UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

DANIELE DE CASSIA RODRIGUES PICCO

ESTUDO DA CONCENTRAÇÃO E ATIVIDADE DA ANIDRASE CARBÔNICA VI NA SALIVA E BIOFILME DE CRIANÇAS EM IDADE ESCOLAR E SUA RELAÇÃO COM A CÁRIE DENTÁRIA

STUDY OF CONCENTRATION AND ACTIVITY OF CARBONIC ANHYDRASE VI IN SALIVA AND BIOFILM OF CHILDREN IN SCHOOL AGE AND ITS RELATION TO TOOTH DECAY

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Odontologia, na área de Odontopediatria.

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Orientadora: Profa. Dra. Marinês Nobre dos Santos Uchôa

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Um coração vibrante, energizado pelo sorriso de Deus e sustentado pelo entendimento de que Ele se deleita em você é o melhor e necessário para toda a vida, a fonte de onde tudo o mais flui.

“O homem semeia hoje a causa, Deus amanhã amadurece o efeito.”

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“Que sua colheita seja abundante e eterna, e o sorriso da felicidade e do sucesso enfeite os seus lábios.”

Lauro Trevisan

RESUMO

A anidrase carbônica VI (AC VI) é a principal enzima responsável pela manutenção da capacidade tampão no meio bucal. Não há relatos na literatura sobre a relação entre a atividade dessa isoenzima, pH, fluxo e capacidade tampão e a prevalência de cárie, e se a atividade da AC VI pode ser empregada como indicador de risco de cárie. Os objetivos desta tese foram: 1)Realizar uma revisão sistemática da literatura 2)Determinar a concentração e atividade da AC VI na saliva e biofilme de crianças com cárie (C) e livres de cárie (LC) bem como o fluxo salivar, pH e capacidade tamponante; e 3)Investigar a relação entre essas variáveis na saliva e biofilme e a cárie dental. Para isto, 74 escolares de 7 a 9 anos foram divididos em 2 grupos. Foi realizado exame clínico, coleta de saliva e biofilme. Os ensaios da concentração da AC VI foram realizados por Elisa. A análise da atividade da AC VI por zimografia. O fluxo salivar foi expresso em mL/min. O pH foi determinado por um medidor de pH portátil, e o pH do biofilme por eletrodo específico. A capacidade tamponante da saliva foi medida eletrometricamente. Os dados foram submetidos ao teste t de Student e Mann-Whitney, além da análise de correlação de Pearson e Spearman (α=0,05). A análise bivariada foi realizada com o objetivo de verificar associações entre a variável dependente e independentes. Na modelagem multivariada, as associações entre as variáveis dependentes e independentes foram expressas como odds ratios com intervalos de confiança de 95%. Os resultados mostraram valores significativamente maiores de fluxo, pH e capacidade tampão salivar no grupo LC. O pH do biofilme também foi maior no grupo LC. A concentração da AC VI na saliva e no biofilme foi significativamente maior no grupo LC. Já a atividade da AC VI foi significativamente maior no grupo C na saliva e biofilme. Observou-se correlação negativa entre a capacidade tampão salivar e a cárie dentária. Além disso, observou-se uma correlação positiva entre a concentração e atividade da AC VI na saliva e uma correlação negativa entre a capacidade tampão e a atividade da isoenzima no grupo C. No grupo LC foi observada correlação negativa entre o pH e concentração da AC VI. Um menor fluxo salivar e uma maior atividade da isoenzima foram significativamente associados com a cárie. Para a placa dental, foi observada correlação negativa entre atividade e concentração da AC VI e entre o pH e a atividade da AC VI no grupo LC. No grupo C foi observada correlação negativa entre o pH e a concentração da AC VI. Conclui-se que a AC VI mostrou estar mais ativa na saliva e placa dental de crianças com cárie ativa. Uma maior concentração da AC VI na saliva e placa dental de crianças livres de cárie sugere que como são menos expostas a desafios cariogênicos, o sistema carbonato seria menos ativado. A AC VI na saliva e placa dentária pode ser considerada uma proteína anti-cáries, além de um biomarcador para a cárie dentária.

Palavras-chave: Anidrases Carbônicas. Saliva. Placa dentária. Cárie dentária.

ABSTRACT

The carbonic anhydrase VI (CA VI) is the main enzyme responsible for maintaining the buffer capacity in the oral environment. There are no reports in the literature on the relationship between the activity of this isoenzyme, pH, flow and buffering capacity and the prevalence of caries, and if the activity of CA VI can be employed as caries risk indicator. The objectives of this thesis were: 1) Conduct a systematic review of the literature 2) Determine the concentration and activity of CA VI in saliva and dental plaque of children with caries (C) and caries-free (CF) and the salivary flow, pH and buffering capacity; and 3) To investigate the relationship between these variables in saliva and dental plaque and tooth decay. For this, 74 students aged 7 to 9 years old were divided into 2 groups. It performed clinical examination, saliva collection and biofilm. The assays of the concentration of CA VI was performed by Elisa. The analysis of the CA VI activity by zymography. The salivary flow rate was expressed in ml/min. The salivary pH was determined by a portable pHmeter and the plaque pH with a selective electrode. The buffering capacity of saliva was measured eletrometrically. Data were subjected to Student's t test and Mann-Whitney, besides the Pearson and Spearman correlation analysis ($\alpha = 0.05$). A bivariate analysis was performed in order to verify associations between the dependent and independent variables. In multivariate modeling, associations between the dependent and independent variables were expressed as odds ratios with 95% confidence intervals. The results showed significantly higher values of flow, salivar pH and buffering capacity in the CF group. The pH of the biofilm was also higher in the CF group. The concentration of CA VI in saliva and plaque were significantly higher in the LC group. The activity of CA VI was significantly higher in group C in saliva and plaque. There was a negative correlation between salivary buffering capacity and tooth decay. Moreover, a positive correlation was observed between the concentration and activity of CA VI in saliva and a negative correlation between the buffer capacity and isoenzyme activity in group C. In the CF group there was a negative correlation between pH and concentration of CA VI. A minor salivary flow and increased isoenzyme activity were significantly associated with caries. For dental plaque, it was observed negative correlation between activity and concentration of CA VI and between pH and activity of CA VI in the LC group. In the C group there was a negative correlation between pH and concentration of the CA VI. It concludes that the CA VI proved to be more active in saliva and dental plaque of children with active decay. Greater concentration of CA VI in saliva and dental plaque of caries-free children suggests that they are less exposed to cariogenic challenges, the system carbonate would be less active. The CA VI in saliva and dental plaque can be considered an anti-caries protein, as well as a biomarker for dental caries.

Keywords: Carbonic anhydrases. Saliva. Dental plaque. Dental caries.

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária continua sendo um grave problema de saúde pública devido à sua natureza multifatorial, de alto custo de tratamento, e é a principal causa de dor e perda de dentes o que afeta a qualidade de vida de crianças e adolescentes (Warren, 2009; Ditmyer, 2010). De acordo com evidências científicas, a cárie dentária é uma doença açúcar dependente, modulada por vários fatores adicionais tais como microrganismos orais, ácidos, propriedades diferenciais dos dentes e o fluxo salivar, que modifica o efeito primário dos açucares (Sheiham, 1987; Scheutz, 1999; Sheiham, 2015). Evidências atuais mostram que a cárie dentária é uma doença complexa modulada por fatores genéticos, comportamentais, sociais e ambientais (Masood et al., 2012). Considerando esta complexa etiologia da cárie dentária, o maior desafio para os epidemiologistas tem sido identificar possíveis fatores determinantes e preditores da cárie dentária, com o objetivo de adotar medidas de saúde pública apropriadas para prevenir e controlar a doença e suas desagradáveis consequências.

Além dos fatores microbianos, vários componentes salivares também estão relacionados à etiologia da cárie, como fluxo, viscosidade, pH e capacidade tamponante, os quais desempenham um papel importante na prevenção, início e progressão da doença. Além disso, imunoglobulinas salivares incluindo IgA, IgG e IgM, ajudam na prevenção da cárie devido aos seus efeitos antibacterianos (Shifa et al., 2008).

Além da saliva, a placa dentária, que tem recebido várias definições, é claramente um biofilme, isto é, um grupo de microorganismos incorporados em uma matriz anexa à superfície dos dentes. Todos os biofilmes têm pelo menos uma propriedade em comum, ou seja, a presença de uma matriz bacteriana (Bowen et al., 2011). No biofilme cariogênico, há uma predominância dos streptococcus do grupo mutans, principalmente S. mutans e outros microorganismos acidúricos, os quais produzirão ácidos que poderão resultar na desmineralização do dente.

No que diz respeito à placa dentária, hábitos alimentares de alta frequência de exposição ao açúcar e consequente metabolismo bacteriano que leva à formação de ácido, resultam em aumento da acidogenicidade da placa, que pode influenciar o processo de cárie. Kumar et al. (2011) reportam que a queda do pH da saliva e da placa dental podem ser atribuídos à concentração de microorganismos cariogênicos na placa dental ou cavidade bucal. Neste contexto, os mecanismos salivares tamponantes desempenham papel importante na

normalização do pH no meio bucal o mais rápido possível (Tenovuo, 1997). A maior capacidade tampão salivar pode ser atribuída à maior concentração de bicarbonato. O bicarbonato contribui com aproximadamente 80% da capacidade tampão da saliva humana, e é encontrado em concentrações mais elevadas na saliva estimulada devido à maior proporção de secreções das glândulas parótidas (Gittings et al., 2015).

As isoenzimas anidrases carbônicas (AC) são metaloisoenzimas de zinco que participam da manutenção da homeostase do pH em vários processos fisiológicos do corpo humano, que incluem acidificação renal e do trato reprodutivo masculino, reabsorção óssea, respiração, gliconeogênese, transdução de sinal, calcificação, formação de ácido gástrico e muitos outros processos fisiológicos e patológicos (Sly et al., 1995). As anidrases carbônicas são uma classe de isoenzimas que tem como função a manutenção da homeostase do pH em tecidos e fluidos corporais, catalisando a hidratação do dióxido de carbono na reação $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. Existem oito proteínas AC citosólicas (I, II, III, VII, VIII, X, XI, XIII), duas proteínas de matriz mitocondrial (VA e VB), uma proteína secretada (VI), duas proteínas glicosilfosfatidilinositol (GPI) ancoradas (IV e XV), e três proteínas transmembrana (IX, XII, XIV) (Frost, 2014).

A AC VI presente na saliva é adsorvida à superfície dental e se torna ativa para catalisar a conversão de bicarbonato salivar e íons hidrogênio produzidos por bactérias cariogênicas na película do esmalte, em dióxido de carbono e água (Leinonen, et al., 1999). Assim, tem sido sugerido que o mecanismo pelo qual a AC VI protege os dentes, dá-se pela aceleração da remoção do ácido do microambiente local tanto da película quanto do biofilme dental (Leinonen, 1999; Kimoto, 2006). Neste sentido, pesquisas têm investigado a relação entre a concentração da AC VI salivar a experiência de cárie. O estudo de Szabó (1974) mostrou uma maior atividade da AC VI na saliva de crianças livres de cárie. A investigação realizada por Kivelä et al. (1999) mostrou que a baixa concentração de AC VI está associada com maior índice de cárie, além de mostrar uma correlação negativa entre a concentração da AC VI e o índice CPO-D em indivíduos com pobre higiene oral. Ozturk et al. (2008), não encontraram diferença significativa na concentração da AC VI quando adultos jovens com cárie e livres de cárie foram comparados. Frassetto et al. (Frasseto et al., 2012), mostraram que a atividade da AC VI é maior na saliva de crianças cárie ativas. De acordo com Yan et al. (2014), a detecção preliminar de proteínas diferenciais, como a AC VI, pode estabelecer um fundamento para a pesquisa de biomarcadores de susceptibilidade à cárie dentária. Em relação à placa dentária, de acordo com Kimoto et al. (2006), a AC VI está concentrada na placa dental e Peres et al. (2010)

refere que a presença da AC VI contribui para a neutralização do ácido da placa, principalmente na saliva estimulada, cujo tamponamento é realizado principalmente pelo bicarbonato.

Observou-se, no entanto, que não existiam relatos na literatura sobre pesquisas que tenham avaliado a correlação entre a concentração/atividade desta isoenzima na saliva e na placa dental. Além disso, não observamos na literatura trabalhos que tenham investigado a participação da AC VI como indicador de risco de cárie.

Assim, os objetivos gerais desta tese foram investigar a função da AC VI na cavidade bucal, determinando sua concentração na saliva e placa dental de escolares com cárie, além de realizar uma análise quantitativa da atividade desta isoenzima, já que uma alta concentração da AC VI não significaria necessariamente que toda a isoenzima presente no meio estaria ativa. Assim, a determinação da atividade da AC VI e não apenas de sua concentração proporcionaria mais uma evidência do efeito desta isoenzima em proteger a superfície dental contra a cárie dentária.

Dessa forma, esta pesquisa foi realizada com os seguintes objetivos específicos: 1) Realizar uma revisão sistemática da literatura sobre as evidências científicas disponíveis a respeito da AC VI e a relação entre a expressão / atividade desta isoenzima na saliva e placa dental e a experiência de cárie; 2) Determinar a concentração e atividade da AC VI na saliva e na placa dental de crianças com cárie, bem como o fluxo salivar, pH e capacidade tamponante; e 3) Investigar a relação entre estas variáveis a cárie dentária.

2 ARTIGOS

2.1 Carbonic anhydrase VI in saliva and biofilm: a systematic review

Artigo submetido ao periódigo Journal of Dentistry (Anexo 3).

Short title: Systematic review of CA VI.

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Carbonic anhydrase VI in saliva and biofilm: a systematic review

Abstract

Objetive: The aim of this paper was to undertake a systematic review regarding the relationship between the expression/activity of CA VI in saliva and dental plaque and caries experience. **Data:** The literature from August 1968 to November 2015 was searched on the Medline/Pubmed and Cochrane Library. **Sources:** The terms used were "carbonic anhydrase VI", "saliva", "dental plaque" and "dental caries". Inclusion criteria: (1) studies performed in children, (2) type of study: analysis of concentration and/or activity of carbonic anhydrase VI, (3) outcome: relationship between concentration and / or activity of AC VI in saliva, dental plaque and dental caries and (4) language: English. The studies were assessed for methodological quality using the PEDro scale. **Study selection:** The initial search identified 119 references. In the first phase, from the reading of title and abstract, 102 articles were excluded. During the second phase, from the 17 remained studies, 15 were excluded for not considering the dictates of bioequivalence, criteria of inclusion and exclusion, type of study and outcome, and 2 articles were submitted to the methodological analysis. The Kappa coefficient evidenced a good inter-examiner agreement between researchers (75.2%). Finally, both articles were selected for this review work. **Conclusions:** The literature shows conflicting and mixed results regarding the potential protection of CA VI against tooth decay. The selected studies showed opposite results regarding the activity of CA VI and caries experience. More well designed clinical studies, preferably, are needed to examine the effectiveness of this isoenzyme in the oral physiology.

Key words: Carbonic anhydrase VI; saliva; dental plaque; dental caries.

Introduction

Carbonic anhydrase VI catalyzes the reversible hydration of CO_2 ($CO_2+H_20\leftrightarrow HCO_3+H^+$), which allows this enzyme to regulate intra- and extra-cellular concentrations of CO_2, H^+ and HCO_3-. Decades of research have implicated CA in a broad range of physiological processes including gas exchange at the air water interface, transport of CO_2 and HCO_3- across membranes, biosynthetic reactions in metabolically active tissue, acid–base balance, secretion, calcification, signal transduction, oncogenesis, proliferation, among the many that have been reported [1]. These seemingly disconnected functions are mediated by specific isoforms in the CA family. Sixteen members of this family have been identified which have distinct tissue-specific expression, kinetic properties, and sensitivity to inhibitors [2]. It appears unlikely that this family will be expanded further as searches of genomic databases have not identified any additional CA sequences [3]. Among those identified, there are eight cytosolic proteins (CA I, CA II, CA III, CA VII, CA VIII, CA X, CA XI, CA XIII), two mitochondrial matrix proteins (CA VA, CA VB), one secreted protein (CA VI), two glycosylphosphatidylinositol (GPI)-anchored proteins (CA IV and CA XV), and three transmembrane proteins (CA IX, CA XII, CA XIV) [1]. The CA isoenzymes show considerable divergence in the DNA sequence, chromosomal localization and the enzymatic properties [4].

Carbonic anhydrase VI (CA VI) is the only secretory isoenzyme of CA genes family of mammals [5]. The CA VI is secreted into saliva by the serous acinar cells of the parotid and submandibular glands [6]. According to early investigation [5], CA VI seems to be one of the key enzymes in oral physiology, and the rate and efficiency of a biochemical reaction are determined by the level of expression and activity status of the appropriate enzyme [7]. Although the CA VI was originally characterized as an iso-enzyme able to adjust the salivary pH or the buffering capacity, some studies indicate that these variables are not directly related to the concentration of CA VI in saliva [8,9]. It is possible that besides the concentration, the activity of CA VI in saliva may also be influenced by other variables such as genetic polymorphism found in the coding sequences that may modulate the activity of this isoenzyme [10].

Concerning dental biofilm, dietary habits as a high frequency of sugar exposure and frequent acid formation by metabolism of the microbial flora on dental surfaces, can result in dental plaque acidogenicity, which may influence the carious process [11]. Some works suggested a consistent relationship between biofilm acidogenicity factors and dental caries [12,13], however,

other studies have cast some doubt on this association [14,15,16]. Considering CA VI in dental plaque, early investigation by Kimoto et al.,[17] demonstrated its activity in dental plaque and Peres et al., [10] claimed that the CA VI presence contributes to the neutralization of plaque acid, mainly in stimulated saliva whose buffering is mainly performed by bicarbonate.

Regarding the association of CA VI with dental caries, different results have been provided by the literature. The study of Szabó [18] showed that the activity of CA VI was higher in the saliva of caries-free children than in the caries active group. The investigation performed by Kivelä et al. [6] has shown that a low CA VI concentration is associated with higher caries index, besides a negative correlation between CA VI concentration and DMFT index in individuals with poor hygiene. Ozturk et al. [19] found no significant difference in CA VI concentration when caries and caries-free young adults were compared. Frasseto et al. [16] showed that CA VI activity is higher in saliva of caries active children. According to Yan et al., [20] the preliminary detection of differential proteins, including CA VI, may lay some foundation for biomarker research of dental caries susceptibility.

The recognized clinical importance of this isoenzyme is confronted with the variability of information presented by the available scientific evidence. Hence, the aim of this paper is to conduct a systematic review of the literature on the protective potential of carbonic anhydrase VI against tooth decay. This review provides a summary of evidence related to relationship between CA VI concentration/activity in saliva and dental plaque, by applying explicit methods and systematic search, critical assessment and synthesis of the selected information. The available scientific evidence of CA VI, as well as the relationship between the expression / activity of CA VI in saliva and/or dental plaque and caries experience were collected.

Materials and methods

Protocol registration

This systematic review followed a guide to summarize careful scientific evidence [21]. The study protocol was not registered. Because this study was a systematic review, ethical approval was not required from the institutional review board of the Piracicaba Dental School, University of Campinas - Piracicaba, SP, Brazil.

Question addressed by this review

What is the scientific evidence on the relationship between the expression / activity of CA VI in saliva or dental plaque and caries experience?

Data/Sources - Literature search

Papers from August 1968 to November 2015 were sought and obtained from online Medline/PubMed and Cochrane Library databases. The main terms used in the search were: (carbonic anhydrase VI) AND saliva) AND dental plaque) OR biofilm) AND dental caries) AND children. The search was limited to articles written in English.

Study selection - Evaluation of scientific papers

The eligibility of the selected studies was determined by reading the title and abstract of the articles identified by the search. If the abstracts were missing, the full-text articles were printed. All papers that appeared to meet the inclusion criteria were selected. Papers were included if English was the publication language.

In the initial search strategy, articles were identified by two authors according to the following inclusion criteria: (1) studies performed in children, (2) type of study: analysis of concentration and/or activity of carbonic anhydrase VI in saliva and/or dental plaque, (3) outcome: relationship between concentration and/or activity of AC VI in saliva and/or dental plaque and dental caries (4) language: English.

The studies that met the inclusion criteria were assessed for methodological quality using the PEDro scale, based on the Delphi list developed by Verhagen and colleagues at the Department of Epidemiology, University of Maastricht [22]. This scale has a 10 points scale, including assessment criteria for internal validity and presentation of statistical analysis. For each criterion defined on the scale, the value of one (1) was regarded as the presence of indicators of the quality of the evidence presented, and zero (0) was attributed to the absence of these indicators according to predetermined criteria for methodology and performance, as defined in Table 1. Papers that had low methodological quality (score lower than 3) would be excluded as well as the ones that had been published in repeated databases, or were considered irrelevant to this review work. Before evaluating papers, the inter-examiner agreement was determined using Kappa calculation and considering all scores of the PEDro scale. With regards to Kappa value, an agreement coefficient greater than 0.61% should be obtained [23]. Disagreements between researchers were solved by discussion and consensus.

Results

The initial literature search yielded 119 references (117 from Medline/Pubmed and 2 from Cochrane Library). In the first phase of the review, 102 references were excluded just by reading the title and abstracts. In the second phase, 17 articles were subjected to a critical reading of full text (Fig. 1).

A full reading of the 17 selected articles allowed us to identify 14 papers as outcome measure in the study population that did not consider the dictates of bioequivalence, criteria of inclusion and exclusion, type of study and outcome. In addition, one topical review was not submitted to the methodological quality analysis by the PEDro scale because it was not a common research work with methodology, results and statistical analysis and thus, were finally excluded. The 2 selected articles were analyzed for methodological quality by the PEDro scale based on the Delphi list.

In the analysis of methodological quality, studies that presented a score lower than 3 would be excluded. At the end of reading the papers in full, none of the articles showed score lower than 3 after analysis of methodological quality by the PEDro scale. Then, the two articles were included in this systematic review. The Kappa value inter-examiner obtained was 75.2%.

In table 2, it can be seen a few more outstanding features of the remaining 2 articles which fulfilled the inclusion criteria and predetermined requirements for this review work as previously described: kind of population, type of study, outcome and language.

Discussion

Saliva, modulated by the ecosystem, plays a critical role in the homeostasis of the oral cavity [24]. Its functions include lubricating the food bolus; protecting against virus, bacteria and fungi; repairing oral mucosa; buffer capacity and remineralizing teeth. It has buffer and neutralizing capacity against acids produced by microorganisms or in the diet, enabling a relatively constant pH to be maintained in bacterial plaque and the oral cavity. It constantly provides calcium and phosphates, which are needed for remineralization processes. Salivary

buffer capacity depends on bicarbonate concentration and its remineralization capacity depends on calcium and phosphate concentrations. Both are correlated with saliva flow rate. Reduced salivary flow rate is a risk factor for caries. Salivary secretion rate, which varies according to the type, strength and duration of the stimulus, is the main factor affecting its composition [25].

The protein concentration in saliva is approximately 2 mg/ml, about one thirtieth of that of the plasma, so that a few amino acids still have significant buffering effect in the usual pH of the oral cavity. The salivary proteins are also involved in modulating colonization of microbes on the teeth and soft tissue in the oral mucosa and chemical modulation salivary calcium and phosphate [26]. The salivary proteins also participate in the formation of the acquired pellicle, which is not only protective, but also influences the initial colonization of the microbial teeth. The production of bases from basic amino acids and peptides in saliva may aid in neutralizing acids in the plaque. Collectively, the salivary proteins, show broad range of functional activities that can assist in maintaining the integrity of the mouth, as well as offering protection against oral and non-oral microbial infections [27].

The existence of carbonic anhydrase in human saliva has been known for 60 years[28], but to this day, little is known about the physiological role and the importance of CA VI in saliva of children. Salivary carbonic anhydrase, a zinc metalloenzyme is the only know secreted isoenzyme of the CA family, which has been detected in the saliva secreted by the serous acinar cells of mammalian parotid and submandibular glands. It catalyzes the reaction that bicarbonate ions neutralize the acids formed by plaque bacteria ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). By catalyzing this reaction, CA VI is believed to provide a greater buffering capacity to saliva by penetrating dental biofilm and facilitating acid neutralization by salivary bicarbonate [17]. There is evidence suggesting that salivary CA is a multifunctional enzyme, which affects taste bud growth, protection of the teeth from caries, and acts as an anti-inflammatory agent [19].

Salivary carbonic anhydrase VI appears to protect teeth from caries via mechanisms other than direct regulation of salivary pH and buffering capacity [29]. In this sense, several researches have investigated the relationship between the CA VI concentration and caries experience [6,19,20,30] and between the CA VI activity and caries experience [16,18]. The relationship among caries, pH and buffering effect of the saliva has frequently been examined and some investigators have found that the buffering capacity and pH show a difference when comparing caries-active and caries-free groups [31,32]. As the literature have shown conflicting evidence on the relationship between the concentration and activity of CA VI in saliva or dental plaque of

subjects with caries and caries-free, this systematic literature review was conducted to critically evaluate this issue.

The study of Szabó [18] was the oldest of this systematic review and made the minimum acceptable level score based on the methodological analysis. Their study was performed with school children and adolescents aged 7 to14 years old and showed that in both stimulated and unstimulated saliva, CA VI activity was higher in saliva of caries-free children than in the caries-active children living under standardized conditions. However, the authors were not able to find a significant difference in CA VI activity in saliva of caries and caries-free subjects when they investigated children who lived and had their meals with their family at home. These results give emphasis to the importance of selecting subjects whose nutrition and living conditions are easily controllable, identical, and constant for at least 2 $^{1}/_{2}$ years. On the other side, a homogenous sample may not represent the CA VI behavior in the whole population. However, the analysis of activity is important since a high concentration of the isoenzyme CAVI in saliva may not necessarily means that all isoenzyme present in the media is active to catalyze the most important buffer system in the oral cavity and thus accelerate the neutralization of acid from the local environment of the tooth surface.

In this regard, the investigation performed by Frasseto et al. [16], determined the activity of CA VI in the saliva of 45.3–80.3 months preschool children and investigated the relationship between dental caries and salivary CA VI activity, salivary flow rate as well as biofilm pH before and after a 20% sucrose rinse. In this study, the prerinse CA VI activity was higher in saliva of children with dental caries and difference was close to the significance level ($p=0.0516$). This result does not agree with those reported by Szabó [18], who found a higher activity of CAVI in 7- to 14-year-old caries-free children than in children having caries. These findings also differ from those of Kivelä et al.[6], who demonstrated a low but significant negative correlation between the CAVI concentration in saliva of young adults and DMFT index. However, it should be emphasized that while the methods of analysis employed by the previous authors were able to determine just the expression of salivary CAVI, in the study of Frasseto et al.[16] the authors used the zymography method [33] to quantitatively determine the activity of CA VI in saliva. In their study, Frasseto and co-workers [16] also demonstrated that children age and variation of CA VI activity variables (ΔCAVI - difference between postrinse CAVI activity and prerinse CAVI activity) were significantly associated with dental caries. Regarding the CA VI activity after a 20% sucrose rinse, a significant decrease was observed in the saliva of the children with caries. One possible explanation for these results could be that CA VI catalyzes

the reaction of $HCO_3^- + H^+ \leftrightarrow CO_2 + H_20$ in both directions in a way that it may neutralize or acidify the media depending on the conditions in the oral cavity. Another possible explanation for these findings is that the children with caries may have had a high daily sugar consumption since it is know that this sugar consumption pattern is significantly correlated with early childhood caries [34,35]. Another result of this study was that the variation of CA VI activity was higher in saliva of caries active children. This result is related to the fact that while a significantly lower postrinse CA VI acitivity was found in caries group, a numerical increase in isoenzyme activity was observed in the caries-free group. This difference behavior of CA VI activity may partially be explained by genetic polimorphisms of the CA VI gene [10]. Additionally, no correlation was found between biofilm pH and dental caries. Early works suggested a consistent relationship between biofilm acidogenicity factors and dental caries [36]. Moreover, no correlation was found between biofilm pH and the postrinse CA VI activity. These findings may suggest that CA VI is not directly involved in the regulation of the biofilm pH.

Conclusions

In conclusion, available data on the scientific evidence about carbonic anhydrase VI (CA VI), as well as the relationship between the expression / activity of CA VI in saliva and dental plaque and caries experience suggest that there is an association between the expression / activity of CA VI in saliva and caries experience. However, continued research is needed to confirm the role of this iso-enzyme in modulating the caries dynamic process. Furthermore, future longitunal studies are strongly encouraged to evaluate if considering the relevance of CA VI in saliva and biofilme, this iso-enzime can predict caries development.

References

1. Frost SC. Physiological functions of the alpha class of carbonic anhydrases. *Subcellular Biochemistry* 2014; **75**: 9-30.

2. Supuran CT. Carbonic anhydrases–an overview. *Current Pharmaceutical Design* 2008; **14(7):** 603-614.

3. Hilvo M, Innocenti A, Monti SM, De Simone G, Supuran CT, Parkkila S. Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. *Current Pharmaceutical Design* 2008; **14(7):** 672-678.

4. Aidar M, Marques R, Valjakka J, Mononen N, Lehtimäki T, Parkkila S, de Souza AP, Line SR. Effect of genetic polymorphisms in CA6 gene on the expression and catalytic activity of human salivary carbonic anhydrase VI. Caries research 2013; **47(5)**: 414-20.

5. Kivela J, Parkkila S, Parkkila AK, Leinonen J, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI. *The Journal of Physiology* 1999; **520 Pt 2:** 315-320.

6. Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H .A low concentration of carbonic anhydrase isoenzyme VI in whole saliva is associated with caries prevalence. Caries research 1999; **33(3):** 178-184.

7. Zeng Y. The functional consequences of relative substrate specificity in complex biochemical systems. *Frontiers in Genetics* 2011; **2:** 65.

8. Kivelä J, Parkkila S, Metteri J, Parkkila AK, Toivanen A, Rajaniemi H. Salivary carbonic anhydrase VI concentration and its relation to basic characteristics of saliva in young men. *Acta Physiologica Scandinavica* 1997; **161(2):** 221-225.

9. Parkkila S, Parkkila AK, Vierjoki T, Ståhlberg T, Rajaniemi H. Competitive time-resolved immunofluorometric assay for quantifying carbonic anhydrase VI in saliva. *Clinical Chemistry* 1993; **39(10):** 2154-7.

10. Peres RC, Camargo G, Mofatto LS, Cortellazzi KL, Santos MC, Nobre-dos-Santos M, Bergamaschi CC, Line SR. Association of polymorphisms in the carbonic anhydrase 6 gene with salivary buffer capacity, dental plaque pH, and caries index in children aged 7-9 years. *The Pharmacogenomics Journal* 2010; **10(2):** 114-119.

11. Peres MA, Sheiham A, Liu P, Demarco FF, Silva AE, Assunção MC, Menezes AM, Barros FC, Peres KG. Sugar Consumption and Changes in Dental Caries from Childhood to Adolescence. *Journal of Dental Research* 2016; pii: 0022034515625907.

12. Fosdick LS, Englander HR, Hoerman KC, Kesel RG. A comparison of pH values of in vivo dental plaque after sucrose and sorbitol mouth rinses. *The Journal of the American Dental Association* 1957, **55(2)**: 191-5.

13. Stephan RM. Some local factors in the development of cavities; plaques, acidity, aciduric bacteria, proteolytic bacteria. *Journal of Dental Research* 1948; **27(1)**: 96-100.

14. Fejerskov O, Scheie AA, Manji F. The effect of sucrose on plaque pH in the primary and permanent dentition of caries-inactive and -active Kenyan children. *Journal of Dental Research* 1992; **71(1):** 25-31.

15. Dong YM, Pearce EI, Yue L, Larsen MJ, Gao XJ, Wang JD. Plaque pH and associated parameters in relation to caries. *Caries Research* 1999; **33(6)**: 428-36.

16. Frasseto F, Parisotto TM, Peres RC, Marques MR, Line SR, Nobre Dos Santos M. Relationship among salivary carbonic anhydrase VI activity and flow rate, biofilme pH and caries in primary dentition. *Caries research* 2012; **46(3):** 194-200.

17. Kimoto M, Kishino M, Yura Y, Ogawa Y. A role of salivary carbonic anhydrase VI in dental plaque. *Archives of Oral Biology* 2006; **51(2):** 117-122.

18. Szabó I. Carbonic Anhydrase Activity in the saliva of children and its relation to caries activity. *Caries Research* 1974; **8(2):** 187-191.

19. Oztürk LK, Furuncuoğlu H, Atala MH, Uluköylü O, Akyüz S, Yarat A. Association between dental-oral health in young adults and salivary glutathione, lipid peroxidation and sialic acid levels and carbonic anhydrase activity. *Brazilian Journal of Medical and Biological Research* 2008; **41(11):** 956-959.

20. Yan G, Huang W, Xue H, Jia Y, Yang D. Relationship between dental caries and salivary proteome by electrospray ionization ion-trap tandem mass spectrometry in children aged 6 to 8 years. *Hua Xi Kou Qiang Yi Xue Za Zhi* 2014; **32(3)**: 297-302.

21. Sampaio RF, Mancini, MC. Systematic review of studies: a guide to summary careful scientific evidence. *Brazilian Journal of Physical Therapy* 2007; **11(1)**: 83-89.

22. Verhagen AP, de Vet HC, de Bie RA, Kessels AG, Boers M, Bouter LM, Knipschild PG. The Delphi List: A Criteria List for Quality Assessment of Randomized Clinical Trials for Conducting Systematic Reviews Developed by Delphi Consensus. *Journal of Clinical Epidemiology* 1998; **51(12):** 1235–1241.

23. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33(1):** 159-74.

24. Carpenter GH. The secretion, components, and properties of saliva. *Annual Review of Food Science and Technology* 2013; **4:** 267-276.

25. Rodríguez PN, Martínez Reinoso J, Gamba CA, Salgado PA, Mateo MT, Manto Mdel C, Molgatini SL, Iglesias V, Argentieri ÁB. Association among salivary flow rate, caries risk and nutritional status in pre-schoolers. *Acta Odontológica Latinoamericana* 2015; **28(2):** 185-91.

26. Edgar M, Dawes C, O´Mullane, D. Saliva e Saúde bucal - Composição, Funções e efeitos protetores. São Paulo: Santos, 2010.

27. Tenovuo J. Antimicrobial function of human saliva--how important is it for oral health? *Acta Odontologica Scandinavica* 1998; **56(5)**: 250-6.

28. Becks H, Wainwright WW. Human saliva. *Journal of dental research* 1939; 447-456.

29. Leinonen J, Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. *Caries Research* 1999; **33(3):** 185-190.

30. Culp DJ, Robinson B, Parkkila S, Pan PW, Cash MN, Truong HN, Hussey TW, Gullett SL. Oral colonization by streptococcus mutans and caries development in reduced upon deletion of carbonic anhydrase VI expression in saliva. *Biochimica et Biophysica Acta* 2011; **1812(12):** 1567-76.

31. Singh S, Sharma A, Sood PB, Sood A, Zaidi I, Sinha A. Saliva as a prediction tool for dental caries: An in vivo study. *Journal of Oral Biology and Craniofacial Research* 2015, **5(2):** 59-64.

32. Pandey P, Reddy NV, Rao VA, Saxena A, Chaudhary CP. Estimation of salivary flow rate, pH, buffer capacity, calcium, total protein content and total antioxidant capacity in relation to dental caries severity, age and gender. *Contemporary Clinical Dentistry* 2015; **6(Suppl 1)**: S65-71.

33. Kotwica J, Ciuk MA, Joachimiak E, Rowinski S, Cymborowski B, Bebas P. Carbonic anhydrase activity in the vas deferens of the cotton leafworm - Spodoptera littoralis (Lepidoptera: Noctuidae) controlled by circadian clock. *Journal of Physiology and Pharmacology* 2006; **57 Suppl 8**: 107-23.

34. Nobre dos Santos M, Melo dos Santos L, Francisco SB, Cury JA. Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. *Caries Research* 2002; **36(5):** 347-352.

35. Parisotto TM, Steiner-Oliveira C, Duque C, Peres RC, Rodrigues LK, Nobre-dos-Santos M. Relationship among microbiological composition and presence of dental plaque, sugar exposure, social factors and different stages of early childhood caries. *Archives of Oral Biology* 2010; **55(5):** 365-373.

36. Aires CP, Tabchoury CP, Del Bel Cury AA, Koo H, Cury JA. Effect of sucrose concentration on dental biofilm formed in situ and on enamel demineralization. *Caries Research* 2006; **40(1)**: 28-32.

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Tables

Table 1: Indications for the administration of the PEDro scale.

1) Specification of the inclusion criteria (not scored item)
2) Random Allocation
3) Confidentiality allocation
4) Similarity of groups at initial phase or baseline
5) Masking (blinding) of the subjects
6) Masking (blinding) of therapists
7) Masking (blinding) of statistical evaluators
8) Measure at least one primary outcome in 85% of subjects allocated
9) Analysis of intention to treat
10) Comparison between groups of at least one primary outcome
11) Reporting of measures of variability and estimation of the parameters of at least one primary variable.

Table 2. Analysis of studies of CA VI.

Author, year	Type of population	Type of study	Outcome	Score
(Szabó, 1974)	16 schoolchildren aged 7-14 years.	Study of activity of CA VI in saliva of caries and caries-free school children	Higher activity of CA VI in the caries-free children living under standardized conditions.	3
(Frassseto et al., 2012)	30 preschool children aged 3-6 years.	Study of activity of CA VI in saliva of caries and caries-free preschool children.	Higher activity of CA VI in saliva from caries children.	5

Figures

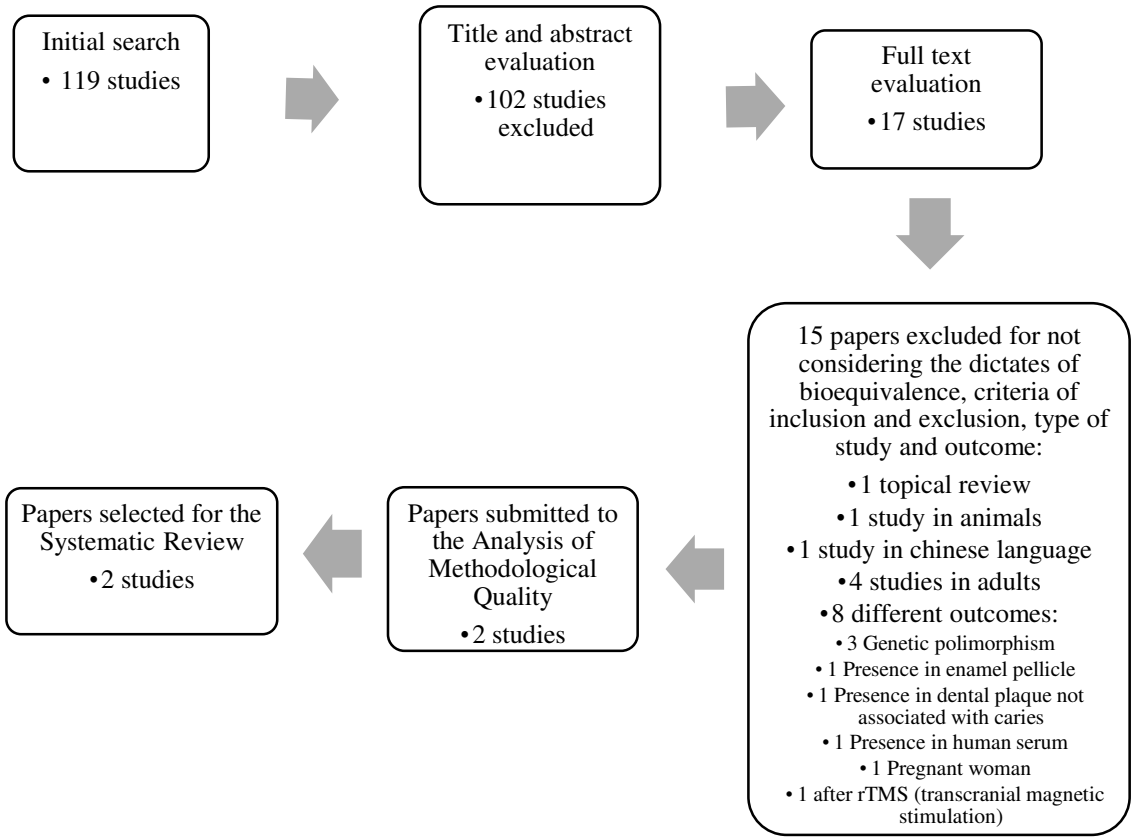

Fig. 1. Flowchart of the article search and study selection.

2.2 Children with higher activity of carbonic anhydrase VI in saliva are more likely to develop dental caries.

Artigo submetido ao periódico Caries Research. (Anexo 4)

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Short title: Carbonic anhydrase VI in saliva of school children.

Key words: Carbonic anhydrases, saliva, dental caries.

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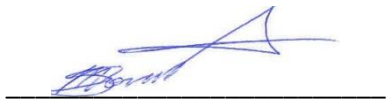
Caries Research

Declaration of Interests

The authors deny any conflicts of interest related to this study.



Marinês Nobre dos Santos

Abstract

Objective: This study aimed to analyze the concentration and activity of CA VI in saliva of school children. We investigated the relationship among caries, CA VI concentration/activity, flow rate, pH and buffering capacity. **Materials and Methods**: Seventy-four school children were divided into caries-free group and caries group. Clinical examinations were conducted by one examiner according to WHO criteria + early caries lesions. Salivary flow rate (SFR), pH and buffering capacity (BC) were analyzed. Salivary CA VI concentration and activity were performed by ELISA and zymography respectively. The data was submitted to Student's t-test and Mann-Whitney apart from Pearson and Spearman correlation analyses. In multivariate modeling, associations between variables were expressed as odds ratios. **Results**: The results showed that SFR, salivary pH as well as BC were significantly higher in saliva of caries-free children. Also, the salivary CA VI concentration was significantly higher in saliva of caries-free children. The salivary CA VI activity was higher in children with caries. We found a negative correlation between BC and dental caries. Also, in caries group we found a positive correlation between concentration and activity of CA VI and negative correlation between BC and CA VI activity. A negative correlation between salivary pH and CA VI concentration was observed in caries free group. High activity of CA and low salivary flow rate are associated with dental caries. **Conclusion**: These results support the conclusion that dental caries is highly affected by activity of CA VI in saliva as well as by the salivary flow rate.

Key words: Carbonic anhydrases, saliva, dental caries.

Introduction

According to recent evidence, dental caries is sugar dependent disease modulated by several additional factors such, oral microorganisms, acids, differential properties of different teeth and salivary flow, that modifies the primary effect of sugars (Sheiham et al., 2015; Scheutz et al., 1999; Sheiham, 1987). Among these additional factors, saliva as a host factor plays an essential role in maintaining the integrity of oral structures by diluting and eliminating sugars and other substances, buffering pH in the oral cavity, balancing demineralization-remineralization process, and exerting its antimicrobial action (Bagherian et al., 2012). Furthermore, the ability of saliva to buffer acids is essential for maintaining pH values in the oral environment. In this regard, bicarbonate ions play a major role in determining the pH and buffering capacity of saliva and this mechanism can help to protect teeth against attack from acids produced by bacteria (Singh et al., 2015).

According to early studies, the carbonic anhydrase VI (CA VI) appears to be one of the key enzymes in the oral physiology in humans and animals (Kimoto et al., 2006; Kaseda et al., 2006; Mau et al., 2010). Carbonic anhydrase is the only isozyme which may be secreted into saliva in order to provide increased salivary buffering capacity, and to promote retention of HCO_3^- in the salivary glands.

Regarding the concentration of CA VI in human saliva quantitative studies have shown that it greatly varies between subjects (Kivelä et al., 1997) and that the rate and efficiency of a biochemical reaction are determined by the level of expression and activity status of the appropriate enzyme (Zeng, 2011). Concerning the relationship between concentration of CAVI and dental caries, conflicting results have been supported by early investigations. The study by Oztürk et al., (2008) found no significant difference in CAVI concentration when caries and caries-free young adults were compared. On the other hand, the investigation performed by Kivelä et al., (1999) has shown that a low CAVI concentration is associated with a higher caries index. In the same way, Szabó (1974) found a higher concentration of CAVI in saliva of 7- to 14-year-old caries-free children than in children with caries. However, the previous investigation by Frasseto et al., (2012) employed the zymography method and quantitatively determined the activity of salivary CAVI. These authors demonstrated that after a cariogenic challenge, the activity of salivary CAVI was higher in pre-school children with dental caries.

Concerning the activity of CA VI in saliva we were not able to find any report in the literature on the possible relationship between the activity and concentration of this isoenzyme in saliva as well as between salivary flow rate, pH and buffering capacity of saliva. In addition, weather activity and concentration CA VI in saliva are significant factors for caries development needs to be determined.

Thus, the aims of this study were to determine the concentration and activity of the isoenzyme CA VI in saliva of children with caries and investigate the relationship between the concentration and the activity of this isoenzyme. We also assess whether there was any correlation between the concentration and activity of this isoenzyme in saliva and tooth decay. We determine salivary flow, pH and buffer capacity and investigate if these salivary properties correlate with the concentration and the activity of this isoenzyme, and to investigate whether there is any correlation between salivary flow rate, pH and buffering capacity of saliva and tooth decay.

Materials and methods

Ethical Considerations

The present study was approved by the Ethical Committee in Research of Piracicaba Dental School/UNICAMP (protocol number 018/2012) and was ethically conducted in accordance with the Declaration of Helsinki (World Medical Association). The schools granted permission for the study and the children's parents signed a written positive consent form.

Sample

We based the sample power calculation (95%; α=5%) on the study previously performed by our group (Frasseto et al., 2012) which used similar methodology and found that activity of CA VI in saliva of caries and caries-free pre-school was 42.75±32.47 and 19.13±16.391 respectively. Sample size was based on the averages for two independent samples and bilateral test (parametric test) by the equation: $n = \frac{(s_1{}^2+s_2{}^2)\left(z_1-\frac{\alpha}{2}+z_1-\beta\right)^2}{(\bar{x}_1-\bar{x}_2)^2}$ = 30,78. Thus, the calculated number of 31 school children were selected to take part in this study. Although this a cross-sectional investigation, the calculated number (31) was increased by 20% to compensate for possible subject drop-out rate. We selected 74 school children aging 07 to 09 years old, from

a public school from the city of Piracicaba, SP, Brazil. The selected children were healthy, with good oral hygiene, good record of general and oral health and were not taking any medication.

Caries assessment

Before the clinical examinations, theoretical discussions using clinical photographic slides were held to give instructions to the examiner about the use of the criteria and the examination method. Following, practicing exercises were carried out and replicate examinations were performed on a random sample of 14 schoolers, with a one-week interval period. This step of training exercises lasted 30h. The intra-examiner agreement measured by Kappa calculation, with regards to all DMFT components including white chalky spot lesions, at the tooth level was 0.68. Dental caries diagnosis was carried out according to the World Health Organization criteria including the early caries lesion (ECL) by visual inspection method, with the aid of clinical mirror a CPITN probe and flashlight after cleaning and drying the teeth with gauze.

Collection of saliva samples

From all children, saliva samples were collected in duplicate at the same time of day by the same dentist. The children were instructed to stop eating or drinking 1 hour prior to sample collection. The salivary secretion was stimulated with a parafilm piece for 6 minutes, and in the first minute, the saliva was swallowed and then spitted in the collection tubes.

Determination of flow rate, pH and buffering capacity of saliva

The volume of stimulated saliva was calculated by the difference in weight between the tubes, before and after saliva collection considering saliva density of 1 mg/ml (Picco et al., 2012). Salivary flow rate was expressed as mL/min.

The salivary pH was determined immediately after saliva collection using a portable Tecnopon pH meter MPa-210/MPA-210P (MS Tecnopon, Piracicaba, SP, Brazil.), previously calibrated with standard solutions of pH 4 and 7, according to the manufacturer's instructions. Following, 1 mL aliquots of saliva samples were stored and chilled for electrometric determination of the buffering capacity; 200 µL aliquots to determine the CA VI activity in saliva by zymography method and 200 µL aliquots to determine the CA VI concentration by Elisa method (Enzyme-Linked Immunosorbent Assay).

For evaluation of buffering capacity, we used the method described by Bouchoucha, et al. (1997). Increments of 10 µL of 0.25 M hydrochloric acid were added tosaliva aliquots and

monitored by microelectrode Accumet® MicroProbe Flexible stem 6" L (Cole Parmer International. Illinois, USA) until reaching pH 4. At each increment, the tube was agitated and the pH determined, resulting in a pH drop curve. The area under the curve (AUC) was then calculated (pH x mmol HCl) using Excel software.

Determination of CA VI activity in saliva

After salivary flow rate measurement, saliva samples were frozen at -40℃ for later analysis of CAVI activity. The determination of CA VI activity was performed by a modified protocol of Kotwica et al., (2006) adjusted to CA VI analysis in saliva in our laboratory. After being thawed, 50 µL of saliva was added to 50 µL of Tris buffer for zymography, and from the total of 100 µL of sample, only 20 µL was placed in each channel of the gel. This material was stirred before being placed on acrylamide gel at 12%/0.8% bisacrylamide, which was ran for 2 h at 140 V and at 4℃. After electrophoresis, the gel was incubated in 0.1% bromothymol blue diluted in 100 mmol/L Tris, pH 8.2, for 10 min. CA VI activity was observed after immersing the gel in distilled deionized water saturated with CO_2. The gels were photographed, and the images were quantified using the ImageJ® software (Collins, 2007) which calculated the luninescence in the area of the band and expressed CA VI activity in numerical values (pixels area).

Determination of CA VI Concentration in Saliva

The analysis of CA VI concentration in saliva was performed using an Enzyme-linked Immunosorbent Assay Kit for carbonic anhydrase VI (Elisa kit SED073Hu 96 tests – Cloud-Clone Corp., Houston, USA) following the manufacturer's instructions. After experimental protocol of the kit, the absorbance was evaluated by an EON spectrometer (Biotek Instruments, Winooski, USA) and the concentration of CA VI in saliva was expressed as nanogram per microlitter (ng/µL).

Statistical analysis

The statistical analysis was performed considering two groups of children with dental caries (C) and caries free (CF) as the dependent variables. The independent variables were: salivary flow rate, pH, buffering capacity, activity and concentration of CA VI in saliva. The D'Agostino-Pearson omnibus normality test was performed to verify the normality of the data (Ayres, 2010). All independent variables were dichotomized based on their median values using the Student T test and Pearson correlation. Since the data did not follow normal distribution, they were subjected to the Mann-Whitney test and Spearman correlation. In addition, the

bivariate analysis (chi-square test) was initially conducted in order to verify possible associations between dependent and independent variables. In the multivariate modeling, associations between the dependent and independent variables were expressed as odds ratios with their respective 95% confidence intervals. The multivariate modeling fitting was assessed by the Hosmer & Lemeshow test. The statistical tests were considered to be significant if p-values were ≤ 0.05. Data were analyzed using the GraphPad Prism 6.01 (GraphPad Software Inc.).

Results

1. Salivary flow

The DMFT index obtained was 3.162, considered moderate by WHO. Figure 1 shows that the salivary flow rate was a significantly higher in caries free children (0,9206 ± 0,2893 n=37) than in caries children (0,5738 ± 0,2806 n=37).

2. Salivary pH and buffering capacity

The data of figure 2 demonstrate that salivary pH was significantly higher in caries-free (7,324 ± 0,1875 n=37) than in children with caries (7,152 ± 0,3039 n=37). The figure 2 also shows a higher salivary buffering capacity in caries-free children (461,6 ± 12,48 n=37) than in caries group (410,6 ± 14,56 n=37).

3. CA VI Activity and concentration in saliva

Data of Figure 3 demonstrates that the activity of this isoenzyme in saliva was significantly higher in children with caries (C: 3,391 ± 2,046 n=37) than in the caries-free children (CF: 1,383 ± 1,076, n=37). From figure 3 also can be seen that concentration of CA VI was significantly higher in saliva of caries free children (CF: 0,8561 ± 0,7141 n=37) than in saliva of children with caries (C: 0,4255 ± 0,3835 n=37).

4. Correlation between Dental Caries and Independent Variables

The results of Table 1 demonstrate that buffering capacity was the only variable that showed a moderate negative correlation with dental caries.

5. Correlation between the independent variables in caries and caries-free children

The results of correlation between the independent variables in both groups are shown in Table 2. This table demonstrates a moderate positive correlation between CA VI activity and concentration in the caries group. Also, a negative weak correlation between pH and CA VI concentration was observed in the caries-free group. Finally, a moderate negative correlation between the buffering capacity and CA VI activity in caries group was observed.

6. Bivariate analysis of the relationship between dental caries and salivary parameters.

In the bivariate analysis of salivary parameters, (Table 3) the factors salivary flow and activity of CA VI presented p values lower than <0.2 and were inserted in the multiple logistic regression model.

7. Multivariate modeling of caries lesions regarding salivary flow and activity of CA VI.

The results showed in Table 4 demonstrate that children with salivary flow rate lower than 1.06 mL/min have 4 four times more chance todevelop caries than children with higher salivary flow rate. In addition, an activity of CA VI in saliva higher than 1.75 turns children 5.03 times more likely to develop caries.

Discussion

To our knowledge no study has been found to investigate the relationship between CA VI concentration and activity as well as the relationship between the activity of this isoenzyme and salivary flow rate, pH and buffering capacity. Carbonic anhydrase VI is a potential drug target for cariogenesis and cancer of the salivary gland. It is the only secreted human CA isozyme which is found in saliva and milk (Kazokaitė et al., 2015). The relevance of carbonic anhydrase VI in oral health still has been under investigated.

Dental caries is a complex and dynamic process in which a multitude of factors influence the initiation and progression of disease. One of the most important factors that influences the development of dental caries is saliva. Saliva can protect tooth tissue in several ways. Factors like salivary flow rate, pH and buffering capacity contribute to this protective role of saliva. After intake of acidic food or drinks saliva acts as a diluting agent, clearing the remnants of acids in the mouth (Zwier et al., 2013).

Regarding salivary parameters, we found a significant difference in stimulated salivary flow rate when children with caries and caries free children were compared. In fact, the salivary flow rate was significantly lower in caries children ($p<0.0001$). A new and interesting result found of our study was that children with salivary flow rate lower than 1.06 mL/min had a 4 times more chance to develop caries than children with a salivary flow rate higher than 1.06 mL/min (Table 4). This result could partially be explained if we consider that appropriate salivary flow rate is crucial for the maintenance of oral health. In this regard, the salivary flow rate has the greatest influence on the rate of salivary clearance. Moreover, early investigation by Dawes (2004), demonstrated that increasing the rate of salivary flow, increases the concentration of protein, sodium, chloride and bicarbonate and decreases the concentration of magnesium and phosphate. The carbonate increase is of high importance to favor it`s diffusion into dental plaque which in turn increases the pH of the plaque and enhances the remineralization of early caries lesions.

In line with this assumption, our study also showed that both salivary pH and buffering capacity were significantly lower in caries active children. It was also observed that the salivary buffering capacity was the only independent variable negatively correlated with dental caries ($r=-0,3363$; $p=0,0449$). These results are in line with Preethi et al., (2010) whose study revealed that when all these parameters were compared in caries free and caries active children, flow rate, pH, buffering capacity were slightly reduced in caries active children. The study of Animireddy et al., (2014) also showed that there was a significant decrease in the mean salivary flow rate, salivary pH and buffering capacity and a significant increase in the salivary viscosity in children with caries activity when caries-free subjects, subjects with minimal caries and subjects with nursing caries were compared. Abbate et al., (2014) evaluate the correlations between unstimulated salivary flow, pH and level of S. mutans, in caries-free and caries-active children and showed that unstimulated salivary flow was significantly lower in the caries group as compared to the caries-free group.

However, although the buffer capacity is intimately associated with the salivary flow rate (Gudkina et al., 2008), some studies provide inconclusive results showing that salivary

parameters as buffer capacity, cariogenic bacteria counts, pH, flow rate and total protein content either as a single test or even combined are unable to predict caries (Vitorino et al., 2006). Alternatively, these salivary parameters have been shown to be more effective in identifying caries-free individuals than those who required treatment (Llena-Puy et al., 2000). However, dental caries is a multifactorial sucrose-biofilm dependent disease that is mediated by a combination of several overlapping factors which must be considered in the role of human saliva in maintaining oral health.

Another result of our study was that the salivary CA VI concentration was significantly higher in caries-free children and the salivary CA VI activity was significantly higher in caries active children. However, the subjects of their study had good oral hygiene and gingival health. regarding the higher avtivity of CA VI in saliva of children with caries our results agree with Frasseto et al., (2012) who showed that the CAVI activity before a 20% sucrose rinse as well as its variation were higher in saliva of children with caries than in caries-free children. However, these results are at odds with those observed by Szabó (1974) who demonstrated that salivary CA VI activity was higher in saliva of 7-to 14-year-old caries-free children than in saliva of children with caries. Considering the higher CA VI concentration in saliva of caries free children, the investigation performed by Kivelä et al., (1999) has shown that a low CA VI concentration is associated with higher caries index, besides a negative correlation between CA VI concentration and DMFT index in individuals with poor hygiene. On the other hand, Oztürk et al., (2008) found no difference in CA VI concentration in saliva of caries and caries-free young adults.

An interesting finding of the present study was that only in caries free children, a significant negative correlation between salivary pH and CA VI concentration was demonstrated (Table 2). Moreover, saliva of caries free children also showed a significantly higher concentration of CA VI (Figure 3). These findings may be an indication that in caries-free children, if the pH decreases, the concentration of CA VI seems to increase, with small change in isoenzyme activity. Findings from the early work by Frasseto et al., (2012), give support to this assumption because after a 20% sucrose rinse, these authors noted only a small increase in the CA VI activity in saliva of caries-free pre-school children.

Regarding children having caries, we detected a significant positive correlation between salivary CA VI concentration and activity (Table 2). This result can partially be explained if we consider that children with caries are more frequently exposed to sucrose, a carbohydrate know to promote a pH drop in the oral cavity. Our results give support to this finding because we

found that, salivary pH was significantly lower in children having caries than in caries-free children. Furthermore, a negative correlation was detected between buffering capacity and CA VI activity. This result was expected because early investigation has shown that during a pH fall and proton concentration increases, the iso-enzyme catalyzes the reaction of $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ and in this way, increase bicarbonate level in saliva to neutralize the media in the oral cavity. According to Öztürk et al., (2012) the increase in iso-enzyme concentration may indicate that more CA VI is available to be activated during cariogenic episodes.

The role played by activity of CA VI in saliva on dental caries was evaluated in this investigation. Interestingly, it should be noted that when the activity of CA VI in saliva was analyzed in the multivariate model, we obtained a significant factor for caries development. In fact, children who showed an activity of CA VI in saliva higher than 1.75 had 5.03 times more chance of presenting caries than children with higher activity of the isoenzyme. At least to our knowledge, this is the first study to investigate the correlation between CA VI activity and concentration in saliva as well as to demonstrate the role of activity of CA VI in saliva in discriminate school children who have a chance of developing dental caries. However, it should be pointed out that this is a cross-sectional study and, therefore, longitudinal investigations considering the child's response to a specific factor during the disease process are necessary to improve the knowledge about the role of salivary parameters in caries development. In this regard, a longitudinal investigation is ongoing in our laboratory.

In conclusion, our data demonstrated that school children with higher activity of CA VI in saliva and lower salivary flow rate are more likely to develop dental caries. In addition, a higher concentration of CA VI and higher pH and buffering capacity associated with a lower activity of this isoenzyme in caries-free group suggests that this isoenzyme is able to neutralize the acids of the oral environment and provide greater protection against tooth decay.

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References

Abbate GM, Borghi D, Passi A, Levrini L. Correlation between unstimulated salivary flow, pH and streptococcus mutans, analysed with real time PCR, in caries-free and caries-active children. Eur J Paediatr Dent. 2014; 15(1): 51-4.

Animireddy D, Reddy Bekkem VT, Vallala P, Kotha SB, Ankireddy S, Mohammad N. Evaluation of pH, buffering capacity, viscosity and flow rate levels of saliva in caries-free, minimal caries and nursing caries children: An in vivo study. Contemp Clin Dent. 2014; 5(3): 324-8.

Ayres M: Normality; in Statistical elements. Belém, Ione Sena, 2010, 2 ed, pp 463-472.

Bagherian A, Asadikaram G. Comparison of some salivary characteristics between children with and without early childhood caries. Indian J Dent Res. 2012; 23(5): 628-32.

Bouchoucha M, Callais F, Renard P, Ekindjian OG, Cugnenc PH, Barbier JP. Relationship between acid neutralization capacity of saliva and gastro-oesophageal reflux. Arch Physiol Biochem. 1997; 105(1): 19-26.

Collins TJ**.** ImageJ for microscopy. Biotechniques. 2007; 43(1 Suppl):25-30.

Dawes C. Factors influencing salivary flow rate and composition; in: Edgar M, Daves C and O´Mullane D: Saliva and Oral Healthy. London, British Associaton, 2004, 3 ed, pp 32-50.

Frasseto F, Parisotto TM, Peres RC, Marques MR, Line SR, Nobre Dos Santos M. Relationship among salivary carbonic anhydrase VI activity and flow rate, biofilm pH and caries in primary dentition. Caries Res. 2012; 46(3): 194-200.

Gudkina J and Brinkmane A. Caries experience in relation to oral hygiene, salivary cariogenic microflora, buffer capacity and secretion rate in 6-year olds and 12 year olds in Riga. Stomatologija. 2008; 10(2): 76-80.

Kaseda M, Ichihara N, Nishita T, Amasaki H, Asari M. Immunohistochemistry of the bovine secretory carbonic anhydrase isozyme (CA-VI) in bovine alimentary canal and major salivary glands. J Vet Med Sci. 2006; 68(2): 131-5.

Kazokaitė J, Milinavičiūtė G, Smirnovienė J, Matulienė J, Matulis D. Intrinsic binding of 4-substituted-2,3,5,6-tetrafluorobenezenesulfonamides to native and recombinant human carbonic anhydrase VI. FEBS J. 2015; 282(5): 972-83.

Kimoto M, Kishino M, Yura Y, Ogawa Y. A role of salivary carbonic anhydrase VI in dental plaque. Archives of Oral Biology. 2006; 51(2): 117-122.

Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H. A low concentration of carbonic anhydrase isoenzyme VI in whole saliva is associated with caries prevalence. Caries Res. 1999; 33(3): 178-84.

Kivelä J, Parkkila S, Metteri J, Parkkila AK, Toivanen A, Rajaniemi H. Salivary carbonic anhydrase VI concentration and its relation to basic characteristics of saliva in young men. Acta Physiol Scand. 1997; 161(2): 221-5.

Kotwica J, Ciuk MA, Joachimiak E, Rowinski S, Cymborowski B, Bebas P. Carbonic anhydrase activity in the vas deferens of the cotton leafworm - Spodoptera littoralis (Lepidoptera: Noctuidae) controlled by circadian clock. J Physiol Pharmacol. 2006; 57 Suppl 8:107-23.

Llena-Puy MC, Montañana-Llorens C, Forner-Navarro L. Cariogenic oral flora and its relation to dental caries. ASDC J Dent Child. 2000; 67(1): 42-6, 9.

Mau M, Kaiser TM, Südekum KH. Carbonic anhydrase II is secreted from bovine parotid glands. Histol Histopathol. 2010; 25(3): 321-9.

Oztürk LK, Furuncuoğlu H, Atala MH, Uluköylü O, Akyüz S, Yarat A. Association between dental-oral health in young adults and salivary glutathione, lipid peroxidation and sialic acid levels and carbonic anhydrase activity. Braz J Med Biol Res. 2008; 41(11): 956-9.

Koç Öztürk L, Ulucan K, Akyüz S, Furuncuoğlu H, Bayer H, Yarat A. The investigation of genetic polymorphisms in the carbonic anhydrase VI gene exon 2 and salivary parameters in type 2 diabetic patients and healthy adults. Mol Biol Rep. 2012; 39(5): 5677-5682.

Picco DC, Costa LF, Delbem AC, Sassaki KT, Sumida DH, Antoniali C. Spontaneously hypertensive rats as experimental model of salivary hypofunction. Arch Oral Biol. 2012; 57(10):1320-6.

Preethi BP, Reshma D, Anand P. Evaluation of Flow Rate, pH, Buffering Capacity, Calcium, Total Proteins and Total Antioxidant Capacity Levels of Saliva in Caries Free and Caries Active Children: An In Vivo Study. Indian J Clin Biochem. 2010; 25(4): 425-8.

Scheutz F, Poulsen S. Determining causation in epidemiology. Community Dent Oral Epidemiol. 1999; 27(3): 161-70.

Sheiham A, James WP. Diet and Dental Caries: The Pivotal Role of Free Sugars Reemphasized. J Dent Res. 2015; 94(10): 1341-7.

Sheiham A. Sucrose and dental caries. Nutr Health. 1987: 5(1-2): 25-9.

Singh S, Sharma A, Sood PB, Sood A, Zaidi I, Sinha A. Saliva as a prediction tool for dental caries: An in vivo study. J Oral Biol Craniofac Res. 2015: 5(2): 59-64.

Szabó I. Carbonic anhydrase activity in the saliva of children and its relation to caries activity. Caries Res. 1974: 8(2): 187-91.

Vitorino R, Calheiros-Lobo MJ, Duarte JA, Domingues P, Amado F. Salivary clinical data and dental caries susceptibility: is there a relationship? Bull Group Int Rech Sci Stomatol Odontol. 2006; 47(1): 27-33.

Zeng Y. The functional consequences of relative substrate specificity in complex biochemical systems. Front Genet. 2011; 2:65.

Zwier N, Huysmans MC, Jager DH, Ruben J, Bronkhorst EM, Truin GJ. Saliva parameters and erosive wear in adolescents. Caries Res. 2013; 47(6): 548-52.

Legends

Table 1. Pearson and Spearman correlation coefficients (r) and probabilities of statistical significance (p) between dental caries and independent variables.

Table 2. Pearson and Spearman correlation coefficients (r) and probabilities of statistical significance (p) between the independent variables studied in caries and caries free children.

Table 3. Bivariate analysis of the relationship between dental caries and salivary parametters.

Table 4. Multivariate modeling of caries lesions regarding salivary flow rate and CA VI activity in children.

Figure 1: Mann Whitney test of salivary flow rate, p<0,0001 in caries and caries free children.

Figure 2: Mann-Whitney test of salivary pH expressed in pH units, p=0,0105; and Unpaired t test with Welch's correction of salivary buffering capacity, p=0,0098 for the caries and caries-free children.

Figure 3: Mann Whitney test of CA VI activity, p < 0,0001; and CA VI concentration, p=0,0006 in saliva of caries and caries-free children.

Tables

Table 1.

Variables	Dental caries	
	r	p
CA VI Activity	0,04582	0,8001[P]
CA VI Concentration	-0,0057	0,9736[P]
Salivary flow	0,09318	0,5833[S]
Salivary pH	0,1033	0,5428[S]
Buffering capacity	-0,3363	0,0449[P]

*[P] Pearson correlation for normal data.

[S] Spearman correlation when data did not follow normal distribution.

Table 2.

Variables	Groups of children			
	Caries		Caries-free	
	r	p	r	p
CA VI Activity x Concentration	0,4466	0,0118[P]	0,0865	0,6210[S]
pH x CA VI Activity	0,0068	0,9698[S]	0,1714	0,3104[S]
pH x CA VI Concentration	0,1621[S]	0,3523[S]	-0,3417	0,0445[S]
Buffering capacity x CA VI Activity	-0,3439	0,0500[P]	-0,1536	0,3640[S]
Buffering capacity x CA VI Concentration	0,0739	0,6730[P]	0,0669	0,7024[S]

*[P] Pearson correlation for normal data.

[S] Spearman correlation when data did not follow normal distribution.

Table 3.

Variables	Caries-free x Caries n(%)		Variables	Caries-free x Caries n(%)	
Salivary flow	p = 0.197		**CA VI Activity**	p = 0.008	
< 1.06	28(44)	35(56)	< 1.75	27(61)	17(39)
≥0.79	10(63)	6(38)	≥ 1.75	11(31)	24(69)
Salivary pH	p = 0.959		**CA VI Concentration**	p=0.471	
< 7.40	26(48)	28(52)	< 1.03	28(46)	33(54)
≥7.40	12(48)	13(52)	≥ 1.03	10(56)	8(44)
Buffering capacity	p= 0.318				
< 497.32	28(45)	34(55)			
≥ 497.32	10(59)	7(41)			

*Significant results were evaluated using the chi-square test or Fisher's exact test (α=0.05).

Table 4.

Variables	Caries lesions				
	No (%)	Yes (%)	OR_{crude} (95%CI)	$OR_{adjusted}$ (95%CI)	Model p-value*
Salivary flow					
< 1.06	28(44)	35(56)	1.96(0.63-6.25)	4.00(1.06-14.28)	0.039
≥1.06	10(63)	6(38)	1	1	
CA VI Activity					
< 1.75	27(61)	17(39)	1	1	
≥ 1.75	11(31)	24(69)	3.93 (1.55 - 10.18)	5.03(1.73-14.59)	0.003

OR = Odds ratio; CI = confidence interval. *Likelihood test with 1 freedom degree = 11.76; p value of the Hosmer and Lemeshow test = 0.94.

Ilustrations

Figure 1.

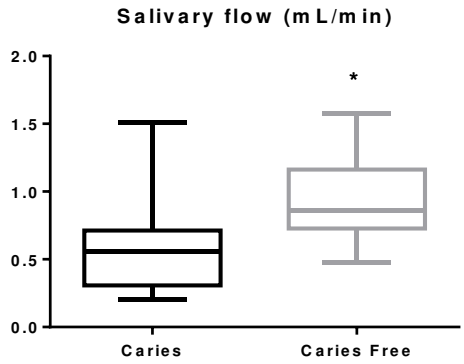


Figure 2.

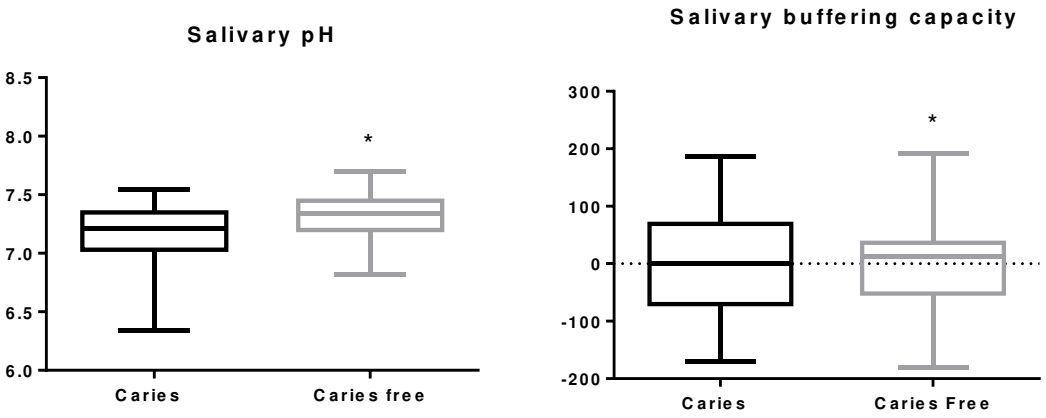
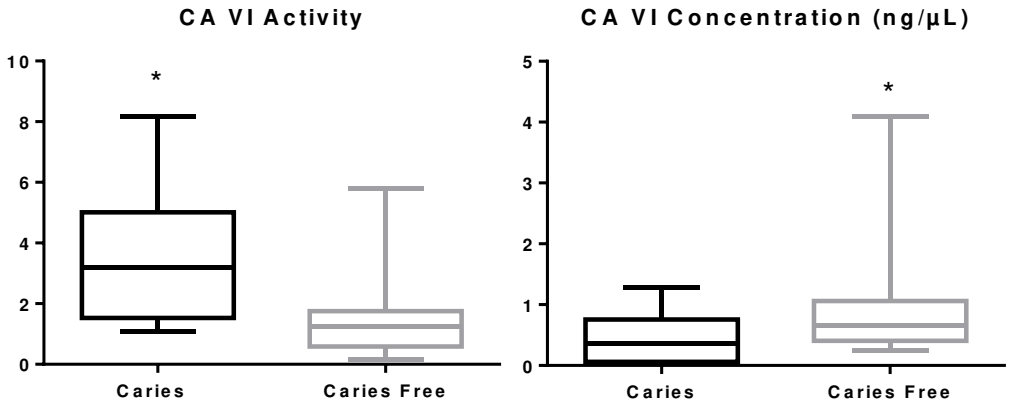


Figure 3.



2.3 Relationship between dental caries and pH, concentration and activity of carbonic anhydrase VI in dental plaque and in school children – A cross-sectional study.

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Conflict of interest

The authors deny any conflict of interest related to this study.

Abstract

Introduction: Carbonic anhydrase VI (CA VI) has been shown to be expressed in saliva and dental plaque. However, information regarding its activity in dental plaque is unknown. **Objective:** This study aimed to perform a quantitative analysis of the concentration and activity of CA VI in dental plaque as well as to determine plaque pH and investigate the relationship between dental caries and activity and concentration of CA VI, and plaque pH in 7-9 year-old school children. **Materials and Methods**: Seventy-four school children were divided into two groups: caries-free group (n=37) and caries group (n=37). Clinical examinations were conducted by one examiner (kappa= 0.684) according to WHO criteria (DMFT) + early caries lesions. Plaque PH was determined from each subject with specific electrode and following, dental plaque was collected for CA VI concentration and activity determination which was performed by ELISA and zymography respectively. The data was submitted to Student's t-test and Mann-Whitney test apart from Pearson and Spearman correlation analyses (α=0.05). **Results:** Dental plaque pH was higher in caries-free than in children with caries. (p=0,0005). Activity of CA VI in dental plaque was significantly higher in children with caries than in caries-free children (p=0,0421). CA VI concentration, was higher concentration in the caries free group (p=0,0335). Additionally, we observed a significant negative correlation between CA VI activity and concentration in caries-free group (r=-0,460, p=0,0206). Besides, we found a negative correlation between plaque pH and CA VI activity in caries-free group (r=-0,4551, p=0,0068) and between pH and CA VI concentration in caries group. **Conclusion:** In conclusion, CA VI was demonstrated to be more active in dental plaque of caries active children to contribute to the neutralization of plaque acid. Moreover, the higher pH and concentration of CA VI in dental plaque of caries-free children suggests that since these individuals are not frequently exposed to cariogenic challenges, the drop of plaque pH doesn`t constantly occur to activate the bicarbonate system to buffer the media.

Key words: Carbonic anhydrase VI, dental plaque pH, dental caries.

Introduction

Bacteria living constantly in the oral cavity are in the form of a biofilm. The biofilm formed on a solid base such as dental enamel, fillings, restorations, orthodontic appliances or obturators is dental plaque. Disturbance of homeostasis of biofilm, excessive growth or increase in the number of acid-forming bacteria leads to the development of the most common diseases of the oral cavity, i.e. dental caries and periodontal disease (Chałas et al., 2015).

According to scientific evidence, dental caries is a sugar-dependent disease modulated by several additional factors, such as oral microorganisms, acids, differential properties of the teeth and the salivary flow, which modifies the primary effect of sugars (Sheiham, 1987; Scheutz, 1999; Sheiham, 2015). An important etiological factor in oral and dental health decline is microbial dental plaque formation (Güngör et al., 1999). The accumulation of dental plaque on the surface of teeth, and the inability to effectively remove it, causes oral health problems due to the toxic by-products of the micro-organisms involved in plaque formation (Acar et al., 2015).

Salivary factors are considered important for dental health, as rampant caries is seen in patients with high salivary hypofunction. In this context, the salivary buffer capacity is a factor of primary importance in maintaining oral homeostasis. The bicarbonate system is the main buffer that contributes to the total buffer capacity of saliva. Bicarbonate ions can neutralize lactic and acetic acids produced by plaque bacteria and reduce demineralization (Peres et al., 2010).

According to recent studies, the carbonic anhydrase VI (CA VI) appears to be one of the key enzymes in the oral physiology in humans and animals (Kimoto, 2006; Kaseda, 2006; Mau, 2010). Carbonic anhydrase VI is the only isozyme which may be secreted into the saliva in order to provide increased buffering capacity, and to promote retention HCO_3^- in level of salivary glands.

Regarding the role of CA VI in saliva early investigations by Oztürk et al. (2008) found no significant difference in CA VI concentration when caries and caries-free young adults were compared. On the other hand, the work performed by Kivelä et al. (1999a) has shown that a low CAVI concentration was associated with a higher caries index. In the same way, Szabó (1974) found a higher concentration of CA VI in saliva of 7- to 14-year-old caries-free children

than in children with caries. However, previous investigation by Frasseto et al. (2012), employed the zymography method (Kotwica et al., 2006) and quantitatively determined the activity of salivary CAVI. These authors demonstrated that after a cariogenic challenge, the activity of salivary CA VI was higher in pre-school children with dental caries.

In this regard the early investigation performed by Kimoto et al. (2006), employed the western blot analysis to qualitatively show that CA VI is concentrated in dental plaque. In a further evidence of CA VI effect in buffering plaque pH, these authors demonstrated when a CA VI specific inhibitor (acetazolamide) was added to sucrose solution, a significant decrease in plaque pH occurred. However, being concentrated in dental plaque may not necessarily mean that all CA VI isoenzyme present in the media is active. Furthermore, knowledge regarding the activity of CA VI in dental plaque is unknown. Thus, determining the activity of CA VI instead of just its concentration would provide further evidence of the effect of this isoenzyme in buffering plaque pH and consequently protecting teeth from dental caries.

Considering the above, the aims of this investigation were to perform a quantitative analysis of the concentration and activity of CA VI in dental plaque as well as to determine plaque pH and investigate the relationship between dental caries and activity and concentration of CA VI, and plaque pH in 7-9 year-old school children.

Materials and Methods

Ethical Considerations

The present study was approved by the Ethical Committee in Research of Piracicaba Dental School/Unicamp (protocol number 018/2012) and was ethically conducted in accordance with the Declaration of Helsinki (World Medical Association). Written consent of parents or guardians of each child participating in the study was obtained.

Sample

We based the sample power calculation (95%; α=5%) on the study previously performed by our group (Frasseto et al., 2012) which used similar methodology and found that activity of CA VI in saliva of caries and caries-free pre-school was 42.75±32.47 and 19.13±16.391 respectively. Sample size was based on the averages for two independent samples and bilateral

test (parametric test) by the equation: $n = \frac{(s_1{}^2+s_2{}^2)\left(z_1-\frac{\alpha}{2}+z_1-\beta\right)^2}{(\bar{x}_1-\bar{x}_2)^2}$ = 30,78. Thus, the calculated number of 31 school children were selected to take part in this study. Although this was cross-sectional investigation, the calculated number (31) was increased by 20% to compensate for possible subject drop-out rate. Thus, we selected 74 school children aging 07 to 09 years old, from a public school from the city of Piracicaba, Brazil. The selected children were healthy, with good oral hygiene, good record of general and oral health and were not taking any medication. All children had healthy gums with no redness, swelling or bleeding.

Calibration of the examiner and caries diagnosis

Before the clinical examinations, theoretical discussions using clinical photographic slides were held to give instructions to the examiner about the use of the criteria and the examination method. Following, practicing exercises were carried out and replicate examinations were performed on a random sample of 14 schoolers, with a one-week interval period. This step of training exercises lasted 30h. The intra-examiner agreement measured by Kappa calculation, with regards to all DMFT components including white chalky spot lesions, at the tooth level was 0.68. Dental caries diagnosis was carried out by visual inspection method, with the aid of clinical mirror a CPITN probe a flashlight after cleaning and drying the teeth with gauze, according to the World Health Organization criteria including the early caries lesion (ECL).

Plaque pH determination

Children were instructed to interrupt the toothbrushing procedures 48 hours before the collections. Plaque samples (about 1 mg) were obtained with a sterile inoculation loop from all accessible buccal surfaces of incisors and molars. Each plaque sample was suspended in 20 µL of distilled water in a plastic micro eppendorf and frozen at -40℃ until subsequent analyzes. The pH was read with a glass combination electrode (PerpHect® ROSS®; Thermo Scientific Orion) previously calibrated with standard buffer solutions at pH 4 and 7.

Determination of CA VI activity in dental plaque

The determination of CA VI activity was performed by a modified protocol of Kotwica et al. (Kotwica et al., 2006), adapted to CA VI in dental plaque in our laboratory. 2 µg of dental plaque was collected and suspended in 40 µL of electrophoresis sample buffer and frozen at -40℃. After being thawed, 10 µL of electrophoresis sample buffer and 50 µL of sample was placed in each channel of the gel. This material was stirred before being placed on acrylamide

gel at 12%/0,8% bisacrylamide, which was run for 2:30 h at 140 V at 4℃. After electrophoresis, the gel was incubated in 0,1% bromothymol blue diluted in 100 mmol/L Tris, pH 8,2, for 10 min. CA VI activity was determined after immersing the gel in distilled deionized water saturated with CO_2. The gels were photographed, and the images were quantified (pixels area) using ImageJ® software (Collins, 2007).

Determination of CA VI Concentration in dental plaque

For this analysis, we firstly performed the protein extract from the biofilm. To each sample (2 µg biofilm), 200 mg of silica beads and 130 ul of distilled water. This sample was stirred in bead better apparatus for 1 and a half minute (45 seconds ON - 1 minute ice - ON 45 seconds - 1 minute ice) and the volume was then passed without silica beads to an eppendorf tube and stored at -40℃ until the day of subsequent analysis. The analysis of CA VI concentration in dental plaque was obtained using an Enzyme-linked Immunosorbent Assay Kit for carbonic anhydrase VI (Elisa kit SED073Hu 96 tests – Cloud-Clone Corp.) following the manufacturer's instructions. After experimental protocol of the kit, the absorbance was evaluated by EON spectrometer (Biotek Instruments, Winooski, USA) and the concentration of CA VI was expressed as nanogram per microliter (ng/µL).

Statistical analysis

The statistical analysis was performed considering two groups of children with dental caries and caries free as the dependent variables. The independent variables were: plaque pH, activity and concentration of CA VI in dental plaque. The D'Agostino-Pearson omnibus normality test was performed to verify the data normality (Ayres, 2012). All variables were dichotomized based on their median values using the Student T test and Mann-Whitney test for parametric and non-parametric data respectively. In addition, the correlation between dental caries and all independent variables under study was assessed by the Pearson and Spearman correlation test. The statistical tests were considered to be significant if p-values were less than 0.05. Data were analyzed using the GraphPad Prism 6.01 (GraphPad Software Inc.).

Results

1. *Plaque pH*

The mean and standard deviation of dental caries was 0,00±0,00 and 3,162±1,385 for caries-free and caries children respectively. Our results showed that plaque pH was significantly higher in caries free group (6,155± 0,4839 n=37) than in children with caries (5,666±0,5534 n=37), p=0,0005. Figure 1 shows a box-plot representation of dental plaque pH.

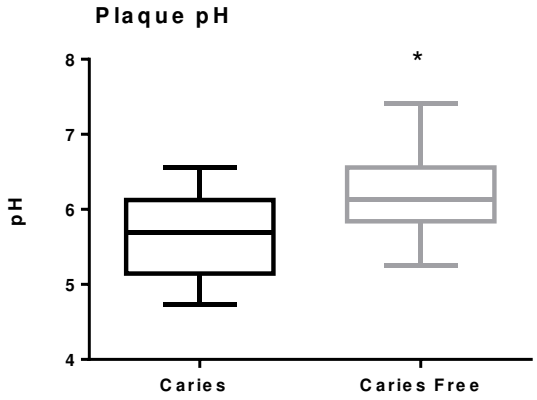

Figure 1: Mann Whitney test of salivary pH, expressed as pH units in caries and caries-free children. p=0,0005.

2. CA VI activity and concentration

Figure 2 shows that the CA VI activity was significantly higher, in children with caries (25,96±16,41 n=34) than in the caries free children (17,65±9,520 n=33). The data of CA VI concentration in dental plaque also in Figure 2, evidenced that the caries free group has a CA VI concentration significantly higher in caries-free children (3,507±4,014 n=25), than the caries group (1,693±1,802 n=26).

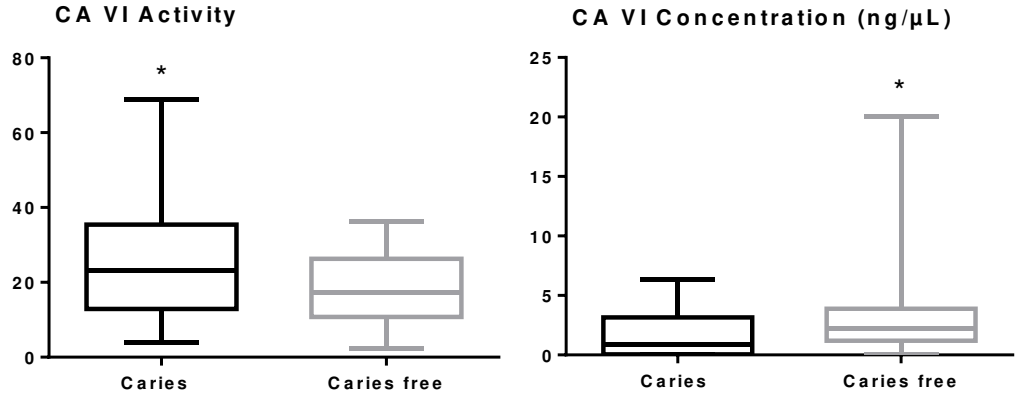

Figure 2: Mann Whitney test of CA VI activity (p=0,0421) and Mann-Whitney test of CA VI concentration (p=0,0335) in dental plaque of caries and caries-free children.

3. Correlation between dental caries and independent variables

The results of correlation between dental caries and all the independent variables analyzed showed that there was no correlation between dental caries and plaque parameter (Table 1).

Table 1. Pearson and Spearman correlation coefficients (r) and probabilities of statistical significance (p) between dental caries and multiple independent variables.

Variables	**Dental caries**	
	r	p
Dental plaque pH	-0,1096	0,5184[S]
CA VI Activity	-0,2326	0,1721[S]
CA VI Concentration	-0,07483	0,7164[P]

*P Pearson correlation for normal data.
S Spearman correlation when data did not follow normal distribution.

4. Correlation among the independent variables in caries and caries-free children

The results of Table 2, shows that in caries-free children, there is a moderate negative correlation between CA VI activity and concentration in dental plaque as well as between pH and CA VI activity. Regarding the caries active group, we detected a negative correlation between pH and CA VI concentration.

Table 2. Pearson and Spearman correlation coefficients (r) and probabilities of statistical significance (p) between the independent variables studied in dental plaque in caries and caries-free children.

Variables	**Groups of children**			
	Caries		**Caries-free**	
	r	p	r	p
CA VI Activity x Concentration	0,04288	0,8352[S]	-0,460	0,02068[S]
pH x CA VI Activity	-0,0113	0,9477[S]	-0,4551	0,00683[P]
pH x CA VI Concentration	-0,4657	0,0164[S]	0,2196	0,29132[S]

*P Pearson correlation for normal data.
S Spearman correlation when data did not follow normal distribution.

Discussion

Caries process is not a static one, instead it is dynamic with interspersed periods of demineralization and remineralization of enamel, intimately related and occurs episodically based upon the presence of cariogenic bacteria in dental plaque and the availability of refined carbohydrates for fermentation to organic acids (Sathe et al., 2014). Saliva controls the pH of dental plaque after exposure to fermentable carbohydrate, and thus helps to prevent dental caries. The salivary effect on the plaque pH is attributed to water that washes away sugars and acids but mainly to carbonate and phosphate buffers that neutralize acids (Kimoto et al., 2006). After the sucrose intake, glycolysis is initiated, and acids are produced by bacteria in plaque. In plaque-enamel interface, occurs an increase of the concentration of hydrogen ions, then the local pH starts to fall (Bonecker et al., 2012). This pH drop should be stopped both by the action of plaque buffers fluid elements as saliva, to contribute to neutralization of plaque acid. This contribution is expected to be much greater in the stimulated saliva whose buffering is performed mainly by bicarbonate. Thus, investigation of concentration and activity of CA VI in dental plaque is relevant to clarify how this ios-enzyme behaves in children having caries.

To figure out how CA VI contributes to the protection of enamel surface from dental caries, we investigated for the first time the activity of this isoenzyme in dental plaque. Dental plaque is the biofilm naturally found on teeth and it is also implicated in dental caries. This disease is associated with shifts in the microbial balance of the biofilm resulting in increased proportions of acid producing and acid tolerating bacteria, especially mutans streptococci and lactobacilli (Marsh, 2010).

The results of the present study demonstrated that CA VI is not only concentrated in plaque but more importantly, the isoenzyme is active to increase acid neutralization after the frequent cariogenic challenges that children with caries are expected to be exposed. Indeed, CA VI activity was significantly higher in plaque of children with caries than in caries-free children. This result suggests that the isoenzyme is being activated more constantly in individuals with caries as a protective mechanism to neutralize the pH of the medium, specifically dental plaque, to provide a greater defense against enamel demineralization. In line with this assumption, the early investigation performed by Frasseto et al. (2012), who showed that before a 20% sucrose rinse, CAVI activity and its variation were higher in the saliva of children with caries than in saliva of caries-free children.

Our findings suggest that CAVI may protect the enamel surface by catalyzing the most important buffer system in the oral cavity, thus accelerating the neutralization of acid from the local environment of the tooth surface (Kivela et al., 1999b). In saliva, bicarbonate diffuses into enamel pellicle and dental plaque and combines with H+ to form carbonic acid essentially instantaneously. In turn, H_2CO_3 dehydrates to CO_2 and H_2O, but the natural rate of this reaction is quite slow (Leinonen et al., 1999). However, CA VI is known to increase the speed of the dehydration reaction up to 13.000 times (Bidani et al., 1988). Our results give support to this mechanism because we found a negative correlation between salivary CA VI activity in saliva and dental plaque (r= -0,3910; p=0,0360 data not shown). Therefore, CAVI in dental biofilm would contribute to neutralization of biofilm acid in the microenvironment of the tooth surface, as a consequence of the buffering performed by salivary bicarbonate and thus would help to prevent dental caries (Leinonen, 1999; Kimoto, 2006).

An interesting finding of this investigation was that we observed a negative correlation between CA VI activity and concentration, only in caries-free children. Furthermore, we also found in this group of children, a significant negative correlation between plaque pH and the CA VI activity (p=0,0068) which means that the higher the plaque pH, the lower the activity of the isoenzyme. These results can be explained considering that caries-free individuals are less frequently exposed to cariogenic carbohydrates and consequently, to lower pH fall in dental plaque (Nobre dos Santos, 2002; Parisotto, 2010), which in turn would activate the CA VI concentrated in dental biofilm (Kivelä et al., 1997). Our data give support to this inference, because we showed that plaque pH of caries-free children was significantly higher than in children with caries (p=0,0005). In this way, acid buffering in dental biofilm provided by the activity of CA VI would not be so necessary in the caries-free children. Regarding the caries group, our results demonstrated a negative correlation between plaque pH and CA VI concentration, even with a higher concentration being observed in caries-free group. These finding suggest that in caries active children, when proton concentration increases as consequence of plaque pH drop, the expression of this isoenzyme would be greater to regulate the actual biofilm pH. This pH regulation would be a consequence of bicarbonate increase in dental plaque since CA VI is known to catalyze the reversal reaction $CO_2 + H_2O \leftrightarrow H^+ + HCO^-_3$ (Kivelä et al., 1997).

Another result of this study was that, plaque pH was significantly higher in caries free children than in children with caries. Although there was no information about the frequency of acidogenic episodes that children were exposed, this finding was expected if we consider

that caries-free children are less exposed to rapidly fermentable carbohydrate, usually sucrose with lower frequency than children with caries. In line with this assumption, early work form Armfield et al. (2013), demonstrated a significant association between caries and sugar-sweetened beverage (SSB) consumption. Moreover, there is evidence suggesting that regular conditions of low pH in plaque select for mutans streptococci and lactobacilli (Marsh, 2006) and it is well known that a high number of cariogenic microorganisms if found in dental plaque of children having caries (Farsi, 2008). Our results are in accordance with Appelgren et al. (2014), who claimed that caries-free individuals had a higher baseline plaque pH compared with caries active individuals in the anterior mandibular region. On the other side, our data are not in line with previous investigations from Peres et al. (2010), Kimoto et al. (2006) and Frasseto et al. (2012), who found no change in plaque pH in caries and caries-free children. However, it should be noted that these authors determined the plaque acidogenicity after a sucrose rinse, an experimental condition distinct from the one we used in the present investigation.

Individuals without caries or those with a minimum of cavities, tend to show a plaque pH at slightly higher break, a higher pH min after consumption of fermentable carbohydrates, and faster return to resting levels when compared to individuals susceptible to cavities. When saliva is deleted, however, the differences between individuals caries free and that one more susceptible to dental caries are less marked and minimum values achieved of pH are lower. These findings indicate the importance of saliva as a determinant of susceptibility to cavities by modifying the plaque pH response (Edgar et al., 2010), which may explain the lack of correlation between the studied variables in dental plaque and tooth decay.

In conclusion, CA VI was demonstrated to be more active in dental plaque of caries active children to contribute to the neutralization of plaque acid. Therefore, CA VI in dental plaque could be considered as a biomarker for dental caries. Moreover, the higher pH and concentration of CA VI in dental plaque of caries-free children suggests that since these individuals are not so frequently exposed to cariogenic challenges, the drop of plaque pH doesn`t constantly occur to activate the bicarbonate system.

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References

Acar M, Türkcan İ, Özdaş T, Bal C, Cingi C. Obstructive sleep apnoea syndrome does not negatively affect oral and dental health. J Laryngol Otol. 2015. Jan;129(1):68-72.

Appelgren L, Dahlén A, Eriksson C, Suksuart N, Dahlén G**.** Dental plaque pH and ureolytic activity in children and adults of a low caries population. Acta Odontol Scand. 2014 Apr;72(3):194-201.

Armfield JM, Spencer AJ, Roberts-Thomson KF, Plastow K. Water fluoridation and the association of sugar-sweetened beverage consumption and dental caries in Australian children. Am J Public Health. 2013 Mar;103(3):494-500.

Ayres M. Normalidade. In: Ayres M. Elementos de Bioestatística. 2 ed. Belém: Ione Sena; 2012. p.463-72.

Bidani A ,Crandall ED. Velocity of CO2 exchanges in the lungs. Annu Rev Physiol. 1988;50:639-52.

Bonecker M, Guedes-Pinto AC, Fernandes FRC. Cárie dentária In: Guedes-Pinto AC. Odontopediatria. São Paulo: Santos, 2012.

Chałas R, Wójcik-Chęcińska I, Woźniak MJ, Grzonka J, Święszkowski W, Kurzydłowski KJ. Dental plaque as a biofilm - a risk in oral cavity and methods to prevent. Postepy Hig Med Dosw (Online). 2015 Oct 13;69:1140-8.

Collins TJ. ImageJ for microscopy. Biotechniques. 2007 Jul;43(1 Suppl):25-30.

Edgar M, Higham SM. Saliva and plaque pH control. In: Edgar M, Dawes C and O´Mullane D. Saliva and oral health. Composition, functions and protective effects. 3 ed. São Paulo: Santos, 2010.

Farsi N. Dental caries in relation to salivary factors in Saudi population groups. J Contemp Dent Pract. 2008 Mar 1;9(3):16-23.

Frasseto F, Parisotto TM, Peres RC, Marques MR, Line SR, Nobre Dos Santos M. Relationship among salivary carbonic anhydrase VI activity and flow rate, biofilme pH and caries in primary dentition. Caries Res. 2012;46(3):194-200.

Güngör K, Tüter G, Bal B. The evaluation of the relationship between educational status and oral health. Gü Dishekimligi Fakultesi Dergisi. 1999; 21-25.

Kaseda M, Ichihara N, Nishita T, Amasaki H, Asari M. Immunohistochemistry of the bovine secretory carbonic anhydrase isozyme (CA-VI) in bovine alimentary canal and major salivary glands. J Vet Med Sci. 2006 Feb;68(2):131-5.

Kimoto M, Kishino M, Yura Y, Ogawa Y. A role of salivary carbonic anhydrase VI in dental plaque. Arch Oral Biol. 2006 Feb;51(2):117-22.

Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H. A low concentration of carbonic anhydrase isoenzyme VI in whole saliva is associated with caries prevalence. Caries Res. 1999a May-Jun;33(3):178-84.

Kivela J, Parkkila S, Parkkila AK, Leinonen J, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI. J Physiol. 1999b Oct 15;520 Pt 2:315-20.b

Kivelä J, Parkkila S, Waheed A, Parkkila AK, Sly WS, Rajaniemi H. Secretory carbonic anhydrase isoenzyme (CA VI) in human serum. Clin Chem. 1997 Dec;43(12):2318-22.

Kotwica J, Ciuk MA, Joachimiak E, Rowinski S, Cymborowski B, Bebas P. Carbonic anhydrase activity in the vas deferens of the cotton leafworm - Spodoptera littoralis (Lepidoptera: Noctuidae) controlled by circadian clock. J Physiol Pharmacol. 2006 Nov;57 Suppl 8:107-23.

Leinonen J, Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. Caries Res. 1999 May-Jun;33(3):185-90.

Marsh PD. Dental plaque as a biofilm and a microbial community - implications for health and disease. BMC Oral Health. 2006 Jun 15;6 Suppl 1:S14.

Marsh PD. Microbiology of dental plaque biofilms and their role in oral health and caries. Dent Clin North Am. 2010 Jul;54(3):441-54.

Mau M, Kaiser TM, Südekum KH. Carbonic anhydrase VI is secreted from bovine parotid glands. Histol Histopathol. 2010 Mar;25(3):321-9.

Nobre dos Santos M, Melo dos Santos L, Francisco SB, Cury JA. Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. Caries Res. 2002 Sep-Oct;36(5):347-52.

Oztürk LK, Furuncuoğlu H, Atala MH, Uluköylü O, Akyüz S, Yarat A. Association between dental-oral health in young adults and salivary glutathione, lipid peroxidation and sialic acid levels and carbonic anhydrase activity. Braz J Med Biol Res. 2008 Nov;41(11):956-9. Epub 2008 Oct 31.

Parisotto TM, Steiner-Oliveira C, Duque C, Peres RC, Rodrigues LK, Nobre-dos-Santos M. Relationship among microbiological composition and presence of dental plaque, sugar

exposure, social factors and different stages of early childhood caries. Arch Oral Biol. 2010 May;55(5):365-73.

Peres RC, Camargo G, Mofatto LS, Cortellazzi KL, Santos MC, Nobre-dos-Santos M, et al. Association of polymorphisms in the carbonic anhydrase 6 gene with salivary buffer capacity, dental plaque pH, and caries index in children aged 7-9 years. Pharmacogenomics J. 2010 Apr;10(2):114-9.

Sathe N, Chakradhar Raju RV, Chandrasekhar V. Effect of three different remineralizing agents on enamel caries formation--an in vitro study. Kathmandu Univ Med J (KUMJ). 2014 Jan-Mar;12(45):16-20.

Scheutz F, Poulsen S. Determining causation in epidemiology. Community Dent Oral Epidemiol. 1999 Jun;27(3):161-70.

Sheiham A, James WP. Diet and Dental Caries: The Pivotal Role of Free Sugars Reemphasized. J Dent Res. 2015 Oct;94(10):1341-7.

Sheiham A. Sucrose and dental caries. Nutr Health. 1987;5(1-2):25-9.

Szabó L. Carbonic anhydrase activity in the saliva of children and its relation to caries activity. Caries Res. 1974;8(2):187-91.

3 DISCUSSÃO

Existe um campo abrangente de pesquisas sobre saliva como líquido diagnóstico. Tem sido empregado não só para indicar a suscetibilidade à cárie, como também para refletir modificações fisiológicas e patológicas que ocorrem na saliva. A saliva está facilmente disponível para coleta e realização de análises não invasivas. Pode ser empregada para monitorar a presença e o nível de hormônios, drogas, anticorpos, microrganismos, íons e proteínas (Whelton, 2010). A anidrase carbônica VI (AC VI) é uma proteína que está presente nas células serosas das glândulas salivares e está envolvida na regulação do pH e na função do paladar, assim como no desenvolvimento das papilas gustativas (Calò et al., 2011). Rotulagens imunohistoquímica e imunogold comprovam que a AC VI está presente nas células acinares serosas das glândulas parótidas e submandibulares (Piras et al., 2012). De acordo com o portal Atlas da Proteína Humana (http://www.proteinatlas.org), a AC VI é uma proteína órgão-específica presente exclusivamente nas glândulas salivares (Hsieh et al., 2015). Estudos sobre a isoenzima anidrase carbônica VI são importantes para a compreensão da fisiologia oral com o objetivo de investigar sistemas que operam na proteção contra a cárie dentária. A importância clínica reconhecida desta isoenzima é confrontada com a variabilidade das informações apresentadas pela evidência científica disponível.

Na revisão sistemática da literatura que realizamos, apenas 2 artigos preencheram os critérios de inclusão e requisitos pré-determinados para este trabalho de revisão, como descrito anteriormente: tipo de população, tipo de estudo, resultado e idioma. Ambos os estudos analisaram a atividade da AC VI na saliva. Isto é particularmente importante uma vez que uma alta concentração da AC VI na saliva ou placa bacteriana pode não significar necessariamente que toda a isoenzima presente no meio está ativa. No entanto, os dois trabalhos apresentaram resultados opostos. Szabó et al. (1974) mostrou que a concentração da AC VI foi maior na saliva de crianças livres de cárie do que no grupo de crianças cárie ativas, especificamente aquelas que vivem sob condições padronizadas. No estudo de Frasseto et al. (2012), uma maior atividade da AC VI foi encontrada na saliva de crianças com cárie dentária. Este estudo também demonstrou que as variáveis que apresentaram associação estatisticamente significativa com a cárie dentária foram idade e variação da atividade da AC VI (ΔACVI). Uma possível explicação para estes resultados pode ser que a AC VI catalisa a reação de $HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$ em ambos os sentidos de modo que pode neutralizar ou acidificar o meio de acordo com as condições no interior da cavidade bucal.

Neste trabalho, nós também avaliamos a atividade da AC VI, assim como sua concentração, tanto na saliva quanto na placa dentária de crianças de 7 a 9 anos de idade. Determinamos a relação entre a concentração e a atividade dessa isoenzima e se existe correlação entre a concentração e a atividade na saliva e na placa e a cárie dentária. Determinamos o fluxo salivar, pH (da saliva e da placa) e capacidade tampão e investigamos se estas propriedades da saliva correlacionam-se com a concentração e a atividade da isoenzima além da cárie dentária.

Os resultados mostraram valores significativamente maiores de fluxo salivar no grupo de crianças livres de cárie. A presença da saliva é vital para a manutenção de tecidos bucais hígidos. A severa redução na produção de saliva resulta não apenas na rápida deterioração da saúde bucal, como também cria um impacto prejudicial sobre a qualidade de vida do doente. Os pacientes que apresentam um menor fluxo salivar experimentam dificuldades para comer, engolir, na fonação, retenção de próteses dentárias, alteração no paladar, higiene bucal, sensação de queimadura na mucosa, infecções bucais, inclusive por *Candida*, e progresso rápido das cáries dentárias (Whelton, 2010).

O pH e capacidade tampão salivar também foram maiores no grupo de crianças livres de cárie. Estes resultados eram esperados, uma vez que estudos recentes da literatura mostram que estes parâmetros são significativamente menores em crianças cárie ativa (Preethi, 2010; Animireddy, 2014; Abbate, 2014). O pH do biofilme também foi maior no grupo livre de cárie. Indivíduos sem cárie, ou aqueles com um baixo índice de cárie, tendem a apresentar um pH da placa em repouso discretamente mais elevado, um mais alto pH mínimo após o consumo de carboidratos fermentáveis, e um retorno mais rápido aos níveis de repouso, quando comparados com indivíduos suscetíveis à cárie (Edgar et al., 2010). A capacidade tampão ajuda a neutralizar o pH da placa após a ingestão de carboidratos fermentáveis, dessa forma, reduzindo o tempo para que ocorra a desmineralização. A velocidade de recuperação do pH da placa após um desafio cariogênico é amplamente determinada pela capacidade de tamponamento e conteúdo de uréia na saliva, além da velocidade do fluxo salivar. A concentração de bicarbonato na saliva depende do fluxo salivar (Picco et al., 2012). A regulação do pH intrabucal pela saliva pode ser amplamente atribuída às ações de neutralização e de tamponamento de seu conteúdo de bicarbonato, com menores contribuições advindas do tampão fosfato e de outros fatores (Edgar et al., 2010). O sistema ácido carbônico/bicarbonato é o principal sistema tampão na saliva estimulada. Os íons hidrogênio e bicarbonato formam

ácido carbônico, o qual forma dióxido de carbono e água, e a anidrase carbônica VI acelera esta reação em até 13.000 vezes do que se ela não estivesse presente no meio (Bidani et al., 1988).

A concentração da AC VI tanto na saliva quanto no biofilme foi significativamente maior no grupo de crianças livres de cárie. Conforme diminui o pH da placa, a concentração de aminoácidos e consequentemente proteínas e amônia na placa também caem rapidamente (Edgar et al., 2010), o que explica estes menores números no grupo com cárie. Já a atividade da AC VI foi significativamente maior na saliva e no biofilme de crianças com cárie ativa. Isto confirma os resultados de um maior pH na saliva e no biofilme de crianças livres de cárie, maior capacidade tampão e maior concentração da AC VI, provendo uma maior proteção neste grupo contra a cárie dentária. No grupo de crianças cárie ativa, a maior atividade da AC VI revela uma maior ação da isoenzima para aumentar a neutralização dos ácidos após os desafios cariogênicos freqüentes a que as crianças com cárie estão expostas.

Neste estudo, encontramos também uma correlação negativa entre a capacidade tampão salivar e a cárie dentária. Uma alta capacidade de tamponamento indica maior capacidade da saliva de neutralizar os ácidos e, dessa forma, maior resistência à desmineralização. Na placa dentária, nenhuma das variáveis estudadas apresentou correlação com a cárie dentária. O bicarbonato tende a se diluir na placa e agir como tampão neutralizando os ácidos nela presentes, permitindo com isso, mais tempo para a remineralização de cáries incipientes (Edgar et al., 2010). Além disso, observamos uma correlação positiva entre a concentração e atividade da AC VI na saliva no grupo com cárie. O aumento no número de proteínas antimicrobianas sugere uma valorização potencial contra patógenos, uma baixa regulação da inflamação, ou ambos (Gillum et al., 2015), ou seja, uma maior atividade, no que parece haver uma maior necessidade de aumento de quantidade desta isoenzima no grupo com cárie ativa juntamente com o aumento na atividade, apesar da maior concentração ter sido observada no grupo livre de cárie. Na placa dental foi observada correlação negativa entre atividade e concentração da AC VI no grupo livre de cárie. Em um indivíduo que não ingeriu alimentos cariogênicos, o que acontece mais no grupo livre de cárie, a fase líquida extracelular da placa dentária (flúido da placa) normalmente está supersaturada de proteínas (Edgar et al., 2010), com menor atividade da isoenzima. Essa condição favorece a remineralização de lesões de cáries incipientes no grupo livre de cárie.

No presente estudo foi observado também uma correlação negativa entre o pH e concentração da AC VI na saliva do grupo livre de cárie. Apesar de apresentar um maior pH salivar, no caso deste pH cair, a concentração da AC VI parece aumentar neste grupo. Toda a

saliva está pronta para desnaturação dentro de um curto espaço de tempo a 37°C por degradação enzimática bacteriana de proteínas salivares, como a AC VI, ou uréia. A liberação de CO_2 da saliva, promovida pela AC VI, resultaria num aumento gradual do pH da saliva, o que teria uma influência crucial sobre a desmineralização da lesão (Fujikawa et al., 2008). Este resultado enfatiza a influência de concentrações minerais e do pH, assim como da concentração de proteínas e macromoléculas salivares, neste caso principalmente a AC VI, no fenômeno de des-remineralização. Na placa dental, esta correlação foi observada no grupo com cárie ativa. Quando na placa dental o número de lactobacilos e menor capacidade tampão estão presentes, o risco de cárie pode aumentar 87,8% (Yildiz et al., 2015) e elevados níveis de produtos finais da decomposição dos aminoácidos, assim como do metabolismo dos carboidratos estão presentes em concentrações muito mais altas na placa do que na saliva (Edgar et al., 2010), o que pode explicar este e outros resultados como concentração e atividade da AC VI apresentarem números significativamente maiores na placa dentária e neste caso, no grupo de crianças com cárie ativa.

Além disso, tivemos como resultado uma correlação negativa entre a capacidade tampão salivar e a atividade da AC VI no grupo de crianças cárie ativa. Isto confirma a menor capacidade tampão e maior atividade desta isoenzima observada neste grupo de crianças. Segundo Picco et al. (2012), além da taxa de fluxo reduzido, que neste trabalho foi observado no grupo de crianças com cárie, a hipofunção salivar é caracterizada por diminuição na capacidade tampão, assim como nas concentrações de compostos orgânicos, neste caso a AC VI, e inorgânicos presentes na saliva. As propriedades físico-químicas da saliva, tais como o pH, a capacidade tampão, cálcio, fósforo, amilase e Streptococcus mutans tem uma relação definida com atividade de cárie (Singh et al., 2015). Na placa dental esta correlação negativa foi observada entre o pH e a atividade da AC VI no grupo livre de cárie, confirmando os resultados de um maior pH e menor atividade da AC VI neste grupo. A base para o amplo espectro de expressão de proteínas é, pelo menos em parte, atribuída a diferenças na regulação dessas proteínas entre grupos (Huang et al., 2015). Os resultados fornecem insights sobre a base microbiológica para as diferenças inter-atividade da AC VI em biofilmes orais e melhoram a nossa compreensão da cárie como uma doença ecologicamente orientada em que o metabolismo da AC VI modera o pH da placa e promove a saúde dental.

Na análise de regressão logística múltipla, o menor fluxo salivar e a maior atividade da isoenzima foram significativamente associados com a cárie dentária. Crianças com fluxo salivar menor do que 1,06 mL/min apresentam 4 vezes mais chances de desenvolver cárie do

que as crianças com uma taxa de fluxo salivar superior a 1,06 mL/min. Da mesma forma, crianças que mostram uma atividade da AC VI na saliva superior a 1,75 apresentam 5,03 vezes mais chances de desenvolver cárie do que aquelas com menores valores da isoenzima. Pelo nosso conhecimento, este é o primeiro estudo a investigar a correlação entre a atividade e concentração da AC VI na saliva e na placa dentária, bem como a demonstrar o papel da atividade de AC VI na saliva em discriminar crianças que têm a chance de desenvolver cárie dentária.

Neste trabalho, mostramos que a isoenzima Anidrase Carbônica VI apresenta resultados conflitantes na literatura sobre sua disponibilidade, atividade e eficácia nos tecidos bucais, sendo necessários estudos clínicos para confirmar sua verdadeira função e importância na saúde bucal e geral. Neste estudo clínico, verificou-se como esta isoenzima se comporta em dois importantes componentes bucais: a saliva e a placa dental. Realizou-se um estudo transversal qualitativo e quantitativo, nos quais avaliou-se a concentração e atividade desta isoenzima, já que o fato da mesma estar presente no meio, não significaria necessariamente que estejivesse ativa. Outra importante realização desta pesquisa foi o estudo sobre a concentração e atividade da AC VI na placa dental, que forneceu resultados até a presente data, ainda não publicados pela literatura.

Para estudos posteriores no nosso laboratório são desejados um maior aprofundamento no estudo da isoenzima AC VI principalmente na placa dental. Assim, estudos clínicos longitudinais são fortemente recomendáveis para fornecer evidencias a respeito do papel da AC VI na predição do risco de cárie em Odontopediatria. Além disso, o estudo de possíveis drogas capazes de potencializar ou inibir o efeito da AC VI no meio bucal são fortemente recomendáveis.

4 CONCLUSÃO

No capítulo 1 pode-se concluir que esta revisão sistemática mostra resultados conflitantes a respeito da relação entre a concentração/atividade da AC VI na saliva e a cárie dentária e mostra a necessidade desta pesquisa continuada para confirmar o papel desta iso-enzima na modulação do processo dinâmico da cárie.

Nos capítulos 2 e 3, a AC VI mostrou estar mais ativa na saliva e placa dental de crianças com cárie ativa. Uma maior concentração da AC VI na saliva e placa dental de crianças livres de cárie sugere maior proteção e como são menos expostas a desafios cariogênicos, o sistema carbonato seria menos ativado. Pelo nosso conhecimento, este é o primeiro estudo a investigar a correlação entre a atividade e a concentração da AC VI na saliva e na placa, bem como demonstrar o papel da atividade da AC VI em discriminar crianças com maior chance de desenvolver cárie dentária. A AC VI na saliva e placa dentária pode ser considerada uma proteína anti-cáries, além de um biomarcador para a cárie dentária.

Embora muitos esforços tenham sido feitos para identificar um teste ou combinação de provas para predizer o desenvolvimento das cáries, nenhum foi encontrado que predissesse precisamente essa doença multifatorial. As análises da concentração e atividade da AC VI na saliva e na placa são indicadores úteis da suscetibilidade às cáries em nível individual, em que podem ser úteis para o monitoramento prospectivo de intervenções preventivas contra cáries e para criar um perfil do paciente com suscetibilidade à cárie.

REFERÊNCIAS

Abbate GM, Borghi D, Passi A, Levrini L. Correlation between unstimulated salivary flow, pH and streptococcus mutans, analysed with real time PCR, in caries-free and caries-active children. Eur J Paediatr Dent. 2014 Mar;15(1):51-4.

Animireddy D, Reddy Bekkem VT, Vallala P, Kotha SB, Ankireddy S, Mohammad N. Evaluation of pH, buffering capacity, viscosity and flow rate levels of saliva in caries-free, minimal caries and nursing caries children: An in vivo study. Contemp Clin Dent. 2014 Jul;5(3):324-8.

Bidani A, Crandall ED. Velocity of CO2 exchanges in the lungs. Annu Rev Physiol. 1988;50:639-52.

Bowen WH, Koo H. Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011;45(1):69-86.

Calò C, Padiglia A, Zonza A, Corrias L, Contu P, Tepper BJ, et al. Polymorphisms in TAS2R38 and the taste bud trophic factor, gustin gene co-operate in modulating PROP taste phenotype. Physiol Behav. 2011 Oct 24;104(5):1065-71.

Ditmyer M, Dounis G, Mobley C, Schwarz E. A case-control study of determinants for high and low dental caries prevalence in Nevada youth. BMC Oral Health. 2010 Nov 11;10:24.

Edgar M, Higham SM. Saliva e controle do pH da placa. In: Edgar M, Dawes C e O´Mullane. Saliva e Saúde Bucal - Composição, Funções e Efeitos Protetores. 3 ed. São Paulo: Santos; 2010.

Edgar M, Dawes C, O´Mullane D. Saliva e Saúde bucal - Composição, Funções e efeitos protetores. 3 ed. São Paulo: Santos; 2010.

Frasseto F, Parisotto TM, Peres RC, Marques MR, Line SR, Nobre Dos Santos M. Relationship among salivary carbonic anhydrase VI activity and flow rate, biofilm pH and caries in primary dentition. Caries Res. 2012;46(3):194-200.

Frost SC. Physiological functions of the alpha class of carbonic anhydrases. Subcell Biochem. 2014;75:9-30.

Fujikawa H, Matsuyama K, Uchiyama A, Nakashima S, Ujiie T. Influence of salivary macromolecules and fluoride on enamel lesion remineralization in vitro. Caries Res. 2008;42(1):37-45.

Gillum TL, Kuennen MR, Castillo MN, Williams NL, Jordan-Patterson AT. Exercise, but not acute sleep loss, increases salivary antimicrobial protein secretion. J Strength Cond Res. 2015 May;29(5):1359-66.

Gittings S, Turnbull N, Henry B, Roberts CJ, Gershkovich P. Characterisation of human saliva as a platform for oral dissolution medium development. Eur J Pharm Biopharm. 2015 Apr;91:16-24.

Hsieh MS, Jeng YM, Jhuang YL, Chou YH, Lin CY. Carbonic anhydrase VI: a novel marker for salivary serous acinar differentiation and its application to discriminate acinic cell carcinoma from mammary analogue secretory carcinoma of the salivary gland. Histopathology. 2015 Jul 25. doi: 10.1111/his.12792.

Huang X, Schulte RM, Burne RA, Nascimento MM. Characterization of the arginolytic microflora provides insights into pH homeostasis in human oral biofilms. Caries Res. 2015;49(2):165-76.

Kimoto M, Kishino M, Yura Y, Ogawa Y. A role of salivary carbonic anhydrase VI in dental plaque. Arch Oral Biol. 2006 Feb;51(2):117-22.

Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H. A low concentration of carbonic anhydrase isoenzyme VI in whole saliva is associated with caries prevalence. Caries Res. 1999 May-Jun;33(3):178-84.

Kumar A, Hedge R, Dixit U. Role of plaque in the clearance of salivary sucrose and its influence on salivary ph. J Indian Soc Pedod Prev Dent. 2011 Oct-Dec;29(4):310-4.

Leinonen J, Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. Caries Res. 1999 May-Jun;33(3):185-90.

Masood M, Yusof N, Hassan MI, Jaafar N. Assessment of dental caries predictors in 6-year-old school children - results from 5-year retrospective cohort study. BMC Public Health. 2012 Nov 16;12:989.

Oztürk LK, Furuncuoğlu H, Atala MH, Uluköylü O, Akyüz S, Yarat A. Association between dental-oral health in young adults and salivary glutathione, lipid peroxidation and sialic acid levels and carbonic anhydrase activity. Braz J Med Biol Res. 2008 Nov;41(11):956-9.

Peres RC, Camargo G, Mofatto LS, Cortellazzi KL, Santos MC, Nobre-dos-Santos M, et al. Association of polymorphisms in the carbonic anhydrase 6 gene with salivary buffer capacity, dental plaque pH, and caries index in children aged 7-9 years. Pharmacogenomics J. 2010 Apr;10(2):114-9.

Picco DC, Costa LF, Delbem AC, Sassaki KT, Sumida DH, Antoniali C. Spontaneously hypertensive rat as experimental model of salivary hypofunction. Arch Oral Biol. 2012 Oct;57(10):1320-6.

Piras M, Tandler B, Barbarossa IT, Piludu M. Immunogold labeling of carbonic anhydrase isozyme (CA-VI) in secretory granules of human parotid glands. Acta Histochem. 2012 Jul;114(4):406-8.

Preethi BP, Reshma D, Anand P. Evaluation of Flow Rate, pH, Buffering Capacity, Calcium, Total Proteins and Total Antioxidant Capacity Levels of Saliva in Caries Free and Caries Active Children: An In Vivo Study. Indian J Clin Biochem. 2010 Oct;25(4):425-8.

Scheutz F, Poulsen S. Determining causation in epidemiology. Community Dent Oral Epidemiol. 1999 Jun;27(3):161-70.

Sheiham A, James WP. Diet and Dental Caries: The Pivotal Role of Free Sugars Reemphasized. J Dent Res. 2015 Oct;94(10):1341-7.

Sheiham A. Sucrose and dental caries. Nutr Health. 1987;5(1-2):25-9.

Shifa S, Muthu MS, Amarlal D, Rathna Prabhu V. Quantitative assessment of IgA levels in the unstimulated whole saliva of caries-free and caries-active children. J Indian Soc Pedod Prev Dent. 2008 Dec;26(4):158-61.

Singh S, Sharma A, Sood PB, Sood A, Zaidi I, Sinha A. Saliva as a prediction tool for dental caries: An in vivo study. J Oral Biol Craniofac Res. 2015 May-Aug;5(2):59-64.

Sly WS, Hu PY. Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu Rev Biochem. 1995;64:375-401.

Szabó I. Carbonic anhydrase activity in the saliva of children and its relation to caries activity. Caries Res. 1974;8(2):187-91.

Tenovuo J. Salivary parameters of relevance for assessing caries activity in individuals and populations. Community Dent Oral Epidemiol. 1997 Feb;25(1):82-6.

Warren JJ, Weber-Gasparoni K, Marshall TA, Drake DR, Dehkordi-Vakil F, Dawson DV, et al. A longitudinal study of dental caries risk among very young low SES children. Community Dent Oral Epidemiol. 2009 Apr;37(2):116-22.

Whelton H. Introdução: anatomia e fisiologia das glândulas salivares. In: Edgar M, Dawes C, O´Mullane D. Saliva e saúde bucal - Composição, funções e efeitos protetores. 3 ed. São Paulo: Santos; 2010.

Yan G, Huang W, Xue H, Jia Y, Yang D. Relationship between dental caries and salivary proteome by electrospray ionization ion-trap tandem mass spectrometry in children aged 6 to 8 years. Hua Xi Kou Qiang Yi Xue Za Zhi. 2014 Jun;32(3):297-302.

Yildiz G, Ermis RB, Calapoglu NS, Celik EU, Türel GY. Gene-environment Interactions in the Etiology of Dental Caries. J Dent Res. 2016 Jan;95(1):74-9.

APÊNDICE 1

Produção bibliográfica da aluna Daniele de Cassia Rodrigues Picco durante o Doutorado

1. **Picco DCR**, Costa L, Delbem A, Sassaki K, Sumida D, & Antoniali C. (2012). Spontaneously hypertensive rats as experimental model of salivary hypofunction. Archives of Oral Biology, 1320-1326.
2. **Picco DCR**, Delbem A, Sassaki K, Sumida D, & Antoniali C. (2014). The effect of chronic treatment with fluoride on salivary activity, tooth and bone in spontaneously hypertensive rats (SHR). Naunyn-Schmiedeberg's Arch Pharmacol, 321-328.
3. **Picco DCR**, Lopes LM, Nobre-dos-Santos M. Carbonic anhydrase VI in saliva and biofilm: a systematic review. 2016. (Journal of Dentistry)
4. **Picco DCR**, Lopes LM, Marques MR, Line SR, Parisotto TM, Nobre-dos-Santos M. Children with higher activity of carbonic anhydrase VI in saliva are more likely to develop dental caries. 2016. (Caries Research)
5. **Picco DCR**, Lopes LM, Nobre-dos-Santos M. Relationship between dental caries and pH, concentration and activity of carbonic anhydrase VI in dental plaque in school children – A cross-sectional study. 2016. (Tese)

ANEXO 1 - Certificado do Comitê de Ética em Pesquisa

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise da concentração e atividade da Anidrase Carbônica VI (CA VI) na saliva e biofilme de crianças em idade escolar"**, protocolo nº 018/2012, dos pesquisadores Daniele de Cassia Rodrigues Picco e Marinês Nobre dos Santos Uchôa, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 17/09/2012.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Analysis of concentration and activity of Carbonic Anhydrase VI (CA VI) in saliva and plaque of children in school age"**, register number 018/2012, of Daniele de Cassia Rodrigues Picco and Marinês Nobre dos Santos Uchôa, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 09/17/2012.

Lívia M. A. Tenuta

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2 - Declaração

Declaração

As cópias dos documentos de minha autoria já submetidos para publicação em revistas científicas sujeitos a arbitragem, que constam da minha tese de Doutorado intitulada "ESTUDO DA CONCENTRAÇÃO E ATIVIDADE DA ANIDRASE CARBÔNICA VI NA SALIVA E BIOFILME DE CRIANÇAS EM IDADE ESCOLAR E SUA RELAÇÃO COM A CÁRIE DENTÁRIA" não infringem os dispositivos da Lei nº 9.610/98, nem o direito autoral de qualquer editora.

Piracicaba, 18 de fevereiro de 2016.

_____

Autora: Daniele de Cassia Rodrigues Picco

RG: 32.908.359-4

Orientadora: Marinês Nobre dos Santos Uchôa

RG: 416.641

ANEXO 3 - Confirmação de envio do artigo para publicação – Journal of Dentistry

Daniele Picco

De: ees.jjod.0.370a90.04d413ac@eesmail.elsevier.com em nome de Journal of Dentistry <JoD@elsevier.com>
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Para: dpicco@outlook.com.br
Assunto: Your recent submission to JJOD

Dear Dr. Daniele Picco,

You have been listed as a Co-Author of the following submission:

Journal: Journal of Dentistry
Corresponding Author: Marines Nobre dos Santos
Co-Authors: Daniele Picco, PhD; Lenita M Lopes, Master;
Title: Carbonic anhydrase VI in saliva and biofilm: a systematic review.

If you did not co-author this submission, please contact the Corresponding Author of this submission at mnobre@unicamp.br; do not follow the link below.

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Journal of Dentistry

ANEXO 4 - Confirmação de envio do artigo para publicação – Caries Research

Daniele Picco

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Para: dpicco@outlook.com.br
Assunto: Computer-Generated E-Mail: Caries Research - Account Created in Manuscript Central

02-Feb-2016

Dear Miss Picco:

A manuscript titled Children with higher activity of carbonic anhydrase VI in saliva are more likely to develop dental caries. (201602004) has been submitted by Miss Daniele Picco to Caries Research.

You are listed as a co-author for this manuscript. The online peer-review system, Manuscript Central, automatically creates a user account for you. Your USER ID and PASSWORD for your account is as follows:

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Yours sincerely,

Caries Research

david.beighton@kcl.ac.uk