Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

GABRIELA NOVO BORGHI

ASSOCIAÇÃO ENTRE BIOMARCADORES SALIVARES, BIOFILME VISÍVEL E A CÁRIE PRECOCE DA INFÂNCIA - UM ESTUDO LONGITUDINAL

ASSOCIATION AMONG SALIVARY BIOMARKERS, VISIBLE BIOFILM AND EARLY CHILDHOOD CARIES - A LONGITUDINAL STUDY

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Odontologia, área de concentração em Odontopediatria.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Dentistry, in Pediatric Dentistry area.

Orientadora: Profa. Dra. MarinêsNobre dos Santos Uchôa

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA GABRIELA NOVO BORGHI E ORIENTADA PELO PROFA. DRA. MARINÊS NOBRE DOS SANTOS UCHÔA

Piracicaba
2016

Agência(s) de fomento e nº(s) de processo(s): Não se aplica.

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

B644a

Borghi, Gabriela Novo, 1989-
Associação entre biomarcadores salivares, biofilme visível e a cárie precoce da infância - um estudo longitudinal / Gabriela Novo Borghi. – Piracicaba, SP : [s.n.], 2016.

Orientador: Marines Nobre dos Santos Uchoa.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Cárie dentária. 2. Alfa-amilase. 3. Anidrases carbônicas. 4. Placas dentárias. 5. Proteínas e peptídeos salivares. I. Nobre dos Santos, Marines,1956-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Association among salivary biomarkers, visible bioflm and early childhood caries - a longitudinal study
Palavras-chave em inglês:
Dental caries
Alpha-amylases
Carbonic anhydrases
Dental plaque
Salivary proteins and peptides
Área de concentração: Odontopediatria
Titulação: Mestra em Odontologia
Banca examinadora:
Marines Nobre dos Santos Uchoa [Orientador]
Juliano Pelim Pessan
Antonio Carlos Pereira
Data de defesa: 26-02-2016
Programa de Pós-Graduação: Odontologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de Mestrado, em sessão pública realizada em 26 de Fevereiro de 2016, considerou a candidata GABRIELA NOVO BORGHI aprovada.

PROFª. DRª. MARINES NOBRE DOS SANTOS UCHOA

PROF. DR. JULIANO PELIM PESSAN

PROF. DR. ANTONIO CARLOS PEREIRA

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATÓRIA

*Agradeço primeiramente a **Deus**, por estar sempre me guiando.*

*Dedico essa conquista aos **meus pais e minha irmã** por todo apoio e incentivo em todos os momentos da minha vida, por me ajudarem a enfrentar as dificuldades e não me deixarem desistir, por serem minha base, meu alicerce, por todo amor, carinho e compreensão que depositam sobre mim.*

A toda minha família por estar sempre torcendo por mim.
*Especialmente a minha avó paterna, **Maria Lúcia**, que faz parte, que vibra e que me apoia em todas as conquistas da minha vida.*

*Ao meu namorado, **Vinicius**, por estar ao meu lado em todos os momentos, por sempre se mostrar presente e participativo, além de todo amor e dedicação que tem por mim, tornando a jornada mais leve e tranquila. Assim como toda sua família.*

Aos meus amigos e amigas que sempre estiveram presentes e que fazem parte de todas as minhas conquistas, mesmo alguns estando longe.

Às minhas amigas queridas

Tássia Bolognesi, Yara Ferreira, Renata Prete, Cristhiane Sueli Quezada Motta, Paola Soares,

Bianca Azevedo, Isabella Bonato por nossa amizade de tantos anos que nos conforta, ampara

enos ajuda a enfrentar todos os desafios.

AGRADECIMENTOS ESPECIAIS

*À **Prof.Dra. Marinês Nobre dos Santos Uchôa**, minha orientadora, agradeço pela orientação, respeito e dedicação.*

*A **Prof. Dra. Thaís Manzano ParisottoUlmer**pelo auxílio com a análise estatística, pela*

disponibilidade em me ajudar mesmo com tantos compromissos.

*A **Prof. Dra. Carolina Steiner Alarcon**pela ajuda na elaboração do manuscrito e tempo disponível*

*A doutoranda **Lívia Pagotto Rodrigues**, pela companhia durante a pesquisa, por todos os ensinamentos, por toda ajuda, por ter me guiado num começo tão difícil e tortuoso.*

*A doutoranda **Lenita Marangoni Lopes** por toda ajuda e ensinamento.*

AGRADECIMENTOS

À Universidade Estadual de Campinas, na pessoa do seu Magnífico Reitor ***Prof. Dr. José Tadeu Jorge****. À Faculdade de Odontologia de Piracicaba, na pessoa do seu Diretor* ***Prof. Dr. Guilherme Elias Pessanha Henriques. À Profa. Dra Cínthia Pereira Machado Tabchoury****, Presidente da Comissão de Pós-Graduação, FOP/UNICAMP. À* ***Profa. Dra. Juliana Trindade Clemente Napimoga****, Coordenadora do Programa de Pós-Graduação em Odontologia, FOP/UNICAMP.*

.

Às professoras do Departamento de Odontologia Infantil, área de Odontopediatria, ***Profa. Dra Fernanda Miori Pascon, Profa. Dra. Regina Maria Puppin Rontani, Profa. Dra. Maria Beatriz Duarte Gavião e Prof. Dra. Caroline Steiner Alarcon****, que contribuírampara meu aprimoramento profissional.*

*Às professoras que foram membro da banca da primeira fase do exame qualificação****Profa. Dra Maria Beatriz Duarte Gavião e Profa. Dra.Taís de Souza Barbosa****, pelassugestões que contribuíram para o aperfeiçoamento deste trabalho.*

Aos professores membro da banca da segunda fase do exame de qualificação ***Profa****.* ***Dra.Maria Beatriz Duarte Gavião, Profa. Dra Carolina Steiner Alarcon e Prof. Dr. Eduardo Almada****obrigada pela colaboração e auxílio, as modificações sugeridas foram muito importantes.*

Ao técnico do laboratório de Odontopediatria ***Marcelo****atenção e prontidão em me auxiliar em todos os momentos.*

À secretária do departamento de Odontologia Infantil ***Shirley Rosana Sbravatti Moreto****, pelo carinho, atenção e prontidão em me auxiliar em todos os momentos.*

Aos queridos ***amigos do mestrado e doutorado****a amizade de vocês foi fundamental durante essesdois anos, vou levar comigo para sempre todos os momentos vividos. Quero agradecer de coração pelo apoio de vocês.*

À todos os ***voluntários*** *que contribuíram participando da pesquisa, graças a vocês foi possível concretizar esse trabalho.*

RESUMO

A prevalência da cárie precoce da infância (CPI) no Brasil é alta e a severidade aumenta com a idade. A análise da literatura evidência que a saliva contém biomarcadores e esses, têm sido correlacionados com a cárie dentária. No entanto, não se tem conhecimento de pesquisas prévias que tenham avaliado se esses biomarcadores salivares podem ser considerados preditores de risco de cárie dentária em pré-escolares. Assim, o presente estudo teve como objetivos:1. Investigar a associação entre a cárie precoce da infância, a presença de biofilme visível nas superfícies vestibulares dos incisivos superiores anteriores e biomarcadores salivares como a α amilase salivar e a anidrase carbônica VI(AC VI); 2. Investigar se biomarcadores salivares como a α amilase salivar e a anidrase carbônica VI, bem como a presença de biofilme visível,são preditores de risco para a cárie precoce da infância. Para isto, a amostra foi constituída de 100pré-escolares de 24 a 48 meses, de ambos os gêneros, que frequentavam creches e pré-escolas municipais na cidade de Piracicaba-SP. As crianças foram submetidas ao exame clínico para determinação do índice de cárie, pelo critério de Nyvad (1999), adaptado para dentes decíduos por Séllos e Sovieiro (2011) e a verificação da presença de biofilme visível.Assim, 02 grupos foram constituídos: Grupo livre de cárie (n=55) e Grupo com cárie (n=45).A seguirrealizou-se a coleta de saliva para análise das proteínas salivares.Foram determinadas a atividade da amilase salivar e a atividade da AC VI,por meio dos métodos Elisa e Zimografia, respectivamente. Após 1 ano, as crianças foram reavaliadas para a determinação do índice de cárie e verificação da presença de biofilme visível. Os resultados obtidos foram submetidos aos testes deMann-Whitney, qui-quadrado, à análise de regressão logística múltipla, além da correlação de Spearman para avaliar a relação entre a cárie e as variáveis estudadas. Todas as análises foram realizadas empregando-se um nível de significância de 5% e um intervalo de confiança de 95%. A atividade da AC VI foi significativamente maior na saliva das crianças do grupo com cárie($p \leq 0,05$). Por outro lado, a atividade da α amilase salivar foi significativamente maior na saliva das crianças livres de cárie ($p < 0,0001$). A presença de biofilme visível aumentou em 3,6 vezes o risco de a criança desenvolver CPI (OR=3,6). Além disso, crianças com atividade da α amilase salivar menor que 122,8 U/mLapresentaram 3,33 vezes maior risco de desenvolver cárie precoce (OR=3,33). Os resultados encontrados nos permitem concluir que a presença de biofilme visível e a menor atividade da α amilase salivarpodem ser consideradas preditores de risco para a cárie precoce da infância.

Palavras chave: Cárie dental, alfa amilase, anidrase carbônica, biofilme dentário e proteínas salivares.

ABSTRACT

The prevalence of early childhood caries (ECC) in Brazil is high and its severity increases with age. The literature shows that saliva contains biomarkers and theyhave been correlated with tooth decay. However,whether these salivary biomarkers can be consideredprecdictorsof dental caries in preschoolers is largely unknown.Thus, this study aimed to:1-Investigate the association between ECC and clinically visible biofilm, and salivary biomarkers as α amylase and carbonic anhydrase VI. 2- Investigate if salivary biomarkers such as α amylase and carbonic anhydrase as well as clinically visible biofilm can predict caries development in preschool children. The sample consisted of 100preschool children 24-48 months, of both genders, who attended kindergartens and municipal preschools in the city of Piracicaba-SP, Brazil. The children underwent clinical examination to determine the decay rate using theNyvad criteria, adapted for primary teeth by Séllos and Sovieiro (2011)and for verification of the presence of visible biofilm. Then, two groups were formed: caries free group (n=55) and group with caries(n=45). Following, saliva samples were collected and the activity of salivary α amylase and carbonic anhydrase VI were determined using Elisa and zymography methods, respectively. After one year, the children were clinically examined again to determine the decay rate and to verify the presence of visible biofilm. The results were subjected toMann-Whitney test, chi-square test as well to multiple logistic regression analysis.In addition, the Spearman correlation test was used to assess the relationship between the decay and the studied variables. All analyses were performed using a significance level of 5% and a 95% confidence interval.The activity of AC VI was significantly higher in the saliva of children in the caries group ($p \leq 0.05$). On the other hand, the activity of salivary α amylase was significantly higher in the saliva caries freechildren ($p < 0.0001$). The presence of visible biofilm increased 3.6 times therisk to develop ECC (OR = 3.6). In addition, children with activity of salivary α amylase lower than 122,8 U/mL had 3.33 times higher risk of developing ECC than children showing higher activity of this protein in their saliva. (OR = 3.33). The results allow us to conclude that the presence of visible biofilm and a lower salivary activity of α amylase may be considered risk predictors for early childhood caries.

Key words: dental caries, alpha amylase, carbonic anhydrase, dental plaque and salivary proteins.

"Não sei... se a vida é curta ou longa demais para nós,
Mas sei que nada do que vivemos tem sentido, se não
tocarmos o coração das pessoas".
Cora Coralina

SUMÁRIO

1 INTRODUÇÃO

A cárie precoce da infância (CPI) é definida como a presença de uma ou mais superfícies dentais cariadas (cavitadas ou não), perdidas ou obturadas (ceo-s) em pré-escolares com idade inferior a 06 anos (AAPD, 2014).

Levantamentos epidemiológicos evidenciaram que no Brasil a CPI apresenta-se como um problema de saúde pública. No último relatório de saúde bucal, Projeto SB Brasil 2010 (Ministério da Saúde), apenas 46,6% das crianças brasileiras, aos cinco anos de idade, se apresentaram livres de cárie na dentição decídua. Ainda, na mesma faixa etária, uma criança brasileira possuía, em média, 2,43 dentes com experiência de cárie, com predomínio do componente cariado, que é responsável por mais de 80% do índice. Estes índices chamam a atenção para a importância de se pesquisar os fatores relacionados com o desenvolvimento e progressão desta doença. Além disso, a população infantil que apresenta CPI, também apresenta maior risco de desenvolvimento de cárie futura, sendo a experiência passada dessa doença considerada um dos preditores de risco mais significativos (Parisotto et al., 2012), demostrado por pesquisa longitudinal recentemente realizada, a qual verificou que pré-escolares com CPI apresentaram riscos 17 e 24 vezes maiores de desenvolverem novas lesões de manchas brancas ativas e de apresentarem lesões de cárie cavitadas, respectivamente (Parisotto et al., 2012).

Os fatores primários relacionados à etiologia do processo carioso e da CPI são a presença de bactérias cariogênicas, carboidratos fermentáveis e hospedeiro ou superfície dentária susceptíveis, que interagem em determinado período de tempo. (Rosenblatt e Harris, 2004; Parissoto et al.,2010; Sheiham e James, 2015; Peres et al., 2016). Dentre esses fatores, a frequência de exposição à sacarose tem sido destacada como responsável pelas alterações orgânicas, inorgânicas (Ashley e Wilson, 1977; Nobre dos Santos et al., 2002) e microbiológicas (Parisotto et al., 2010) no biofilme dentário. Em pré-escolares, um elevado número de bactérias cariogênicas como *Streptococcusmutans*(SM) e *Lactobacillus*, a presença de biofilme dental visível na região anterossuperior dos dentes decíduos, práticas inadequadas de alimentação, nível socioeconômico e escolaridade têm sido identificadas como fatores importantes como indicadores de risco (Selwitz et al., 2007; Oliveira et al., 2008; Takahashi e Nyvad, 2008) e na predição do desenvolvimento à cárie (Parisotto et al., 2010).

A cárie dentáriaé reconhecida comouma doençamultifatorial biofilme-açúcar-dependente (Fejerskov, 2004; Sheiham e James, 2015), causadapor interaçõescomplexas entreas bactériasprodutoras de ácido, carboidratos fermentáveise muitosfatores do hospedeiro, incluindo asaliva(Selwitz et al.,2007).Pesquisas atuais procuram identificar os fatores de risco da cárie dentária, bem como as defesas bucais naturais que podem proteger ou prevenir o desenvolvimento da doença cárie. A saliva, apesar de ser um sistema de defesa efetivo, apresenta uma grande variedade de propriedades e proteínas cujosmecanismos ainda não são completamente conhecidos (Sumatti Bhalla et al.,2010).

A saliva é um importante fator de proteção da cavidade bucal. Dentre suas múltiplas funções, destacam-se o *clearence* promovido pelo fluxo salivar e a manutenção do pH em níveis aceitáveis pela capacidadetamponante, devido principalmente aos tampões bicarbonato e fosfato (Bardow et al., 2000) que servem como fator protetor tanto para a cárie (Bagherian eAsadikaram, 2012), quanto para a erosão dentárias (Hara et al., 2006).

Além de suas propriedades inorgânicas, a composição proteica da saliva tem grande importância uma vez que confere função digestiva, lubrificante e protetora.As proteínas salivares também se ligam à superfície dental formando uma película adquirida que se comporta como uma membrana (Lendenmann et al., 2000; Hara et al., 2006). Outro aspecto salivar importante para a manutenção de um meio bucal saudável diz respeito à concentração de proteínas. A variabilidade das proteínas salivares e suas modificações pós-tradução podem desempenhar um papel significativo na determinação de suas características de proteção à cárie dentária Assim, entende-se que as proteínas da saliva podem contribuir para a avaliação do risco de cárie dentária em crianças e, portanto, para a prevenção desta doença. Dessa forma, o estudo desses marcadores, representa uma área de pesquisa muito relevante para o entendimento da cárie precoce (Banderas-Tarabay et al.,2002).

Dentre as proteínas da saliva a α amilase salivar é a mais abundante na saliva (Singh et al., 2015). Ela pertence à família de proteínas que se apresenta de várias isoformas as quais diferem quanto à carga e a glicolisação. É considerada uma metaloenzima que contém cálcio hidrolisando as quatro ligações de amido em glicose e maltose, sendo esta sua principal função. Tradicionalmente, a α amilase salivar era estudada apenas como uma enzima iniciadora do processo de digestão do amido na cavidade bucal, porém durante os últimos anos, linhas de pesquisas demonstraram que a α amilase salivar tem uma função pouco estudada e até agora negligenciada, que demostra sua capacidade de inibir diretamente o

crescimento de algumas bactérias, além de se ligar especificamente com elevada afinidade a várias bactérias do tipo estreptococos orais (Bosch et al., 2003). Uma vez ligada a essas bactérias a α amilase salivar retém a sua atividade enzimática, além de ser encontrada na película adquirida do esmalte e biofilme dental, podendo mediar a adesão de estreptococos à superfície do dente. Devido a estas características a α amilase salivar pode, a partir de sua aderência as bactérias, contribuir para a formação do biofilme, por meio da digestão do amido (Scannapieco et al., 1993; Singh et al.,2015).Por outro lado, a sua ligação com bactérias cariogênicas na saliva poderia ser considerada protetora se a α amilase for capaz de promover a eliminação bacteriana da cavidade oral. Essas evidências demostram a importância da α amilase salivar no processo dinâmico da cárie dentária, principalmente devido ao seu possível papel protetor (Scannapieco et al., 1993).

Além da α amilase salivar, outra proteína encontrada na saliva e que desempenha um papel importante na dinâmica do processo de cárie é a AC VI. As anidrases carbônicas são consideradas isoenzimas, formadas por polipeptídeos e que, em sua forma nativa, contém um íon zinco fortemente ligado, essencial para a sua atividade catalítica. A atividade da AC na saliva humana foi relatada pela primeira vez por Becks & Wainwright em 1939, é a única isoenzima secretada pelas células acinares serosas das glândulas salivares parótida e submandibular. A AC VI mantém o pH fisiológico catalisando a reação reversível do dioxido de carbono na reação $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+$.Através da sua atividade catalítica, ela aumenta a capacidade tampão da saliva, principalmente do tampão bicarbonato, o tampão mais importante da saliva, regulando o pH do meio bucal, por conseguir penetrar no biofilme e facilitar a neutralização ácida proveniente do metabolismo bacteriano (Kimoto et al., 2006).

O efeito desta proteina sobre a cárie dentária foi pesquisado por Szabó e colaboradores em 1974 que mostrou uma maior concentração da AC VI em crianças livres de cárie do que naquelas com cárie. Da mesma forma, Kivela e coloboradores em 1999 demostraram que baixas concentrações de AC VI na saliva foram associadas à prevalência de cárie dentária, especialmente em indivíduos com higiene bucal deficiente. Sabe-se que os achados na literatura apresentam resultados conflitantes, desta maneira Ozturk e colaboradores em 2008 não observaram diferença siginificativa na concentração de AC VI na saliva e biofilme de adultos divididos em grupo com cárie e livres de cárie. Importante mesmo é que o fato da AC VI estar concentrada na cavidade bucal não indica necessariamente que esta proteína esteja ativa. Portanto mais relevante do que a concentração da isoenzima no

meio, é a sua atividade, já que alguns estudos indicam que sua capacidade de regulação do pH e seu poder de catalisar a reação do tampão bicarbonato não estão diretamente associadas à sua concentração na saliva (Parkkila et al., 1993; Kivela et al., 1997).

Dessa forma, no presente estudo, investigau-se a associação entre a CPI, a presença de biofilme visível e biomarcadores salivares como a AC VI e a alfa amilase salivar, além de investigar se essas variáveis são preditoras de risco para a cárie precoce da infância.

2 ARTIGO: Can alpha amylase, carbonic anhydrase VI in saliva and visible biofilm predict early childhood caries? - a longitudinal study.

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Key words: ***dental caries, alpha amylase, carbonic anhydrase, dental plaque and salivary proteins***

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Abstract

This longitudinal study aimed to investigate the relationship between early childhood caries (ECC) and alpha amylase, carbonic anhydrase VI (CA VI)as well as thepresence of visible biofilmand detect if these factors could predict the risk for ECC development.

One hundred childrenwere divided into two groups: caries group (n=45) and caries-free group (n=55). Visible biofilm on maxillary incisors was recorded, followed by caries diagnosis in preschoolers at baseline and after one yearaccording to Nyvad´s index (1999) adapted to primary teeth by Séllos and Sovieiro (2011). Saliva samples were collectedandactivity of CA VI andα amylasewere determined by zymography and ELISA methods,respectively. After 1 year, dental caries and presence of visible biofilmwere reassessed. Data were analyzed by Shapiro Wilk normality test, Mann Whitney test and Spearman correlation test.Moreover, the chi-square test followed by multiple logistic regression analysis were performed (α=0.05, 95% confidence interval). Activity of CA VI was significantly higher in the saliva of children with caries (p=0.05) andactivity of α amylase was significantly higher in saliva of caries-free children (p <0.0001).In addition, children with activity of αamylase in saliva lower than 122.8U/mL (OR=3.33) andshowingvisible biofilm on maxillary incisors(OR=3.6)showed higher risk of developing caries(p<0.05).

The presence of visible biofilm and salivary activity of α amylase may be considered risk predictors for early childhood caries.

Key words: dental caries, alpha amylase, carbonic anhydrase, biofilm and salivary proteins.

1.Introduction

Early childhood caries (ECC) is a multifactorial sucrose-biofilm dependent disease (Sheiham and James, 2015) and it is considered the most common disease in childhood (Misra et al., 2007. ECC is characterized by the presence of early caries lesions in the tooth cervical region and around the marginal gum of upper incisors, wherein the biofilm accumulates more easily in children younger than 6 years (AAPD, 2014).

With respect to the caries protective functions of saliva, the clearance promoted by salivary flow and pH stability, at acceptable levels stand out, mainly due to bicarbonate and phosphate buffer systems (Bardow et al., 2000; Bagherian and Asadikaram, 2012). In addition to these functions, saliva has proteins, which play an important role in maintaining the homeostasis of the oral environment and in the dynamics of the carious process. CA VI is an

isoenzyme, which maintains the physiological pH by catalyzing the hydration of carbon dioxide in the reaction$CO_2 + H_2O \leftrightarrow HCO_3 + H^+$. The CA VI is secreted into the external medium and its concentration in saliva has been associated with the prevalence of dental caries, particularly in subjects with poor oral hygiene (Kivela et al., 1999).In this regard, early studies haveshown that salivaryCA VI is able to penetrate into the biofilm,to facilitate theneutraliSation of acids from bacteria (Kimoto et al., 2006). However, an elevatedCA VI concentrations in the oral cavity does not necessarily indicate that the enzyme is active, so more important than its concentration, is the enzyme's activity, as some studies indicate that pH adjustment capacity and its power to catalyze the reaction of bicarbonate buffer are not directly related to its concentration in saliva (Parkkila et al., 1993; Kivela et al, 1997). In this regard, to our knowledge only the study performed by Frasseto et al.,(2012) investigated the activity of CA VI in saliva as well as its relationship with ECC. These authors demonstrated that after a 20% sucrose rinse, the activity of salivary CAVI was higher in pre-school children with dental caries.

The salivaryα amylase is one of the most abundant components in saliva, is responsible for the digestion of carbohydrates and 80%of its total volume andis produced by the parotid glands (Singh et al., 2015). It is found in abundance in the acquired pelicle being capable of modulating bacterial colonization, as well as providing additional glucose to the biofilm formation process, and thus, an ally in the caries development. Moreover, it has been shown that this protein is also capable of binding to the membrane of bacteria such as *S. mutans* and *Lactobacillus* and thus promote removal of these bacteria from the oral cavity, which could help to reduce these microorganisms and, consequently, to lower the risk of development of ECC (Scannapieco et al., 1993; Singh et al., 2015). Regarding the relationship between salivaryα amylase and dental caries, a previous studydemonstrated that the concentration of this protein waswas higher in saliva of 4 and 8 year old children with active caries as compared with the caries-free one (Singh et al., 2015). In the same way, Balekjian et al., 1795 showed that parotid salivasamples of caries rampant group had a significantly higherlevel of iso-amylase than saliva of thecaries-resistant children. In addition, higher concentrations of alpha amylase were detected in saliva of caries susceptible young adults(Vitorinoet al., 2006). However, determining just the concentration of this protein in saliva does not provide insightsabout the activity ofalpha amylase in this media. Thus, it would be relevant to investigate how activity of alpha amylase behaves in saliva of children with ECC. Furthermore, whether salivary biomarkers such as CA VI andalpha amylasecan be considered predictors for ECC in preschoolers is largely unknown.

This longitudinal study aimed to investigate the associationbetween early childhood caries and alpha amylase, carbonic anhydrase VIas well as thepresence of visible biofilmand examinedif these factors could predict the risk for ECC development.

2.Material and methods

2.1Ethical considerations

This study was approved by the Ethics Committee in Research of Piracicaba Dental School/University of Campinas, under protocol number 058/2014. Parents or guardians who agreed with the inclusion of the children in the study completed and signed a free and informed consent. In addition, all preschoolers selected to participate in the study agreed to do so through the consent term (term that clearly explains, according to the age range of participants,how the research will be carried outand asking if they agree to participate in the survey).

2.2 Sample

To perform this investigation, we based the sample power calculation (95%; α = 5%) on the study previously performed by our group which used similar methodology and found that activity (±standard deviation) of CA VI in saliva of caries and caries-free groups were 42.75 (±32.47) and 19.13 (±16.39), respectively (Frasseto et al.,2012). Sample size was based on these averages for two independent samples and bilateral test (parametric test) by the equation: $= \frac{(s_1{}^2+s_2{}^2)\left(z_1-\frac{\alpha}{2}+z_1-\beta\right)^2}{(\bar{x}_1-\bar{x}_2)^2}$. Thus, the calculated number of 31 preschool children was chosen to take part in this study. Since this was a longitudinal investigation, the calculated number (31) was increased by 20% to compensate for possible subject drop-out rate, totalizing a minimum of 37 children per group (caries and caries-free groups).

Initially, 316 children wereapproached, but only 100 preschoolersbetween 24-48 months of both genders, regularly attending5municipal preschools in the city of Piracicaba, SP,Brazil,located in the urban area were selected. They were divided in two groups: caries-free (n = 55) and group with caries (n = 45). These preschoolers were initially examined for visible biofilm on maxillary incisors, followed by caries diagnosis at baseline and after one year, 19 children were excluded due to preschool changes. Thus, 81 children were re-examined for verification of the visible biofilm, followed by caries diagnosis to allow caries incrementcalculation (Figure 1).

Piracicaba has a population around 365 thousand inhabitants with fluoridated public water supply (0.7 ppm F) since 1980. The children received, early in the research and in the second evaluation, after one year, a kit containing a toothbrush, fluoride toothpaste (1100 ppm F) and oral hygiene instruction.In addition, children who needed dental treatment were sent to be treatedinthe Pediatric Dentistry Department at the Piracicaba Dental School - University of Campinas.

The inclusion criteria were childrenwith age range between 24-48 months, enrolled in preschools in the city of Piracicaba, SP, Brazil. The exclusion criteria were children syndromes or chronic systemic diseases; parent's refusal to sign the informed consent and children who did not cooperate or agreed to participate.

2.3 Study design

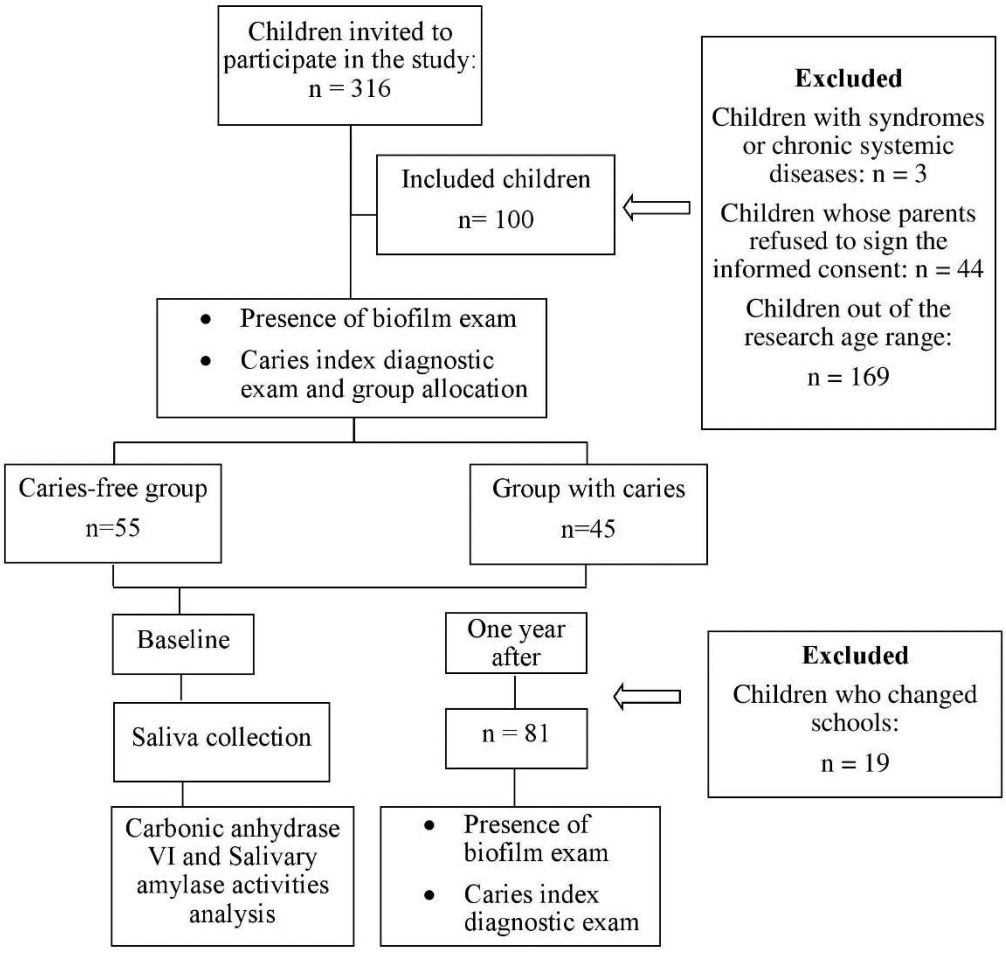


Figure 1. Participant's flow chart distribution and study design.

2.4 Calibration of the examiner

The examinations were performed by only one dentist (G.N.B) previously calibrated, who received the assistance of a helper. For calibration, sessions of theoretical discussions were conducted using clinical images of the diagnostic criteria according to the Nyvadcriteria (Nyvad, 1999) adapted to primary teeth by Séllos and Sovieiro (2011). Following, training exercises were carried out to reinforce what was learned in theoretical discussionswith children who attended the Pediatric Dentistry Clinic at Piracicaba Dental School; Thus, 12 children whose parents had authorized to be clinically examined were selected. This training was conducted by a researcher with experience in epidemiological surveys (TMP).

To determine the degree of intra-examiner agreement, children were selected from daycare centers and preschools included in the survey, when an initial examinationwas carried outand repeated, after one week. The Kappa statistic was then calculated and a Kappa coefficient of 0.81was obtained (Oakley, 2002).

2.5Presence of visible biofilm examination

The presence of visible biofilm was observed on buccal surfaces of the four upper incisors by visual examination (Alaluusua and Malmivirta, 1994) and recorded in the clinical record to be 0 for no visible biofilm and 1 for presence of visible biofilm.

2.6 Determination of the caries index

The diagnosis of dental caries was carried out by visual inspection method, with the aid of a clinical mirror and flashlight according to Nyvad´s index (1999) adapted to primary teeth by Séllos and Sovieiro (2011) (ANEXOS 2, 3 e 4). This caries diagnosis method has the advantage to allow the assessment of the extent and depth of carious lesions from pre-cavitated to deep cavities stage in enamel and dentin lesions, allowing for the evaluation of disease activity. A portable dental unit with a triple syringe, including a portable compressor (Odontocase Basic Line, Rio de Janeiro, Brazil)was used in order to allow the teeth surfaces drying, which facilitated the dental cariesdiagnosis. Prior to examination, the children preformed mechanical removal of biofilmby toothbrushing. The explorer was eventually used to clean any surface or detect dental sealants (Kopycka-Kedzierawski and Billings, 2004). The teeth were considered all erupted when they had gone through the

gingival mucosa.

2.7 Saliva Collection

Whole saliva was collected in the morning, between 8:00 and 10:00 am, at least one hour after food intake. The preschoolers were sitting while the saliva was collected from the mouth floor by suction technique with a disposable pipette. No salivary stimulation was made, but pipettes were often bitten, which probably caused some mechanical stimulation of the salivary flow (Hyyppa et al., 1989). Saliva samples were kept under dry ice cooling and were transported to the Pediatric Dentistry laboratory at Piracicaba Dental School, University of Campinas. Then, they were centrifuged at 16,000 g at 4° C for 10 minutes, separated into aliquots in microcentrifuge tubes, identified and stored at - 80° C until biochemical analyses.

2.8 Determination of the α-amylase activity

The activity of salivary α amylase was measured by means of the enzyme immunoassay kit (ELISA) (Salimetrics, State College, PA, USA). Initially, saliva samples were diluted using the αamylase diluent solution (1: 200 dilution) and brought to stirring. The samples and controls volumes were measured in the same 8μL plate at 37° C temperature. This method uses a chromogenic substrate, colo-2-p-nitrophenol associated with maltotriose. The substrate was preheated to 37° C and added 320 μL of each cell solution was added. The enzymatic action of α amylase produced by the 2-chloro-p-nitrophenol substrate,is directly proportional to the activity of α amylase in the sample. The optical density was performed by the solution absorbance reading at 405 nm for 3 minutes on a spectrophotometer (Bio Tek Instruments, Wiooski, VT, USA) and active of α amylase was expressed as U/mL of saliva.

2.9 Determination of the Carbonic Anhydrase VI activity

The determination of CA VI activity was performed by the zymography method (Kotwica et al. 2006), using a modified protocol. After being thawed, 10 μL of saliva was added to 10 μL of Tris buffer for zymography. The solution material was stirred before being placed on acrylamide gel at 30% and bisacrylamide at 0.8%. After that, 20 μL of this sample was placed in each channel of the gel, which remained for 1:50h at 140 V and at 4°C. Following electrophoresis, the gel was stained for 10 min with 0.1% bromothymol blue. 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 Image J software

(Collins, 2007) which calculated the luminescence in the area of the band and expressed CA VI activity in numerical values (pixels area).

2.10 Statistical analysis

All results were submitted to the Shapiro-Wilk test to assess normality of the data. The dependent variable was dental caries. The independent variables were the presence of visible biofilm, activity of salivary alpha amylase and activity of CA VI, as well as gender and race. The independent variables, carbonic anhydrase VI and salivaryα amylase were analysed using the Mann-Whitney test. In addition, the Spearman correlationteswas used to investigate possible correlation between dental caries and all independent variables.

Moreover,all independent variables were dichotomized, considering their median or presence / absence of biofilm. These variables were initially tested (chi-square test) to investigate possible associations between dependent and independent variables. Multiple logistic regression analysis and relative risk were expressed as odds ratios with their respective 95% confidence intervals to identify the factors that could predict ECC presence.

Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA), significance level was set at 5%.

3.Results

Table 1 shows the general characteristics of the participants in the study. It can be noted that most children are female and that the majority is not white.

Table 1. General characteristics of the participants.

Variables		n(%)
Gender	Male	43(43,00)
	Female	57(57,00)
Ethnicity	White	44(44,00)
	Not white	56(56,00)

n=100

Table 2 shows the mean numbers of surfaces/teethaffected by caries at baseline and at follow-up in thestudied population, as well as the 1-year caries increment.

Table 2. Mean numbers of decayed, missing, and filled surfaces (dmfs) and teeth (dmft) according to Nyvad's Criteria.

Caries index	Baseline (n=100)	Follow-up (n=81)	1 yearincrement (n=81)*
dmfs	7.88 ± 9.98	10.24 ± 14.04	2.24 ± 2.78
dmft	5.28 ± 4.64	5.72 ± 4.57	0.34 ± -0.69

*calculation based only in the 81 children present at the baseline and after one year.

Table 3 shows that the activity of the carbonic anhydrase VI was significantly higher in saliva of children with caries than in those caries-free ($p \leq 0.05$). Also, the activity of salivary alpha amylase was significantlyhigher in saliva of caries-free children ($p < 0.0001$).

Table 3. Means and standard deviations of activity of salivary CA VI and α amylase in caries and caries-free children.

	Caries-freegroup (n = 55)	Groupwith caries (n = 45)	pvalue*
CA VI activity	0.30 ± 0.29	0.65 ± 0.92	$p \leq 0.05$*
α amylase activity	111.6 ± 60.9	66.4 ± 58.7	$p < 0.0001$*

* Mann Whitney test (n=100)

Table 4 shows that there wasno correlation between dental caries and CA VI activity.In addition, a significant negative correlation between dental caries and salivary alpha amylasewas found.

Table 4. Correlation between dental caries and activity of salivaryCA VI / alpha amylase

	r	pvalue
CarbonicAnhydrase VI	0.08	0.39
Alpha amylase	-0.33	0.0008

* Spearman correlation test (n=100)

Table 5 shows thedichotomized independent variables submitted to chi-square test. The variables that had a p value lower than 0.2 in this test were subjected to a multiple logistic regression analysis as expressed in Table 6. From this table it is clear that the presence of visible biofilm on the anterior surfaces of teeth increased by 3.6 times the risk of childrento develop ECC. Moreover, those children who had an activity of salivary alpha amylase less than 122.8 U/mL showed 3:33 times increased risk of developing ECC.

Table5. Bivariate analysis of the relationship between caries, clinical and salivary parameters in preschoolers.

Variables	**CFx CA**	
	N(%)N(%)	
Biofilm	p= 0.004	
Absent	33(73)	12(27)
Present	15(42)	21(58)
Amylase	p = 0.018	
< 122.8	29(31)	28(49)
≥ 122.8	19(79)	5(21)
Anhydrase	p= 0.30	
< 1.5	47(61)	30(39)
≥ 1.5	1(25)	3(75)

*Significant results were evaluated using the chi-square test and Fisher's exact test (α=0.05). CF: caries free group; CA: group withcaries.

Table 6. Multivariate modeling of caries lesions regarding presence of dental biofilm and alpha amylase activity in preschoolers.

Variables	Caries lesions				
	NO (%)	YES (%)	OR_{crude} (95%CI)	$OR_{ajustado}$ (95%CI)	pvalue*
Biofilm					
Absent	33(73)	12(27)	1	1	
Presence	15(42)	21(58)	3.85(1.51-9.81)	3.58(1.37-9.37)	0.009
Activity alpha-amylase					
< 122.8	29(31)	28(49)	3.66(1.20-11.23)	3.33(1.04-10.52)	
≥122.8	19(79)	5(21)	1	1	0.042

OR = Odds ratio; CI = Confidence interval.
* Likelihood testwith 1 freedom degree = 12.96; p value of the Hosmer and Lemeshow test = 0.98.

4. Discussion

Dental caries is recognized as a biofilm-sugar-dependent disease (Sheiham et al., 2015), but there are other factors involved in its development, such as dietary habits, microbiota, the host, oral hygiene and socioeconomic factors. It is known that there is a complex interaction between these factors and when they come into imbalance, the caries process begins (Fejerskov, 2004). This caries process occurs in the same way with the ECC, adding only at a lower age factor, since it affects preschoolers younger than 6 years old (AAPD, 2014).

The visible biofilm buildup in the anterior upper incisors region plays an important and decisive role in the development of the ECC (Parisotto et al., 2010). In this regard, the results of this study showed that after a 1 year follow-up of, the presence of visible biofilm increased by 3.6 times the risk of preschoolers to develop ECC in (Table 6). This result corroborates the findings of Parisotto et al (2010), who demonstrated that the presence of visible biofilm was strongly associated with the ECC, especially with active early caries lesions (OR = 2.6). Likewise, Santos and Sovieiro (2007) indicated that the presence of biofilm would be a visible sign of caries. There are several reasons why a thick biofilm increases the risk of a child developing caries. Research previously carried out demonstrated that the thickness of visible biofilm hinders the influx of ions or ion exchange between the oral fluids and the biofilm fluid which is in intimate contact with the enamel surface during episodes of de- and remineralization. In fact, previous studies have shown that children with ECC have a highly cariogenic biofilm, since it is relatively subsaturated concerning the concentrations of calcium

and fluoride ions in addition to containing high-insoluble polysaccharide concentrations and high levels of cariogenic microorganisms (Nobre dos Santos et al., 2002; Parisotto et al., 2010).

Regarding the carbonic anhydrase VI, the results of present study showed that the activity of this isoenzyme was 53.8% higher in saliva of children with ECC, than in saliva of the caries-free children. These results confirm those obtained by Frasseto et al. (2012), who found higher activity of CA VI in the group with caries. The mechanism that explains the increased activity of CA VI in saliva of preschool children with early childhood caries, refers to the adherence of this isoenzyme to the enamel surface, acting as a regulator of the local pH and thus neutralizer the excess acidity in the oral environment (saliva and biofilm) by the catalysis of the reversible reaction of carbon dioxide $CO_2 + H_2O \leftrightarrow H^+ + HCO_3$ (Leinonen et al., 1999). On the other hand, the study by Szabó (1974) has demonstrated that in caries-free children , the expression of CA VI was higher than in those with active caries lesions. In the same way, the research by Kivelä., et al (1999) has shown a low, but significant correlation between CA VI concentration in the saliva of young adults and caries experience. However, these authors have just performed the qualitative detection of CA VI concentration while in the present study, we have quantitatively determined the activity of CA VI in saliva. Thus, the quantitative analysis of this isoenzyme activity becomes relevant if we consider that the protein expression in saliva does not necessarily means that it is active. Additionally, previous studies have indicated that the pH adjustment capacity and its power to catalyze the reaction of bicarbonate buffer are not directly related to its concentration in saliva (Parkkila et al., 1993; Kivela et al., 1997). Furthermore, our results found no correlation between dental caries and CA VI activity. One possible explanation for this finding could be the high variation of CA VI activity although we used an appropriated number of individuals as evidenced by the *sample power calculation* which showed a power of 0.7700*(85%; α=5%; p=0.0104)*. This high variation has also been reported by previous investigations (Kivela et al., 2003; Frasseto et al., 2012).

Another result found in the present investigation was that the activity of α amylase was 1.7 times higher in the saliva of caries-free preschool children than in saliva of children with caries(p= 0.00001). These data differ from the results obtained by Bezerra and Farias (2003) who evaluated children from 12 to 47 months with ECC and found no significant difference between groups. However, these authors determined the activity of this protein in saliva of only 20 preschool children in each group. Thus this small number of participants may not have been enough for these authors demonstrate a significant difference between the

groups. Our results also differ from those found by Singh et al. (2015), who showed a higher concentration of salivary alpha amylase in saliva of caries active children. However, it should be noted that in their investigation, the authors determined only the enzyme concentration, while in the present study we evaluated the activity of salivary alpha amylase. In addition, a significant negative correlation was found between dental caries and salivary alpha amylase($p = 0.0008$). Furthermore, the results of this study showed that preschoolers who had an activity of salivary alpha amylase lower than 122.8 U/mL showed 3.33 times higher risk of developing early childhood caries than children who had greater activity of the protein. These results are in agreement with Scannapieco et al. (1993) who has demonstrated that salivary alpha amylase is able to bind to cariogenic bacteria and thereby promote their removal from the oral cavity which could help to reduce the level of these microorganisms and hence decrease the risk of ECC development. In this way, we understand that the study of salivary alpha amylase is an example of the potential that salivary proteins have in modulating and exerting several functions in the oral cavity. Moreover, salivary alpha amylase can be connected to the adherence and colonization of cariogenic bacteria, providing additional substrate for the formation of the biofilm, contributing to dental caries occurrence. This fact points out the need for additional studies in order to investigate, in more detail, the mechanisms by which salivary alpha amylase behaves on the dynamic process of caries lesions formation.

In conclusion, the presence of visible biofilm and lower activity of salivary alpha amylase increased by more than 3 times the risk of preschoolers to develop early childhood caries. However, further studies are necessary to investigate the mechanisms by which this protein would act, as regards to early caries development.

5. Conclusion

The results allow us to conclude that the presence of visible biofilm and lower activity of α salivary amylase may be considered risk predictors for early childhood caries.

6.References

1. Alaluusua S, Malmivirta R. Early plaque accumulation--a sign for caries risk in young children. Community Dent Oral Epidemiol. 1994;22:273-6.

2. American Academy of Pediatric Dentistry (AAPD) 2014. Policy on early childhood caries (ECC): classifications, consequences and preventive strategies. Pediatr Dent, 30, 40-3.
3. Bagherian A eAsadikaram G. Comparison of some salivary characteristics between children with and without early childhood caries. Indian J Dent Res. 2012; 23(5):628-32.
4. Balekjian AY, Meyer TS, Montague ME, LongtonRW.Electrophoretic patterns of parotid fluid proteins from cariesresistantand caries-susceptible individuals. J Dent Res.1975;54:850 - 856.
5. Bardow A, Moe D, Nyvad B, Nauntofte B. The buffer capacity and buffer systems of human whole saliva measured without loss of CO_2. Arch Oral Biol. 2000 Jan; 45(1): 01-12.
6. Bezerra e Farias. Salivary antimodes, amylase and protein from children with early childhood caries. Clin Oral Invest, 2003;7: 154-157.
7. Collins TJ: Image J for microscopy. Biotechniques. 2007; (43): 25-30.
8. FejerkovO.Changing paradigms in concepts on dental caries: consequences for orl health care. Caries Res 2004;38:182-91.
9. Frasseto F, Parisotto TM, Peres RCR, Marques MR, Line SRP. Ralationship among salivary carbonic anhydrase VI activity and flow rate, biofilm, pH and caries in primary dentition. Caries Research 2012. 46: 194-200.
10. Hyyppa T, Karhuvaara L, Tenovuo J et al. Antimicrobial factors in whole saliva of humans infants a longitudinal study. Pediatric Dent. 1989; 11:30-36.
11. Kimoto M, Kishino M, Yura Y, Ogawa Y. A role of salivary carbonic anhydrase VI in dental plaque. Archives of Oral Biology. 2006. 51, 117-122.
12. Kivela J, Parkkilaa S, Waheed A, Parkkila AK, Sly WS, Rajaniemi H. Secretory carbonic anhydrase isoenzyme (CA VI) in human serum. Clinical Chemistry. 1997; 43(12), 2318 – 2322.
13. Kivela J, Parkkila S, Parkkila A , Leinonen J, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI. J Phys. 1999; 15 (520) (Pt 2): 315-20.
14. Kivelä J, Laine M, Parkkila S, Rajaniemi H. Salivary carbonic anhydrase VI and its relation to salivary flow rate and buffer capacity in pregnant and non-pregnant women. Arch Oral Biol. 2003;48(8):547-51.

15. Kopycka-Kedzierawski DT, Billings RJ. A longitudinal study of caries onset in initially caries-free children and baseline salivary mutans streptococci levels: a Kaplan-Meier survival analysis. Community Dent Oral Epidemiol. 2004;32(3):201-9.
16. Kotwica J, Ciuk MA, Joachimiak E, Rowinski S, Cymborowski B, BebasP.Carbonic anhydrase activity in the vas deferens of the cotton leafworm –Spodopteralittoralis (Lepidoptera: Noctuidae) controlled by circadian clock. J PhysiolPharmacol. 2006 Nov;57Suppl 8:107-23.
17. Leinonen J, Kivela J, Parkkila S, Parkkila AK. Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. Caries Research 1999. 33: 185-190.
18. Misra S, Tahmassebi J, Brosman M. Early childhood caries - a review. Dent Update. 2007;34:556-8.
19. 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;36(5):347-52.
20. Nyvad B, Machiulskiene V, Baelum V. Reliability of a new caries diagnostic system differentiating between active and inactive caries lesions. Caries Res. 1999;33(4):252-60.
21. Oakley C, Brunette DM. The use of diagnostic data in clinical dental practice. Dent Clin North Am. 2002;46(1):87-115. Review.
22. Odontocase Basic Line, Rio de Janeiro, Brasil.
23. 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;55(5):365-73.
24. Parkilla S, Parkilla AK, Vierjok T, Stahlberg T, Rajaniemi H. Competitive time-resolved imunofluorometric assay for quantifying carbonic anhydrase VI in saliva. ClinChem 1993, 39: 2154-2157.
25. Santos AP &Sovieiro VM .Comparison between a simplified and a conventional biofilm index in relation to caries activity and gingivitis in the primary dentition EurArchsPaediatr Dent 2007 Dec, 8(4): 201-205.
26. Scannapieco FA, Torres G, Levine MJ. Salivary α amylase: Role in Dental Plaque and Caries Formation. Critical Reviews in Oral Biology and Medicine, 1993. 43(3/4): 301 -307.

27. Séllos MC, Sovieiro VM.Reliability of the Nyvad criteria for caries assessment in primary teeth. Eur J Oral Sci 2011 ; 119 : 225-231.
28. Sheiham A, James WP. Diet and Dental Caries: The Pivotal Role of Free Sugars Reemphasized. J Dent Res. 2015 Oct; 94(10):1341-7.
29. 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 BiolCraniofac Res. 2015 May-Aug; 5(2): 59-64.
30. Szabó I. Carbonic anhydrase activity in the saliva of children and its relation to caries activity. Caries Research. 1974; (8): 187-191.
31. Vitorino R, de MoraisGuedes S, Ferreira R, Lobo MJC, Duarte J, Ferrer-Correia AJ,Tomer KB, Domingues PM, Amado FML. Two-dimensional electrophoresis study ofin vitro pellicle formation and dental caries susceptibility. Eur J Oral Sci 2006; 114:147–153.

3 CONCLUSÃO

A presença de biofilme visível e a menor atividade da α amilase salivarpodem ser consideradas preditores de risco para a cárie precoce.

REFERÊNCIAS

1. Ashley FP, Wilson RF. Dental plaque and caries. A 3-year longitudinal study in children. Br Dent J. 1977;142(3):85-91.

2. American Academy of Pediatric Dentistry (AAPD) 2014. Policy on early childhood caries (ECC): classifications, consequences and preventive strategies. Pediatr Dent, 30:40-3.

3. 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.

4. Banderas-Tarabay JA, Zacarias –D'Oleeire IG, Garduño-Estrada R, Aceves-Luna E, González-Begné M. Electrophoretic analysis of whole saliva and prevalence of dental caries- A study in Mexican dental students. Arch Med Res. 2002;33:449-505.

5. Bardow A, Moe D, Nyvad B, Nauntofte B. The buffer capacity and buffer systems of human whole saliva measured without loss of CO_2. Arch Oral Biol. 2000 Jan;45(1):1-12.

6. Becks H,WainwrightWW.Human saliva. J Dent Res. 1939;18:447-56.

7. Bhalla S, Tandon S, Satyamoorthy K. Salivary proteins and early childhood caries: A gel electrophoretic analysis. ComtempClin Dent. 2010;1:17-22.

8. Bosch JA, de Geus EJ, Veerman EC, Hoogstraten J, NieuwAmerongen AV.Innate secretory immunity in response to laboratory stressors that evoke distinct patterns of cardiac autonomic activity. Psychosom Med. 2003 Mar-Apr;65(2):245-58.

9. Fejerkov O.Changing paradigms in concepts on dental caries: consequences for orl health care. Caries Res. 2004;38:182-91.

10. Hara A, Lussi A, Zero D. BiologicalFactors. Dent Erosion. 2006;20(1):88-99.

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

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

13. Kivela J, Parkkila S, Parkkila A, Leinonen J, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI. J Phys. 1999;15(520pt 2):315-20.

14. Lendenmann U, Grogan J, Oppenheim FG. Saliva and dental pellicle – a review. Adv Dent Res. 2000;14(1):22-8.

15. Ministério da Saúde. Projeto SB Brasil 2010 – condições de saúde bucal da população da população brasileira 2002-2003. Disponível em: http://www.cfo.org.br/download/relatorio_sb_brasil_2010.pdf.

16. 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;36(5):347-52.

17. Oliveira LB, Sheiham A, Bönecker M. Exploring the association of dental caries with social factors and nutritional status in Brazilian preschool children. Eur J Oral Sci. 2008;116:37-43.

18. Oztürk LK, Furuncuoglu H, Atala MH, Ulukölü OS, Akyüz S, YaratA.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;(4): 956-9.

19. 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;55(5):365-73.

20. Parisotto TM, Santos MN, Rodrigues LK, Costa LS. Behavior and progression of early carious lesions in early childhood: a 1-year follow-up study. J Dent Child. 2012;79(3):130-5.

21. Parkilla S, Parkilla AK, Vierjok T, Stahlberg T, Rajaniemi H. Competitive time-resolved imunofluorometric assay for quantifying carbonic anhydrase VI in saliva. Clin Chem. 1993, 39:2154-7.

22. Peres MA, Sheiham A, Liul P, Denarco FF, Silva AER, Assunção MC, et al. Sugar comsuption and changes in dental caries from childhood to adolescence. JDent Res. 2016Apr;95(4):388-94. doi: 10.1177/0022034515625907. 1-7.

23. Rosenblatt A, Zarzar P. Breast-feeding and early childhood caries: an assessment among Brazilian infants. Int J Paediatr Dent. 2004;14(6):439-45.

24. Scannapieco FA, Torres G, Levine MJ. Salivary α amylase: Role in Dental Plaque and Caries Formation. Crit Rev Oral Biol Med. 1993;43(3/4):301-7.

25. Selwitz R, Ismail A, Pitts NB. Dental Caries. Lancet. 2007;369(9555):51-9.

26. Sheiham A, James WP. Diet and dental caries: the pivotal role of free sugars reemphasized. J Dent Res. 2015 Oct;94(10):1341-7.

27. 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 BiolCraniofac Res. 2015 May-Aug;5(2):59-64.

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

29. Takahashi N, Nyvad B. Caries ecology revisited: microbial dynamics and the caries process. Caries Res 2008;42:409-18.

ANEXOS

Anexo 1 – Certificado de 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 **"Associação entre a cárie precoce da infância, capacidade tampão e biomarcadores salivares, presença de biofilme dentário e variáveis ambientais - Estudo longitudinal em pré-escolares de 24 a 36 meses"**, protocolo nº 058/2014, dos pesquisadores Gabriela Novo Borghi 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 16/07/2014.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Association among early childhood caries, buffer capacity and biomarkers, presence of dental biofilm and environmental factors - Longitudinal study in 24 to 36- month-old preschool children "**, register number 058/2014, of Gabriela Novo Borghi 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 on Jul 16, 2014.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Profa. Dra. Lívia Maria Andaló Tenuta
Coordenadora
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.

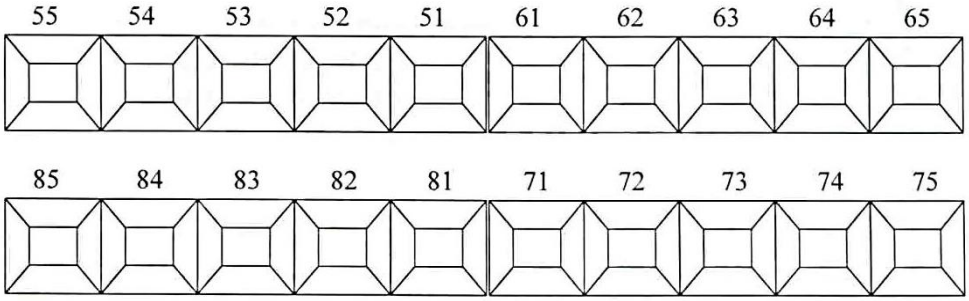
Anexo 2 – Ficha Clínica

FICHA CLÍNICA

Nome:_____Nº da Ficha:_____
Creche:_____Data exame:_____

Data nasc.:_____Idade (meses):_____Sexo: (F) (M): Cor: (B) (N) (P)

0- Sadio
1- Cárie ativa (superfície intacta)
2- Cárie ativa (superfície descontínua)
3- Cárie ativa (cavidade)
4- Cárie inativa (superfície intacta)
5- Cárie inativa (superfície descontínua)
6- Cárie inativa (cavidade)
7- Restaurada (superfície sadia)
8- Restaurada + cárie ativa
9- Restaurada + cárie inativa



Biofilme visível nas superfícies vestibulares dos incisivos superiores:_____

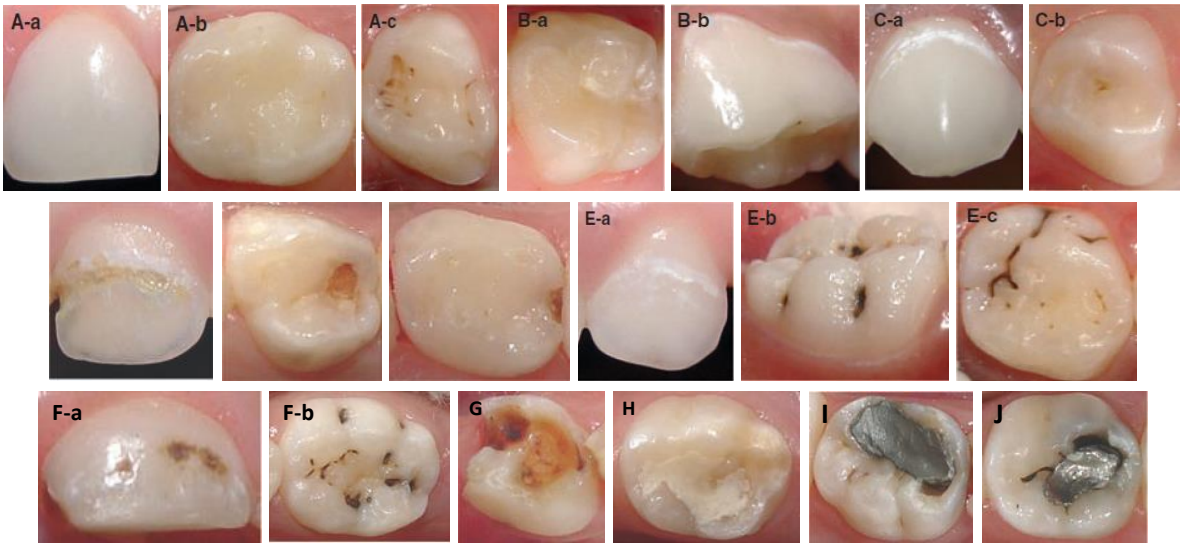
0: biofilme ausente ou não visível;
1: biofilme visível

Anexo 3 - Quadro1 - Escores de acordo com critério diagnóstico Nyvad

Score	*Category*	*Criteria*
0	Sound	Normal enamel translucency and texture (slight staining allowed in otherwise sound fissure).
1	Active caries (intact surface)	Surface of enamel is whitish/yellowish **opaque** with **loss of luster**; feels **rough** when the tip of the probe is moved gently across the surface; generally covered with plaque. No clinically detectable loss of substance. Smooth surface: Caries lesion typically located close to gingival margin. Fissure/pit: Intact fissure morphology; lesion extending along the walls of the fissure.
2	Active caries (surface discontinuity)	Same criteria as score 1. Localized surface defect (microcavity) in enamel only. No undermined enamel or softened floor detectable with the explorer.
3	Active caries (cavity)	Enamel/dentin cavity easily visible with the naked eye; surface of cavity feels **soft** or **leathery** on gentle probing. There may or may not be pulpal involvement.
4	Inactive caries (intact surface)	Surface of enamel is whitish, brownish or black. Enamel may be **shiny** and feels **hard** and **smooth** when the tip of the probe is moved gently across the surface. No clinically detectable loss of substance. Smooth surface: Caries lesion typically located at some distance from gingival margin. Fissure/pit: Intact fissure morphology; lesion extending along the walls of the fissure.
5	Inactive caries (surface discontinuity)	Same criteria as score 4. Localized surface defect (microcavity) in enamel only. No undermined enamel or softened floor detectable with the explorer.
6	Inactive caries (cavity)	Enamel/dentin cavity easily visible with the naked eye; surface of cavity may be **shiny** and feels **hard** on probing with gentle pressure. No pulpal involvement.
7	Filling (sound surface)	
8	Filling + active caries	Caries lesion may be cavitated or non-cavitated.
9	Filling + inactive caries	Caries lesion may be cavitated or non-cavitated.

Fonte: Nyvad et al. (1999).

Anexo 4 - Fotos do critério diagnóstico de cárie



Fonte:SélloseSovieiro (2011), adaptado de Nyvad et al. (1999).