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PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

SARAH FLORINDO DE FIGUEIREDO GUEDES

PROTEÍNAS SALIVARES COMO BIOMARCADORES PARA CÁRIE DENTÁRIA

FORTALEZA

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, como requisito parcial à obtenção do título de mestre em Odontologia. Área de concentração: Clínica Odontológica.

Orientadora: Profa. Dra. Lidiany Karla Azevedo Rodrigues.

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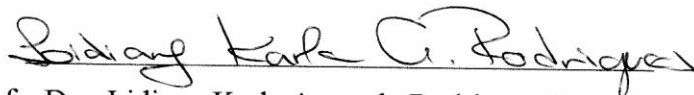
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BANCA EXAMINADORA

Profa. Dra. Lidiany Karla Azevedo Rodrigues (Orientadora)
Universidade Federal do Ceará (UFC)

Profa. Dra. Simone Duarte
New York University (NYU)

Prof. Dr. Arlindo de Alencar Araripe Noronha Moura
Universidade Federal do Ceará (UFC)

A Deus.

Aos meus pais, Izabel e Luiz.
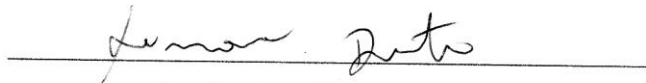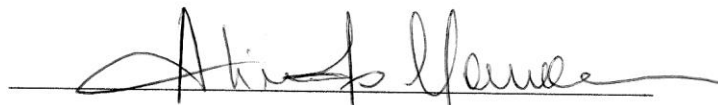
Aos meus irmãos, Isabela e Luizinho.

Ao meu marido, Anderson.

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A DEUS, por sua eterna presença em minha vida.

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"A mente que se abre a uma nova idéia
jamais voltará ao seu tamanho original."

Albert Einstein

RESUMO

O número de estudos que correlacionam proteínas salivares e cárie dentária têm aumentado consideravelmente, no entanto, não há elementos suficientes que permitam qualificá-las como biomarcadores específicos para a doença. O objetivo do estudo foi realizar uma análise comparativa das proteínas da saliva de pacientes com cárie precoce da infância (CPI) em diferentes níveis de severidade, visando à identificação de potenciais biomarcadores salivares específicos para diagnóstico desta patologia. Metodologia: 1) busca eletrônica foi realizada na base de dados *PubMed Medline* aplicando os termos: "cárie" e "proteínas e peptídeos salivares". Cento e vinte estudos foram identificados e 10 selecionados para a revisão; 2) Cento e vinte e seis crianças (24-71 meses de idade) foram selecionadas. As crianças foram examinadas de acordo com o índice visual ICDAS II e divididas em 3 grupos (n = 42): Grupo livre de cárie (CF) (ICDAS 0), Grupo cárie de esmalte (EC) (ICDAS 1, 2 e 3) e Grupo cárie dentina (DC) (ICDAS 5 e 6). As amostras foram digeridas e analisadas por espectrometria de massa de varrimento multiplexado alternativo (MSE). As análises foram realizadas com *TransOmics Software*, Anova e *Volcano plot v-plot* (p<0,05). Resultados: 1) Noventa por cento de artigos mostraram uma relação entre proteínas salivares e presença ou ausência de cárie; 2) Trezentos e uma proteínas foram identificadas e quantificadas. Destas, 122 proteínas foram expressas com diferença estatística significante em pelo menos um grupo: 84 foram identificados nos 3 grupos; uma proteína foi expressa apenas no CF e outro no EC; 11 foram expressas apenas no CF e EC, 15 no CF e DC, 10 no EC e DC. Das 122 proteínas, 54 proteínas foram, pelo menos, 5 vezes mais expressas na comparação entre os grupos e foram consideradas como candidatas a biomarcadores. Destas 54, 18 proteínas com potencial mecanismo de ação relacionado a cárie dentária foram selecionadas e analisadas. Conclusão: A relação entre proteínas salivares e presença ou ausência de cárie é notória e já está estabelecida. Neste estudo,18 proteínas apresentaram uma relação com a CPI, e a SAA1, AIMP1, IL36A, IL36G e CAH1 possuem um maior potencial para serem consideradas biomarcadores da cárie dentária. No entanto, estudos longitudinais ainda precisam ser realizados, a fim de estabelecer o papel de cada uma das proteínas estudadas e consolidar o seu papel como um biomarcador.

Palavras-chave: Cárie. Saliva. Proteínas. Peptídeos.

ABSTRACT

The number of studies that correlate salivary proteins and dental caries have increased considerably, however, there is not sufficient evidence to classify them as specific biomarkers for the disease. The aim of the study was to conduct a comparative analysis of saliva proteins of patients with early childhood caries (CPI) at different levels of severity, in order to identify specific potential salivary biomarkers for diagnosis of this pathology. Methodology: 1) electronic search was performed in PubMed Medline database by applying the terms "carie" and "protein and salivary peptides." One hundred and twenty studies were identified and 10 selected for review; 2) One hundred twenty-six children (24-71 months old) were selected. The children were examined in accordance with the visual index ICDAS II and divided into 3 groups (n = 42): Group caries-free (CF) (ICDAS 0), Group enamel caries (EC) (ICDAS 1, 2 and 3) and Group caries dentin (DC) (ICDAS 5 and 6).Then, the saliva was collected, processed and stored. The samples were digested and analyzed for mass spectrometry alternative multiplexed scanning (MSE). Analyses were performed with TransOmics Software, ANOVA and Volcano plot v-plot ($p<0.05$). Results: 1) ninety percent out of articles showed a relationship between salivary proteins and the presence or absence of caries; 2) three hundred one proteins were identified and quantified, among them, 122 proteins were statistically significant different at least in one group: 84 were identified in all 3 groups; one protein was expressed only in CF and other in EC; 11 were expressed only in CF and EC, 15 in CF and DC, 10 in EC and DC. Of the 122 proteins, 54 proteins were at least 5 times more expressed in the comparison between the groups and were considered as candidates for biomarkers. From these 54, 18 proteins with potential mechanism of action related to dental caries were selected and analyzed. Conclusion: The relationship between salivary proteins and the presence or absence of caries is notorious and is already established. In this study, 18 proteins showed a relationship with the CPI, and SAA1, AIMP1, IL36A, IL36G and CAH1 have a greater potential to be considered biomarkers of dental caries. However, longitudinal studies have yet to be performed in order to establish the role of each protein studied and consolidate its role as a biomarker.

Keywords: Dental caries. Saliva. Proteins and peptides.

SUMÁRIO

RESUMO

ABSTRACT

1 INTRODUÇÃO GERAL

A cárie dentária é caracterizada pela desmineralização dos tecidos duros dentários e alguns fatores são determinantes para o seu aparecimento: o acúmulo de biofilme na superfície do dente, o frequente consumo de carboidratos fermentáveis e a susceptibilidade do indivíduo, após um período de tempo (LOESCHE, 1986; MARSH, 1991). Em crianças, apresenta-se como a doença crônica mais prevalente da infância (de SILVA-SANIGORSKI *et al.*, 2010). Apesar de haver disponibilidade de medidas preventivas como o uso de fluoretos, de selantes e conhecimento a respeito da etiologia bacteriana da doença, há uma grande necessidade de descobertas que permitam gerar imunidade, prevenção e/ou propiciar tratamento da doença cárie na área científica.

A cárie precoce da infância (CPI), conhecida mundialmente pelo termo "*Early Childhood Caries*'' (ECC), é definida como a presença de uma ou mais superfícies cariadas (lesões cavitadas ou não-cavitadas), perdidas (devido à cárie) ou restauradas em qualquer dente decíduo em uma criança com menos de seis anos de idade. A presença de padrões atípicos, progressivos, agudos ou rampantes desta doença é designada cárie precoce da infância severa. Em crianças de pouca idade, a grande quantidade de bactérias cariogênicas como estreptococos mutans e lactobacilos, a presença de biofilme dental, práticas inapropriadas de alimentação e condição sócio-econômica têm sido identificadas como fatores predisponentes ao desenvolvimento de cárie (BERKOWITZ, 2003; AAPD, 2009).

Com o advento de métodos e sistemas de diagnósticos para a cárie dentária ao longo dos últimos anos, houve a inserção do ICDAS II (*International Caries Detection & Assessment System*), que é um sistema de detecção de cárie baseado na inspeção visual e inclui avaliação de lesões cariosas cavitadas e não-cavitadas. Esse sistema foi desenvolvido para uso na prática clínica, bem como em pesquisas clínicas e epidemiológicas (PITTS, 2004). O método provou ser bem aceito e praticável, baseado na sua padronização de critérios e possibilidade de comparação com outros índices (SHOAIB *et al.*, 2009).

A saliva é um líquido incolor filamentoso, presente na cavidade oral, e devido a características individuais e a íntima relação com o esmalte dentário é muito importante no controle da formação do biofilme e na patogênese da cárie dentária, ou seja, é um elemento fundamental para o desenvolvimento ou prevenção desta patologia (AREIAS *et al*., 2011). Ela

apresenta várias funções, sendo a mais relevante à ação física de limpeza que realiza na cavidade oral, além de lubrificar os tecidos bucais, permitindo a fonética, a alimentação e a deglutição. Também atua como protetora do esmalte e da mucosa oral.

Alguns aspectos da saliva são comumente relacionados com a doença cárie, dentre eles, o pH, o fluxo e a capacidade tampão (KRASSE, 1988). No entanto, ela possui diversas proteínas antimicrobianas que estão diretamente relacionadas com a prevenção dessa doença: sistema peroxidase e mieloperoxidase (inibe o metabolismo da célula e diminui a capacidade das bactérias em produzir ácidos), lisozima (quebra da parede celular de algumas bactérias), lactoferrina (extrai ferro que algumas bactérias necessitam para crescer), aglutininas: glicoproteínas, mucinas, IgA secretora, β2-microglobulina e fibronectina (promovem aglutinação e remoção física de bactérias), histidina (atividade antibacteriana e antifúngica) e imunoglobulinas: IgG, IgA secretora e IgM (previnem adesão microbiana; ativam o sistema complemento) (TENOVUO & LAGERLOF, 1994).

Como o sangue, a saliva é um fluido complexo que contém uma variedade de enzimas, hormônios, anticorpos, constituintes antimicrobianos e citocinas (ZELLES *et al*., 1995; REHAK *et al*., 2000). Assim, a maioria dos compostos encontrados no sangue está também presente na saliva, podendo esta refletir o estado fisiológico do corpo, inclusive variações emocionais, endócrinas, nutricionais e metabólicas. Consequentemente, esse líquido é uma fonte para o monitoramento da saúde sistêmica e também bucal (SPIELMANN & WONG, 2011).

A análise da saliva pode potencialmente auxiliar no diagnóstico precoce, monitoramento da progressão e/ou a resposta ao tratamento de muitas doenças, como a doença periodontal, e avaliar o risco de cárie dentária (KORNMAN *et al*., 1997; BAUGHAN *et al*., 2000; SOCRANSKY *et al*., 2000). Além disso, a natureza não invasiva da coleta de amostra salivar reduz o desconforto e a ansiedade do paciente. Essas vantagens têm despertado a atenção pesquisadores que procuram simplificar o procedimento de diagnóstico em fluídos corporais (HU *et al.*, 2006; GIUSTI, *et al.*, 2007; HU *et al.*, 2007). Um bom método diagnóstico deve ter alta sensibilidade, especificidade e funcionalidade, e atender aos requisitos de alto rendimento, portabilidade e baixo custo para uma posterior aplicação clínica.

Estudos envolvendo proteínas salivares (GHAFOURI, *et al.*, 2003; PERRY, *et al.*, 2007; ZHANG *et al.*, 2009) têm mostrado que, além das principais famílias de proteínas salivares, a saliva contém centenas de pequenas proteínas ou peptídeos e ácidos nucléicos que estão presentes em baixas concentrações, mas podem desempenhar um papel importante no diagnóstico de doenças. Com os avanços biotecnológicos das últimas décadas, houve o desenvolvimento de tecnologias capazes de analisar esses peptídeos disponíveis em baixas concentrações, o que trouxe vantagens inestimáveis em todos os campos de conhecimento da área da saúde. A maioria desses esforços têm se concentrado em avanço tecnológico e desenvolvimento de estudos, a fim de identificar biomarcadores que sinalizem determinadas doenças e atendam aos critérios de diagnósticos salivares.

A importância de se analisar as proteínas diretamente é que elas desempenham as funções fisiológicas e patológicas nas células, além disso, estão sujeitas às modificações pós-traducionais, as quais vão influenciar o enovelamento correto para formação da estrutura tridimensional, a localização, a interação com outras proteínas, formando a estrutura quaternária, muitas vezes fundamental para se tornarem funcionais.

A identificação de proteínas em um meio biológico complexo, como a saliva, pode ser realizada por diferentes métodos, dentre eles a espectrometria de massa (MS) merece destaque devido à sua alta sensibilidade (HU *et al*., 2006; RAMACHANDRAN *et al*., 2006). O desenvolvimento da tecnologia de MS levou a uma nova era na fabricação de biomarcadores, o que potencialmente terá um enorme impacto sobre diagnóstico e terapia de doenças. A MS permite examinar detalhadamente um proteoma salivar em um curto período de tempo. A presença ou a ausência de biomarcadores múltiplos num proteoma salivar, alterado por doenças ou intervenções, pode ser detectada com a MS (LEE & WONG, 2009; CAPOROSSI *et al.*, 2010; AL-TARAWNEH *et al*., 2011;). O resultado da análise de MS é um mapa peptídico ou "*fingerprinting*", expresso em relação as suas razões de m/z (massa/carga), que quando comparado com banco de dados, fornece dados sobre a identificação e modificações pós-traducionais da proteína em questão.

O número de estudos tentando relacionar proteínas salivares e cárie tem aumentado consideravelmente, mas ainda não há elementos suficientes para estabelecer proteínas salivares como biomarcadores para a cárie dentária (MARTINS *et al*., 2013). Assim, esta dissertação objetivou revisar, a partir da literatura, a relação entre cárie dentária e proteínas salivares, comparando indivíduos com e sem experiência de cárie e realizar uma análise

comparativa das proteínas da saliva de pacientes com cárie precoce da infância (CPI) em diferentes níveis de severidade e pacientes sem cárie, visando à identificação de potenciais biomarcadores salivares específicos para diagnóstico da cárie.

2 PROPOSIÇÃO

Essa dissertação será apresentada em capítulos, tendo como objetivos:

Capítulo 1: Revisar, a partir da literatura, a relação entre cárie dentária e proteínas salivares, comparando indivíduos com e sem experiência de cárie.

Capítulo 2: Realizar uma análise comparativa da expressão do proteoma da saliva de pacientes com cárie precoce da infância (CPI) em diferentes níveis de severidade e pacientes sem cárie, visando à identificação de potenciais biomarcadores salivares específicos para diagnóstico da cárie.

3 CAPÍTULOS

REGIMENTO INTERNO

Esta dissertação está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato. Por se tratar de pesquisa envolvendo seres humanos, o projeto de pesquisa do trabalho referente ao capítulo 2 foi submetido à apreciação do Comitê de Ética em Pesquisa da Universidade Federal do Ceará, tendo sido aprovado (ANEXO A). Assim sendo, esta dissertação é composta de dois capítulos contendo artigos a serem submetidos para publicação em revistas científicas, conforme descrito abaixo:

Capítulo 1

"Salivary proteins as a tool for diagnostic of dental caries – A systematic review" Sarah F. F. Guedes, Beatriz G. Neves, Daniela S. Bezerra, Myrna M. A. Frota, Lidiany K. A. Rodrigues. Este artigo será submetido à publicação no periódico "Brazilian Oral Research".

Capítulo 2

"Differential expression proteomics of saliva from children with caries at different stages of severity" Sarah F. F. Guedes, Beatriz G. Neves, Daniela B. Silva, Gustavo H. M. F. Souza, Maria I. F. Guedes, Lidiany K. A. Rodrigues. Este artigo será submetido à publicação no periódico "Journal of Proteomics".

3.1 CAPÍTULO 1

Salivary proteins as a tool for diagnostic of dental caries – A systematic review

Sarah F. F. Guedes[1], Beatriz G. Neves[1], Daniela S. Bezerra[1], Myrna M. A. Frota[1], Lidiany K. A. Rodrigues[1].

[1]Post-graduation Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Brazil, w/n Cap Francisco Pedro Street - Rodolfo Teófilo, Zip Code: 60430-170, Phone-+558533668410, Fax- +558533668232.

Correspondence: Lidiany K. A. Rodrigues (Email: lidianykarla@yahoo.com), Professor, Coordinator, Postgraduate Program in Dentistry, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, Ceará, Brazil. Cap. Francisco Pedro St - Zip Code: 60430-170, Phone- #+558533668410/Fax- #+558533668232

ABSTRACT

The number of studies trying to correlate salivary proteins and dental caries has considerably increased, but until now there are no sufficient elements to establish salivary proteins as biomarkers for this disease. Thus, we can evaluate the possibility whether salivary proteins can be considered biomarkers for dental caries or not and if there is already identified salivary proteins that have the potential to become, in the near future, biomarkers of dental caries. The present study aimed to develop a systematic review based on the relationship between dental caries and salivary proteins by comparing subjects with and without caries experience. Electronic search was performed in the PubMed Medline database applying the following combinations of MeSH (Medical Subject Heading) terms: "dental caries" and "salivary proteins and peptides". From 120 identified studies, 10 were included in this systematic review. Ninety percent out of articles showed a relationship between salivary proteins and the presence or absence of caries. Although a notorious relationship between salivary proteins and the presence or absence of caries is established, there is insufficient evidence to make some salivary protein eligible as biomarker for caries.

Keywords: Dental caries, salivary proteins and peptides.

INTRODUCTION

Dental caries is the demineralization of dental hard tissues and factors such as the biofilm accumulation of on the tooth surface and the frequent consumption of fermentable carbohydrates are involved in its origin[1,2] Although, there is availability of preventive measures such as the use of fluorides, sealants and knowledge about the bacterial etiology of the disease, there is a great need for new discoveries that allow generating immunity, prevention and/or provide treatment of caries in science.

Saliva is corporal fluid very associated to tooth integrity due to some aspects, such as pH, flow and buffering capacity.[3] Besides, as blood, saliva is a complex liquid that contains a variety of enzymes, hormones, antibodies, cytokines and antimicrobial constituents.[4,5] Therefore, most of the compounds found in the blood are also present in saliva, which may reflect the physiological state of the body, including emotional, endocrine, nutritional and metabolic changes, being an important source for monitoring systemic and oral health.[6] Several antimicrobial proteins are directly related to the prevention of this disease for example, peroxidase and myeloperoxidase system, which inhibit cell metabolism and decrease the ability of bacteria to produce acids; lysozyme (that breaks cell wall of some bacteria; lactoferrin related to releasing ofiron, a necessary ion for growing some kinds of bacteria need; agglutinins, glycoproteins, mucins, secretory IgA, β2-microglobulin and fibronectin, which promote agglutination and physical removal of bacteria; antibacterial and antifungal histidine, and immunoglobulins as IgG, secretory IgA and IgM associated with prevention of microbial adhesion and activation of the complement system.[7]

The analysis of saliva can potentially aid in obtaining early diagnosis, monitoring the progression and/or the response to treatment of many diseases such as periodontal disease, as well as to evaluate the risk of dental caries.[8,9,10] Furthermore, the noninvasive nature of the salivary sample collection reduces patient anxiety and discomfort. These advantages have attracted the attention of researchers seeking to simplify the diagnostic procedure in bodily fluids. [11,12,13]

Especially in dental caries, saliva could be used as an important pathway of caries diagnostics in populations with high prevalence of the disease or caries polarization, a condition in which a small proportion of people manifests the majority of disease.[14] Therefore, salivary biomarkers could facilitate the early detection and apply prevention forms to reduce caries and costs with therapeutic methods.

The number of studies trying to correlate salivary proteins and dental caries has considerably increased, however there is no enough scientific evidence to establish salivary proteins as biomarkers for caries.[15] Therefore, the present study aimed to develop a systematic review based on the relation between dental caries and salivary proteins by comparing persons without and with caries. Thus, we can evaluate the possibility of whether salivary proteins can be considered biomarkers for dental caries or not and if there is already identified salivary proteins that have the potential to become, in the near future, biomarkers of dental caries.

METHODS

This systematic review included controlled observational studies that evaluated the relation between salivary proteins and dental caries.

Search strategy

Electronic research was performed in the PubMed Medline database applying the following combinations of MeSH (Medical Subject Heading) terms: "dental caries" and "salivary proteins and peptides".

The database research was performed by one reviewer who included studies published between 1976 and March, 2014. All studies were limited to "humans". The reviewer checked, independently, the title and abstract of each identified study. Those studies which could not be included in selection criteria were discarded from the research. Then, the full text of each remaining study was analyzed. The relevant studies were selected and those that fulfilled the inclusion criteria were selected for this systematic review.

Study selection criteria

Controlled observational studies which sample was composed by a group of caries-resistant subjects (DMF or dmf=0) and another group of caries-susceptible subjects (DMF or dmf>0) were initially selected. Only studies with healthy individuals who were not taking any medicine that could affect the salivary composition were included. The exclusion criteria comprehended: subjects medically compromissed; no comparisons between subjects without and with; reviews and case reports. There was no age restriction.

RESULTS

The electronic search resulted in 120 non-duplicate records. After checking title, abstract and full texts of the identified studies, 110 articles were excluded (Figure 1) and 10 articles were selected for this systematic review (Table 1). The studies selected for this review were those that best correlated tooth decay and proteins present in saliva.
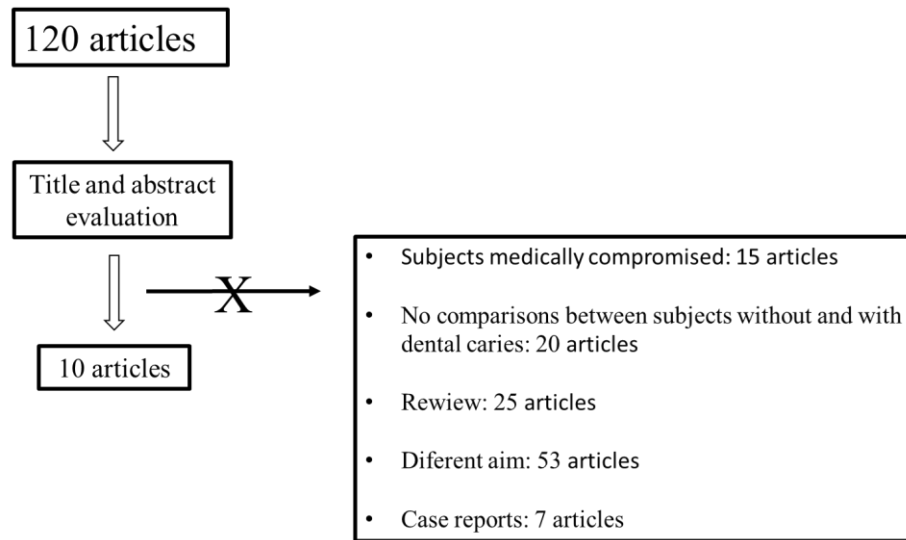

Figure 1. Stages of the studies selection process.

Table 1. Papers selected for the systematic review

Authors and year	Han et al., 2013	Ribeiro et al., 2013	Zehetbauer et al., 2009	Jonasson et al., 2007
Objective	To determine the association of salivary glutathione with dental caries and cariogenic bacteria	To evaluate the salivary peptide profile of children with and without ECC and its association with caries experience	To perform a qualitative comparison of major salivary protein profiles between children with ECC and caries-free controls	To examine the association of the gp-340 I to III polymorphisms with caries experience and adhesion of *S. mutans*
Sample size (n)	257	106	50	38
Age	6-14 years	10–71 months	Not reported	12 years
Caries index	DMF-T	DMF-T DMF-S	Not reported	DMF-S
Groups	Not reported	Children who had (CE, DMFT > 0, n = 48) and had not experienced dental caries (CF, DMFT =0, n = 58)	Children who had been diagnosed with ECC (ECC group, n=30) and children who were free of carious lesions (CF group, n=20)	Children with high (mean baseline DMFS = 5.0, n = 19) or low (baseline DMFS = 0, n = 19) caries experiences
Type of saliva	SWS	UWS SWS	UWS	SWS

Saliva collection and preparation	All the subjects were instructed to refrain from eating or drinking for a minimum of 2 h before the saliva samples were collected Individuals rinsed their mouths with water, and SWS samples were collected using 50 ml, ice-chilled, Falcon screw cap tubes. SWS samples were obtained after subjects had chewed for 5 min on wax blocks provided in the commercial kits	UWS was collected during 60 s, and supernatants were lyophilized and stored at 80°C for later peptide analysis. SWS was collected by asking patients to chew on a standard piece of Parafilm® during 60 s, stored in sterile microcentrifuge tubes, and used for microbiological analysis	Whole saliva was collected by expectoration into a sterile polypropylene vial at the time-point of caries examination. For children who were not able to expectorate into a vial, saliva was collected by manual suction with the use of sterile disposable polypropylene Pasteur pipettes. No intake of food had taken place for at least 2 h before the collection of the saliva sample. Samples were stored on ice and further processed not later than 30 min after collection	Parotid saliva, collected on ice using Lashley cups and 3 % citric acid stimulation, was stored frozen (-80°C) in aliquots for subsequent Db and gp-340 phenotyping and the ability to mediate adhesion of S. mutans to saliva-coated hydroxyapatite
Salivary parameters	Glutathione	Salivary peptide profile	Salivary peptide profile	gp-340 I to III
Data analysis	X^2 T-test ANOVA and linear regression	Binary logistic regression Student's t-test	The comparison of patterns was performed using the image master 1D data-base software, v. 4.0. Similarity coefficients were calculated by the software according to the method of Pearson	Student's unpaired t test (2 groups) or ANOVA followed by Tukey's test (>2 groups)
Results	Salivary GSH level was significantly associated with salivary LB level, DMFT and DT, and the relationships were positively linear	Three peptides were significantly related with caries experience. HNP-3 (α-defensin 3) ($P = 0.019$) and HBD-3 (β-defensin 3) ($P = 0.034$) reduced the chances of experiencing ECC. Proline-rich peptides IB-4 significantly increased caries experience ($P = 0.035$). Age ($P = 0.020$) and MS counts ($P = 0.036$) increased caries experience; however, gender was not associated with dental caries ($P = 0.877$)	The results attest a uniform expression of the major protein components in children's saliva, regardless of the clinical manifestation of ECC, and thus pave the way for further detailed investigations of more subtle differences in the salivary proteome	The results reveal a positive association of gp-340 I with both caries experience and saliva adhesion of S. mutans, and that purified gp-340 I protein mediates increased AgI/II-mediated adhesion of S. mutans
Other considerations	More comprehensive and detailed prospective studies are needed to clarify the causality between salivary glutathione levels and dental caries	Future in-depth studies to evaluate the function of specific salivary peptides in ECC may help elucidate host-related aspects associated with this disease	It is now indicated to test specifically for differential expression of relevant salivary proteins in relation to ECC by employing advanced proteomics techniques	Further studies on host polymorphisms and their evaluation in disease profiling and risk assessment using larger clinical samples may reveal the usefulness of the gp-340 and other host polymorphisms in risk assessment of caries in a clinical setting
Authors and year	**Vitorino et al., 2006**	**Tao et al., 2005**	**Vitorino et al., 2005**	**Farias et al., 2003**
Objective	Evaluate the influence of salivary protein composition on in vitro dental pellicle formation and its possible correlation with dental caries	Determine a possible correlation between caries prevalence in children and salivary concentrations of the antimicrobial peptides human beta-defensin-3 (hBD-3), the cathelicidin LL37, and the alpha-defensins HNP1-3 (a mixture of HNP1, 2, 3)	Correlate peptide saliva composition with dental caries susceptibility through the analysis of saliva and hydroxyapatite-adsorbed salivary peptides samples	Analyze the organic composition of saliva from children without dental caries and children with ECC
Sample size (n)	32	149	20	40
Age	18-29 years	11-15 years	Not reported	12 to 47 months

Caries index	DMF-T	Not reported	DMF-T	DMF-S
Groups	Caries-free (CF, n=16, DMFT=0) and caries susceptible (CS, n=16, DMFT ranging from 3 to 12) with treated caries	Examiners were instructed to rank subjects separately for active caries and for filled surfaces as follows: 0, no decayed or filled surfaces (n=53); 1, mild (one or two affected surfaces – n=37); 2, moderate (three to six affected surfaces – n=39); 3, severe (more than six affected surfaces – n=20)	Caries-free (DMFT = 0) and caries- susceptible (DMFT > 0)	Group I, caries-free children (n=20); Group II, children with ECC (n=20)
Type of saliva	UWS	UWS	UWS	UWS
Saliva collection and preparation	Each volunteer washed his mouth twice with water before saliva collection. Collection of UWS was performed by direct draining into a saliva collection tube. Immediately after collection, UWS was centrifuged at 13,200 g for 20 min (4°C) to remove insoluble material and divided in two fractions for 2D gel electrophoresis analysis and for in vitro assays	UWS was collected (3 to 5 ml), the detergent Nonidet P-40 (Sigma, St. Louis, MO) was added to a final concentration of 0.1%, and the sample was frozen for later analysis	After collection UWS was divided into two fractions. To fraction 1, 5 µL of a protease inhibitor cocktail were added to avoid proteolytic activity with 300 µL of guanidine and the mixture was incubated for 2 h at 37°C under continuous stirring; the second fraction was centrifuged at 12,000 g for 20 min at 4°C to remove particulate matter	Two samples of whole saliva, 1.5 ml each, were collected from the floor of the mouth of each child using a soft, disposable plastic pipette for aspiration. All samples were collected between 8:00 and 11:00 a.m., and the time spent on the procedure did not exceed 25 min. The samples were codified, stored in Eppendorf plastic tubes in an icebox at 0 °C, and carried immediately to the laboratory, where the immunological and biochemical assays were performed
Salivary parameters	Salivary peptide profile in vitro	HBD-3 LL37 HNP1-3	Salivary peptide profile	IgA, IgG and IgM Amylase activity Total protein concentrations
Data analysis	Student's t-test Spearman correlation	Kruskal-Wallis nonparametric test	MassLynx version 3.5 Software (MaxEnt 1 and MaxEnt 3)	U Mann-Whitney test
Results	The results obtained from the WS and HA experiments were similar. From the matrix of correlations, it can be seen that DMFT presents a significantly negative statistical correlation (between 60 and 80%) with cystatins (S, SN2 and SAIII), acidic PRPs and lipocalin-1 for WS. A similar trend is also observed for HA pellicle. A positive significant correlation was found for IgA, amylase, and lactoferrin in saliva and HA pellicle	Levels of LL37 and hBD-3 did not correlate with caries experience and HNP1-3 levels were significantly higher in children with no caries than in children with caries	Data analysis has allowed us to verify a strong correlation between large amounts phosphopeptides (PRP1/3, histatin 1 and statherin), and the absence of dental caries, which reinforces the importance of these peptides in the maintenance of tooth integrity	Children with ECC presented significantly higher levels of total salivary IgA and IgG, while the mean values of amylase activity, total protein concentrations and total IgM were similar between the groups
Other considerations	Not reported	Low salivary levels of HNP1-3 may represent a biological factor that contributes to caries susceptibility	In the caries-susceptible group a high number of peptide fragments was observed, suggesting a high proteolytic activity	The presence of ECC was associated with an increase in total salivary IgA

Authors and year	Sikorska et al., 2002	Ayad et al., 2000
Objective	Determine the potential connection between certain agents of the salivary defence system and the level of dental caries in a group of teenagers	Investigate the association between the peptide composition of human parotid saliva and dental decay (caries) experience
Sample size (n)	83	18
Age	15 years	DMFS = 0 (mean age = 59.2) and DMFS > 0 (mean age = 51.2)

Caries index	DMF-S	DMF-S
Groups	Not reported	Caries-free (DMFS = 0; n = 9) and caries- susceptible (DMFS > 0; n= 9)
Type of saliva	UWS	SWS
Saliva collection and preparation	The subjects had been instructed to refrain from eating (particularly sweets), drinking or using breath fresheners for a minimum of 2h before the saliva was collected. Unstimulated whole saliva was collected into sterile test tubes on ice in the morning and maintained at 4°C during centrifugation. The saliva was not collected in fractions. The collected samples were centrifuged at 5000 g for 15min, transferred to tubes in 0.5 ml amounts, and frozen at -70°C	All samples were collected between 9 a.m. and 12. The subjects were requested not to eat 2 hrs prior to their appointment. Gustatory-stimulated secretions were obtained by means of sugar-free lemon drops. Secretions collected during the first 2 min were discarded. Saliva was collected by means of a modified Lashley cup that fits over the duct of the parotid glands and is retained by means of a vacuum drawn seal, into a polypropylene tube set in crushed ice. Subjects were asked to collect at least 25 mL of saliva, and for most subjects, this took approximately 30 min. The specimens were returned to the laboratory and immediately frozen at -20°C until the time of analysis
Salivary parameters	Lactoferrin Secretory IgA (SIgA) α1 proteinase inhibitor	Proline-rich Peptides
Data analysis	Kruskal–Wallis test Spearman test	Mann-Whitney test
Results	A significant relationship was found between the decayed surface index and the levels of lactoferrin, SIgA and a lactoferrin; saliva; SIgA 1 proteinase inhibitor in the UWS	Sequence analysis identified 18 peptides that appear to be proteolytic cleavage products of the basic prolinerich proteins IB-4, IB-5, IB-7, IB-8b, and P-B. The peptides that were more abundant in saliva obtained from the caries-free group differed from those isolated from the caries-susceptible group. The median peptide concentration of one possible precursor protein, IB-7, was found to be higher in saliva collected from caries-free individuals than in that from caries-susceptible individuals
Other considerations	The decayed surface index rose with increases in the levels of lactoferrin, SIgA and α1 proteinase inhibitor, the relationship with α1 proteinase inhibitor being strongest	Collectively, our results indicate that proteolytic processing of parotid salivary proteins differs among individuals who have remained caries-free and those who have experienced dental decay

DMF-T = decayed, missing or filled teeth; DMFS = decayed, missing and filled surfaces; SWS = stimulated whole saliva; UWS = unstimulated whole saliva; WS= whole saliva; LB = Lactobacilli; DT= decayed tooth; ECC = early childhood caries.

DISCUSSION

Saliva is the first line of defense against dental caries. The composition and physiology of saliva clearly influence oral health.[15] For this reason, some components of saliva, such as proteins, have been more thoroughly studied. Han et al.[16] investigated the association of salivary glutathione (GSH) with dental caries and cariogenic bacteria. The major physiological role of GSH in organisms is to ensure redox homeostasis and protection against toxic chemicals. In bacteria, GSH protects against acid stress and osmotic stresses. It was reported that, in the presence of GSH, Lactobacillus spp. exhibited increased growth rates. The results show that a high level of salivary GSH could be a protective factor for Lactobacillus spp. in saliva and, consequently, could increase the chances of caries progression. The authors suggest that GSH concentration in saliva can be used as a biomarker for evaluating the dental caries status in children. However, there is insufficient evidence to

support the relationship of the mechanism of action of GSH with the protection of Lactobacillus spp. nor with increased odds of progression of caries.

Ribeiro et al.[17] evaluated the salivary peptide profile of 106 children with and without early childhood caries (ECC) and its association with caries experience (dmft > 0). Identification of molecular masses suggested the presence of three peptides significantly related with caries experience: HNP-3 (α-defensin 3) and HBD-3 (β-defensin 3) reduced the chances of experiencing ECC and proline-rich peptides IB-4 (PRPIB-4) significantly increased caries experience. They concluded that specific salivary peptides of CF (children with-out caries experience) or CE (children with caries experience) children in early childhood predispose to a higher or lower risk of caries experience. Already described in the literature that defensins present in saliva (HNP-3 and HBD-3) are antimicrobial peptides that play a role in innate immunity, and most likely are protective factors of caries.[18] However, the results of the relationship between PRPIB-4 and caries are contradictory because Ayad et al.[19] said PRPIB-4 were over expressed in individuals caries-free.

Zehetbauer et al.[20] made a qualitative comparison of major salivary protein profiles between children with ECC and caries-free controls. Saliva was collected from 30 children with ECC and, after separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, was compared with saliva from 20 caries-free controls for the general composition of proteins by means of silver staining, glycoprotein staining, and lectin blotting. Gels and blots were analysed using densitometry, and the protein-banding patterns resulting from the individuals' samples were compared by image analysis for the presence or absence of protein bands. The results attest a uniform expression of the major protein components in children's saliva, regardless of the clinical manifestation of ECC, and thus pave the way for further detailed investigations of more subtle differences in the salivary proteome.

Although Ribeiro and Zehetbauer presented similar aims, several factors may have influenced the different results. The sample size was quite different in the two studies, the criteria for assessing the presence or absence of caries and the level of severity of the lesions was not well established, the type of saliva and saliva such as the collection was performed in the two studies differed. It is also possible that SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis used for Zehetbauer may not be sensitive enough to detect putative subtle differences among some less-abundant proteins.

Jonasson et al.[21] examined the association of the receptor cysteine-rich glycoprotein gp-340, which mediates adhesion of *Streptococcus mutans* (microorganism implicated in caries) gp-340 I to III polymorphisms with caries experience and *S. mutans* adhesion. The results reveal a positive association of gp-340 I with both caries experience and saliva adhesion of *S. mutans*, and that purified gp-340 I protein mediates increased antigen I/II-mediated adhesion of *S. mutans*. Thus, Gp-340 I was considered as a protein related to caries susceptibility.

Vitorino et al.[22] through a proteomic approach to evaluate the influence of salivary protein composition on *in vitro* dental pellicle formation and its possible correlation with dental caries. Whole saliva, collected from caries-free and caries- susceptible subjects, was analyzed by two-dimensional electrophoresis, and protein spots were identified by mass spectrometry. Data analysis of salivary protein composition showed a statistically significant correlation between the quantity of acidic proline-rich proteins (PRPs), lipocalin, cystatin SN and cystatin S, and samples from the caries-free group of subjects [decayed, missing or filled teeth (DMFT) = 0]. Samples from subjects with a high DMFT index appear to be correlated with high levels of amylase, immunoglobulin A, and lactoferrin. As cystatins are known physiological inhibitors of cathepsins, the higher quantities of lipocalin, and cystatins S and SN found in the samples from the caries-free subjects suggest that inhibition of proteolytic events on other salivary proteins may indirectly provide tooth protection. The correlation between higher levels of the phosphorylated acidic PRPs 1/2 with samples from the caries-free group also suggests a protective role for these proteins.

Tao et al.[23] determined a possible correlation between caries prevalence in children and salivary concentrations of the antimicrobial peptides (AMPs) human beta-defensin-3 (HBD-3), the cathelicidin LL37, and the alpha-defensins HNP1-3 (a mixture of HNP1, 2, 3). AMPs are natural antibiotics that provide a first line of defense against a wide spectrum of pathogens.[24] One hundred forty-nine children were examined and examiners rated subjects separately for active caries and for filled surfaces and subsequently unstimulated saliva was collected. The results showed that levels of LL37 and HBD-3 were not correlated with caries experience and HNP1-3 levels were significantly higher in children with no caries than in children with caries. They concluded that low salivary levels of HNP1-3 may represent a biological factor that contributes to caries susceptibility. These results partially agree with those found by Ribeiro et al.[17] since this study HBD-3 (β-defensin 3) reduced the chances of experiencing ECC and Tao et al. did not correlate with caries experience. This partial

correlation by the different techniques have been used for peptide analysis, immunoassays used Tao et al. while the Ribeiro used liquid chromatography-mass spectrometry (LC-MS).

Vitorino et al.[25] correlated peptide saliva composition with dental caries susceptibility through the analysis of saliva and hydroxyapatite-adsorbed salivary peptides samples. Saliva samples were obtained from 20 volunteers divided into 2 groups, a caries-free and a caries-susceptible group, and were analysed using HPLC-MS. Data analysis showed a strong correlation between large amounts phosphopeptides (PRP1/3, histatin 1 and statherin), and the absence of dental caries, which reinforces the importance of these peptides in the maintenance of tooth integrity. In addition, in the caries-susceptible group a high number of peptide fragments was observed, suggesting a high proteolytic activity.

Farias et al.[26] analyzed the organic composition of saliva from children without dental caries and children with ECC. Two groups of 20 children varying in age from 12 to 47 months were selected: Group I, caries-free children; Group II, children with ECC. Measurements of total salivary IgA, IgG and IgM were performed by using nephelometric techniques, while total protein concentrations and amylase activity were determined by colorimetric techniques. Children with ECC presented significantly higher levels of total salivary IgA and IgG, while the mean values of amylase activity, total protein concentrations and total IgM were similar between the groups. The presence of ECC was associated with an increase in total salivary IgA.

Sikorska et al.[27] determined the potential connection between certain agents of the salivary defense system and the level of dental caries in a group of teenagers. The agents of saliva were investigated: lactoferrin (has a direct bacteriostatic effect), secretory IgA (SIgA – reduces the adherence of bacteria to tooth surfaces and oral mucosa) and α1 proteinase inhibitor (is a positive acute phase protein, the concentration of which increases 2–5 times during acute phase reaction; it is also the main inhibitor of elastase). Eighty-three volunteers (15-year-old) participated in the study. A significant relationship was found between the decayed surface index and the levels of lactoferrin, SIgA and a α1 proteinase inhibitor in the saliva. The decayed surface index rose with increases in the levels of lactoferrin, SIgA and α1 proteinase inhibitor, the relationship with α1 proteinase inhibitor strongest one. The increase in the levels of defense elements saliva (lactoferrin, SIgA and α1 proteinase inhibitor) probably occurs by the body's attempt defender some infectious process such as caries.

Ayad et al.[28] investigated the association between the peptide composition of human parotid saliva and dental decay (caries) experience. Eighteen volunteers participated in the

study, 9 caries-free individuals and 9 with caries experience. Sequence analysis identified 18 peptides that appear to be proteolytic cleavage products of the basic proline rich proteins IB-4, IB-5, IB-7, IB-8b, and P-B. The peptides that were more abundant in saliva obtained from the caries-free group differed from those isolated from the caries-susceptible group. The median peptide concentration of one possible precursor protein, IB-7, was found to be higher in saliva collected from caries-free individuals than in that from caries-susceptible individuals. Although differences were found in the phenotypes of proline-rich proteins expressed by these groups of caries-free and caries-susceptible subjects, no statistically significant associations were observed among proline-rich phenotypes and the level of any peptide. Collectively, the results indicate that proteolytic processing of parotid salivary proteins differs among individuals who have remained caries-free and those who have experienced dental decay.

Even with some promising results, in future studies should be employed more sensitive proteomic methods that exist today. In addition, a broader analysis of the proteomic profile of the saliva for individuals with caries lesions at different levels of severity should be performed, in order to better understand the correlation of certain proteins and the different stages of caries.

CONCLUSION

Ninety percent of the articles showed a relationship between salivary proteins and the presence or absence of caries. Although the studies established a relationship between salivary proteins and the presence or absence of caries, there is insufficient evidence to conclude that certain salivary proteins can be considered biomarkers for caries.

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3.2 CAPÍTULO 2

Proteomics of saliva from children with caries at different stages of severity

Sarah F. F. Guedes[1], Beatriz G. Neves[1], Daniela S. Bezerra[1], Gustavo H. M. F. Souza[2], Izabel F. F. Guedes[3], Arlindo A. A. N. Moura[4], Adriana Bassini[5], Lidiany K. A. Rodrigues[1].

[1]Post-graduation Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Brazil.

[2]Waters Corporation, MS Applications Development Laboratory, Brazil.

[3]Laboratory of Human Biochemistry, State University of Ceara, Brazil.

[4]Post-graduation Program, Faculty of Zootechnology, Federal University of Ceará, Brazil.

[5]Laboratory of Protein Biochemistry, Federal University of State of Rio de Janeiro, Brazil.

Correspondence: Lidiany K. A. Rodrigues (Email: lidianykarla@yahoo.com), Professor, Coordinator, Postgraduate Program in Dentistry, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, Ceará, Brazil. Cap. Francisco Pedro St - Zip Code: 60430-170, Phone- #+558533668410/Fax- #+558533668232

ABSTRACT

The number of studies trying to correlate salivary proteins and dental caries has considerably increased, however, there is no enough scientific evidence to establish specific salivary proteins as biomarkers for caries. Therefore, the present study aimed to perform a comparative analysis of proteins from the saliva of patients with early childhood caries (ECC) at different levels of severity, in order to identify potential specific salivary biomarkers for diagnosis of caries. For the study, 126 children (2-6 years old) were selected. Initially, the children were examined according to the visual index ICDAS II and divided into 3 groups (n = 42): Caries-free group (CF) (ICDAS 0), Enamel caries group (EC) (ICDAS 1, 2 and 3) and Dentine caries group (DC) (ICDAS 5 and 6). Then, the saliva was collected, processed and stored. The samples were digested and analyzed for mass spectrometry alternative multiplexed scanning (MSE). Analyses were performed with TransOmics Software and ANOVA and Volcano plot v-plot ($p<0.05$). Three hundred one proteins were identified and quantified, among them, 122 proteins were statistically significant different at least in one group: 84 were identified in all 3 groups; one protein was expressed only in CF and other in EC; 11 were expressed only in CF and EC, 15 in CF and DC, 10 in EC and DC. Of the 122 proteins, 54 proteins were at least 5 times more expressed in the comparison between the groups and were considered as candidates for biomarkers. From these 54, 18 proteins with potential mechanism of action related to dental caries were selected and analyzed. Concluding, these 18 proteins presented correlation with ECC, and SAA1, AIMP1, IL36A, and IL36G CAH1 may have increased potential to be considered as caries biomarkers. However, longitudinal studies still need to be made in order to establish the role of each of the proteins studied and to consolidate its role as a biomarker.

Keywords: Dental caries. Saliva. Proteins and peptides.

INTRODUCTION

Dental caries is a complex disease, characterized by demineralization of tooth structure by bacterial organic acids from the dental biofilm and by salivary proteins adsorbed on mineral tooth surface.[1] Early childhood caries (ECC) is defined as the presence of one or more decayed surfaces (non-cavitated or cavitated lesions), missing due to caries or restored in any primary tooth in a child under six years old. In young children, the large amount of cariogenic bacteria, the presence of biofilm, inappropriate feeding practices, environment and socioeconomic status have been identified as predisposing factors to the development of caries.[2]

With the advent of diagnostic methods and systems to identify dental caries over the past few years there has been the insertion of ICDAS II (International Caries Detection & Assessment System), which is a caries detection system based on visual inspection and includes assessment carious lesions cavitated and non-cavitated. This system was developed to be used in clinical practice and in clinical and epidemiological research.[3] The method proved to be feasible and well accepted, based on its standardized criteria and comparability with other indexes.[4]

Saliva is a fluid complex that contains a variety of enzymes, hormones, antibodies, cytokines and antimicrobial constituents.[5,6] Thus, most of the compounds found in the blood are also present in the saliva, which may reflect the physiological state of the body, including emotional, endocrine, nutritional and metabolic changes. Consequently, this fluid is a source for monitoring systemic and oral health.[7] The analysis of saliva can potentially aid in obtaining early diagnosis, monitoring the progression and/or the response to treatment of many diseases such as periodontal disease, as well as to evaluate the risk of dental caries.[8,9,10] Furthermore, the noninvasive nature of salivary sample collection reduces patient anxiety and discomfort. These advantages have attracted the attention of researchers seeking to simplify the diagnostic procedure in bodily fluids.[11,12,13]

In the last decades, many biotechnological advances have been established, bringing invaluable advantages in all fields of human health. The identification of proteins in complex biological samples such as saliva can be carried out by different methods. However, due to the high sensitivity, mass spectrometry has achieved an important highlight in the proteomic analysis.[12] The development of mass spectrometry (MS) technology has led to a new era in manufacturing biomarkers and should potentially have a substantial impact on diagnosis and therapy of diseases. Thus, the presence or absence of multiple biomarkers in salivary proteome,

modified by diseases or interventions, can be detected with the MS.[14,15] The importance of identifying biomarkers for tooth decay occurs due to the high number of lesions per patient for the same portion of the population. An early diagnosis could reduce the average incidence of caries per patient and the average cost of prevention. Considering this scenario, the number of studies trying to correlate salivary proteins and dental caries has considerably increased, however, there is no enough scientific evidence to establish specific salivary proteins as biomarkers for caries.[16] Therefore, the present study was conducted to perform a comparative analysis of proteins from the saliva of patients with ECC at different levels of severity, in order to identify potential specific salivary biomarkers for diagnosis of caries.

METHODS

Ethics statement

The Ethics Committee of the Federal University of Ceará State (COMEPE/UFC) (Protocol Number 158/2011) (ANEXO A) approved the study protocol. Written consents were obtained from the parents of all subjects and samples were taken only after receiving the approval from the children and their parents.

Subject and experimental design

One hundred and twenty-six children with and without early childhood caries were recruited for this study from nurseries and public schools in the city of Fortaleza, located in the state of Ceará. Selected children were from 2 to 6 years old. Patients were excluded from the study if they had a significant medical disease, received an antimicrobial treatment within 3 months or presented carious lesions classified with score 4 ICDAS II. The selected subjects did not present salivary gland diseases or systemic diseases or spontaneous symptoms associated with the caries lesions. A formal consent of the respective child and family and the willingness to participate was used as inclusion condition. All children involved in this study were enrolled in a dental care program that included routine professional monitoring of their oral health status. Such program is maintained until the present time.

The presence of dental caries was assessed using the International Caries Detection and Assessment System (ICDAS).[3,17] Three calibrated examiners conducted dental examinations. A professional oral cleaning was performed prior to clinical examination. The examiners used a

WHO periodontal probe, mirror, gauze and adequate illumination during the clinical evaluation. Three hundred children were examined and, from this larger group, 126 were selected for the study according to ICDAS scores: Caries-free group (CF) (n = 42; ICDAS 0); Enamel caries group (EC) (n = 42; ICDAS 1, 2 and 3); Dentine caries group (DC) (n = 42; ICDAS 5 and 6).

Saliva colletion

One hour before the collection of saliva, patients brushed their teeth with non-abrasive toothpaste and remained without ingestion of any food or liquid.[18] Fifteen minutes before the collection, all patients did a rinse with potable water. For the collection procedure, the patients remained seated inside an air conditioned room that had also been protected with organic seal bags to ensure the purity of samples. Then, the researcher in charge of sample collection placed a Children's Swab (SCS) (Salimetrics LLC Company, State College, Pennsylvania, USA) in the patient's mouth that was chewed during 90 seconds. After this time, the swab was removed from the child's mouth and placed in a Swab Storage Tube (Salimetrics LLC Company). The tube containing the swab was immediately centrifuged at 3,000 g for 15 minutes (CT6E Hitachi Koki Company, Minato-ku, Tokyo, Japan). Then, the supernatant was removed from the tube and placed in a Cryovial (W985872-100 Wheaton, Millville, NJ, USA) correctly identified. The Cryovial was immediately stored on ice and subsequently dried at -80°C.

Proteomic Analysis

Prior to proteomic analysis, pools of saliva samples were made, each pool containing 50 μl of saliva per individual of each group (n = 42 samples/group). For these pools, albumin and immunoglobulin G were depleted using a specific column (Albumin e IgG Depletion SpinTrap - GE Healthcare, Uppsala, Sweden) and total protein concentration was determined using a filtration device Amicon Ultra-4 10K (Merck Milipore, Carrigtwohill, Ireland). After protein concentrations were estimated, 50 μl of each sample, corresponding to 50 μg of protein, was washed with in RapiGest at 0.05% (w/v) (Waters Corporation, USA) in 50 mM ammonium bicarbonate (Sigma Aldrich, Hamburg, Germany) in order to hydrolyse the proteins. Then, samples were reduced using 100 mM dithitreitol (Sigma Aldrich) at 60ºC for 30 min and alkylated for 30 min at 25ºC in the presence of 300 mM iodoacetamide (Sigma Aldrich, USA). Proteolytic digestion proceeded at a 1:50 (w/w) ratio with an overnight incubation at 37 ºC by the addition of sequencing grade trypsin (Promega, Madison, WI, USA). Following this trifluoroacetic acid (TFA) was added to the samples to hydrolyze the RapiGest and the solutions

incubated at 37 °C for 90 min before being vortexed and centrifuged. Samples were diluted 1:1 with 10fmol/ μL of yeast alcohol dehydrogenase internal standard tryptic digest (Waters Corporation, USA) before mass spectrometry analysis.[19]

For the analysis, 500 ng of protein digest from each pool were separated using nanoscale chromatography performed with a nanoACQUITY UPLC (Waters Corporation, USA). One-dimensional reversed phase (RP) nanoACQUITY experiments with trapping were performed. Mobile phases A and B were water containing 0.1% (v/v) formic acid and acetonitrile containing 0.1% (v/v) formic acid respectively. Following de salting of the peptides on a Symmetry C18 5 μm, 2 cm x 180 μm trap column, (Waters Corporation, USA) a reversed phase gradient was employed to separate peptides using 5 to 35% acetonitrile over 90 minutes on a 25 cm x 75 μm analytical RP column (Waters Corporation, USA) at a flow rate of 300 nl/min and a constant temperature of 35ºC.[19] Analysis of the complex peptide mixtures was performed using a SYNAPT G2-S HDMS mass spectrometer (Waters, Manchester UK) operated in a data-independent manner coupled with ion mobility (HDMSE). The mass spectrometer was operated in positive ESI resolution mode with resolution of >20,000 FWHM. In all experiments the mass spectrometer was programmed to step between low energy (4 eV) and elevated (14-40 eV) collision energies on the Triwave collision cell, using a scan time of 0.9 s per function over 50-2000 m/z. All samples were analyzed in triplicates.

Protein identifications and quantification information were obtained using PLGS by searching UNIPROT human database. The dilution of the samples 1:1 with an ADH (yeast) tryptic digest of a known concentration allowed the data processing software not only to identify the components of the complex mixture, but also to calculate the estimated amounts of identified proteins. The analysis was carried out with minimum requirements for specificity: at the peptide level, at least 3 peptides per protein, and a maximum 4% False Discovery Rate (FDR) at the protein level for all quantified proteins, for each replicate of each sample. Furthermore, proteins were required to be identified in replicate injections.

Statistical analysis

Expression analyses were performed with TransOmics Software (Nonlinear Dynamics – Waters, UK) and ANOVA ($p < 0.05$) and Volcano plot V-plot.

RESULTS

For the 1D-MSE analysis, collectively, 301 quantified proteins (0% FDR) were identified in the saliva samples and 122 proteins were statistically significant different among the three groups of children. The majority of the proteins (84) were identified in all three groups indicating a high overlap in saliva proteins. One protein was expressed only in CF and another in EC, while 11 were expressed only in CF and EC, 15 in CF and DC, 10 in EC and DC. Figure 1 shows a Venn diagram with the number of proteins from each group and their overlaps among the three groups of patients. Of the 122 proteins mentioned above, 54 proteins were at least 5 times more expressed in the comparison between the groups and were considered as candidates for biomarkers. From these 54, 18 proteins with potential mechanism of action related to dental caries were selected (Table 1).

Within this group of 18 proteins, eight were detected with greater expression in caries-free patients (Figure 2). The Lymphocyte-specific protein 1 (LSP1), Glutathione synthetase (GSHB) and Zinc finger protein 511 (ZN511) were more expressed in CF, with no statistical differences between groups EC and DC. Alpha-1-acid glycoprotein 2 (A1AG2), Serpin B3 (SPB3), Glucose-6-phosphate isomerase (G6PI) and SH2 domain-containing adapter protein F (SHF) were more expressed in the CF, but again had their increased levels in DC, and one, the Phosphoglyceric acid mutase (PGAM1), had their lowest expression levels when the caries severity was increasing.

Peroxiredoxin-4 (PRDX4) and Myeloblastin (PRTN3) were more expressed in CF and DC with no significant statistical variation (Figure 3). NK cell receptor 2B4 (CD244) has much greater expression in EC patientes than in children with no caries and with dentine caries, but the amount of such molecule in these tow groups were also significantly distinct (Figure 4). Alpha-amylase 2B (AMY2B) and Gamma-enolase (ENOG) were expressed in CF and EC, but were expressed much more in DC (Figure 5).

According to Figure 6, Serum amyloid A-1 protein (SAA1) and Aminoacyl tRNA synthase complex-interacting multifunctional protein 1 (AIMP1) were more expressed in the groups with caries (EC and DC). Figure 7, in turn, shows proteins with more expression with increasing severity of dental caries: Interleukin-36G (IL36G), Interleukin-36A (IL36A) and Carbonic anhydrase 1 (CAH1).
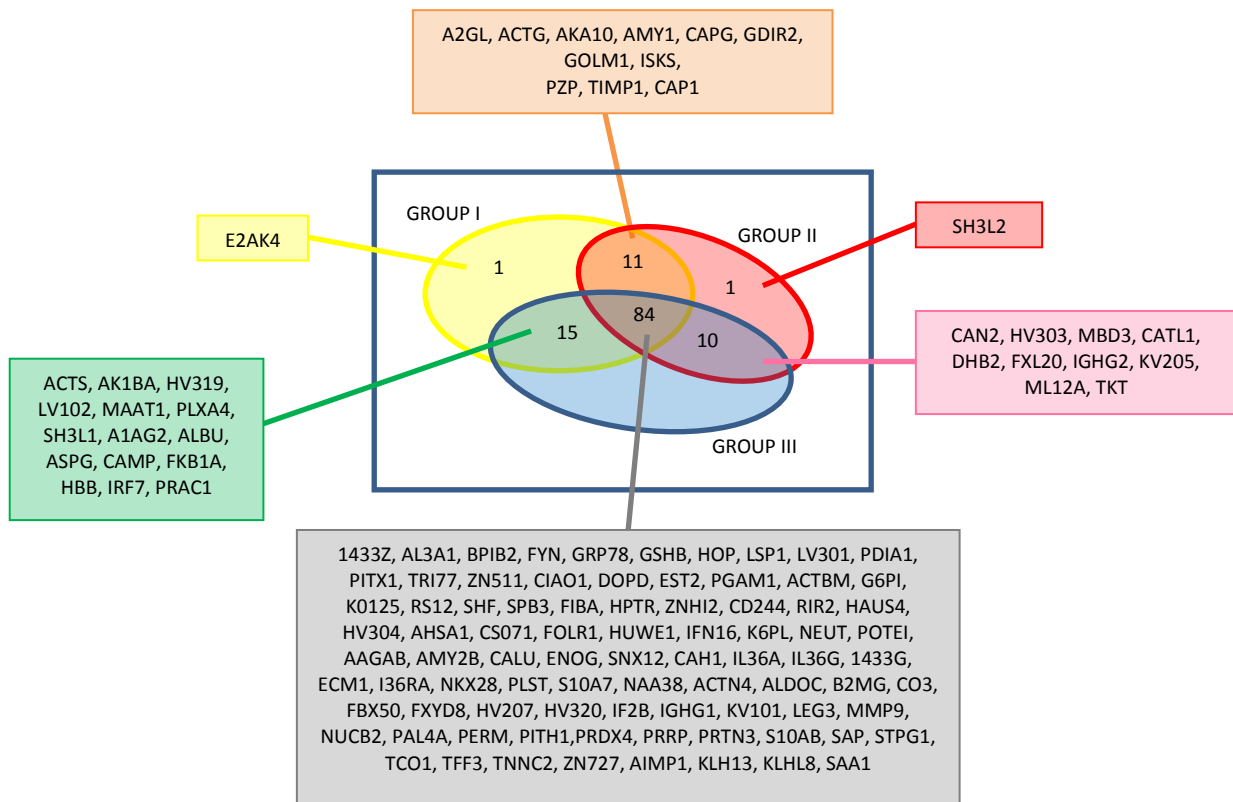
Figure 1. Venn diagram of salivary proteins identified in each group and across groups.
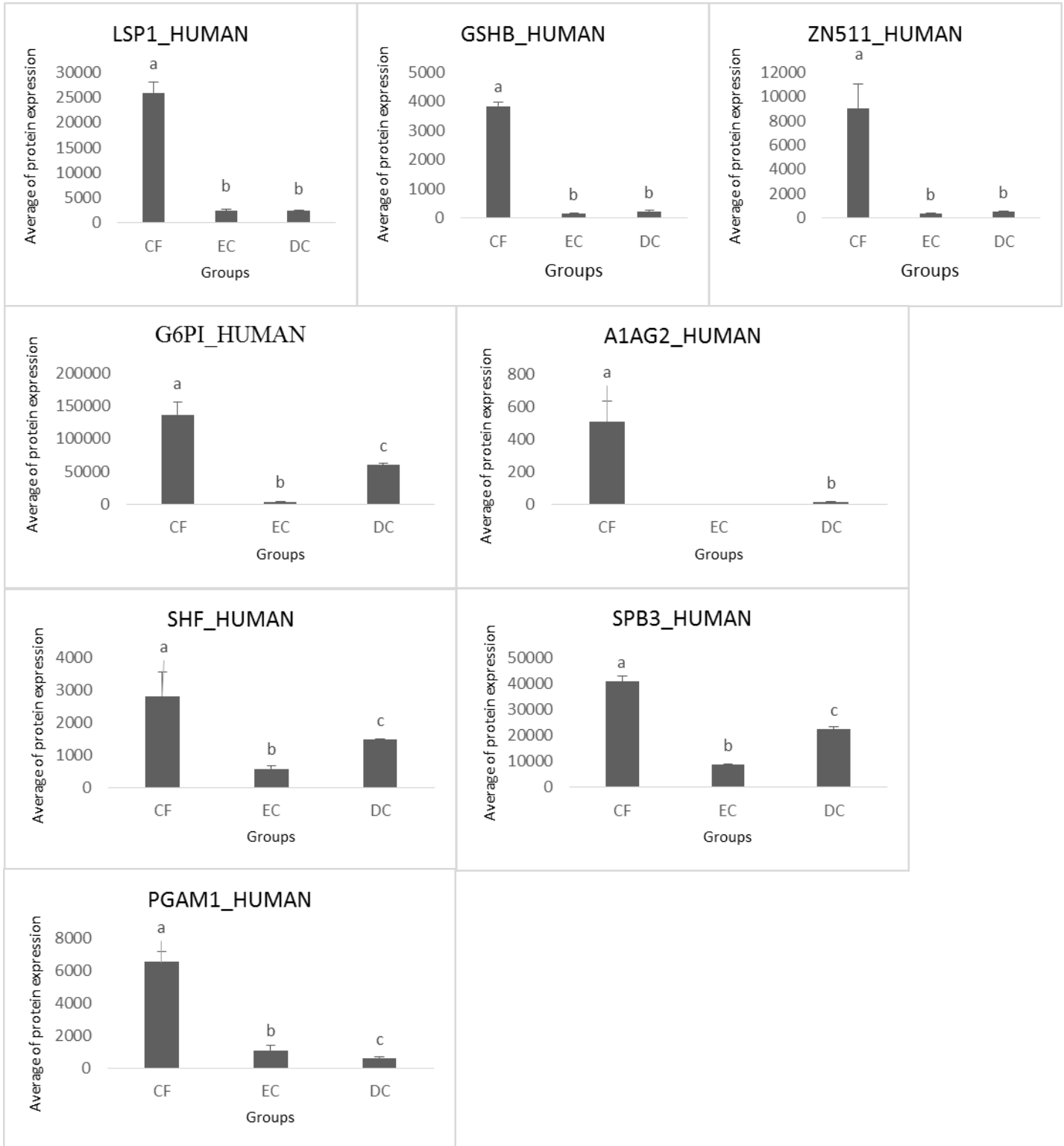
Figure 2. Expression of proteins in saliva from caries-free children (CF), children with enamel caries (EC) and dentine caries (DC). Different letters represent significant statistical variations (p < 0.05). Protein names refer to those described in Table 1.
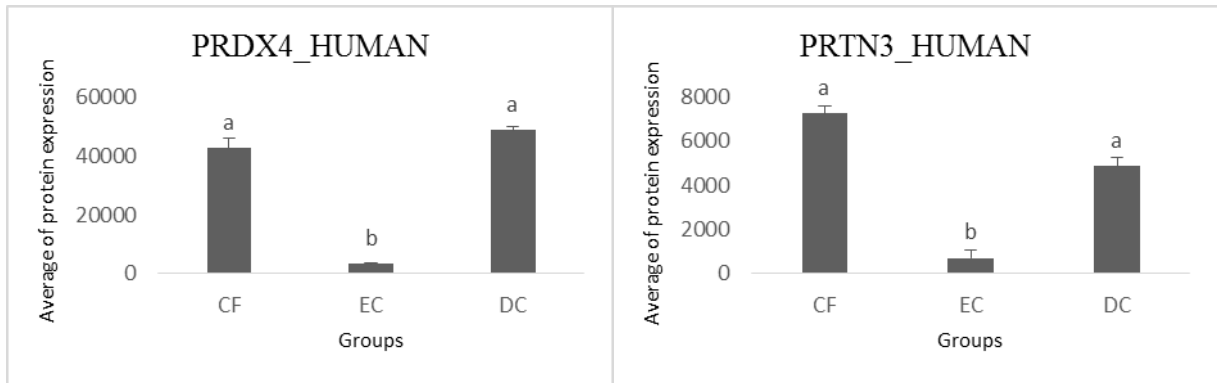
Figure 3. Expression of proteins in saliva from caries-free children (CF), children with enamel caries (EC) and dentine caries (DC). Different letters represent significant statistical variations ($p < 0.05$). Protein names refer to those described in Table 1.

Figure 4. Expression of CD244 protein in saliva from caries-free children (CF), children with enamel caries (EC) and dentine caries (DC). Different letters represent significant statistical variations ($p < 0.05$). Protein name refer to those described in Table 1.
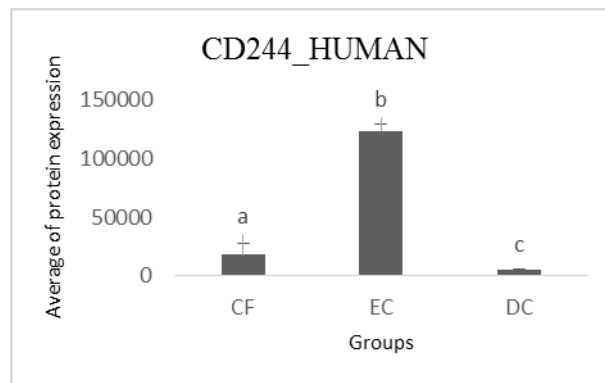
Figure 5. Expression of proteins in saliva from caries-free children (CF), children with enamel caries (EC) and dentine caries (DC). Different letters represent significant statistical variations (p < 0.05). Protein names refer to those described in Table 1.
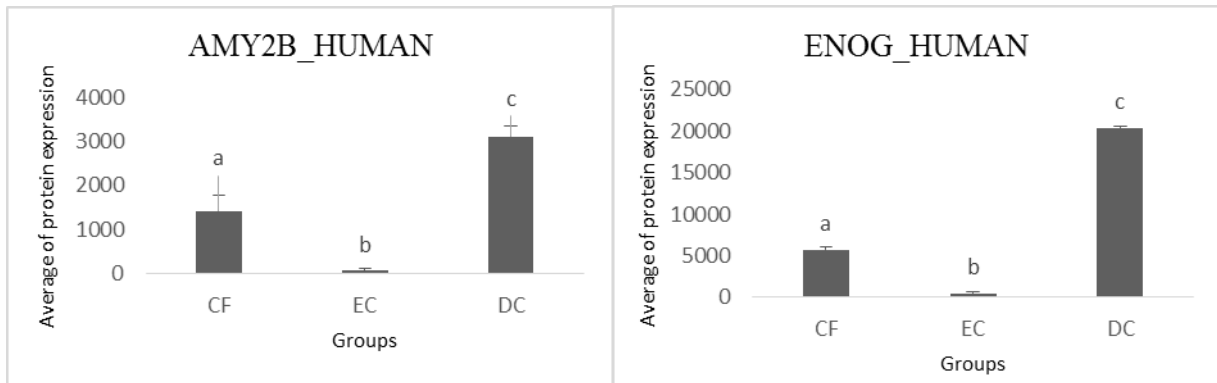

Figure 6. Expression of proteins in saliva from caries-free children (CF), children with enamel caries (EC) and dentine caries (DC). Different letters represent significant statistical variations (p < 0.05). Protein names refer to those described in Table 1.
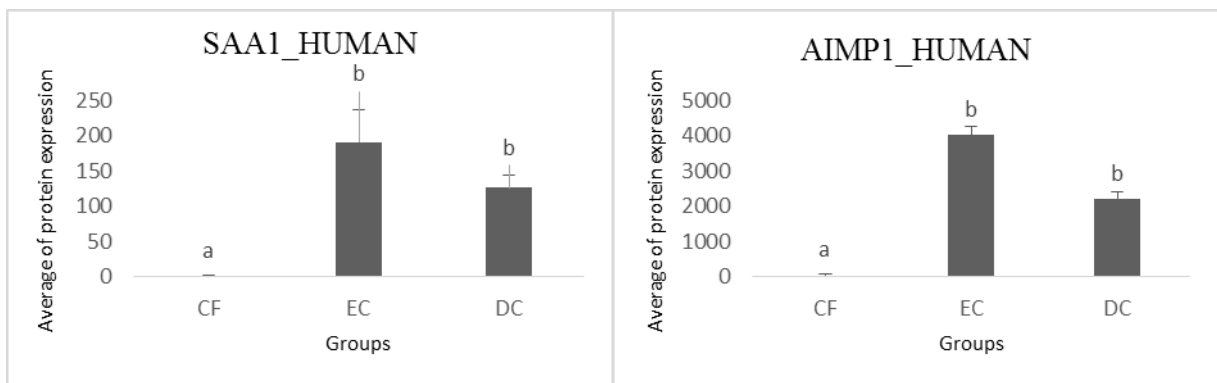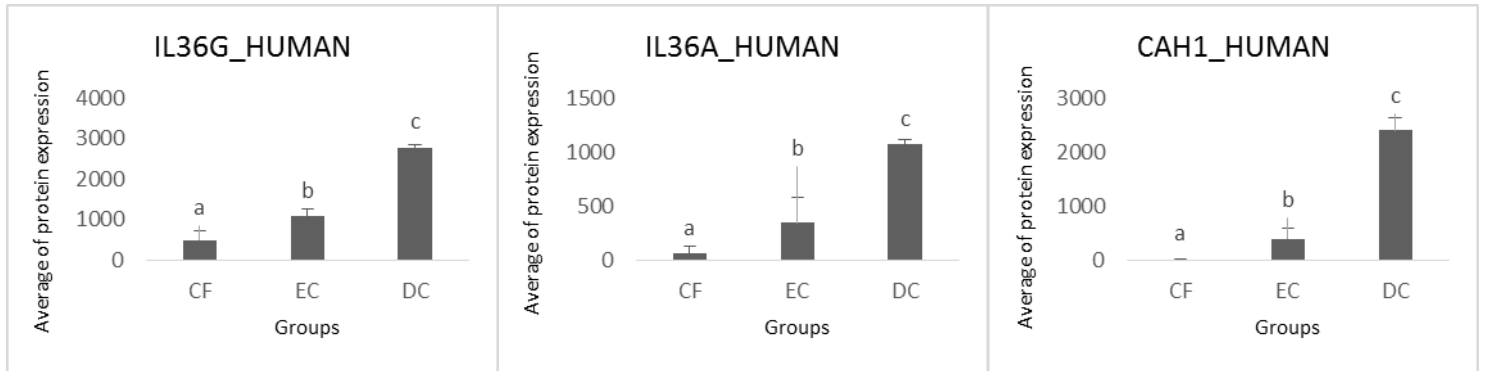
Figure 7. Expression of proteins in saliva from caries-free children (CF), children with enamel caries (EC) and dentine caries (DC). Different letters represent significant statistical variations ($p < 0.05$). Protein names refer to those described in Table 1.

Table 1. Proteins with potential mechanism of action related to dental caries

Name	Abbreviation
Lymphocyte-specific protein 1	LSP1_HUMAN
Glutathione synthetase	GSHB_HUMAN
Zinc finger protein 511	ZN511_HUMAN
Glucose-6-phosphate isomerase	G6PI_HUMAN
Alpha-1-acid glycoprotein 2	A1AG2_HUMAN
SH2 domain-containing adapter protein F	SHF_HUMAN
Serpin B3	SPB3_HUMAN
Phosphoglyceric acid mutase	PGAM1_HUMAN
Peroxiredoxin-4	PRDX4_HUMAN
Myeloblastin	PRTN3_HUMAN
NK cell receptor 2B4	CD244_HUMAN
Alpha-amylase 2B	AMY2B_HUMAN
Gamma-enolase	ENOG_HUMAN
Serum amyloid A-1 protein	SAA1_HUMAN
Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	AIMP1_HUMAN
Interleukin-36G	IL36G_HUMAN
Interleukin-36A	IL36A_HUMAN
Carbonic anhydrase 1	CAH1_HUMAN

DISCUSSION

Recent developments of mass spectrometry methodologies associated with tools of bioinformatics have opened new avenues for the characterization of protein profiles of biological samples. Using such technologies, we have conducted the first global analysis of the saliva proteome in children with early childhood caries in different stages of severity. Some attempts to conduct such analysis have already been made before [20,21] but with limited success, probably because of the use of less sensitive techniques and the lack of patients with caries at an early stage (enamel caries - ICDAS 1, 2 and 3). In the present study, thus, we have successfully employed a proteome approach to identify specific salivary biomarkers of specific types of caries in six-year old children.

The identification of saliva proteins occurred by using a very sensitive technique and preliminary standardization of sampling that was conducted in an environment inside a refrigerated and protected with organic seal bags room with specific swabs, tubes free of contamination that could interfere with the analysis, centrifugation of samples and immediate cooling to prevent degradation of proteins by temperature. Depletion of albumin and immunoglobulin G was important to avoid masking of low molecular weight proteins and enable their identification. [19]

The proteins Eukaryotic translation initiation factor (E2AK4) and SH3 domain-binding glutamic acid-rich-like protein 2 (SH3L2) were expressed exclusively in groups CF and EC, respectively (Figure 1). Although there is no report in the literature that links these proteins and caries, a deeper investigation of them can clarify their functioning as a biomarker for resistance of caries (E2AK4) and enamel caries (SH3L2).

LSP1, ZN511 and GSHB were more expressed in CF, with no statistically significant difference between EC and DC (Figure 2A). The LSP1 is involved in the primary defense mechanism of the body and there are no reports correlating LSP1 with caries. The ZN511 is involved in transcriptional regulation Proline-rich acidic protein 1 (PRAP1). PRAP1 has been described as being related to remineralization phenomena occurring at the tooth surface[22], which may confirm its precursor (ZN511) as protective factor against caries. The GSHB deserves to be a protein involved in defense of saliva system, could be as a suppressor of pathogens that cause oxidative stress or inflammation, as previous described by Öztürk et al. However, contradictory results are found by Han et al. for the relationship between the level of salivary GSHB and caries.[23,24] Han et al. believes that a high level of salivary GSH could

be a protective factor for Lactobacillus spp. in saliva and thus promote dental caries. Thus, with regard to dental caries, our results suggest a protective effect for these proteins.

Of the eight proteins, four (A1AG2, SPB3, G6PI and SHF) were more expressed in the CF, but again had their increased levels in DC, and one, the PGAM1, had their expression lowest levels when the caries severity was increasing (Figure 2B and 2C). The A1AG2 appears to function in modulating the activity of the immune system during the acute-phase reaction and SPB3 acts as a protease inhibitor which also modulate the host immune response. G6PI is an enzyme involved in activation mechanisms of the host primary immune response while SHF operates in the secondary immune response. This suggests that in healthy individuals this group of proteins is well expressed by not being requested and has diminished with the onset of an infectious process (enamel caries), increasing their levels with the increase of injury (dentine caries), but without expression levels recover to levels of healthy subjects. The PGAM1 stimulates immunoglobulin production that may have an important role in innate host defense.[25] This protein appears to be related to protection of healthy subjects once its expression decreases with increasing severity of the disease up to be less expressed in children with dentine caries.

PRDX4 and PRTN3 were more expressed in CF and DC with no significant statistical variation (Figure 3). The PRXD antioxidant family comprises six isoforms that have recently been shown to have important functions in cellular antioxidant defence[26] and PRTN3 is serine protease that degrades elastin, fibronectin, laminin, vitronectin, and collagen types I, III, and IV (in vitro).[27] The most expressed of PRTN3 in DC can be explained by the dentinal degradation caused by caries Which performs its role in the lysis of collagen. However, its greatest expression in CF, as well as the relationship of PRDX4 with this disease still need to be better investigated and appear to be an unexpected result.

For the group that had enamel caries (EC), CD244 was more expressed (Figure 4). The CD244 is a transmembrane protein belonging to the Ig superfamily, which can also be expressed by CD8+ T cells. This protein may be related to the infectious process started with their increased expression early enamel lesions. For the their behavior on other groups can not be a role defined for it.

AMY2B is secreted protein that hydrolyze 1,4-alpha-glucoside bonds in oligosaccharides and polysaccharides. High levels of AMY2B appear to be correlated with high levels caries.[30] Already in relation ENOG there is no report in the literature that links these proteins and caries. AMY2B and ENOG were expressed in CF and EC, but were

expressed much more in DC (Figure 5). This suggests that in healthy individuals this group of proteins is well expressed by not being requested and has diminished with the onset of an infectious process (enamel caries), increasing their levels with the increase of injury (dentine caries), expression surpassing levels in healthy subjects.

SAA1 and AIMP1 were more expressed in the groups with caries (EC and DC) (Figure 6). SAA1 is related to acute-phase reactions is one of the major proteins produced by hepatocytes through induction, and the induced SAA is subsequently released to the blood circulation. Within the first 2–3 days after initiation of an acute-phase response, the plasma level of SAA can often increase by 1000-fold, which follows trauma and infection. Inflammatory cytokines including IL-1β, IL-6, and TNF-α contribute to the induction of SAA. SAA is a biomarker for inflammatory diseases and is associated with the pathogenesis of chronic diseases. These environmental changes include microbial infection, tissue injury, and immunological disorders.[28] AIMP1 is involved in the stimulation of inflammatory responses after proteolytic cleavage, in the control of angiogenesis and wound healing.[29] These proteins probably perform an important role as biomarkers of dental caries indicating that their presence is related to the presence of the disease.

Figure 7 shows the proteins that had their increased expression with increasing severity of dental caries: IL36G, IL36A and CAH1. IL36G and IL36A are a pro-inflammatory cytokine that plays an important role in the pathophysiology of several diseases, but there is not described in the literature, the relationship of these cytokines with caries. Carbonic anhydrases (CAs) are a large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide. They participate in a variety of biological processes, including respiration, calcification, acid-base balance, bone resorption, and the formation of aqueous humor, cerebrospinal fluid, saliva, and gastric acid. They show extensive diversity in tissue distribution and in their subcellular localization. [30] CA VI has been widely studied their function in caries having a protective role.[31] This protective role was not observed in the present study, since its presence was detected similarly in all 3 groups. However, CAH1 also appears to play a role against the disease since its expression is higher the greater the severity of the disease indicating that their presence may be important against the disease.

CONCLUSION

The 18 proteins presented correlation with ECC, and SAA1, AIMP1, IL36A, and IL36G CAH1 may have increased potential to be considered as caries biomarkers. However, longitudinal studies still need to be made in order to establish the role of each of the proteins studied and to consolidate its role as a biomarker.

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4 CONCLUSÃO GERAL

A relação entre proteínas salivares e presença ou ausência de cárie é notória e já está estabelecida. Neste estudo, 18 proteínas apresentaram uma relação com a CPI, e a SAA1, AIMP1, IL36A, IL36G e CAH1 possuem um maior potencial para serem consideradas biomarcadores da cárie dentária. No entanto, estudos longitudinais ainda precisam ser realizados, a fim de estabelecer o papel de cada uma das proteínas estudadas e consolidar o seu papel como um biomarcador.

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ANEXO A

Universidade Federal do Ceará
Comitê de Ética em Pesquisa

Of. N° 221/11   Fortaleza, 29 de agosto de 2011
Protocolo COMEPE n° 158/ 11
Pesquisador responsável: Beatriz Gonçalves Neves
Título do Projeto: "Determinação de indicadores de risco relacionados a diferentes estágios da cárie precoce na infância"

Levamos ao conhecimento de V.Sª. que o Comitê de Ética em Pesquisa da Universidade Federal do Ceará – COMEPE, dentro das normas que regulamentam a pesquisa em seres humanos, do Conselho Nacional de Saúde – Ministério da Saúde, Resolução n° 196 de 10 de outubro de 1996 e complementares, aprovou o protocolo e o TCLE do projeto supracitado na reunião do dia 18 de agosto de 2011.

Outrossim, informamos, que o pesquisador deverá se comprometer a enviar o relatório final do referido projeto.

Atenciosamente,

Dr. Fernando A. Frota Bezerra
Coordenador do Comitê
de Ética em Pesquisa
COMEPE/UFC