improving phagocytosis.<sup>10</sup> The precursor of HNPs 1–4 is eliminated by proteinase-3 (PR3) during the differentiation and maturation of neutrophils, and active HNPs 1–4 are reserved in the azurophilic granules of neutrophils to be released in the presence of inflammation.<sup>11</sup> Proteinase-3 is a serine protease

that is involved in the inflammatory reactions associated with several infectious and non-infectious diseases; however, little is known regarding its role in the process of dental caries.<sup>12</sup>

The association between salivary immunological biomarkers and ECC has been evaluated in some previous studies<sup>2,8</sup>; however, few of them have shown the relationship between S-ECC and the salivary levels of IL-8 and PR3. The confirmation of this relationship by well-designed clinical studies could make the analysis of these salivary cytokines a useful tool for determining the severity of dental caries and the dental pulp status,<sup>13</sup> evaluating the efficacy of caries prevention protocols, and assessing children's susceptibility to dental caries.<sup>2,12,14,15</sup>

The present study aimed to assess the relationship between S-ECC and its treatment and the salivary concentrations of PR3 and IL-8 as pro-inflammatory cytokines related to the function of neutrophils. In addition, the possible risk factors associated with S-ECC were determined.

## Material and methods

### Participants

The present study was conducted at the Department of Pediatric Dentistry of the Shahid Beheshti University of Medical Sciences, Tehran, Iran, between April 2018 and December 2018. Children aged 36–60 months were recruited using the convenience sampling method and divided into 2 groups: S-ECC patients; and caries-free children who served as controls. The participants were included in the study according to the following criteria: complete physical and mental health; no confounding history of systemic diseases; no consumption of local or systemic drugs during the past 2 months; the absence of any gingival inflammation or periodontal disease; and the absence of any exfoliating primary teeth or erupting permanent teeth at the time of this study. The sample size was calculated based on previous studies to be 25 in each group ( $\alpha = 5\%$  and a power of 90%)<sup>16</sup>; however, in the S-ECC group, 35 patients were initially recruited to compensate for the possible sample loss during the follow-up period and to improve the validity of the study.

Each participant was enrolled in the study after reading, understanding and completing the written informed consent document by their parent. Furthermore, a questionnaire was completed by the parents in order to assess their educational status and to determine their children's dietary habits.

The research was designed and performed in accordance with the Declaration of Helsinki, and was approved by the institutional committee for ethics in research (IR.SBMU.DRC.REC.1397.036).

## Clinical examinations

Caries-free children (i.e., the control group) were selected according to the clinical screening examinations performed at 5 randomly selected kindergartens in Tehran, Iran. For improved visibility, the teeth were dried with sterile gauze and examined under adequate artificial lighting, using disposable explorers and dental mirrors (Asia Dental, Tehran, Iran). In order to ensure the absence of interproximal caries, only children with open tooth contacts were enrolled.

Children affected with S-ECC were selected from among the patients referred to the Department of Pediatric Dentistry of the Shahid Beheshti University of Medical Sciences, following clinical and radiographic examinations. Participants were selected according to the definition of S-ECC by the American Academy of Pediatric Dentistry (AAPD), i.e., the presence of one or more decayed, missing (due to caries) or filled smooth surfaces in primary maxillary anterior teeth, or a dmfs score  $\geq 4$  at the age of 3 years,  $\geq 5$  at the age of 4, or  $\geq 6$  at the age of 5.<sup>2</sup> The dmfs score of each child was recorded. The plaque index (PI) of each patient was also determined using the Greene and Vermillion's simplified oral hygiene index (OHIS), which consists of the simplified debris and calculus indices (DI-S and CI-S).<sup>17,18</sup> The modified version of this index for primary dentition, introduced by Miglani et al., was utilized in this study; it evaluates the buccal surfaces of the second primary molars and central incisors in the upper right and lower left quadrants.<sup>17</sup>

## Saliva sampling

In both groups, approx. 1 mL of unstimulated whole resting saliva was collected by passive drooling for 5 min.<sup>19</sup> Saliva samples were all collected between 9 a.m. and 11 a.m. to minimize the effect of the circadian rhythm on the composition of saliva.<sup>2</sup> The children were requested to refrain from eating, drinking, toothbrushing, and using dental floss for 2 h before sampling.<sup>9</sup> The samples were collected into capped, sterile, pre-chilled microtubes, and coded. The microtubes were placed on dry ice to prevent the hydrolysis of salivary proteins, and were immediately transferred to the Laboratory of Immunology. The saliva samples were then centrifuged (Eppendorf® centrifuge, model 5415; Eppendorf, Hamburg, Germany) for 10 min at 6,000 rpm. The supernatant from each sample was carefully transferred to a new microtube by using a sampler and stored at  $-70^{\circ}\text{C}$  until further use. Subsequently, the patients in the S-ECC group received all the required restorative treatment, in addition to preventive procedures, such as oral hygiene instruction, nutritional counseling, and professional prophylaxis and fluoride therapy. All procedures were performed by an experienced post-graduate student of pediatric dentistry. Post-treatment saliva sampling was performed 6–8 weeks after the completion of restorative treatment, and the samples were stored under the same conditions as mentioned above.

## Investigating the salivary concentrations of PR3 and IL-8

The salivary concentrations of IL-8 and PR3 were determined through the enzyme-linked immunosorbent assay (ELISA). For this purpose, the samples were kept at room temperature to thaw, and the ELISA test was performed according to the instructions provided in the ELISA kits of human IL-8 (Human Interleukin-8 ELISA Kit; MyBioSource, San Diego, USA; item code: MBS772139) and PR3 (Human Proteinase-3 Antibody ELISA Kit; MyBioSource; item code: MBS773125). The plates containing the saliva samples were transferred to the ELISA microplate reader (Anthos 2020; Biochrom Ltd., Waterbeach, UK) for spectrophotometric analysis at a wavelength of 450 nm. All analyses were performed by 2 experienced immunologists who were blinded to the sample groups. Inter-examiner reliability was evaluated using Cohen's kappa coefficient ( $\kappa = 0.8$ ).

The optical density (OD) values were converted to the concentration levels according to the respective standard curve provided by the manufacturer. Then, the salivary levels of IL-8 and PR3 were quantified and reported in picograms per milliliter (pg/mL).

## Statistical analysis

Data was analyzed using the IBM SPSS Statistics for Windows software, v. 21.0 (IBM Corp., Armonk, USA). Numerical data was presented as mean and standard deviation ( $M \pm SD$ ). The Kolmogorov–Smirnov test was used to assess the normality of distribution of the salivary cytokine concentrations. The independent  $t$  test, the  $\chi^2$  test and Fisher's exact test were used to compare the 2 groups. The paired  $t$  test was also used to evaluate the treatment effect in the S-ECC group. The potential risk factors related to S-ECC were determined using the multiple logistic regression model. The level of significance was set at 0.05.

## Results

A total number of 60 children were initially included in the study – 35 S-ECC-affected and 25 caries-free controls. However, 10 patients in the S-ECC group dropped out of the study because of uncooperative behavior or not being available for post-treatment sampling. Therefore, the data gathered from 25 children in each group was subjected to statistical analysis.

Table 1 presents the patients' background information on age, gender, the dmfs score, PI, maternal education, and the nighttime breast/formula feeding duration and type. The independent  $t$  test revealed a significant difference between the 2 groups in terms of PI ( $p < 0.001$ ). Furthermore, the mean values for the patient age were

significantly higher in the S-ECC group than in the control group ( $p = 0.006$ ). Conversely, there was no significant difference in gender distribution between the 2 groups, as revealed by the  $\chi^2$  test ( $p = 0.569$ ). The results of the  $\chi^2$  test also showed that the maternal educational levels were significantly higher in the control group than in the S-ECC group ( $p = 0.009$ ); however, such a significant difference was not observed among fathers ( $p = 0.061$ ). The mean duration of nighttime breast/formula feeding in the S-ECC group was approx. 5.6 months longer as compared to the caries-free group, which was considered a significant difference ( $p = 0.002$ ). However, the type of nighttime feeding (i.e., breast vs. formula) did not differ significantly between the groups ( $p = 0.479$ ).

Table 2 presents the mean salivary concentrations of IL-8 and PR3 in the 2 groups. Due to the normal distribution of the salivary cytokine levels in each group at both time points shown by the Kolmogorov–Smirnov test ( $p > 0.05$ ), the independent  $t$  test and the paired  $t$  test were used for further comparisons. The initial mean salivary concentrations of IL-8 and PR3 in the S-ECC group were significantly higher than in the caries-free group ( $p < 0.001$ ). After controlling for the confounding effect of age with the use of the analysis of covariance (ANCOVA), the 2 groups still showed significant differences regarding the salivary levels of IL-8 and PR3 ( $p < 0.001$ ).

As shown in Table 3, in the S-ECC group, a significant reduction occurred in the cytokine levels following restorative treatment as compared to the baseline values ( $p < 0.001$ ), although the levels were still significantly higher than in the caries-free group ( $p = 0.030$  for IL-8, and  $p = 0.002$  for PR3).

Table 1. Patients' background information

Group	Age [months] $M \pm SD$	Gender $n$ (%)		dmfs score $M \pm SD$	PI $M \pm SD$	Maternal educational level $n$ (%)			Nighttime feeding duration [months] $M \pm SD$	Nighttime feeding type $n$ (%)		
		boys	girls			high school diploma	bachelor's degree	master's degree or higher		breast	formula	both
S-ECC ( $n = 25$ )	53.9 $\pm$ 7.2	13 (52)	12 (48)	11.8 $\pm$ 3.2	1.44 $\pm$ 0.46	14 (56)	5 (20)	6 (24)	18.1 $\pm$ 6.9	15 (60)	2 (8)	8 (32)
Caries-free ( $n = 25$ )	40.5 $\pm$ 6.3	15 (60)	10 (40)	0	0.93 $\pm$ 0.34	4 (16)	12 (48)	9 (36)	12.5 $\pm$ 4.6	18 (72)	0	7 (28)
$p$ -value	0.006*	0.569		<0.05*	<0.001*	0.009*			0.002*	0.475		

$M$  – mean;  $SD$  – standard deviation; dmfs – decayed, missed or filled surfaces in primary dentition; PI – plaque index; S-ECC – severe early childhood caries; \* statistically significant.

Table 2. Initial concentrations of interleukin-8 (IL-8) and protease-3 (PR3) [pg/mL] in the study groups

Cytokine concentration [pg/mL]	Group	$M \pm SD$	$SE$	$p$ -value	F (ANCOVA)	$p$ -value (ANCOVA)
IL-8	S-ECC ( $n = 25$ )	35.84 $\pm$ 4.39	0.87	<0.001*	122.347	<0.001*
	caries-free ( $n = 25$ )	20.00 $\pm$ 3.88	0.77			
PR3	S-ECC ( $n = 25$ )	242.48 $\pm$ 54.33	10.86	<0.001*	122.347	<0.001*
	caries-free ( $n = 25$ )	158.73 $\pm$ 32.92	6.58			

$SE$  – standard error; \* statistically significant.

The determination of the possible risk factors associated with S-ECC with the use of multiple logistic regression models revealed that PI ( $p = 0.018$ ) and the duration of nighttime breast/formula feeding ( $p = 0.021$ ) had a significant influence on the development of S-ECC. The odds ratio (OR) for the duration of nighttime feeding was calculated to be 1.3, meaning that each further month increased the incidence of S-ECC by 30% (Table 4).

Table 3. Pre- and post-treatment concentrations of interleukin-8 (IL-8) and protease-3 (PR3) [pg/mL] in the severe early childhood caries (S-ECC) group

Cytokine concentration [pg/mL]	Time point	$M \pm SD$	$SE$	$p$ -value
IL-8	pre-treatment	35.84 $\pm$ 4.39	0.87	<0.001*
	post-treatment	24.68 $\pm$ 4.92	0.98	
PR3	pre-treatment	242.48 $\pm$ 54.33	10.86	<0.001*
	post-treatment	184.72 $\pm$ 37.86	7.57	

\* statistically significant.

Table 4. Possible risk factors for severe early childhood caries (S-ECC)

Risk factors	Wald $\chi^2$ statistic	$p$ -value	OR
Age	3.495	0.062	1.17
Gender	0.702	0.402	0.44
PI	5.611	0.018*	32.72
Maternal educational level	0.701	0.704	–
Paternal educational level	0.013	0.994	–
Nighttime feeding duration	5.337	0.021*	1.26
Nighttime feeding type (breast/formula)	0.900	0.956	–

OR – odds ratio; \* statistically significant.

## Discussion

Saliva plays an essential role in the development and progression of dental caries owing to its continuous and direct contact with the teeth, as well as the presence of various immune-related factors.<sup>20</sup> The analysis of salivary cytokines is considered an appropriate, non-invasive method for monitoring oral conditions.<sup>9</sup> When performed before and after the treatment of dental caries, the cytokine levels can be regarded as a suitable caries assessment tool and prognostic biomarkers.<sup>2</sup>

The present study aimed to assess the salivary concentrations of PR3 and IL-8 as pro-inflammatory cytokines related to the neutrophil function in caries-free and S-ECC-affected children, and revealed significantly higher salivary levels of IL-8 and PR3 in the S-ECC group in comparison with the control group. Restoration treatment in the S-ECC group resulted in a significant decrease in the salivary concentrations of these cytokines, although their levels were still higher as compared to the caries-free group, indicating the continuation of inflammatory stimulation.

Few clinical studies have investigated the relationship between IL-8 and dental caries. Gornowicz et al. reported significantly higher levels of pro-inflammatory cytokines, i.e., IL-8, in adolescents having dental caries as compared to caries-free ones, confirming the role of IL-8 as an essential chemokine in neutrophil chemotaxis.<sup>9</sup> Zhao et al. similarly showed a significantly higher concentration of IL-8 in patients having active carious lesions.<sup>19</sup> In another case–control study by Sharma et al., the salivary level of IL-8 was shown to be significantly higher in ECC-affected children as compared to caries-free controls.<sup>2</sup> Although a significant reduction in the IL-8 levels occurred following dental restorations, the concentrations were still significantly higher than the corresponding values in the caries-free group.<sup>2</sup> The results of these investigations are consistent with those of the present study.

On the other hand, Seyedmajidi et al. found no significant differences in the salivary levels of IL-8 among caries-free, ECC- and S-ECC-affected children.<sup>8</sup> The controversy between their results and the present study could be attributed to the differences in the age or mean dmfs score of the patients enrolled, and the utilization of non-parametric tests for statistical analysis as opposed to the less conservative parametric tests used in the present study.

The reduction in the levels of IL-8 pro-inflammatory mediator following a decrease in the microbial load may be due to the fact that cariogenic microorganisms are mainly Gram-positive, and their products, such as lipoteichoic acid (LTA), which is abundantly found in cariogenic streptococci, stimulate toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain (NOD) proteins, leading to the considerable production of IL-8.<sup>21,22</sup> It is worth noting that due to the incapability of the complement system to eliminate Gram-positive

bacteria because of their thick cell walls, the involvement of neutrophils as the primary phagocytes of the immune system is imperative.

Limited studies have been conducted regarding the relationship between the salivary levels of PR3 and dental caries. Yang et al. reported lower levels of PR3 in patients having dental caries as compared to the caries-free group, and found an inverse relationship between the salivary concentration of PR3 and the severity of caries,<sup>12</sup> which is in contrast to our results. This difference can be justified by a lower mean dmfs score of patients in their study (i.e., 6.3 vs. 11.8 in the present study). One of the main tasks of PR3 is the production and activation of antimicrobial peptides.<sup>23</sup> Thus, as long as dental caries is present as a source of infectious bacteria, higher salivary levels and activity of PR3 are expected. Similar findings were reported in previous studies regarding periodontitis.<sup>24,25</sup> Since PR3 serves as a regulator of the immune system and inflammatory reactions, its increased level in the S-ECC group can be considered a mechanism of host immunity to confront bacteria and prevent uncontrolled inflammatory responses.

To the best of our knowledge, the effect of treating carious lesions on the salivary levels of PR3 has not been investigated in any previous studies. Considering the post-treatment reduction in the salivary levels of IL-8 as a neutrophil chemotactic factor in the present study, the subsequent reduction in the PR3 levels can be attributed to decreased migration of neutrophils, as the primary source of PR3, to the oral cavity. Furthermore, the post-treatment care employed in the present study, including oral hygiene instruction, nutritional counseling, and professional prophylaxis and fluoride therapy, may be responsible for further reduction of the IL-8 and PR3 levels in the S-ECC group following restorative treatment. In the present study, the time interval between pre- and post-treatment sampling was decided to be 6–8 weeks. This decision was made according to a recommendation by Sharma et al. for using longer periods in future studies, since the concentrations of salivary markers are affected by this time interval, and elevated cytokine levels are expected with more extended duration.<sup>2</sup>

Considering the limitations of the present study and the small number of clinical studies evaluating the relationship between IL-8 and PR3 and dental caries, further studies with larger sample sizes and longer follow-up duration are required to obtain more definitive conclusions regarding this issue.

## Conclusions

The salivary levels of IL-8 and PR3 were significantly affected by the presence of dental caries in children. Therefore, the analysis of these salivary cytokines can be considered a suitable, non-invasive method for the determination of the caries risk and treatment effectiveness.

## Ethics approval and consent to participate

The research was designed and performed in accordance with the Declaration of Helsinki, and was approved by the institutional committee for ethics in research (IR.SBMU.DRC.REC.1397.036). Each participant was enrolled in the study after reading, understanding and completing the written informed consent document by their parent.

## Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Consent for publication

Not applicable.

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