



Relationship between the IgA antibody response against *Streptococcus mutans* GbpB and severity of dental caries in childhood



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ABSTRACT

Objective: Explore the associations between the severity of dental caries in childhood, mutans streptococci (MS) levels and IgA antibody response against *Streptococcus mutans* GbpB. Moreover, other caries-related etiological factors were also investigated.

Design: 36–60 month-old children were grouped into Caries-Free (CF, n = 19), Early Childhood Caries (ECC, n = 17) and Severe Early Childhood Caries (S-ECC, n = 21). Data from socio-economic-cultural status, oral hygiene habits and dietary patterns were obtained from a questionnaire and a food-frequency diary filled out by parents. Saliva was collected from children for microbiological analysis and detection of salivary IgA antibody reactive with *S. mutans* GbpB in western blot.

Results: S-ECC children had reduced family income compared to those with ECC and CF. There was difference between CF and caries groups (ECC and S-ECC) in MS counts. Positive correlations between salivary IgA antibody response against GbpB and MS counts were found when the entire population was evaluated. When children with high MS counts were compared, S-ECC group showed significantly lower IgA antibody levels to GbpB compared to CF group. This finding was not observed for the ECC group.

Conclusions: This study suggests that children with S-ECC have reduced salivary IgA immune responses to *S. mutans* GbpB, potentially compromising their ability to modify MS infection and its cariogenic potential. Furthermore, a reduced family income and high levels of MS were also associated with S-ECC.

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1. Introduction

Dental caries is an infectious disease that results from the dissolution of tooth mineral by acids derived from bacterial fermentation of sucrose and other dietary carbohydrates (Loesche, 1996). When occurring in young children, this disease is called early childhood caries (ECC). According to the American Academy of Pediatric Dentistry (2014) ECC is characterized by the presence of one or more decayed (noncavitated or cavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth of children under the age of six. However, in children younger than

three years of age, any sign of smooth-surface caries is indicative of severe early childhood caries (S-ECC). From ages three through five, one or more cavitated, missing (due to caries) or filled smooth surfaces in primary maxillary anterior teeth or a decayed, missing or filled score of greater than or equal to four (age 3), five (age 4) or six (age 5) surfaces also constitutes severe S-ECC. The disease can progress and lead to destruction of the primary dentition affecting negatively children's physical and mental health, as well as increasing the risk of new caries lesions in the permanent dentition (Ng & Chase, 2013; Isaksson, Alm, Koch, Birkhed, & Wendt, 2013).

The mutans streptococci (MS) group, mainly *Streptococcus mutans*, is most strongly associated with the pathogenic process of ECC because of its high presence in the biofilms and saliva of the affected children (Ge, Caufield, Fisch, & Li, 2008; Parisotto, Steiner-Oliveira, Silva, Rodrigues, & Nobre-dos-Santos, 2010b). Three important cell-associated antigens (Ags) are correlated directly with the ability of *S. mutans* to adhere and accumulate in the tooth

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surfaces forming dental biofilm: antigen I/II (Agl/II); glucosyltransferases (GtfB, GtfC, GtfD) and glucan-binding proteins (GbpA, GbpB, GbpC, GbpD) (Smith, 2002). Ag I/II promotes interaction of bacterial proteins and host-derived components; Gtf catalyzes glucan synthesis and Gbps increase the binding of *S. mutans* to each other and to glucans deposited on tooth surfaces, contributing to the sucrose-dependent adherence to teeth (Smith, 2002). Several studies have demonstrated that induction of specific antibodies against these antigens, mainly GtfB/C and GbpB, can prevent the development of dental caries in animal models (Jespersgaard et al., 1999; Koga, Oho, Shimazaki, & Nakano, 2002; Smith & Taubman, 1996) and modify infection in humans (Smith & Taubman, 1987).

Salivary immunoglobulins, particularly secretory IgA have major importance in the resistance of the mucosa to oral infections. The secretory IgA immune response represents the first line of adaptive immune defense against mutans streptococci, blocking microbial adhesins and potentially reducing oral colonization with this cariogenic microflora. Salivary IgA has also been shown to enhance the activity of several enzymes such as lactoferrins and lysozymes (Law, Seow, & Townsend, 2007). A clinical study showed that the IgA antibody response against *S. mutans* GbpB was predominant in the first year of life and frequently correlated with the delay in the oral infection with *S. mutans* (Nogueira, Alves, Napimoga, Smith, & Mattos-Graner, 2005). The intensity of IgA patterns against antigens of *S. mutans* was previously studied and lower levels of salivary IgA against GbpB were associated with higher caries risk (Parisotto et al., 2011). However, the relationship between IgA antibody levels against this antigen and different levels of caries has not yet been evaluated. The objective of this study was to explore the associations among the severity of dental caries in childhood, mutans streptococci levels and IgA response against *S. mutans* GbpB. Moreover, caries-related etiological factors were also investigated. The null hypotheses formulated was that immune response, MS levels, socio-economic-cultural aspects, hygiene and dietary habits presented by ECC and S-ECC are not different from that exhibited by CF children.

2. Methods

2.1. Subjects

The study population comprised 36- to 60-month-old children who attended the four public nursery schools in the city of Araçatuba, São Paulo, Brazil. The city's population has access to public water supply with fluoride level of 0.7 ppm. Children's parents as well as administrators at the preschools involved granted written permission for the study which was previously approved by the Research Ethics Committee of Araçatuba Dental School—Univ. Estadual Paulista (UNESP), Brazil (CAAE # 13079213.4.0000.5420).

Data from socio-economic-cultural status, including family income and mother's education level and from dietary habits were obtained from a questionnaire and a food-frequency diary filled out by parents. The diet reflected food consumption of three consecutive days during the workweek, because in the weekend the diet can be highly variable. In addition to the diet data reported by parents, the preschool staff provided information about infant feeding in the school period, as many of the children were in school full time. This diet chart included the time of day that the children ate and drank anything and the content of all meals and snacks. The daily frequencies of total sugar exposure and consumption from baby bottles was calculated from chart data (Parisotto et al., 2010a).

Clinical examinations were performed at the school by a single calibrated examiner (NHC) previously training (Kappa value of

0.75) using mouth mirrors and probes under natural light. Decayed, missing and filled surfaces in deciduous teeth (dmfs) were counted in each child. The exclusion criteria for this study were: children suffering from systemic disease or using long-term medications or antibiotics less than one month before the examination and children with mucosal lesions. Children with only restored or missing teeth were also excluded. Preschoolers who had white chalky spot lesions were excluded from caries free group. All children were encouraged and instructed on dental hygiene and received all other necessary oral care. The inclusion criteria of this study comprise oral/systemic health children from both genders, 36–60 months of age. Initially sixty children were selected. Three of them were absent from schools when saliva was collected. So, fifty-seven children were divided into three groups according to oral health status: caries-free group (CF) (n = 19), early childhood caries group (ECC) (n = 17) and severe early childhood caries group (S-ECC) (n = 21). ECC was defined for this study as the presence of 1 through 3 decayed tooth surface (cavitated lesions) in primary teeth, S-ECC was defined as the presence of decayed surfaces score of ≥ 4 (age 3 years), ≥ 5 (age 4 years), with at least one smooth-surface carious lesion (American Academy of Pediatric Dentistry, 2014).

2.2. Saliva samples

About 2 ml of unstimulated saliva was collected from children at afternoon and at least 1 h after feeding. Tubes were transported on ice to the laboratory and processed within 1 h. After agitation, one hundred microliters of saliva were separated for microbiological procedures. The remaining saliva was clarified by centrifugation at 10000 rpm at 4 °C for 10 min. The supernatants were collected and 250 mM EDTA was added to minimize salivary IgA aggregation. Aliquots of 50 μ l of each saliva samples were frozen at -70 °C until immunological analysis (Nogueira et al., 2005).

2.3. Microbiological procedures

Aliquots of saliva were homogenized by vortexing for 1 min and the suspensions were serially diluted in 0.9% NaCl solution. Each dilution was cultivated in triplicate on Mitis Salivaris Agar (Difco Laboratories, Detroit, MI, USA) with 0.2 U/mL bacitracin for isolation of mutans streptococci (MS). All plates were incubated at 37 °C for 48 h in a 5% CO₂ atmosphere. After 48 h, the total number of colony-forming units (CFU) was counted using a stereoscopic microscope. Results were expressed as CFU/ml.

2.4. Total salivary IgA level measurement

The concentration of total IgA in saliva samples were determined by an enzyme-linked immunosorbent assay (ELISA) kit using a commercially available analysis kit (Mabtech Inc., Cincinnati OH, USA) and following the manufacturer's instructions.

2.5. Western blot analysis of salivary antibody to mutans GbpB

In order to analyze the influence of patterns of specificity of IgA response to *S. mutans* GbpB, levels of MS infection and caries status, Western blot assays were performed using saliva samples from children and tested against Ags extracted from a standard *S. mutans* strain (ATCC 25175). For Ags preparation, colonies of *S. mutans* from fresh Brain Heart Infusion Agar (BHI, Difco) were inoculated in 5 ml BHI broth and incubated for 18 h. Bacterial cells were then harvested from 1 ml of cultures previously adjusted to an absorbance of 1.0 (A550 nm). Cells were resuspended in TE containing 100- μ m-diameter zirconia/silica beads and mechanically disrupted using a Mini-BeadBeater (BioSpec) at maximum

speed (2800 rpm) for 1-min pulses, three times, with a 30-s rest on ice between pulses. Cell pellets were then boiled in Laemmli buffer for 5 min, and protein extracts were separated by centrifugation at 4°C (10000 rpm for 4 min). Protein concentrations were determined by the method of Bradford and a total of 16 µg of protein extract was used for Western blot analysis (Nogueira et al., 2005). Ags extracts were loaded per lane, separated by sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After transference, membranes were washed and blocked overnight at 4°C (in Tris-buffered saline–Tween, pH 7.5, 5% nonfat milk). Incubations with saliva samples diluted 1:100 were performed at room temperature for 2 h. As negative controls, membranes were incubated only with blocking buffer, and as positive controls, membranes were incubated with a standard saliva sample obtained from an adult subject whose pattern of reaction with *S. mutans* antigen extracts had been previously measured. The secondary antibody was HRP–Goat Anti-Human IgA (1:4000 dilution) (Invitrogen, Life Technologies, USA). Immunoreactive bands were detected by autoradiography using ECL chemiluminescent substrate reagent kit (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. X-ray films were scanned in a transilluminator using a White Light Converter Plate (UVP, LLC, Upland, CA, USA) and the patterns of antigen recognition, including the number and intensity of reactive bands were analyzed with UVP Image software. The molecular weight of *S. mutans* GbpB was about 60 kDa. Migration position of GbpB were determined in parallel western blot assays performed with specific polyclonal rat antiserum to GbpB (Smith & Taubman, 1996).

2.6. Statistical analysis

The statistical analysis was performed considering caries status: CF, ECC and S-ECC as the dependent variables. The comparisons among the groups were performed according to data distribution, considering Kolmogorov-Smirnov Z tests. ANOVA and Tukey tests were applied for age and sugar intake. Kruskal-Wallis and Mann-Whitney tests were applied for gender comparison, family income, mother's education level, artificial (bottle) feeding, mutans streptococci/lactobacilli counts and total IgA levels. Medians and ranges of bacterial counts were expressed as log (CFU + 1) and the constant 1 was added to CFU counts, when

the sample showed zero CFUs. Pearson correlation tests were conducted to compare IgA levels against *S. mutans* GbpB and bacterial counts for the entire population. The reactivities of salivary IgA antibody with *S. mutans* GbpB were compared with respect to caries severity (CF, ECC and S-ECC) and mutans streptococci levels (low mutans streptococci – LMS and high mutans streptococci–HMS) using Mann-Whitney tests.

3. Results

Means/standard deviations obtained for caries levels (dmfs) were: CF (0), ECC (2 ± 1.06) and S-ECC (23.43 ± 17.17). Considering white spots lesions, dmfs were: CF (0), ECC (2.94 ± 1.67) and S-ECC (28.28 ± 18.76). There were no statistical differences among the groups in relation to age, gender, mother's education level, diet habits (artificial feeding and sugar intake) and total IgA levels (Table 1). Families of S-ECC children had reduced income compared to families of ECC and CF children showing a relationship between high scores of caries and economic condition of family. Statistical differences between CF × ECC and CF × S-ECC children were observed for mutans streptococci (MS) counts. S-ECC children were heavily colonized by MS. There was a gradual

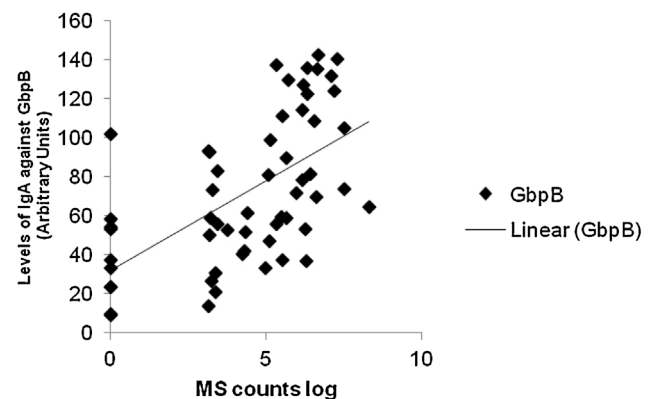


Fig. 1. Scatter plot showing correlation between levels of IgA antibody reactive with GbpB and mutans streptococci (MS). The analysis was carried out for the total population, regardless the caries status. Positive Pearson correlation ($R=0.583$, $p=0.00$) was observed for MS counts.

Table 1

Comparative analysis between the severity of early childhood caries and related etiological factors.

		CF	ECC	S-ECC	p value
Dmfs (mean ± SD)		0 ^a	2 ± 1.06^b	23.43 ± 17.17^c	0.00
Age (months) Mean ± SD		46.26 ± 5.05^a	45.94 ± 9.83^a	48.10 ± 8.59^a	0.664
Gender (%)	Female	47.37 ^A	35.29 ^A	52.38 ^A	0.571
	Male	52.63	64.71	47.62	
Family income per month	≤R\$ 1448.00 [*]	41.1 ^A	25 ^B	64.71 ^C	0.05
Mother's education (%)	Up to 8 years	37.50 ^A	35.71 ^A	47.62 ^A	0.737
Mother's help with tooth brushing (%)		82.35 ^A	80.0 ^A	65.0 ^A	0.961
Bottle feeding (%)		82.36 ^A	66.67 ^A	85 ^A	0.692
Sugar intake (mean ± SD)	liquid	4.21 ± 1.37^a	4.02 ± 1.18^a	4.56 ± 1.38^a	0.465
	solid	2.70 ± 0.90^a	3.17 ± 1.43^a	2.97 ± 1.40^a	0.565
	total	6.88 ± 1.62^a	7.19 ± 1.66^a	7.53 ± 2.32^a	0.579
mutans streptococci median (range)	(log UFC + 1)	3.35 (0–7.29) ^A	3.74 (0–6.66) ^A	5.63 (3.15–8.3) ^B	0.012
total lactobacilli median (range)	(log UFC + 1)	0 (0–6.79) ^A	3.30 (0–6.47) ^A	1.97 (0–6.72) ^A	0.447
Total IgA (ng/ml) Median (range)		99.15(24.52–114.24) ^A	83.24 (32.27–114.56) ^A	88.04 (18.17–109.31) ^A	0.125

^{a,b,c} Different lower case letters show statistical difference among the groups, according to ANOVA and Tukey tests.

^{A,B,C} Different upper case letters show statistical difference among the groups, according to Kruskal-Wallis and Mann-Whitney tests.

CF—caries free, ECC—early childhood caries, S-ECC—severe early childhood caries.

^{*} R\$—Brazilian real. 1 US\$ ~ R\$ 3.50 (2015, August).

Bold represents values of $p < 0.05$.

Table 2

Medians (range) of total salivary IgA, distributed according to mutans streptococci levels.

	MS levels ^{a,b}		<i>p</i> value
	LMS	HMS	
CF	101.9 (24.52–114.24)	91.05 (60.04–107.43)	0.375
ECC	81.93 (32.27–107.43)	84.55 (48.02–114.56)	0.341
S-ECC	98.92 (18.17–104.14)	84.10 (26.01–109.31)	0.357
<i>p</i> value	0.380	0.243	

^a There was no statistical difference among groups of children (columns), considering each MS levels separately (LMS or HMS), using Kruskal-Wallis tests.

^b There was no statistical difference inside each group of children (rows), comparing MS levels (LMS x HMS), using Mann-Whitney tests.

increase in the MS count with respect to the severity of the disease (Table 1). Considering the total population, positive correlations between salivary IgA levels to GbpB and MS counts were found (Fig. 1). For these reasons, children were paired according to levels of MS within each group. Considering the mean of MS counts of the population, the groups of children (CF, ECC and S-ECC) were subdivided as follows: LMS – low mutans streptococci count (with MS counts $\leq \log 4$ CFU/ml) and HMS – high mutans streptococci count (with MS counts $\geq \log 5$ CFU/ml). Comparing LMS and HMS, there was no significant difference among the groups in relation to total IgA levels (Table 2). The levels of IgA antibody reactive with GbpB were lower in S-ECC compared to ECC and CF children, only for HMS (Fig. 2). IgA antibody levels to GbpB increased for all groups, when compared with the same groups of children (CF, ECC and S-ECC) with LMS and HMS counts (Fig. 2). However, S-ECC children with high mutans streptococci counts showed significantly lower IgA antibody levels to GbpB compared to the CF group with HMS. No statistically significant differences were observed comparing ECC with the other groups with HMS (Fig. 2).

4. Discussion

Early childhood caries has a complex etiology with biological, behavioral, and socioeconomic influences (Arora, Schwarz, & Blinkhorn, 2011; Ng & Chase, 2013). For this reason, in this study, some factors determining oral health were concomitantly analyzed. S-ECC children had a reduced family income compared to CF and ECC groups, however, no difference among the groups were found in relation to mother's education level. High prevalence of dental caries in children with adverse socio-economic conditions was previously confirmed by another studies (Oliveira, Sheiham, & Bönecker, 2008; Parisotto et al., 2010a). However, discrepancy in relation to level of mother's education was found in the literature, with children whose mothers had less than 8 years of education with high caries levels (Oliveira et al., 2008) and absence of difference between caries-free and caries group (Parisotto et al., 2010a), in agreement with our study. This lack of difference probably occurred because children were selected from the schools with similar social characteristics. A strong association between high frequency of sugar exposure and occurrence of dental caries has been established yet (Kalsbeek & Verrips, 1994; Milgrom et al., 2000; Parisotto et al., 2010a). However, the present study did not find a significant difference in sugar exposure among the groups, similar to found in another study (Ohlund et al., 2007) which it was not correlated caries status with sugar intake frequency. One possible explanation for this finding is that the responses from questionnaires may have reflected present, not historical experience, which would have better revealed habits during the period when caries had started.

Several previous studies found mutans streptococcus is a significant factor for the presence of ECC (Ge et al., 2008; Milgrom

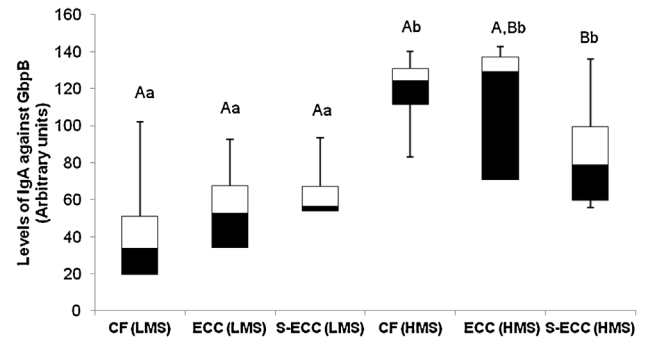


Fig. 2. Box plots of the western blot reactivity of salivary IgA antibody with *S. mutans* GbpB among the groups of children, distributed according to caries status (CF, ECC and S-ECC) and mutans streptococci levels (LMS and HMS). Bars indicate minimum and maximum values. Black and white boxes indicate lower and upper quartiles, respectively. Line in the middle of boxes is median. A—Different upper case letters show statistical difference among groups of children considering each MS level separately (LMS or HMS), according to Mann-Whitney tests. For example: GbpB CF (LMS) x GbpB ECC (LMS).

a—Different lower case letters show statistical difference inside each group of children, comparing MS level (LMS x HMS), according to Mann-Whitney tests. For example: GbpB CF (LMS) x GbpB CF (HMS).

CF—caries free, ECC—early childhood caries, S-ECC—severe early childhood caries. LMS— Low mutans streptococcus levels.

HMS – High mutans streptococcus levels.

et al., 2000; Ohlund et al., 2007). Our results showed that S-ECC children were highly colonized by mutans streptococci compared with CF and ECC children. This information confirmed data from literature which have demonstrated that children with high *S. mutans* counts have more risk to develop dental caries (Ge et al., 2008; Milgrom et al., 2000; Ohlund et al., 2007). A recent systematic review and meta-analysis showed that there is scientific evidence of *S. mutans* transmission from mother to child especially when the mother is the primary caregiver (da Silva Bastos et al., 2015), although 18% of bacterial strains were from an unknown source (Kozai et al., 1999). The American Academy of Pediatric Dentistry (2014) recommended the reduction of mutans streptococci levels of mothers, primary caregivers and sibling(s), educating them on avoiding saliva-sharing behaviors (e.g., sharing spoons, cups and other utensils, cleaning a dropped pacifier or toy with their mouth), to reduce the child's risk of ECC.

The literature has shown that a better immune response to oral microorganisms, mainly *S. mutans*, may be a protective factor against the development of dental caries (Davidopoulou, Diza, Menexes, & Kalfas, 2012; Nogueira et al., 2005; Tao et al., 2005). This observation may be valid for both innate and adaptive immune responses. Reduced levels of some antimicrobial peptides, components of innate immune response, are associated with caries in childhood (Davidopoulou et al., 2012; Tao et al., 2005). The action of salivary IgA against specific surface proteins of cariogenic bacteria such as *S. mutans* has been the subject of many studies (Nogueira et al., 2007, 2005; Parisotto et al., 2011), with focus on GbpB and Gtf expression. These proteins may be targets in the development of vaccines against dental caries (Kim et al., 2011; Smith & Taubman, 1996). The immunization with GbpB induced an immune response that interfered with the accumulation of *S. mutans* and reduced the levels of dental caries in rats (Smith & Taubman, 1996). The caries protection resulting from immunization of rats with Gtf was lower than observed after immunization with *S. mutans* GbpB. The same study showed that saliva of sham-immunized/*S. mutans* infected rats contained antibody to GbpB in saliva at the end of the experiment, indicating that infection with *S. mutans* can induce an immune response to this antigen.

Preschoolers with a lower baseline level of salivary IgA antibody reactive with GbpB had 7.5 higher risk to develop caries, but any differences between CF and caries group (ECC mixed with S-ECC) in relation to salivary IgA against *S. mutans* antigens (GbpB, Gtf) was found (Parisotto et al., 2011). The results of the current study are partially in accordance with this mentioned study. We did not find a difference in IgA antibody levels against GbpB between CF and ECC children, regardless of their mutans streptococci levels. However, difference was found in anti-GbpB antibodies levels between CF and S-ECC children, considering only children with high mutans streptococci levels (HMS), suggesting that reduced levels of IgA against GbpB may be related to high severity of dental caries. The absence of difference in total salivary IgA levels among the groups reinforces that heavily-infected S-ECC children have a reduced immune response against GbpB, and this immunological failure could have contributed to the severity of caries status. Study demonstrated that salivary IgA levels to *S. mutans* antigens were higher in a caries-free group than in caries group and persisted in children from 3 to 11 years old (Bolton & Hlava, 1982) demonstrated that a recent study comparing specific IgA levels in three-year old children revealed increased concentrations of anti-*S. mutans* IgA and anti-*S. sanguinis* IgA in children who were culture positive for *S. mutans* compared with those who were culture negative (Malcolm et al., 2014).

Children infected with *S. mutans* showed a delay in the immune response to the *S. mutans* GbpB antigen during the first year of life (Nogueira et al., 2007). This study paired children according to mutans streptococci infection and they were divided in two groups: infected or non-infected children. In the current study, children were paired according to the mutans streptococci (MS) counts (CFU/ml) because a positive correlation was observed for this variable and IgA response to *S. mutans* GbpB, for the total population, besides no children had zero *S. mutans* counts and can be considered non-infected. Then it was obviously expected that with the increasing of bacterial infection, higher host immune response to pathogen will occur (Nogueira et al., 2007).

The importance of GbpB for *S. mutans* viability has been studied by several investigators (Duque et al., 2011; Fujita, Matsumoto-Nakano, Inagaki, & Ooshima, 2007; Matsumoto-Nakano, Fujita, & Ooshima, 2007). Using a GbpB-deficient mutant strain, authors suggested that GbpB may have an important role in cell-wall construction, as well as in the cell separation and cell-wall maintenance in *S. mutans*, similar to murein hydrolases (Fujita et al., 2007). Furthermore, a GbpB-deficient mutant was more sensitive to acid pH in the acid killing assays (Matsumoto-Nakano et al., 2007), had decreased autolysis, increased cell hydrophobicity, and increased sensitivity to antibiotics and osmotic and oxidative stresses (Duque et al., 2011). These functions, associated with binding of *S. mutans* to glucans deposited on tooth surfaces, highlight the important role of GbpB in the biofilm formation and survival of *S. mutans* in the oral cavity (Matsumoto-Nakano et al., 2007). Thus a good strategy to control dental caries could be the interference in the virulence factors of *S. mutans*, such as GbpB, by means the development of vaccines to reduce its cariogenicity (Smith & Mattos-Graner, 2008).

In conclusion, this study suggests that children with severe early childhood caries and high levels of mutans streptococci have reduced salivary IgA response to *S. mutans* GbpB showing that this parameter may influence the severity of dental caries in childhood. Furthermore, a reduced family income and high levels of MS were also associated with S-ECC.

Conflict of interest

The authors declare that they have no conflict of interest.

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