UNIVERSIDADE FEDERAL DO RIO DE JANEIRO
Centro de Ciências da Saúde
Faculdade de Odontologia

Tatiana Kelly da Silva Fidalgo

COMPONENTES SALIVARES E FATORES DE RISCO ASSOCIADOS À CÁRIE DENTÁRIA

Rio de Janeiro
2014

UNIVERSIDADE FEDERAL DO RIO DE JANEIRO
Centro de Ciências da Saúde
Faculdade de Odontologia

Tatiana Kelly da Silva Fidalgo

COMPONENTES SALIVARES E FATORES DE RISCO ASSOCIADOS À CÁRIE DENTÁRIA

Tese submetida ao corpo docente da Faculdade de Odontologia da Universidade Federal do Rio de Janeiro como parte dos requisitos para obtenção do título de Doutor em Odontologia (Odontopediatria).

Orientadores:
Profª Drª Ivete Pomarico Ribeiro de Souza
Profª Titular da Disciplina de Odontopediatria da FO/UFRJ

Profª Drª Ana Paula Canedo Valente
Profª Associada da Disciplina de Bioquímica do Instituto de Bioquímica Médica/UFRJ

Profª Drª Liana Bastos Freitas Fernandes
Profª Visitante da Disciplina de Odontopediatria da FO/UFRJ

Rio de Janeiro
2014

FICHA CATALOGRÁFICA

Fidalgo, Tatiana Kelly da Silva
Componentes salivares e fatores de risco associados à carie dentária / Tatiana Kelly da Silva Fidalgo. – Rio de Janeiro: Faculdade de Odontologia, 2014.
xxiii, 133 f. : il. ; 31 cm.
Orientadores: Ivete Pomarico Ribeiro de Souza, Ana Paula Canedo Valente, Liana Bastos Freitas Fernandes.
Tese (doutorado) - UFRJ, FM, Programa de Pós-graduação em Odontologia, Odontopediatria, 2014.
Referências bibliográficas: f. 107 – 113.
1. Saliva - microbiologia. 2. Saliva - química. 3. Cárie Dentária - etiologia. 4. Metaboloma - fisiologia. 5. Imunoglobulina A Secretora. 6. Lipídeos. 7. Espectroscopia de Ressonância Magnética. 8. Fatores de Risco. 9. Criança. 10. Pre-escolar. 11. Revisão. 12. Odontopediatria - Tese. I. Souza, Ivete Pomarico Ribeiro de. II. Valente, Ana Paula Canedo. III. Fernandes, Liana Bastos Freitas. IV. Universidade Federal do Rio de Janeiro, FM, Programa de Pós-graduação em Odontologia, Odontopediatria. V. Título.

FOLHA DE APROVAÇÃO

TATIANA KELLY DA SILVA FIDALGO

"COMPONENTES SALIVARES E FATORES DE RISCO ASSOCIADOS À CÁRIE DENTÁRIA"

Tese de Doutorado submetida ao Programa de Pós-Graduação em Odontologia (Odontopediatria), Faculdade de Odontologia, Universidade Federal do Rio de Janeiro-UFRJ, como parte dos requisitos necessários à obtenção do título de **Doutor em Odontologia**(**Odontopediatria**).

Rio de Janeiro, _____/_____/ 2014.

Profa. Dra. Ivete Pomarico Ribeiro de Souza
DO–Professor Titular do Departamento de Odontopediatria e Ortodontia da FO-UFRJ

Profa. Dra. Laura Salignac de Souza Guimarães Primo
DO–Professor Adjunto do Departamento de Odontopediatria e Ortodontia da FO-UFRJ

Profa. Dra. Lucianne Cople Maia de Faria
DO–Professor Titular do Departamento de Odontopediatria e Ortodontia da FO-UFRJ

Profa. Dra. Vera Lígia Vieira Mendes Soviero
DO–Professor Adjunto da Universidade do Estado do Rio de Janeiro-UERJ

Profa. Dra. Raquel Assed Bezerra da Silva
DO–Professora Associada da Disciplina de Odontopediatria do Departamento de Clínica Infantil da Faculdade de Odontologia de Ribeirão Preto-USP

DEDICATÓRIA

A Deus
Por tornar realidade os meus sonhos...
"Porque desde a antiguidade não se ouviu, nem com ouvidos se percebeu, nem com os olhos se viu um Deus além de ti que trabalha para aquele que nele espera."
(Isaías 64:4)

À minha família,

Minha mãe Claudia Macedo, avó Neuza Macedo, tia Alece Richardelli e bisavó Adália Richardelli (in memorian) que sonharam o meu sonho e foram meus alicerces durante todos esses anos. Obrigada por me educarem e se esmerarem não apenas para que eu tivesse uma formação melhor, mas para que eu fosse um ser humano melhor. Amo vocês!

AGRADECIMENTOS

- Às minhas irmãs **Bianca Medonça** e **Bruna Mendonça** que sempre me incentivaram e estiveram presentes me apoiando e compreendendo minhas muitas atividades acadêmicas.

- À minha prima-irmã **Alessandra Richardelli** que se alegra com as minhas vitórias e não me poupa palavras de ânimo e coragem.

"Enfim, ser a irmã mais velha é uma das melhores coisas que existe no mundo. No entanto, chega um momento em que a diferença de idade ela não é mais considerada, é como se todos estivem no mesmo patamar. Um ajudando ao outro, com suas potencialidades e competências. Cada um com seu perfil, cada um com suas características particulares, com o mesmo compromisso: amar e respeitar o outro e ajudá-lo a vencer aos desafios da vida."
(Autor desconhecido)

- Ao meu tio **Márcio Batista**, um dos meus grandes incentivadores; minha prima **Verônica Richardelli**, ao marido **Guilherme Xavier** e priminhos **Gabi** e **Igor** por todas as travessuras de final de semana!

- Aos meus queridos amigos **Aline Jardim**, **Carla Caetano**, **Danniel Dreux**, **Eurico Souza**, **Flávia Castro**, **Juilberto Martins**, **Rafaela Castro**, **Jefferson Moraes**, **Lívia Caetano**, **Raquel Diniz**, **Rafael Coutinho** e **Vanessa Targino** pelas orações, companheirismo, carinho e amizade.

"Quem tem um amigo, mesmo que um só, não importa onde se encontre, jamais sofrerá de solidão; poderá morrer de saudades, mas não estará só."
(Amyr Klink)

- À minha amiga **Lívia Mourão**, minha eterna dupla de faculdade que durante e após a graduação nunca poupou palavras e gestos de amizade, só tenho boas recordações suas!

"Saber encontrar a alegria na alegria dos outros, é o segredo da felicidade."
(Georges Bernanos)

- À minha amiga-irmã **Roberta Barcelos**, você sem dúvidas é uma das responsáveis por eu ter chegado até aqui! Sua amizade é um dos melhores e

mais preciosos presentes que Deus poderia ter me concedido durante minha formação acadêmica. Agradeço pelo companheirismo e cuidado ao longo de todos esses anos. Não poderia deixar de mencionar sua família, igualmente especial: tia **Zélia Barcelos**, **Gynna Barcelos**, **Isac Barcelos** e **Renato Sampaio**. Obrigada pelo carinho e apoio constante, também amo muito vocês. A **Ana Helena** tem muita sorte por nascer cercada por pessoas tão especiais.

"Amiga é uma bênção que vem do coração de Deus pra gente cuidar. É assim que você é pra mim, como uma pérola que eu mergulhei pra encontrar. É assim que você é pra mim, um tesouro que pra sempre eu vou guardar. Eu acredito em você, eu acredito nos sonhos de Deus pra tua vida. Amiga, eu oro por você porque a tua vitória também é minha." (Fernanda Brum)

- Aos meus amigos-irmãos **Matheus Pithon** e **Rogério Lacerda**, como irmãos mais velhos cuidam tanto de mim, aconselhando e desejando o meu bem. Mesmo a algumas milhas de distância, estamos sempre próximos, unidos pela força dessa amizade. Admiro e aprendo muito com vocês. Obrigada por me impulsionarem a crescer, sonhar e realizar. A felicidade de vocês é a minha felicidade! Amo muito vocês.

A amizade é uma predisposição recíproca que torna dois seres igualmente ciosos da felicidade um do outro.
(Platão)

- Aos amigos do mestrado **Andrea Lips**, **Camila Nassur**, **Clarissa Avelar**, **Daniela Soares**, **Elaine Amorin**, **Helena Romanos**, **Luciana Pereira**, **Marina Siqueira**, **Nashalie Alencar**, **Queila Oliveira**, **Renata Otero**, **Sabrina Loren**, **Tacíria Braga**, **Thais Alves**, **Thais Soares**, **Yedda Rosário** e pela convivência agradável, sempre muito solícitos e gentis. Vocês foram essenciais!

- Aos amigos **Adrielle Mangabeira** e **Thiago Isidro** que fazem os meus dias mais felizes e me fazem ter a certeza que "sorrir sempre é o melhor remédio"! Como mencionei, já me apeguei ao lado nordestino do Departamento.

- Em especial, agradeço à **Priscila Almeida** pela amizade. Não me lembro de ter medido esforços para me ver bem. Dividimos conquistas, angústias, incertezas e felicidades. Compartilhamos amostras e o trabalho até tarde da

noite, madrugada e no raiar do dia. Sua alegria contagiante tornava a rotina mais agradável. Você foi uma grande surpresa, um grande presente!

- Aos amigos do doutorado **Ana Karla**, **Christiane Cruz**, **Claudia Tavares**, **Cristiana Aroeira**, **Erika Kuchler**, **João Farinhas**, **Lívia Azeredo**, **Marcia Thomaz**, **Marlus Cajazeira**, **Thais Soares**, **Marcello Roter**, **Michele Lenzi**, **Michelle Ammari**, **Rafael Pedro**, **Raquel Santos**, **Valeria Abreu**, mesmo em meio à correria, foi muito bom dividir esse tempo e espaço com vocês. Vou levá-los comigo no coração.

- Em especial, agradeço à **Adílis Alexandria** pela amizade, parceria, companheirismo, pelas conversas filosóficas e ideias compartilhadas. Você é muito querida.

- À amiga **Viviane Pierro**, por quem tenho tanto carinho. Uma pessoa determinada e batalhadora que me ensinou muito com sua dedicação.

"Cada um que passa em nossa vida, passa sozinho, mas não vai só, nem nos deixa sós; leva um pouco de nós mesmos, deixa um pouco de si mesmo."
(Antoine de Saint-Exupéry)

- Às companheiras da linha de pesquisa **Priscila Almeida**, **Luciana Pereira**, **Luciana Pomarico**, **Carla Martins**, **Livia Oliveira** e **Valeria Abreu** por aceitarem o desafio e vivenciarem as mesmas dificuldades e alegrias desse projeto tão inovador.

"Prefiro mergulhar no desconhecido a viver na monotonia do saber que se acomoda."
(Leo Cruz)

- Aos funcionários e amigos da disciplina de Odontopediatria: **Andrea**, **Isabel**, **João Carlos**, **Katia Seixas**, **Luiza Queiroz**, **Maria José (Zezé), Mery**, **Robson**, **Rose** e **Patricia** por sempre estarem próximo, auxiliando no que fosse possível, sempre com bom humor e boa vontade, mais que funcionários, tornaram-se amigos.
- Aos amigos que fiz no laboratório do CNRMN: **Adolfo Moraes**, **Aline Batista**, **Anwar Iqbal**, **Carolina Cruzeiro**, **Fabrício Cruz**, **Gisele Amorim**, **Karen Santos**, **Luciana Machado**, **Mariana Quezado**, **Thalita Lopes**, **Rosane**

Andrade e **Viviane Paula** pelo auxílio e entusiasmo desde o meu mestrado. Vocês têm a habilidade de transformar dias difíceis em agradáveis tardes de alegria e bom humor. Obrigada pela convivência tão agradável!

"O bom humor espalha mais felicidade que todas as riquezas do mundo. Vem do hábito de olhar para as coisas com esperança e de esperar o melhor e não o pior."
(Alfred Montapert)

- À **Profª Drª Lucianne Cople** com quem tive o grande prazer de conviver e dividir alegrias, tristezas, conquistas e perdas. Sou muitíssimo feliz por ter tido o privilégio de participar de tantos projetos idealizados por você que trouxeram tantos avanços para a nossa FO-UFRJ. Obrigada por acreditar e confiar sempre em mim. Digo e repito: você tem uma capacidade incrível de me gerar uma inquietude que me faz avançar; agradeço também por isso! Se mantenha assim do jeitinho que você é: brilhante, transparente, entusiasta e intensa em tudo que faz!

"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis."
(Fernando Pessoa)

- À **Profª Drª Laura Primo** uma das grandes responsáveis por eu ter chegado até aqui! Obrigada por ter me permitido permear por esse mundinho mágico. Agradeço por ter visto em mim potencial, por sempre acreditar. E mesmo trabalhando em outra linha de pesquisa, você nunca me poupou palavras de ânimo para me impulsionar até aqui. Você é muito querida e quero sempre ser a "agregada das pulpectomias"

"Eis o meu segredo: só se vê bem com o coração. O essencial é invisível aos olhos."
(Antoine de Saint-Exupéry)

- À **Profª Drª Glória Castro** que com um senso de humanidade ímpar sempre está por perto dos alunos e pacientes doando seu tempo e atenção. Admiro sua competência e serenidade. Obrigada pelo aprendizado ao longo do curso.

"Três paixões, simples mas irresistivelmente fortes, governam minha vida o desejo imenso de amar, a procura do conhecimento e a insuportável compaixão pelo sofrimento da humanidade."
(Bertrand Russel)

- Às **Prof[as] Nena** e **Fátima** que nunca recusaram um sorriso, mesmo em momentos adversos. Vocês são sinônimos de felicidade e exemplo de amor pelo que fazem. Como a clínica era animada com vocês por perto!

Gosto de gente bem humorada, de riso fácil, de abraço apertado. Gente de coração grande que faz amigos só pela amizade e ama só pelo amor!
(Tamara Nascimento)

- Às fonoaudiólogas **Cíntia** e **Fernanda** e à psicóloga **Neyde** pela ajuda profissional e palavras constantes de incentivo.

- Aos **Profs Drs Andrea Quirino**, **João Farinhas**, **Kátia Dias**, **Maristela Portela**, **Silvia Alencar**, **Vanessa Moraes** obrigada pelos conhecimentos transmitidos, por toda atenção, palavras de carinho.

"Felicidade! É inútil buscá-la em qualquer outro lugar que não seja no calor das relações humanas. Só um bom amigo pode levar-nos pela mão e nos libertar."
(Antoine de Saint-Exupéry)

- À **Prof[a] Dr[a] Andrea Antonio** por sempre ter palavras gentis, por compartilhar ideias e a vivência no laboratório. Obrigada pelo carinho que sempre demonstrou ter para comigo. Admiro muito a sua determinação e comprometimento com tudo o que faz.

É graça divina começar bem. Graça maior é persistir na caminhada certa. Mas graça das graças é não desistir nunca.
(Dom Helder Camara)

- Ao **Prof Dr Rogerio Gleiser** por quem tenho tanto carinho e admiração. Sempre se mostrou disposto a ajudar no que fosse preciso. Nunca economizou seu tempo e disposição para ler meus trabalhos e opinar, mesmo não sendo da linha de pesquisa. Agradeço muito por todo incentivo desde o mestrado.

"Tudo o que um sonho precisa para ser realizado é alguém que acredite que ele possa ser realizado."
(Roberto Shinyashiki)

- Ao **Prof Dr Marcelo Castro** que me acolheu desde o curso de extensão em Odontopediatria na FO-UFRJ. Foi ele quem me deu a primeira oportunidade na docência, como professora de seu curso de extensão. Sempre com um

jeito irreverente, consegue transformar o ambiente ao seu redor e tornar o dia-a-dia mais leve. Obrigada por todo apoio!

"Transportai um punhado de terra todos os dias e fareis uma montanha."
(Confúcio)

À equipe de professores da FO-UERJ **Profs Ana Paula Pires**, **Branca Vieira**, **Fernanda Fidalgo**, **Luiz Flavio Moliterno**, **Marialice**, **Michele Lenzi**, **Mirian Souchois, Sonia** e **Vera Campos** por me permitir fazer parte dessa equipe, pelo convívio harmonioso e enriquecedor. Em especial, à Prof[a] **Vera Sovieiro** com quem aprendi lições não apenas para a clínica, mas para a vida. Te adimiro demais como pessoa e profissional!

"Ensinar não é transferir conhecimento, mas criar as possibilidades para a sua própria produção ou a sua construção." (Paulo Freire)

- À equipe de professoras e amigas da FO-UVA **Andrea Valente**, **Lucia Andrade**, **Marcia Thomaz** e **Patricia Tannure** com quem divido o trabalho e momentos de descontração. Foi uma grata surpresa o trabalho ao lado vocês; tenho muito orgulho da nossa equipe! Obrigada pela confiança e amizade, vocês são especiais!

Escolhe um trabalho de que gostes, e não terás que trabalhar nem um dia na tua vida.
(Confúcio)

- Aos **alunos** da FO-UFRJ, FO-UERJ e da especialização da FO-UVA que me deram a oportunidade de compartilhar conhecimentos e participar da formação de cada um. Essa experiência tem sido muito prazerosa e gratificante; e só me fazem ter a certeza que estou no caminho certo.

A alegria não chega apenas no encontro do achado, mas faz parte do processo da busca. E ensinar e aprender não pode dar-se fora da procura, fora da boniteza e da alegria.
(Paulo Freire)

- Aos **pequenos pacientes**, sou grata por permitirem entrar em seus pequenos mundos.

"Pequena criança, pura e confiante, volto a ser quando meus olhos encontram os olhos de pequenos infantes."
(Autor desconhecido)

- À **FO-UFRJ** que me acolheu e, de fato, se transformou na minha segunda casa. Lembro bem quando cheguei cheia de sonhos à FO-UFRJ há exatos dez anos. Não sabia o que estaria por vir e, apesar de jovem, a única certeza que tinha era a paixão pela pesquisa e docência. Nessa casa construí amizades sólidas que vou levar para vida toda, além de ter conquistado tudo o que tenho hoje. Com o decorrer do tempo, aqui me graduei, me tornei mestre e, agora, doutora. Tenho muito orgulho de carregar essa bandeira!

  "Na universidade se ensina porque se pesquisa"
  (Carlos Chagas)

- À **CAPES** e posteriormente à **FAPERJ** pela bolsa de estudos concedida.

AGRADECIMENTO ESPECIAL

- À **Profª Drª Liana Fernandes** pelo apoio constante, por estar sempre por perto desde os momentos mais felizes aos mais difíceis. Por diversos momentos suas palavras me deram força e coragem para prosseguir. Sem você nada disso teria sido possível. Agradeço por todo incentivo, por todo gesto de entrega e generosidade. Obrigada por sonhar junto comigo e por ousar a realizar. Certamente, juntas chegamos mais longe!

  "Muitas coisas não ousamos empreender por parecerem difíceis; entretanto, são difíceis porque não ousamos empreendê-las."
  (Lucius Annaeus Seneca)

- À **Profª Drª Ana Paula Valente** e ao **Prof Dr Fábio Lacerda**, que me receberam tão bem e não apenas me abriram as portas do laboratório, mas me fizeram sentir muito à vontade nele. Obrigada por me proporcionarem essa experiência, sem dúvida foi uma oportunidade única e um voto de confiança muito importante pra mim. Admiro a entrega de vocês e amor pelo que fazem. Obrigada por me confiarem esse projeto e por terem contribuído de todas as formas para que ele fosse bem sucedido!

  "O que está em jogo é muito mais que realizar coisas; é transformar a própria existência em um ato criador.
  (Miriam Subirana)

- À **Profª Drª Ivete Pomarico**, por quem tenho imensa admiração, carinho e respeito. Ao longo desses anos pude observar e aprender com o exemplo de uma líder integra e sábia que não mede esforços para o crescimento do Departamento de Odontopediatria e Ortodontia da FO-UFRJ. O que me emociona nessa relação orientador-orientando é que além do cuidado com os trabalhos, ela se preocupa com o bem-estar e crescimento do aluno. Tenho muito orgulho de ter sido sua orientanda. E como agradecer por tantas oportunidades ímpares a mim concedidas? Sou realmente muito grata a senhora por tudo! Obrigada por ter acreditado e, mais que isso, ter investido no meu potencial.

  "E se ainda eu não consigo explicar você pra mim, eu simplesmente aceito e agradeço."
  (Marla de Queiroz)

"Suba o primeiro degrau com fé. Não é necessário que você veja toda a escada. Apenas dê o primeiro passo."
(Martin Luther King Jr.)

"Não tenho palavras pra agradecer Tua bondade
Dia após dia me cercas com fidelidade
Nunca me deixes esquecer
Que tudo o que tenho, tudo o que sou
O que vier a ser vem de Ti Senhor"
(Diante do Trono)

RESUMO

FIDALGO, Tatiana Kelly da Silva. **Componentes salivares e fatores de risco associados à cárie dentária.** Rio de Janeiro, 2014. Tese (Doutorado em Odontologia – Área de concentração: Odontopediatria) – Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2014.

O objetivo do presente estudo foi analisar os componentes salivares e microbiota associada à cárie. Foram realizadas duas revisões sistemáticas da literatura para avaliação da associação de IgA-s não específica e lipídios com cárie dentária. Para os estudos experimentais, foi aplicado um questionário direcionado aos pais das crianças até 71 meses de idade da Clínica de Odontopediatria da FO-UFRJ a fim de avaliar dados demográficos, hábitos de dieta e higiene. Para avaliação de cárie, adotou-se o índice de superfícies cariadas, estraídas e obturadas (ceos) para dentes decíduos. A saliva total não estimulada de crianças com e sem cárie foi coletada para avaliação da microbiota e dos metabólitos de baixo peso molecular. As crianças com cárie foram submetidas a tratamento dentário. Foram avaliados os parâmetros salivares de crianças com cárie antes do tratamento, após 7 dias, 1 mês, 2 meses e 3 meses decorridos do tratamento. Durante a coleta da saliva total não estimulada, o tempo foi contabilizado a fim de avaliar o fluxo salivar. Após a coleta, as amostras de saliva foram plaqueadas a fim de quantificar os níveis de *Streptococcus muta*ns e *Lactobacillus sp*. O restante da saliva foi submetido à análise por Ressonância Magnética Nuclear (RMN) por meio da aquisição de espectros ^{1}H-RMN. Procedeu-se a análise estatística não paramétrica para a microbiota e fluxo salivar e os dados de RMN foram submetidos ao o método dos mínimos quadrados parciais para análise discriminante. Com relação às revisões sistemáticas, foi demonstrado que indivíduos com cárie apresentavam níveis aumentados de IgA-s e de lipídeos. O estudo experimental demonstrou que crianças com cárie apresentavam tempo prolongado de amamentação artificial com conteúdo cariogênico ($p > 0,05$). Foram observadas maiores contagens de microrganismos e metabólitos como propionato, ácido graxo, acetato e butirato em crianças com cárie ($p < 0.05$). Após o tratamento dentário, houve uma redução da microbiota e dos metabólitos, no entanto ainda eram superiores comparados aos de crianças que nunca tiveram cárie ($p > 0,05$). O presente estudo demonstrou diferenças salivares em indivíduos com cárie antes e após o tratamento dentário.

DESCRITORES: Saliva, Metaboloma, Cárie dentária, Lipídeo, IgA, Criança, Espectroscopia de Ressonância Magnética.

SUMMARY

FIDALGO, Tatiana Kelly da Silva. **Salivary components and risk factors associated to dental caries.** Rio de Janeiro, 2014. Tese (Doutorado em Odontologia – Área de concentração: Odontopediatria) – Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2014.

The aim of this study was to analyze the salivary components and microbiota associated with dental caries. It was performed two systematic reviews of the literature to assess the association between unspecific s-IgA and lipids with dental caries. For experimental study, a questionnaire was applied each child until 71 months of age from Pediatric dentistry clinic of FO-UFRJ parent to assess demographics, dietary habits, and hygiene. For evaluation of caries, it was adopted the index for decayed, missing and filled surface for primary teeth(dmft). Unstimulated whole saliva of healthy children with and without caries was collected for evaluation of microbiota and low molecular weigh metabolites. Children with caries were submitted to dental treatment. The salivary parameters were evaluated after 7 days, 1 month, 2 months, and 3 months after dental treatment. During the collection of unstimulated whole saliva, the time was recorded to assess salivary flow rate. After saliva collection, the samples were plated in agar to quantify the levels of *Streptococcus mutans* and *Lactobacillus sp*. The remaining saliva was analyzed by Nuclear Magnetic Resonance (NMR) by acquiring ^{1}H-NMR spectra. Nonparametric statistical analysis was preceded for microbiota and salivary flow rate and NMR data were submitted to partial least squared discrimnant analysis. The systematic review showed that subjects with dental caries had increased levels of s-IgA and lipids. Regarding experimental study, children with caries had prolonged artificial breastfeeding with cariogenic content ($p > 0.05$). Higher counts of microorganisms and metabolites, such as propionate, fatty acid, acetate, and butyrate were observed in caries children ($p < 0.05$). After dental treatment there was a reduction in microbiota and metabolites levels, however it was still higher in comparison to children who never had caries experience ($p > 0.05$). The present study demonstrated salivary differences between subjects with dental caries before and after dental treatment.

KEY-WORDS: Saliva, Metabolome, Tooth caries, Lipids, IgA, Children, Magnetic Resonance Spectroscopy.

RESUMEN

FIDALGO, Tatiana Kelly da Silva. **Componentes salival y factores de riesgo asociados con la caries dental.** Rio de Janeiro, 2014. Tese (Doutorado em Odontologia – Área de concentração: Odontopediatria) – Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2014.

El objetivo de este estudio fue analizar los componentes salivales y microbiota asociadas con caries. Se recolecto saliva no estimulada de los niños sanos con y sin caries. Dos revisiones sistemáticas de la literatura se llevaron a cabo para evaluar la asociación de s-IgA. Para estudios experimentales, fue realizado un cuestionario a los padres de los niños hasta 71 meses de edad Clinic de Odontología Pediátrica del FO-UFRJ para evaluar datos demografícos, los hábitos alimentarios y de higiene. Para la evaluación de las caries, fue adoptada el índice para superficies cariadas, extraídas y obturadas para los dientes temporales (ceos). Los niños con caries dental fueron sometidos a tratamiento. Se evaluaron los parámetros salivales después de 7 días, 1 mes, 2 meses y 3 meses de tratamiento transcurrido para evaluar la microbiota y metabolitos de bajo peso molecular. Durante la recogida de toda la saliva no estimulada, se registró el tiempo para evaluar el flujo salival. Después de la recogida, las muestras de saliva se colocaron en placas para cuantificar los niveles de *Streptococcus mutans* y *Lactobacillus sp*. El resto de la saliva se analizó mediante Resonancia Magnética Nuclear (RMN) mediante la adquisición de espectros de ^{1}H - RMN. Procedió a un análisis estadístico paramétrico de la microbiota salival, flujo y datos de RMN fueron sometidos a método de los mínimos cuadrados parciales para el análisis discriminante. Las revisiones sistemáticas de la literatura demostrado que los individuos con caries presentaron niveles aumentados de lípidos y s-IgA. El studio experimentale demonstró que los niños con caries habían prolongado la lactancia materna con el contenido cariogénico artificial ($p > 0,05$). Se observaron recuentos altos de microorganismos y metabolitos, tales como propionato, butirato, ácido graso y acetato en los niños com caries ($p < 0,05$). Después del tratamiento dental se observo una reducción de la microbiota y metabolitos, pero fueron aún más altos en comparación con los niños que nunca tuvieron caries ($p > 0,05$). El presente estudio demostró diferencias salivales en individuos con caries antes e después Del tratamiento dental.

PALABRAS CLAVE: Saliva, Metaboloma, Caries dentales, Lípidos, IgA, Niño, Espectroscopia de Resonancia Magnética.

LISTA DE FIGURAS

Artigo 1

Artigo 2

Artigo 3

LISTA DE TABELAS

Artigo 1

Artigo 2

Artigo 4

LISTA DE SIGLAS

CD14	Cluster of differentiation 14
ceo-s	Cariado, perdido, obturado – dente decíduo
CPMG	Carr–Purcell–Meiboom–Gill
D_2O	Deuterium oxide
DSS	Sodium2,2-Dimethyl-2-Silspentane-5-Sulfonate
FO-UERJ	Faculdade de Odontologia da Universidade Estadual do Rio de Janeiro
FO-UFRJ	Faculdade de Odontologia da Universidade Federal do Rio de Janeiro
FO-UVA	Faculdade de Odontologia da Universidade Veiga de Almeida
FID	Free Induction Decay
Gbpb	Glucosyltransferase (Glucosiltransferase)
IESC	Instituto de Estudos em Saúde Coletiva
IPPMG	Instituto de Puericultura e Pediatria Martagão Gesteira
IgA	Imunoglobulina A
IgA-s	Imunoglobulina A secretória
µl	Microlitro
ml	Mililitro
MG-1	Mucin glycoprotein 1 (Mucoglicoproteína 1)
NMR	Nuclear Magnetic Resonance
1D ^{1}H-NMR	One dimensional spectrum of Nuclear Magnetic Resonance
RMN	Ressonância Magnética Nuclear
PLS-DA	Partial least squared-discrimnant analysis
PRP	Protein rich prolin (proteína rica em prolina)
TCLE	Termo de Consentimento Livre e Esclarecido
TOCSY	^{1}H-^{1}H total correlation

LISTA DE SÍMBOLOS

δ	Chemical Shift (Deslocamento químico)
=	Igual
±	Mais ou menos
>	Maior que
<	Menor que

Sumário

1 INTRODUÇÃO

A cárie dentária é a doença crônica mais comum na infância (Misra, Tahmassebi et al., 2007). A evolução da doença é capaz de causar grande destruição das superfícies dentárias ou até mesmo sua perda, podendo resultar em complicações locais, sistêmicas, psicológicas e sociais. A cárie precoce de infância é um termo utilizado para substituir as designações cárie de mamadeira, cárie do aleitamento e cárie rampante (Păsăreanu, 2007). Esta, caracteriza-se pelo rápido desenvolvimento da lesão com a presença de uma ou mais cavidades nos dentes decíduos de crianças com idade inferior ou igual a 71 meses (Wyne, 1996; Rosenblatt e Zarzar, 2002; Peretz e Gluck, 2006; Aapd, 2011). Em algumas populações, é considerada um problema de saúde pública, uma vez que observa-se o fenômeno da polarização da doença. Suas consequências podem afetar a curto e longo prazo a qualidade de vida das crianças acometidas e de seus responsáveis (Martins-Junior, Vieira-Andrade *et al.*, 2013).

Para a prevenção dessa doença, é necessário conhecer sua etiologia e os fatores de risco para o seu desenvolvimento (Touger-Decker e Van Loveren, 2003). A literatura destaca distintas variáveis que associam-se a esta problemática, como: a microbiota, a dieta, o hospedeiro, fatores socioeconomicosociais, estrutura dental, utilização de fluoretos e possíveis alterações de metabólitos salivares que promovem o desequilíbrio bioquímico da cavidade bucal (Valaitis, Hesch et al., 2000; Baginska e Stokowska, 2006; Schroth, Brothwell et al., 2007; Irigoyen Camacho, Sanchez Perez et al., 2009; Svec, Sedlacek et al., 2009). No processo de cárie, o biofilme dental compreende um microecosistema de microrganismos que apresentam características fisiológicas que favorecem a colonização por meio das suas propriedades que propiciam a adesão e resistência a baixos níveis de pH (Gudino, Rojas *et al.*, 2007). Dentre a microbiota presente, os *Streptococcus mutans* são frequentemente isolados de lesões cavitadas de cárie. Quando presentes em ambiente rico em sacarose, proveniente da dieta, são altamente acidogênicos, tornando estes microrganismos agentes patognêicos principais o início da cárie dentária. Os *Lactobacillus sp* destacam-se pela progressão da doença e são

selecionados posteriormente pela acidez do microambiente (Mattos-Graner, Smith *et al.*, 2000; Nobre Dos Santos, Melo Dos Santos *et al.*, 2002; Takahashi e Nyvad, 2008).

Nesse contexto, a saliva exerce um importante papel na homeostase da cavidade bucal. As mudanças salivares em geral podem modular as alterações nos tecidos dentais. Isto porque as modificações bioquímicas no fluido salivar podem influenciar no risco à cárie dentária, alterando não somente a viscosidade, o pH e a capacidade tampão, mas também os componentes presentes neste biofluido (Dale, Tao *et al.*, 2006). A literatura relata que a variabilidade nas proteínas salivares pode exercer um importante papel na determinação da resposta imune não específica, realizando sua função protetora contra a cárie dentária (Banderas-Tarabay, Zacarias-D'oleire *et al.*, 2002). Neste sentido, tem sido demonstrado que a formação de complexos entre moléculas como MG-1, amilase salivar, PRPs, e a estaterina são determinantes na iniciação do biofilme e na instalação da cárie dentária (Nieuw Amerongen, Oderkerk *et al.*, 1987). Para atividade de cárie subclínica é possível detectar a ausência da proteína solúvel CD14, possível biomarcador por estar envolvida na resposta imune inata (Bergandi, Defabianis *et al.*, 2007) que retorna a seus níveis normais após restauração das lesões cavitadas. No que tange a resposta do hospedeiro em detrimento da interação microrganismo-hospedeiro na cavidade bucal, componentes da resposta imune exercem importante papel na produção de fatores de proteção específicos contra determinados antígenos, como as imunoglobulinas A secretória (IgA-s). A IgA-s é responsável pela primeira linha de imunidade adaptativa contra os antígenos do *Streptococcus mutans*. Além disso, a IgA-s pode favorecer a atividade de enzimas como a lactoferrina, a peroxidase e a lisozima, que atuam na atividade antimicrobiana, neutralizando virus e toxinas e inativando enzimas associadas a colonização do *streptococcus mutans* (Law, Seow *et al.*, 2007). Nogueira *et* al. (2008) demonstraram que a presença expressiva de resposta imunológica de IgA-s salivar para GbpB pode ocorrer durante o primeiro ano de vida. Também foi demonstrado que esta resposta estava diretamente associada ao atraso na infecção com *Streptococcus mutans* (Parisotto, King et al., 2011). Alguns autores relataram níveis mais elevados de IgA-s em indivíduos sem cárie comparados aos com atividade de cárie, sugerindo uma função de proteção eficaz (Orstavik e Brandtzaeg, 1975; Rose, Gregory et al., 1994; Fernandes, Nagao et al., 1995). Por outro lado, outros autores não observaram correlação entre

atividade de cárie e os níveis de IgA-s (Krasse e Gahnberg, 1983; Olsson e Svanberg, 1991). Apesar do grande número de investigações sobre o tema, ainda não é possível afirmar se os altos níveis de IgA-s atuam como um fator de proteção ou como marcadores da presença da doença, constituindo uma resposta do hospedeiro aos microrganismos (Shifa, Muthu *et al.*, 2008; Thaweboon, Thaweboon *et al.*, 2008; Kirtaniya, Chawla *et al.*, 2009; Chawda, Chaduvula *et al.*, 2010; Chopra, Jadhav *et al.*, 2011; Parisotto, King *et al.*, 2011; Ranadheer, Nayak *et al.*, 2011; Bagherian e Asadikaram, 2012; Omar, Khattab *et al.*, 2012).

Embora a cárie dentária ainda seja muito prevalente, é muito comum algumas crianças permanecem livres de cárie, mesmo sem hábitos adequados de higiene, assim como crianças com boa higiene bucal apresentarem elevado ceod ou CPOD (World Health Organization. World Health Organization. Oral health surveys: basic methods, 1997). Este tem sido um tema de grande interesse no campo da pesquisa odontológica durante décadas. Apesar da grande quantidade de informações sobre a etiologia macromolecular que envolve a doença e crescentes estudos sobre componentes salivares envolvidos nesse processo, ainda são escassos os dados disponíveis sobre os metabólitos de baixo peso molecular e sua relação com a saúde bucal (Hardt, Thomas *et al.*, 2005; Fidalgo, Freitas-Fernandes *et al.*, 2013). Estudos recentes têm demonstrado o potencial diagnóstico de metabolitos salivares de baixo peso molecular voltado para avaliação do estado sistêmico (Brindle, Antti *et al.*, 2002; Silwood, Lynch *et al.*, 2002; Takeda, Stretch *et al.*, 2009). Neste contexto, pela primeira vez, Fidalgo et al. (2013) observaram metabolitos salivares característicos de indivíduos com cárie dentária, como ácido graxo, acetato, butirato, propionato e o aumento de açúcares, sendo possível classificar crianças com e sem cárie por meio desses metabólitos. Entretanto, assim como o IgA-s, não se sabe se esses metabólitos traduzem uma suscetibilidade do hospedeiro ou se são resultantes da atividade da doença. Diante do que foi exposto, torna-se relevante avaliar se existe associação entre os componentes salivares e cárie dentária, assim como avaliar possíveis fatores de risco essa doença.

2 PROPOSIÇÃO

2.1 Objetivo Geral

- Identificar componentes salivares associados à cárie, assim como fatores de risco para a doença.

2.2 Objetivos Específicos

- Avaliar por meio de revisão sistemática da literatura se os níveis de IgA-s salivar estão relacionados à cárie dentária;

- Avaliar por meio de revisão sistemática da literatura se lipídeos salivares estão relacionados à doença cárie;

- Avaliar os níveis de metabólitos salivares de baixo peso molecular de crianças saudáveis e com cárie de acometimento precoce antes e após o tratamento dentário;

- Avaliar os fatores de risco associados à cárie dentária em crianças saudáveis com e sem cárie dentária.

3 DELINEAMENTO DA PESQUISA

Algumas crianças permanecem livres de cárie, mesmo sem hábitos adequados de higiene, assim como outras crianças com hábitos de higiene mais rígidos apresentam elevado índice de cárie. Partindo-se da hipótese de que componentes imunológicos do hospedeiro podem modular a instalação da cárie dentária, o primeiro estudo consistiu em uma revisão sistemática da literatura sobre o papel protetor da IgA-s salivar em relação à cárie dentária. Para a revisão, uma busca eletrônica e manual foi realizada em cinco bases de dados, sendo elas PubMed, Isi Web of Science, Scopus, Cochrane e Lilacs com a pesquisa manual das referências dos artigos selecionados. A avaliação da qualidade dos artigos incluídos foi realizada após os mesmos terem preenchido os critérios de inclusão. Inicialmente foram filtrados 314 resumos, sendo que 15 preencheram os critérios de inclusão: presença de grupo controle e caso, método ideal de diagnóstico de cárie, analise da concentração de IgA-s de ambos os grupos e análise estatística Após a leitura dos artigos, um foi excluído devido à falta de grupo controle. Após a avaliação da qualidade metodológica, sete artigos foram incluídos na meta-análise para avaliação estatística dos trabalhos.

O segundo estudo teve por objetivo avaliar um dos componentes relacionados à cárie dentária, os lipídeos. Esse componente salivar, ainda pouco explorado na literatura, foi encontrado em maiores quantidades em crianças com cárie (Fidalgo, Freitas-Fernandes *et al.*, 2013). Assim, esse estudo objetivou fazer uma revisão sistemática da literatura a fim de avaliar a associação de lipídeos em geral com a atividade de cárie dentária. Para tanto, foi realizada uma busca sistemática e manual da literatura nas bases de dado PubMed, Web of Science, Cochrane e Ovid. A elegibilidade dos estudos foi determinada após a leitura dos resumos dos artigos identificados a partir dos bancos de dados eletrônicos. A avaliação da qualidade foi realizada por meio da classificação dos artigos selecionados em A, B ou C (qualidade metodológica alta, moderada e baixa, respectivamente). Após a leitura de 65 resumos para verificar se eles preencheram os critérios de inclusão, foram incluídos 5 artigos. Posteriormente, dois artigos foram excluídos, sendo um devido à duplicidade da amostra e o outro, em função da avaliação lipídica ter sido realizada em biofilme, ao invés de saliva. Os três artigos remanescentes foram, em seguida,

avaliados e classificados de acordo com sua qualidade metodológica e risco de vieses.

O terceiro estudo objetivou avaliar os metabólitos salivares de crianças com e sem cárie antes e após tratamento dentário por meio de RMN. Este estudo foi realizado após a aprovação pelos Comitês de Ética do IPPMG e do Instituto de Estudos em Saúde Coletiva (IPPMG parecer 23/07 e IESC parecer 130/09; Anexo 1 e 2, páginas 114 e 115, respectivamente). Previamente às entrevistas (Apêndice 1, página 127), todos os participantes assinavam o Termo de Consentimento Livre e Esclarecido (Apêndice 2, página 130).

Para esse experimento, foram avaliados os metabólitos salivares relacionados à cárie antes e após tratamento dentário, utilizando como ferramenta a RMN. Foram avaliados os metabólitos descritos como potenciais biomarcadores de cárie dentária, descritos previamente por Fidalgo et al. (2013) (Anexo 3, página 116). Este estudo caracteriza-se como sendo do tipo clínico controlado de caráter longitudinal. A seleção da amostra adotou o critério de amostra por conveniência. Os critérios de inclusão foram crianças na dentição decídua até 71 meses de idade sem alterações sistêmicas, sem doença periodontal ou demais alterações bucais. Os participantes também não deveriam fazer uso de agentes antimicrobianos durante 3 meses anteriores à coleta salivar, assim como jejum 1 hora previamente à coleta. As crianças de ambos os grupos pertenciam à creche-escola do Instituto de Puericultura e Pediatria Martagão Gesteira (IPPMG) e à clínica de Odontopediatria (FO-UFRJ). Foram examinadas e incluídas inicialmente no estudo 57 crianças com e sem cárie, sendo excluídas 14 crianças devido às mudanças no plano de tratamento e também devido ao início de terapia antibiótica antes do final do tratamento dentário. Aos participantes do estudo foi disponibilizado tratamento odontológico, quando indicado, conforme as necessidades encontradas, e realizadas medidas preventivas como fluorterapia e controle do biofilme dental. Aos pais e responsáveis foram fornecidas instruções sobre higiene bucal e orientações dietéticas. Inicialmente as crianças eram examinadas para constatar a presença ou ausência de cárie por inspeção visual em cadeira odontológica e com iluminação artificial. A seguir, a saliva total não estimulada era coletada com o paciente sentado na cadeira odontológica. A coleta era realizada por meio de pipetador automático devido às crianças de baixa idade não possuirem aptidão para expectoração. Posteriormente, a criança era submetida

a exame clínico mais detalhado por meio de sonda de ponta romba e espelho dental. Foi utilizado o índice de superfícies cariadas, perdidas e obturadas (ceo-s), conforme preconizado pela Organização Mundial da Saúde (OMS) (World Health Organization. World Health Organization. Oral health surveys: basic methods, 1997). Exames radiográficos eram realizados para avaliação das lesões cariosas e para descartar a possibilidade de envolvimento pulpar. Foram excluídas do estudo crianças que apresentavam superfícies restauradas e dentes com envolvimento pulpar ou extração indicada. As crianças com cárie eram submetidas a tratamento odontológico restaurador com resina composta (THP, Dentisply, USA) por ser um material mais inerte e com elevada resistência. Após 7 dias, 1 mês, 2 meses e 3 meses retornavam para realização de novo exame clínico para avaliação de reocorrência de cárie e nova coleta salivar.

As crianças de todos os grupos eram submetidas à coleta salivar após exame clínico, sendo 50µL de amostra pura e diluídas a 10^{-1}, 10^{-2} e 10^{-3} plaqueadas em 10 mL de meio ágar Mitis salivarius (Difco, Detroit, USA) com bacitracina, tellurito e suplementação de 15% de sacarore para *Streptococcus mutans*. Para Lactobacillus sp, 50 µL de amostra salivar pura e diluídas a 10^{-1} e 10^{-2} foram plaqueados em 10 mL de meio ágar Rogosa (Difco, Detroit, USA). As placas foram mantidas a 37°C, em microaerofilia por 48 horas. Após esse período, as colônias foram contabilizadas. O restante das amostras foi centrifugado (Centrifuge 5417C/5417R, Eppendorf, Hamburg-Germany) a 10.000*g* durante 60 minutos, a 4º C no Laboratório Multidisciplinar de Pesquisa em Odontologia (LMPO) da FO-UFRJ. Esta etapa objetivou a remoção de componentes não solúveis da amostra, além de grande parte dos microorganismos. O sobrenadante foi transferido em alíquota de 600µL para três tubos (Ependorffs, Hamburg-Germany) que foram armazenadas no congelador a -80 ºC até o momento da análise em RMN (Silwood, Lynch *et al.*, 2002). Essa baixa temperatura é suficientemente baixa para que a degradação se mantenha desprezível.

Previamente a análise em RMN, as amostras foram novamente centrifugadas a 3.000*g* durante 10 minutos, a 4º C A amostra final era composta de 610 µL, sendo 540µL de saliva, 60 µL de água deuterada (D_2O; Cambridge Isotope Laboratories inc., USA) e 10 µL de Dodecil Sulfonato de Sódio a 5mM (DSS; Sigma-Aldrich, Milwaukee, USA). O DSS é a referência para o deslocamento químico de hidrogênio,

δ = 0 ppm. Os espectros foram obtidos em um aparelho de RMN 400 MHz (Bruker Biospin, Rheinstetten, Germany), a 25ºC no Centro Nacional de Ressonância Magnética Nuclear (CNRMN). Nos experimentos de RMN de alta resolução com ondas pulsadas, os sinais de decaimento livre de indução (FID - *Free Induction Decay*) de vários pulsos podem ser somados (Scans), a fim de obter-se uma melhor relação sinal/ruído e, consequentemente, uma melhor resolução do espectro de RMN. Padronizou-se espectros 1D-^{1}H CPMG (Carr–Purcell–Meiboom–Gill) com 1024 scans para o hidrogênio. Utilizou-se ainda a sequencia de pulso PRESAT para a pressaturação do sinal da água (localizado a 4.7 ppm). Experimentos 1D-^{31}P CPMG com 512 scans também foram obtidos a fim de avaliar o pH das amostras salivares. Para tanto, foi obtida a equação da reta após realização da curva padrão (Apêndice 3, página 131). A curva padrão foi confeccionada por soluções de fosfatos baseadas na equação de Henderson-Hasselbalch com pH de 5,8 a 7,8 e com variação de pH de 0,1. Após a aquisição dos espectros, os dados de intensidade de cada pico de hidrogênio do espectro 1D-^{1}H CPMG foram extraídos por meio de um programa computacional (AMIX, Bruker Biospin, Rheinstetten, Germany) e processados estatisticamente. Para as análises, também foi utilizado o Metaboanalyst 2.0 (www.metaboanalyst.ca). O assinalamento foi realizados utilizando como referência o banco de dados Human Metabolome Database[1], os assinalamentos realizados por Silwood et al. 2002 e o pacote computacional Chenomx NMR Suite (Chenomx Inc., Edmonton, AB, Canadá). Algumas amostras também foram submetidas à técnica ^{1}H-^{1}H-TOCSY para visualização de ambiguidades e, para confirmação dos assinalamentos, foram realizados experimentos com a adição de compostos puros. A espectroscopia de ressonância magnética nuclear baseia-se na medida da absorção da radiação eletromagnética por um núcleo atômico, com spin diferente de zero, sob a influência de um campo magnético (Abrahan e Loftus, 1978). A técnica de ressonância magnética Nuclear segue melhor detalhada no Apêndice 4 (página 132).

O quarto estudo objetivou avaliar fatores de risco clínicos e microbiológicos relacionados á cárie dentária, como dieta, hábitos de higiene, utilização de fluoretos,

[1]Human Metabolome database (http://www.hmdb.ca/search/spectra?type=nmr_search) é um banco de dados que contem informações detalhadas sobre mais de 7900 metabólitos encontrados no corpo humano. Destina-se a utilização para aplicações em metabolômica, química clínica, descoberta de biomarcadores e educação geral.

avaliação do fluxo salivar e microbiota cariogênica. Para tanto, previamente a consulta, os responsáveis eram submetidos a uma entrevista contendo perguntas abertas e fechadas sobre as variáveis acima mencionadas (Apêndice 1, página 127) e também teve aprovação do comitê de ética em pesquisa local (IPPMG parecer 23/07 e IESC parecer 130/09; Anexo 1 e 2, páginas 114 e 115, respectivamente). A coleta salivar e análise da microbiota foi realizada como mencionado no estudo 3, assim como o tratamento dentário no grupo de crianças com cárie.

4 DESENVOLVIMENTO DA PESQUISA

4.1 **ARTIGO 1:** The relationship between unspecific s-IgA and dental caries: a systematic review and meta-analysis**.**

Tatiana Kelly da Silva Fidalgo[1]
Michelle Ammari[1,2]
Liana Bastos Freitas-Fernandes[3]
Claudia Trindade Mattos[4]
Ivete Pomarico Ribeiro de Souza[5]
Lucianne Cople Maia[5]

[1]PhD student, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil;
[2]Department of Specific Training, School of Dentistry, Universidade Federal Fluminense, Nova Friburgo, Brazil;
[3]Visiting Professor, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil;
[4]Adjunct Professor, Dental Clinics Department, School of Dentistry, Universidade Federal Fluminense, Niterói, Brazil;
[5]Chairman Professor, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil;

Correspondence author
Lucianne Cople Maia
Email: rorefa@terra.com.br
Disciplina de Odontopediatria da FO-UFRJ
Caixa Postal: 68066 - Cidade Universitária - CCS
CEP.: 21941-971 - Rio de Janeiro – RJ – Brasil

ABSTRACT

This systematic review and meta-analysis focused on evaluating the possible association of s-IgA levels and dental caries. An electronic and manual search was performed in PubMed, ISI Web of Science, Scopus, Cochrane Library, and Lilacs with supplemental hand search of the references of retrieved articles. Quality assessment and data extraction of the included articles were performed. Meta-analysis of pooled data was performed through RevMan software after a sensitivity analysis. From 314 abstracts, 14 fulfilled the inclusion criteria. After reading the full articles, one of them was excluded due to the lack of a control group. Seven studies were included in the meta-analysis and the heterogeneity among the studies (I^2) was 41%. The pooled meta-analysis demonstrated higher levels of s-IgA in the caries active group ($p < 0.00001$) than in the control group with a mean difference and confidence interval of 0.27 [0.17 – 0.38]. Based on these findings, there is evidence that support the presence of increased s-IgA levels in caries-active subjects.

Key-words: Saliva; Dental caries; IgA; Systematic review; Meta-analysis.

1. INTRODUCTION

Dental caries is a prevalent oral disease that is resulted from chronic exposure to the imbalance of multiple risk and protective factors over time.[1] The saliva is the major important intrinsic regulator host factor of dental caries which provides physical and biological defensive mechanisms.[2-4] Humoral immunologic response can regulate caries activity, especially salivary secretory immunoglobulin A (s-IgA). The ability of the pathogen to bind on salivary pellicle is the principal event to oral disease installation.[5] The s-IgA prevents the adherence of cariogenic microorganisms to hard surfaces and besides of inhibition the activity of glucosyltransferases (Gtf), it neutralizes viruses and toxins, inactivates enzymes, exclude antigen in saliva, and prevents activities which may affect cariogenic microrganisms colonization.[6]

The secretory immune response is microorganism-specific. In addition, this local sensibilization can lead to a cross-reacting epitopes potentializing the response already present.[7] Exposure to cariogenic microbiota leads to the secretory immune components against several microorganism epitopes and this binding is responsible to start the immunological response.[8] The binding reduces the free s-IgA in saliva from caries-active subjects in comparison with caries resistant ones, presumably being a salivary indicator of dental caries activity. The titration of salivary s-IgA levels as a caries diagnostic tool is largely explored in the literature, for both unspecific and especific s-IgA,[9-11] such as against *Streptococcus mutans* epitopes.[12, 13]

However, previous investigations have reported contradictory results regarding the association of salivary levels of s-IgA and dental caries. Some authors reported higher levels of salivary s-IgA in caries-resistant individuals in comparison to caries-susceptible ones, suggesting an effective protective function of this immunoglobulin.[10-12] On the other hand, some other investigations did not observe association between the presence of dental caries and salivary s-IgA levels.[9, 14] Based on the lack of conclusive information on dental caries immunity, this systematic review and meta-analysis focused on the evaluation of the possible association of s-IgA levels and dental caries.

2. MATERIAL AND METHOD

This systematic review and meta-analysis was registered in PROSPERO database (PROSPERO registry number: CRD42013005502).

2.1 Design and search strategy

The search process was performed independently by two examiners (TKSF and MMA) under the guidance of a librarian. The Cochrane Library, MEDLINE-PubMed, ISI Web of Knowledge, Scopus, Lilacs databases were searched for articles published until January 2014, without language restriction. The search strategy included appropriate changes in the key-words and followed the syntax rules of each database. The main key-words used were ‘‘saliva’’ (MeSH/DeCS), ‘‘immunoglobulin A’’ (MeSH), ‘‘caries’’ (uniterm), and ‘‘IgA’’ (uniterm). The booleans operators “AND” and “OR” were applied to combine the key-words. Specific related terms used and their combinations for each database are described in Table 1. Experts were also contacted to identify unpublished and ongoing studies. The searches were complemented by screening the references of selected articles to find any that did not appear in the database search.

Table 1: Search strategy in databases

Database	Mesh or Key-word
PubMed	#1 Search - (Saliva* [Title/Abstract]) AND (Caries [Title/Abstract]) AND (IgA [Title/Abstract]) #2 Search - (Saliva* [Title/Abstract]) AND (Caries [Title/Abstract]) AND (Immunoglobulin A [Title/Abstract]) #1 or #2 search
ISI Web of Science	#1 Search - Saliva* AND Caries AND IgA #2 Search - Saliva* AND Caries AND Immunoglobulin A
Cochrane	#1 Search - Saliva* AND Caries AND IgA OR #2 Search - Saliva* AND Caries AND Immunoglobulin A
Scopus	#1 Search - Saliva [Article Title/Abstract/Keyword] AND Caries [Article Title/Abstract/Keyword] AND IgA [Article Title/Abstract/Keyword] #2 Search - Immunoglobulin A [Article Title/Abstract/Keyword] – limited by Article as “Document type”
Lilacs	#1 Search - Saliva AND caries AND IgA #2 Search - Saliva AND caries AND Immunoglobulin A

2.2 Selection criteria

The inclusion criteria comprised clinical investigations with case (presence of dental caries) and control (absence of dental caries) group; a caries diagnostic method; with evaluation of unspecific s-IgA concentration by using tests for both groups (case and control) in humans, healthy subjects, and with statistical analyses. For dental caries assessment, it was included studies that applied dmft/DMFT or dmfs/DMFS index, in accordance with the World Health Organization criteria (WHO).[15] The Population, Exposition, Comparisons, Outcome, and Study design (PECOS) are explored in Table 2, as well as the null hypothesis.

Table 2: PECOS format and null hypothesis

Population	Patients with and without dental caries lesions
Exposition	Dental Caries
Comparison	Presence of IgA levels in saliva from subjects with and without dental caries lesions
Outcome	Levels of salivary IgA
Study design	Cross sectional
Null hypothesis	There is no difference between salivary IgA levels from subjects with and without dental caries

Case reports, case series, descriptive studies, review articles, opinion articles, letters, and articles that did not measure dental caries were excluded.

Studies which the IgA levels did not correspond to the aims of this review, such as specific ones against *S. mutans*, *Lactobacillus* and others, were excluded from this review. All records electronically identified were scanned by title and abstract. Eligibility of the selected studies was determined by reading the title and abstracts of the articles identified from the electronic databases. Full articles were retrieved and examined when their title and abstract did not provide enough information for a definite decision. Articles appearing in more than one database search were considered only once.

2.3 Quality assessment and control of bias and data extraction

After the inclusion of the abstracts that fulfilled the selection criteria and verification of their eligibility by reading the complete articles, the studies were submitted to the quality assessment.

The methodological quality assessment and control of bias of the studies were independently evaluated by two authors (TKSF and MMA). Full texts of all articles were obtained of all articles identified and judged. When any differences between the two readers occurred, it was solved by consensus. If relevant data were missing, the authors of the articles in question were contacted for additional information.

The quality assessment and bias control was carried out according to the guidelines described by Fowkes and Fulton.[16] This quality assessment allows the ranking of cross-sectional, cohort, controlled trial, and case-control studies. The guide provide a standardized approach to quality assessment and cover patient spectrum, disease progression bias, verification bias, review bias, clinical review bias, test execution, study withdrawals and indeterminate results. The checklist includes questions on study design, study sample representativeness, characteristics of the control group, quality of measurements and outcomes, completeness and distorting influences. When checking the criteria for each guideline, the importance of fails in terms of their expected effect on the results was scored as major (++) or minor (+), and a decision was made as to whether the methods were adequate to produce useful information or not. The confounding factors and bias were also scored. For items where the question was not applicable, “NA” was registered. This quality check provides summary questions for the soundness assessment.

The data of included papers were compiled and the following data were extracted: age of participants, sample size, caries index, s-IgA levels, analytic test used, dilution, kind of saliva, and statistic analysis.

Publication bias was assessed though the funnel plots by using the RevMan (Review Manager - RevMan - Computer program. Version 5.2. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012).

2.4 Meta-analysis

The meta-analysis was performed using the RevMan software (Review Manager - RevMan - Computer program. Version 5.2. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). The papers that presented the mean concentration of IgA, standard deviation, and the number of subjects for each group were included in the analysis. Since concentration was presented in different units, all measures were converted to µg/mL. Additionally, as different dilutions were used to perform ELISA analysis, these values were converted to percentage.

For each study, to calculate the percentage of IgA concentration we considered 100% the higher IgA concentration value (caries-free or caries-active

group) and after that we calculated the percentage for the lower concentration. The standard deviation was also converted in values compatible with IgA levels mantaining the same ratio according to the original values. In the studies that evaluated baseline and follow-up, the values from baseline were used. For the studies that evaluated individuals with low, moderate, and high caries, we used IgA values involved with the highest number of caries. A subgroup analysis was also performed grouping the studies into age groups (children and adults). Heterogeneity was assessed using the I^2 index, with significance set at $p < 0.01$. Since heterogeneity was significant, a sensitivity analysis was performed to explore the influence of the low quality studies on pooled data.

3. RESULTS

The search strategy (Figure 1) retrieved 703 articles. After duplicate separation, 314 studies remained in the review. After title and abstract reading, initially, 15[13, 17-30] papers fulfilled eligibility criteria and were selected for full text reading. After that, one study[17] was excluded after reading full paper due to absence of control group. The remained 14 studies were submitted to the quality assessment (Table 3). The summary questions presenting the soundness of studies showed that 14 studies[13, 18-26, 28-30] presented confounding factors. The most important confounding factor was the lack of distinction between decayed tooth/tooth surface and missing or filled tooth/tooth surface.

A summary of the characteristics of each included study and detailed findings are available in Table 4. In all retrieved studies, unstimulated and stimulated saliva were collected from the children and adult subjects. The caries index was also evaluated. The s-IgA levels were mainly assessed by ELISA assay using different dilutions rates.

The funnel plot of 13 articles that presented s-IgA concentrations values demonstrated a similar distribution of included studies and absence of publication bias (Figure 2). The Figure 3 shows the pooled meta-analysis of all thirteen studies[13, 19-24, 26-31] showed significant heterogeneity ($P < 0.00001$, $I^2 = 98\%$). Sensitivity analysis detected six studies that were mainly responsible for the heterogeneity.

Hence, sensitivity analysis was conducted, thereby avoiding heterogeneity. Figure 4, shows the included seven studies with a acceptable heterogeneity (I^2 = 41%).[13, 22, 24, 26-29] The caries-free group was composed by 102 subjects and the caries active by 201. The pooled meta-analysis demonstrated a higher levels of s-IgA in caries active group ($p < 0.00001$) with mean difference and confidence interval of 0.27 [0.17 – 0.38].

The analysis of subgroup according to the age showed high heterogeneity even after sensitivity test ($I^2 > 80\%$). Thus the subgroup analysis was not possible to be performed.

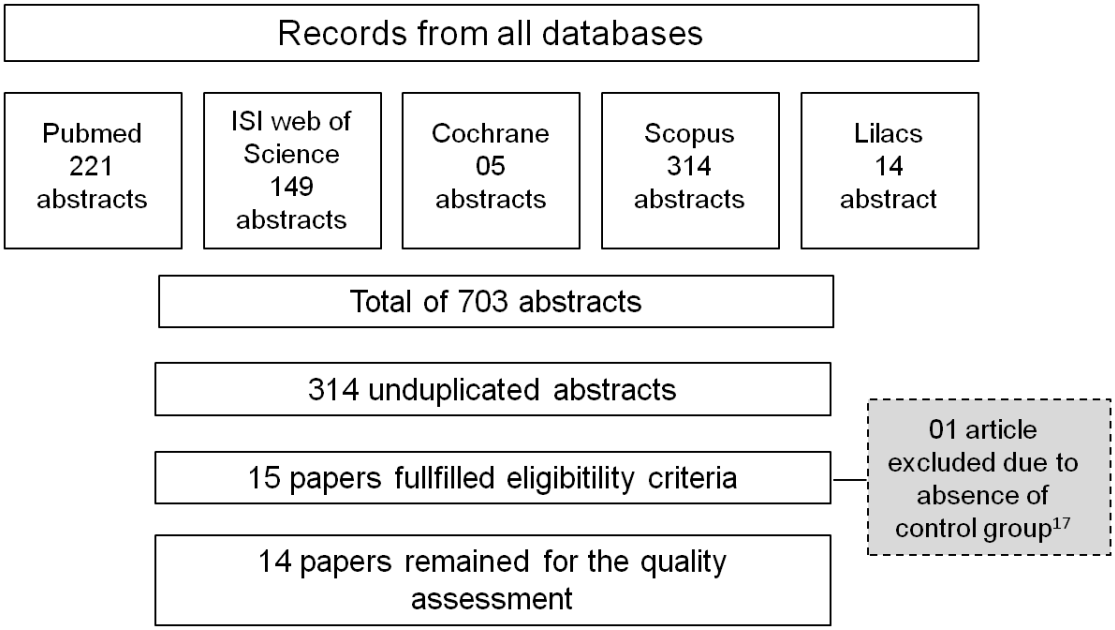


Figure 1: Flow diagram of literature search and selected papers.

Table 3: Methodological checklist for quality assessment and control of bias

Guideline	Checklist	Hagh et al.[18]	Bagherian et al.[19]	Ranadheer et al.[20]	Parisotto et al.[21]	Chopra et al.[22]	Thaweboon et al.[23]	Farias et al.[24]	Sikorska et al.[25]	Kirtaniya et al.[13]	Chawda et al.[26]	Omar et al.[27]	Hocini et al.[28]	Pal et al.[29]	Priya et al.[30]
Study design appropriate?	Cross sectional (Prevalence)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Cohort (Prognosis)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Controlled trial (Treatment)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Cohort, case-control, cross-sectional (Cause)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Study sample representative?	Source of sample	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sampling method	0	0	0	+	+	+	+	+	+	0	+	+	0	0
	Sample size	0	0	0	0	0	0	0	+	0	0	0	0	0	0
	Entry criteria and exclusions	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control group acceptable?	Definition of control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Source of control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Matching/randomization	0	0	0	+	+	+	+	+	+	0	+	+	+	+
	Comparable characteristics	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Quality of	Validity	0	0	0	0	0	0	0	0	0	0	0	0	0	0

measurements and outcomes?	**Reproducibility**	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Blindness	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Quality control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Completeness?	**Compliance**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Drop out	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Death	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Missing data	0	+	0	0	0	+	0	+	+	+	+	0	+	0
Distorting influence?	**Extraneous treatments**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	0
	Contamination	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	0
	Changes over time	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Confounding factors	+	+	+	+	+	+	+	+	+	+	0	+	+	+
	Distortion reduced by analysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Summary questions	**Bias - Are the results erroneously biased in a certain direction?**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Confounding - Are there any serious confounding or other distorting influences?	+	+	+	+	+	+	+	+	+	+	0	+	+	+
	Chance - Is it likely that the results occurred by chance?	0	0	0	0	0	0	0	+	0	0	0	0	0	0

Table 4: Detailed findings of retrieved studies.

Author, Year	Subjects							Saliva collection	Dilution	Test	Statistic test and p-value
	Caries-active				Caries-free						
	Age	Sample size	Caries index	s-IgA levels (µg/ml)	Age	Sample size	s-IgA levels (µg/ml)				
Hagh et al.[18], 2013	26.8 ± 5.61 years	25	11.12 ± 1.62 DMFT	60.2 ± 7.60	28.5 ± 7.07 years	15	123.2 ± 19.90	Unstimulated	1:20	ELISA	Kruskal–Wallis (P = 0.009)
Bagherian et al.[19], 2012	59.4 ± 12.09 months	45	9.3 ± 3.6 dmft	1,961.40 ± 1,000.70	60.9 ± 8.8 months	45	1,484.50 ± 811.60	Unstimulated	Missing data	ELISA	Pearson's and Spearman's rho correlation (p = 0.015)
Ranadheer et al.[20], 2011	12 – 18 years	20	4.5 ± 0.5 dmft	117.60 ± 18.0	12 – 18 years	20	75.90 ± 24.80	Unstimulated	1:100	ELISA	Pearson correlation (p = 0.050)
Parisotto et al.[21], 2011	Baseline: 3 – 4 years 1-year follow-up: 4 -5 years	17	> 3.0 dmft	Baseline: 150.30 ± 40.06 1-year follow-up: 181.97 ± 34.18	Baseline: 3 – 4 years 1-year follow-up: 4 -5 years	23	Baseline: 132.22 ± 19.09 1-year follow-up: 150.30 ± 40.06	Unstimulated	1:500	ELISA	Mann-Whitney (p = 0.0118)
Chopra et al.[22], 2011	24 - 55 years	88	> 3.0 DMFT	774 ± 47	24 - 55 years	14	727 ± 409	Unstimulated	1:2,000	ELISA	ANOVA (p > 0.050)
Thaweboon et al.[23], 2008	92.46 ± 19.19 months	15	> 5.0 dmft/DMFT	114.96 ± 34.24	92.73 ± 19.86 months	15	86.47 ± 43.23	Mecanical stimulus	Missing data	ELISA	Mann-Whitney (p < 0.050)
Farias et al.[24], 2003	12 to 47 months	20	16.4 ± 8.9 dmft	3.25 ± 2.10	12 - 47 months	20	5.04 ± 4.50	Unstimulated	Missing data	Nephelometry	Mann-Whitney (p < 0.050)
Sikorska et al.[25], 2002	15years and 7months ± 3months	83 (all subjects)	14.53 ± 8.51 DMFS	higher	15years and 7 months ± 3months	83 (all subjects)	lower	Unstimulated	1:40	ELISA	Analysis of variance for multiple re-gression (p < 0.016)
Kirtaniya et al.[13], 2012	6 to 14 years	36	Low: 1.7 ± 0.48 (n = 11)	Low: 0.43 ± 0.13	6 to 14 years	11	0.49 ± 0.14	Unstimulated	Missing data	ELISA	Missing data about

			Moderate: 3.6 ± 0.52 (n = 10) High: 8.8 ± 3.59 DMFT/dmft (n = 10)	Moderate: 0.39 ± 0.06 High: 0.35 ± 0.14							statistical test Caries free x low (p > 0.05) Caries free x Moderate (p > 0.05) Caries free x High (p < 0.05)
Chawda et al.[26], 2011	4-8 years	Low: 10 High: 10	Low: 1-5 dmft High: 6-10 dmft	Low: 186.60 ± 48.40 High: 166.30 ± 30.40	4-8 years	10	243.60 ± 48.70	Stimulated	Missing data	ELISA	ANOVA Caries free x Low: p = 0.018 Caries free x High: p = 0.001
Omar et al.[27], 2012	3 to 6 years	Low: 11 Moderate: 13 High: 11	Low: 1-3 Moderate: 4-6 High: >6 dmft	Low: 1.09±0.25 Moderate: 0.72±0.36 High: 0.45±0.29	3 to 6 years	10	0.81±0.38	Unstimulated	Missing data	ELISA	2-tailed Pearson's correlation test (p < 0.05)
Hocini et al.[28], 1993	20 to 63 years	21	> 10 DMFT	34.2 ± 20.9	20 to 64 years	22	31.4 ± 36.1	Unstimulated	1:2,000 to 1:8,000)	ELISA	Mann-Whitney (p > 0.050)
Pal et al.[29], 2013	9.67 ± 2.47	15	6.60 ± 2.10 dmft/DMFT	144.13 ± 20.85	19.0 ± 2.59	15	213.63 ± 28.67	Unstimulated	Missing data	ELISA	ANOVA (p < 0.05)
Priya et al.[30], 2013	7 to 12 years	15	> 5 DMFT/dmft	130.07 ± 15.5	7 to 12 years	15	119.0 ± 15.8	Unstimulated	X 1000	ELISA	t test (p = 0.05)

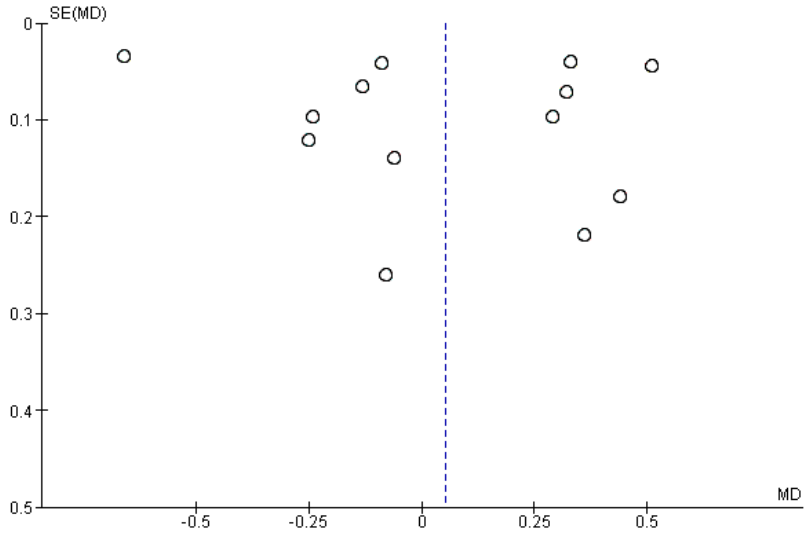


Figure 2: Funnel plot of comparison between IgA levels from caries-free and caries-active subjects**.**

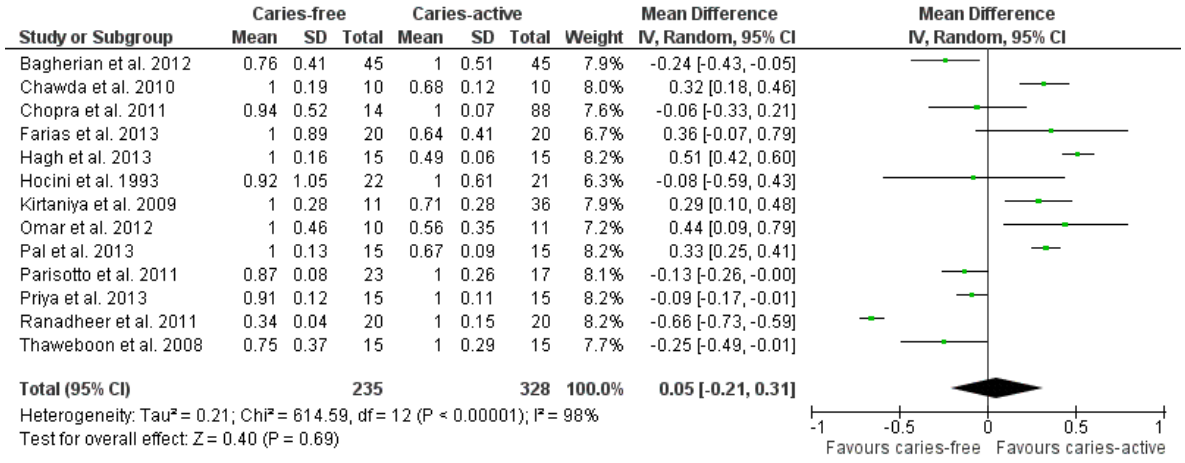


Figure 3: Forest plot of salivary IgA concentration in caries-free and caries-active subjects of 13 articles.

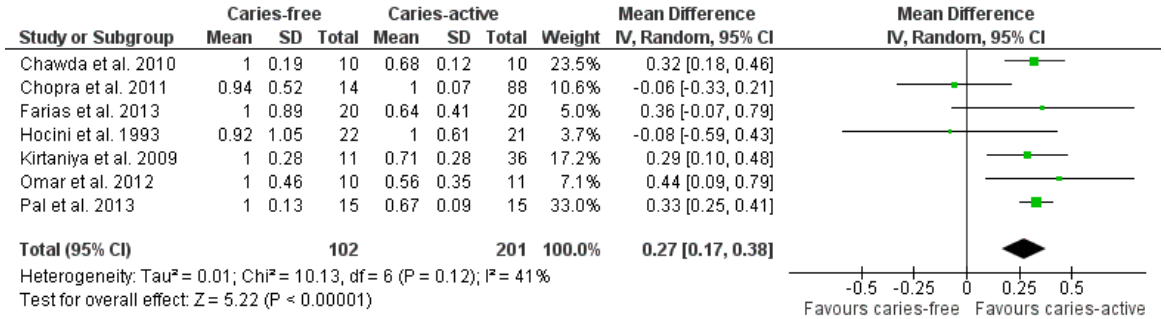


Figure 4: Forest plot of salivary IgA concentration in caries-free and caries-active subjects of 7 articles remained after sensitivity test**.**

4. DISCUSSION

Host genetic differences and their phenotype effect saliva characteristics and can be a reasonable explanation why some children do not develop caries, even with poor dietary and hygiene habits.[19, 21] Whole saliva and its composition has an important biological function in maintaining oral health.[32] Saliva presents plasmatic components that are available in this fluid through crevicular fluid. This biofluid carry out important metabolites of physiologic system to determine the status of health and disease for both oral and systemic condition.[2, 33] In this context, the salivary glands provide the most important source of s-IgA in the upper tracts and many factors can influence it concentration.[34]

The aim of this systematic review was to evaluate if there is a correlation between salivary s-IgA levels and the presence of dental caries. Although the analytic test does not consisted on exclusion criteria, major included studies used a confidence method for the assessment of s-IgA. ELISA methodology is an immunoassay for antibody detection that provides a combination of sensitivity, specificity, detection limit, precision, reproducible, and accurate. In addition, most of studies used a commercial kit and followed the manufacture instructions.

After reviewing all articles identified in the search, we retained 14 studies after inclusion criteria. One study that did not include a control group without dental caries was excluded of this systematic review.[17] After selection of studies that fulfilled the eligibility criteria, the quality assessment was applied. There are few guidelines developed for rank quality of evidence for prevalence studies. The quality assessment applied in this systematic review present a comprehensive judgment of methodology and bias.[16] The guide provide a standardized approach to quality assessment such as cover patient spectrum, verification bias, clinical review bias, test execution, study withdrawals, and indeterminate results.

One difficult in performing the meta-analysis was the discrepant values of s-IgA, even though most studies used the same analytic method. In addition, the dilutions were different among studies. In order to make these values comparable, they were transformed in percentage as well as their standard deviation. These discrepant values can be explained by the inter-individual different exposure to antigen. From the seven studies included in this meta-analysis after sensitivity

analysis, five showed higher s-IgA concentration in the caries group and the other 2 showed the opposite. This finding demonstrates that this immunoglobulin is associated to the response of immunological system to the disease. Salivary IgA reflect a previous exposure of the host to cariogenic microorganisms. Otherwise, some authors found increased s-IgA in caries-free group.[19-23, 28, 30] It is suggested that these subjects could be in contact with high levels of microorganisms, but did not developed dental caries. The secretory immune system provides local immune protection against cariogenic organisms, much other factor are responsible to prevent or induce dental caries, such as salivary composition, flow rate, oral hygiene, sugar consumption and others. In addition, it is suggested that absence of continuous stimulation by treatment of dental caries, leads the immunological titers levels to decline.[35]

According to Omar et al,[27] although the s-IgA level was significantly higher in caries-free subjects, the reverse was observed in children with low caries experience, since s-IgA levels in this group was significantly higher than the control group. As one of the immune factors, IgA may increase in response to mild exposition of dental caries as a form of a protective mechanism of the body against caries attack. This author[27] also suggested that, it may be more realistic to relate s-IgA concentration to the decayed component (d) of caries index rather than total caries index (dmft) score. This study showed a significant correlation between the decayed component and sIgA. Unfortunately, the major of studies evaluated the completed index and not assessed decayed component separately which consists on a confounding factor.

Parissoto et al[21] found high concentration of total s-IgA in children with dental caries. In addition, preschoolers with a lower baseline level of salivary IgA antibody reactive to *Streptococcus mutans* had 7.5 higher chances to develop caries during the period of study. This finding suggests exposition to the caries stimulates the production of s-IgA and also that specific antibodies could play a role in oral/bacterial homeostasis. These authors evaluated children with and without caries in two moments, the baseline and after the five years of follow-up. Children with dental caries presented higher levels of s-IgA in the both moments. After follow-up the s-IgA levels were increased. For the author, the children were beginning the mixed dentition transition that could be linked to maturation of salivary glands as part of general development of systems of the body.

Koga-Ito et al[36] also found increased concentrations of s-IgA in young adults in comparison to children. For this reason, it is important to point out the ages of subjects of the studies. It was tried to perform a subgroup analysis based on ages, however it was not possible due to the great heterogeneity resulted from the variability in concentration and sample procedures. Even discrepant concentrations, the aim of this mata-analysis was not establish standard concentration for saliva s-IgA; and it was possible to demonstrate the association between dental caries and s-IgA levels.

The immunological system trend to maturate over the age.[37] Previous studies demonstrated that secretory IgA levels increase with age.[37-40] Parisotto et al.[21] that performed an IgA-s longitudinal evaluation and suggest that the increased IgA-s levels over the time is explained by the mixed dentition transition and the growth process that could be related to maturation of salivary glands as part of general development of systems of the body. In the current study it was not possible to evaluated subgroups according to age due to the high heterogeneity. Although the younger subjects present lower IgA-s levels it was not considered a bias since the control group match in age with caries-active subjects.

The retrieved studies showed that the most of included studies demonstrated a higher s-IgA concentration in caries active subjects. Therefore, based on the results of this systematic review and meta-analysis, it can be concluded that there is evidence in the literature showing an association between caries and the increased levels of s-IgA. It is suggested that more prospective studies should be conducted in larger populations to evaluate if children that develop dental caries had increased s-IgA levels before disease.

REFERENCES

1. AAPD. Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies. Pediatr Dent. 2008;30(7 Suppl):40-3.

2. Fidalgo TKS, Freitas-Fernandes LB, Angeli R, Muniz AMS, Gonsalves E, Santos R, et al. Salivary metabolite signatures of children with and without dental caries lesions. Metabolomics. 2013;9(3):657-66.

3. Lenander-Lumikari M, Loimaranta V. Saliva and dental caries. Adv Dent Res. 2000 Dec;1440-7.

4. Llena-Puy C. The role of saliva in maintaining oral health and as an aid to diagnosis. Med Oral Patol Oral Cir Bucal. 2006 Aug;11(5):E449-55.

5. Banas JA, Vickerman MM. Glucan-binding proteins of the oral streptococci. Crit Rev Oral Biol Med. 2003;14(2):89-99.

6. Smith DJ, Mattos-Graner RO. Secretory immunity following mutans streptococcal infection or immunization. Curr Top Microbiol Immunol. 2008;319131-56.

7. Nogueira RD, King WF, Gunda G, Culshaw S, Taubman MA, Mattos-Graner RO, et al. Mutans streptococcal infection induces salivary antibody to virulence proteins and associated functional domains. Infect Immun. 2008 Aug;76(8):3606-13.

8. Hannig C, Hannig M. The oral cavity--a key system to understand substratum-dependent bioadhesion on solid surfaces in man. Clin Oral Investig. 2009 Jun;13(2):123-39.

9. Krasse B, Gahnberg L. Available immunoglobulin A antibodies in mouth rinses and implantation of Streptococcus mutans. Infect Immun. 1983 Sep;41(3):1360-2.

10. Orstavik D, Brandtzaeg P. Secretion of parotid IgA in relation to gingival inflammation and dental caries experience in man. Arch Oral Biol. 1975 Nov;20(11):701-4.

11. Rose PT, Gregory RL, Gfell LE, Hughes CV. IgA antibodies to Streptococcus mutans in caries-resistant and -susceptible children. Pediatr Dent. 1994 Jul-Aug;16(4):272-5.

12. Fernandes FR, Nagao AT, Zelante F, Carneiro-Sampaio MM. Dental caries and salivary anti-Streptococcus mutans antibodies in IgA deficient children. Adv Exp Med Biol. 1995;371B1145-8.

13. Kirtaniya BC, Chawla HS, Tiwari A, Ganguly NK, Sachdev V. Natural prevalence of antibody titres to GTF of S. mutans in saliva in high and low caries active children. J Indian Soc Pedod Prev Dent. 2009 Jul-Sep;27(3):135-8.

14. Olsson J, Svanberg M. Oral implantation in man of Streptococcus mutans in relation to salivary IgA activity. Scand J Dent Res. 1991 Dec;99(6):489-97.

15. World Health Organization. Oral health surveys, basic methods. *4th edn World Health Organization*. Geneva 1997.

16. Fowkes FG, Fulton PM. Critical appraisal of published research: introductory guidelines. Bmj. 1991 May 11;302(6785):1136-40.

17. Jentsch H, Beetke E, Gocke R. Salivary analyses and caries increment over 4 years: an approach by cluster analysis. Clin Oral Investig. 2004 Sep;8(3):156-60.

18. Golpasand Hagh L, Zakavi F, Ansarifar S, Ghasemzadeh O, Solgi G. Association of dental caries and salivary sIgA with tobacco smoking. Aust Dent J. 2013 Jun;58(2):219-23.

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

20. Ranadheer E, Nayak UA, Reddy NV, Rao VA. The relationship between salivary IgA levels and dental caries in children. J Indian Soc Pedod Prev Dent. 2011 Apr-Jun;29(2):106-12.

21. Parisotto TM, King WF, Duque C, Mattos-Graner RO, Steiner-Oliveira C, Nobre-Dos-Santos M, et al. Immunological and microbiologic changes during caries development in young children. Caries Res. 2011;45(4):377-85.

22. Chopra M, Jadhav S, Venugopalan A, Hegde V, Chopra A. Salivary immunoglobulin A in rheumatoid arthritis (RA) with focus on dental caries: a cross-sectional study. Clin Rheumatol. 2011 Feb;31(2):247-50.

23. Thaweboon S, Thaweboon B, Nakornchai S, Jitmaitree S. Salivary secretory IgA, pH, flow rates, mutans streptococci and Candida in children with rampant caries. Southeast Asian J Trop Med Public Health. 2008 Sep;39(5):893-9.

24. de Farias DG, Bezerra AC. Salivary antibodies, amylase and protein from children with early childhood caries. Clin Oral Investig. 2003 Sep;7(3):154-7.

25. Sikorska MH, Mielnik-Blaszczak M, Kapec E. The relationship between the levels of SigA, lactoferrin and alpha(1) proteinase inhibitor in saliva and permanent dentition caries in 15-year-olds. Oral Microbiol Immunol. 2002 Oct;17(5):272-6.

26. Chawda JG, Chaduvula N, Patel HR, Jain SS, Lala AK. Salivary SIgA and dental caries activity. Indian Pediatr. 2010 Sep;48(9):719-21.

27. Omar OM, Khattab NM, Rashed LA. Glucosyltransferase B, immunoglobulin a, and caries experience among a group of Egyptian preschool children. J Dent Child (Chic). 2012 May-Aug;79(2):63-8.

28. Hocini H, Iscaki S, Bouvet JP, Pillot J. Unexpectedly high levels of some presumably protective secretory immunoglobulin A antibodies to dental plaque bacteria in salivas of both caries-resistant and caries-susceptible subjects. Infect Immun. 1993 Sep;61(9):3597-604.

29. Pal S, Mitra M, Mishra J, Saha S, Bhattacharya B. Correlation of total salivary secretory immunoglobulin A (SIgA) and mutans specific SIgA in children having different caries status. J Indian Soc Pedod Prev Dent. 2013 Oct-Dec;31(4):270-4.

30. Priya PR, Asokan S, Karthick K, Reddy NV, Rao VA. Effect of dental treatments on salivary immunoglobulin A of children with and without dental caries: a comparative study. Indian J Dent Res. 2013 May-Jun;24(3):394.

31. Hagh LG, Zakavi F, Ansarifar S, Ghasemzadeh O, Solgi G. Association of dental caries and salivary sIgA with tobacco smoking. Aust Dent J. 2013 Jun;58(2):219-23.

32. Zehetbauer S, Wojahn T, Hiller KA, Schmalz G, Ruhl S. Resemblance of salivary protein profiles between children with early childhood caries and caries-free controls. Eur J Oral Sci. 2009 Aug;117(4):369-73.

33. Helmerhorst EJ, Oppenheim FG. Saliva: a dynamic proteome. J Dent Res. 2007 Aug;86(8):680-93.

34. Kugler J, Hess M, Haake D. Secretion of salivary immunoglobulin A in relation to age, saliva flow, mood states, secretion of albumin, cortisol, and catecholamines in saliva. J Clin Immunol. 1992 Jan;12(1):45-9.

35. Challacombe SJ. Serum and salivary antibodies to Streptococcus mutans in relation to the development and treatment of human dental caries. Arch Oral Biol. 1980;25(7):495-502.

36. Koga-Ito CY, Martins CA, Balducci I, Jorge AO. Correlation among mutans streptococci counts, dental caries, and IgA to Streptococcus mutans in saliva. Braz Oral Res. 2004 Oct-Dec;18(4):350-5.

37. Fageras M, Tomicic S, Voor T, Bjorksten B, Jenmalm MC. Slow salivary secretory IgA maturation may relate to low microbial pressure and allergic symptoms in sensitized children. Pediatr Res. 2011 Dec;70(6):572-7.

38. Childers NK, Greenleaf C, Li F, Dasanayake AP, Powell WD, Michalek SM. Effect of age on immunoglobulin A subclass distribution in human parotid saliva. Oral Microbiol Immunol. 2003 Oct;18(5):298-301.

39. Everhart DL, Bamgboye PO, Schwartz MS. Salivary anti-Streptococcus mutans changes over a six-month period in children ages two-five years. J Dent Res. 1982 Feb;61(2):386-90.

40. Weemaes C, Klasen I, Goertz J, Beldhuis-Valkis M, Olafsson O, Haraldsson A. Development of immunoglobulin A in infancy and childhood. Scand J Immunol. 2003 Dec;58(6):642-8.

4.2 **ARTIGO 2-** Do Salivary Lipids Influence Dental caries Suscetibility? A Systematic Review.

Tatiana Kelly da Silva Fidalgo[1]
Valéria Abreu[1]
Liana Bastos Freitas-Fernandes[2]
Ivete Pomarico Ribeiro de Souza[3]
Lucianne Cople Maia[4]

[1]PhD student, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;

[2]Postdoctoral student, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;

[3]Chairman Professor, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;

[4]Adjunct Professor, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;

da Silva Fidalgo et al., 1:12
http://dx.doi.org/10.4172/scientificreports.580

Open Access Scientific Reports

Review Article Open Access

Do Salivary Lipids Influence Dental Caries Susceptibility? A Systematic Review

Tatiana Kelly da Silva Fidalgo[1], Valéria Abreu[1], Liana Bastos Freitas-Fernandes[2], Ivete Pomarico Ribeiro de Souza[3] and Lucianne Cople Maia[4]*

[1]PhD Student, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
[2]Postdoctoral Student, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
[3]Chairman Professor, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
[4]Adjunct Professor, Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Abstract

This study aimed to appraise the association between salivary lipids and caries experience through a systematic review. A computerized and manual systematic search was made of the PubMed, Web of Science, Cochrane and OVID databases. The key MeSH (Medical Subject Headings) terms used were: Saliva and Dental caries and Lipid or Cholesterol or Diglyceride or Fatty acids or Glycolipids or Phospholipids. Eligibility of the selected studies was determined by reading the abstracts of the articles identified from the electronic databases. A quality assessment was carried out classifying the selected articles into A, B or C (high, moderate, and low methodological quality, respectively). After reading 65 titles/abstracts to verify whether they met the inclusion criteria 05 titles/abstracts remained. The selected articles were then carefully read and ranked according to their methodological quality and risk of bias. The results showed higher concentration of total lipids, cholesterol, free fatty acids, glycolipids, glycerides, neutral lipids, phospholipids, and triacylglyceride in caries subjects than caries free. According to the methodological quality and risk of bias, this systematic review indicates a moderate association between dental caries and salivary lipid content.

Keywords: Dental caries; Lipid; Saliva; Systematic review

Introduction

Saliva is a complex biofluid that has important functions in oral homeostasis and therefore its composition is related to systemic and oral physiological conditions [1,2]. Physiological, pathological and environmental factors can cause changes in salivary composition that can be correlated to disease susceptibility and can also reflect advanced stages of diseases [3]. Many saliva constituents including proteins, carbohydrates, lipids, and ions interact under fine regulation to fulfill such important tasks [4-6]. The most frequent lipids in saliva are glycolipids, neutral lipids and phospholipids [7].

Salivary lipids are mostly of glandular origin, although cholesterol and some fatty acids are believed to come directly from serum [8]. Local and systemic disorders may disturb or interrupt these complex balanced functions, which can lead to mucosal and tooth damage. Lipids originate from several membranes such as secretory vesicles, microsomes, lipid rafts, and other plasma and intracellular membrane fragments of lysed cells and bacteria [7,9,10].

A large part of the salivary lipids are associated with proteins, especially to high molecular weight glycoproteins and to proline-rich proteins (PRPs) [11]. Despite the great amount of information concerning salivary peptides and protein compositions and their well defined functions in the caries process [3,12-14], the available data about salivary lipids and their relationship to oral conditions is still inconclusive. However some studies affirm a positive association to caries experience [6,15-19]. The present systematic review was conducted in an attempt to support this positive association between high salivary lipid content and caries experience.

Materials and Methods

Search strategy

The extensive literature search strategy carried out was based on PubMed, Web of Science, Cochrane and OVID databases and all articles published before December 2012 were considered for review. The MeSH (Medical Subject Headings) terms used were: Saliva and Dental caries and Lipid or Cholesterol or Diglyceride or Fatty acids or Glycolipids or Phospholipids. Selected article references were hand searched in order to extend the search to other relevant articles.

Grey literature was also searched. In the last stage of the search process the websites of the major dental journals Archives of Oral Biology, Caries Research, Journal of Dental Research, Journal of Dentistry, European Journal of Oral Science, Journal of the American Dental Association and Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics were searched.

Selection criteria

The inclusion criteria comprised clinical investigations with one case and one control group; a caries diagnostic method; with evaluation of lipid concentration by using tests for both groups (case and control), and with statistical analyses. Case reports, case series, descriptive studies, review articles, opinion articles, letters, and articles that did not correspond to the aims of this review were excluded. All records electronically identified were scanned by title and abstract. Eligibility of the selected studies was determined by reading the abstracts of the articles identified from the electronic databases. Articles appearing in more than one database search were considered only once. Two authors independently assessed the methodological quality of the trials and the retrieved data. In cases of discrepancies, a decision was

***Corresponding author:** Lucianne Cople Maia, Disciplina de Odontopediatria da FO-UFRJ, Caixa Postal: 68066 - Cidade Universitária - CCS, CEP.: 21941-971 - Rio de Janeiro – RJ – Brazil, E-mail: rorefa@terra.com.br

Received December 14, 2012; **Published** December 23, 2012

Citation: da Silva Fidalgo TK, Abreu V, Freitas-Fernandes LB, de Souza IPR, Maia LC (2012) Do Salivary Lipids Influence Dental Caries Susceptibility? A Systematic Review. 1:580 doi:10.4172/scientificreports.580

made by consensus. Full texts were obtained of all articles identified and judged as being potentially relevant. A consensus was reached if relevant data were missing and/or the authors of the articles in question were contacted for additional information.

Quality assessment and control of bias

The methodological quality and control of bias of the studies were evaluated. The following questions were applied to each selected study: (a) Are the demographic data described? (b) Is the sample size satisfactory? (c) Is the study design correct? (d) Was the caries group diagnosed correctly? (e) Was the lipid detection method applied corrected? (f) Was the statistical analysis applied correctly? Each reviewer classified the study as: A, when answer was "yes" to at least five questions (low risk of bias); B, when answer was "yes" to four questions (moderate risk of bias); C, when answer was "yes" to three, two, one or no questions (high risk of bias).

Results

The table 1 shows the database search strategy yielded a total of 73 titles/abstracts from PubMed; 10 articles from Web of Science; 03 from Cochrane; 09 from Ovid; 00 (none) different articles from the reference list of the previous search and none from gray literature. All the articles retrieved from the databases were repeated in the PubMed.

After reading the 73 titles/abstracts, 05 titles/abstracts were considered to meet the inclusion criteria. The selected articles were then carefully read and ranked as shown in table 2. Two articles were excluded, one due to an overlapping sample [17] and the other due to appraisal of the lipid content from dental plaque and not from saliva [15].

An illustrative diagram of the electronic search and selected articles is represented in figure 1. Only observational studies were found using the mentioned search strategy.

A summary of the main findings of each selected study is shown in table 3. Tomita et al. [6] assessed salivary lipid content from stimulated whole saliva. This was not compared to subjects with caries lesion because the objective of this assessment was to correlate the concentration of metabolites in saliva and type of stimulus, mechanical (chewing gun base) and chemical (citric acid in different concentrations). For this reason only data from the parotid gland was included in this study.

MeSH / Database	PubMed	Web of Science	Cochrane	Ovid
Lipid	73	07	02	07
Cholesterol	06	04	01	00
Diglyceride	00	00	00	00
Fatty acids	30	03	00	03
Glycolipids	04	00	00	00
Phospholipids	08	00	00	00
Total *	65	10	03	09

*After exclusion of duplicated articles.

Table 1: Database search strategy consisted on the MeSH (Medical Subject Headings) terms Saliva AND Dental caries AND the following MeSH terms.

Article	[1]Demographic data	[2]Sample size	[3]Study design	[4]Caries diagnosis	[5]Lipid detection
Tomita et al. [6]	Appropriate	Appropriate	Appropriate	Inappropriate	Appropriate
Slomiani et al. [16]	Inappropriate	Inappropriate	Appropriate	Appropriate	Appropriate
Slomiani et al. [19]	Inappropriate	Inappropriate	Appropriate	Appropriate	Appropriate
*Slomiani et al. [17]	NA	NA	NA	NA	NA
**Murthy et al. [15]	NA	NA	NA	NA	NA

NA = not assessed. *Duplicated sample; **Assessment of lipid content from dental biofilm.

Table 2: Articles selected according to inclusion criteria and quality assessment.

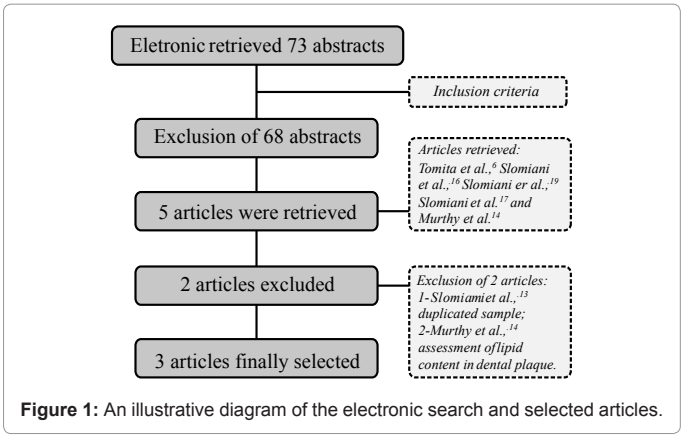


Figure 1: An illustrative diagram of the electronic search and selected articles.

Table 4 shows the lipid concentrations of the selected studies. The concentration of free fatty acid and total lipids was evaluated in the three studies [6,16,19]. Glycolipids, cholesterol and cholesterol esters levels from the submandibular gland were not statistically higher in caries subjects. The other lipid composition presented statistically higher levels in caries subjects in comparison to caries resistant ones. The findings suggest that there is a positive association between dental caries and salivary lipid content with moderate evidence.

Discussion

Although some studies have shown positive association of increased lipid levels to caries experience [6,15-19], until now there has been no scientific evidence that supports this association. The present systematic review evaluated the positive association between salivary lipid and caries experience. All included articles were observational and no experimental clinical studies were found. However, all included studies are old, all fulfill the inclusion criteria and demonstrated the possible association of dental caries with salivary lipids. Since, the studies applied valid methodology and statistic analysis, the fact of be old studies does not reduce the scientific value of the studies. On the other hand, this finding suggests that there is a need to conduct controlled experimental studies.

The function of lipids in saliva is still controversial. The salivary glands have a considerable capacity for biosynthesis of phospholipids and triglycerides in a short period of time [20,21]. The active participation of lipids in salivary secretory processes is thought to initiate as a part of the Golgi complex and then as a part of the microtubule system [22], and this process could be modified by different conditions such as in caries patients.

None of the included articles were blinded. However other parameters such as the correct collection of samples and separation of lipid content were fulfilled. The included articles [6,16,19] evaluated the lipid content of the parotid and/or submandibular gland. Only Slomiani et al. [16] compared lipids from different glands when submandibular and parotid lipid contents were appraised. Although the sample of three included articles was considered small [16,19], the findings showed that total lipids from the submandibular gland were

Article	Year	Subjects		Caries assessment		Method			Outcome
		Caries free/ caries (Sample size; n)	Population (years)	Caries free subjects	Caries subjects	Saliva	Test used	Statistical method	Lipid content
Tomita et al. [1]	2008	22/22	20-21	3.0 ± 1.6 DMFT (Clinical exam)	12.3 ± 3.7 DMFT (Clinical exam)	Parotid (stimulated with chewing gun base)	Chromatography	t test; p<0.05	Caries was statistically higher than caries-free
Slomiani et al. [16]	1982	10/10	Above 24	0 DMFS (Clinical and radiographic exam)	15-45 DMFS (Clinical and radiographic exam)	Parotid and submandibular (stimulated with citric acid)	Chromatography	t test; p<0.05	Caries was statistically higher than caries-free; except for glycolipids and cholesterol from submandibular gland
Slomiani et al. [19]	1986	05/05	Data not found	0 DMFS (Clinical and radiographic exam)	15-45 DMFS (Clinical and radiographic exam)	Submandibular (stimulated with citric acid)	Chromatography	t test; p<0.05	Caries was statistically higher than caries-free; except for cholesterol ester

Table 3: Detailed descriptions of the selected studies.

Salivary sample		
Article	**Parotid - caries-free/caries**	**Submandibular - caries-free/caries**
Tomita et al. [1]	-Total lipids: 3.80 ± 1.00/ 5.0 ± 1.10** -Free fatty acids: 1.30 ± 0.20/2.3 ± 0.10** -Neutral lipids: 3.10 ± 0.20/4.4 ± 0.40** -Triacylglyceride: 0.70 ± 0.070/1.00 ± 0.10**	Data not evaluated
Slomiani et al. [16]	-Total lipids: 4.81 ± 0.28/ 7.63 ± 0.57** -Cholesterol: 0.44 ± 0.07/0.51 ± 0.15* -Cholesterol esters: 0.46 ± 0.08/1.42 ± 0.35** -Free fatty acids: 1.32 ± 0.22/2.33 ± 0.41** -Glycolipids: 1.27 ± 0.08/1.21± 0.13** -Mono/diglycerides: 0.09 ± 0.02/0.11± 0.03* -Neutral lipids: : 2.89 ± 0.34/5.35 ± 0.58** -Phospholipids: 0.09 ± 0.02/0.12 ± 0.03* -Triacylglyceride: 0.58 ± 0.11/0.98 ± 0.14**	-Total lipids: 5.20 ± 0.37/ 8.01 ± 0.32** -Cholesterol: 0.50 ± 0.10/0.51 ± 0.14 -Cholesterol esters: 0.62 ± 0.13/1.26 ± 0.37** -Free fatty acids: 1.39 ± 0.11/2.34 ± 0.30** -Glycolipids: 1.46 ± 0.23/1.56 ± 0.29 -Mono/diglycerides: 0.12 ± 0.03/0.19 ± 0.04* -Neutral lipids: : 3.23 ± 0.40/5.64 ± 0.52** -Phospholipids: 0.10 ± 0.02/0.5 ± 0.03** -Triacylglyceride: 0.60 ± 0.15/1.34 ± 0.19**
#Slomiani et al. [19]	Data not evaluated	-Cholesterol: 2.04 ± 0.16/1.33 ± 0.12* -Cholesterol esters: 0.91 ± 0.11/0.71 ± 0.09 -Free fatty acids: 5.29 ± 0.47/6.77 ± 0.70** -Glycolipids: 2.40 ± 0.22/3.61 ± 0.42* -Mono/diglycerides: 0.06 ± 0.02/0.30 ± 0.10* -Phospholipids: 1.73 ± 0.15/2.89 ± 0.21* -Triglycerides: 2.49 ± 0.25/4.68 ± 0.41*

*p<0.01 and **p<0.05; t test; #The data were expressed in mg/100mg glycoprotein.

Table 4: Quantification and statistical analysis of lipid contents of salivary samples.

statistically higher than from the parotid gland, both in caries free and caries subjects.

The dynamic changes in lipid levels was associated to the biofilm maturation and an increase of neutral and phospholipids contents and a decrease of glycolipids in the plaque [15] were found. All included articles that used chromatography to quantify the lipids, demonstrated that most of the lipid composition presented statistically higher levels in caries subjects than in caries free subjects. This positive association between caries experience and an increase of total lipid levels was shown in all included articles [6,16,19]. However glycolipids, cholesterol and cholesterol esters levels from the submandibular gland were not statistically higher in caries subjects. Cholesterol is thought to derivate from plasma and the clearance of compounds from plasma into saliva may involve several processes such as ultrafiltration through gap junctions between cells of secretary units and low molecular weight lipids such as cholesterol are involved [23].

Some theories for the positive correlation between dental caries and lipid levels in saliva have been suggested. The most accepted theory is defended by Slomiany et al. [19] and Tomita et al. [6]. Based on the fact that fatty acids and lipids are present in the region of mucus glycoproteins of salivary pellicle of tooth surfaces, the effect of caries development by inhibition of acid diffusion is increased [18]. Moreover, the salivary lipids vary according to biofilm maturation and this process is accompanied by an increase of neutral and phospholipid contents [17]. However, higher salivary lipid concentration in caries subjects presents higher lipid content in dental plaques and this has a considerably greater capacity to retard lactic acid diffusion that determines the susceptibility of the tooth surface to demineralization [17]. The increased levels of lipid content in saliva from subjects with dental caries suggest the salivary content as potential biomarker for dental caries, that could be useful for the clinical field due to evaluate the risk of the patient develop dental caries.

Other theories are also accepted, such as the effect of lipids on the physical-chemical properties of saliva, viscosity and solubility [24]. A theory that defends the ablity of lipids to enhance glucosyltransferase activity, associated to cariogenicity of oral microorganisms. In addition, the presence of lipids in saliva modify the hydrophobicity force of bacteria surfaces and facilities its adsorption on tooth surfaces [25]. Although, there are many theories for the association between dental caries and lipid levels in saliva, the effect of lipids on cariogenic challenge was not evaluated in the selected studies [6,16,19], suggesting

the need to carry out experimental studies to appraise the function of salivary lipids during the caries process.

Conclusions

The results presented in this systematic review indicate a positive association between dental caries and salivary lipid content with moderate evidence. However, the present findings suggest the need to conduct controlled experimental studies with larger sample sizes and high methodological rigor.

References

1. Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M (2010) Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. Metabolomics 6: 78-95.
2. Takeda I, Stretch C, Barnaby P, Bhatnager K, Rankin K, et al. (2009) Understanding the human salivary metabolome. NMR Biomed 6: 577-584.
3. Bergandi L, Defabianis P, Re F, Preti G, Aldieri E, et al. (2007) Absence of soluble CD14 in saliva of young patients with dental caries. Eur J Oral Sci 115: 93-96.
4. de Almeida Pdel V, Gregio AM, Machado MA, de Lima AA, Azevedo LR (2008) Saliva composition and functions: a comprehensive review. J Contemp Dent Pract 9: 72-80.
5. Greabu M, Battino M, Mohora M, Totan A, Didilescu A, et al. (2009) Saliva--a diagnostic window to the body, both in health and in disease. J Med Life 2: 124-132.
6. Tomita Y, Miyake N, Yamanaka S (2008) Lipids in human parotid saliva with regard to caries experience. J Oleo Sci 57:115-121.
7. Slomiany BL, Murty VL, Aono M, Slomiany A, Mandel ID (1983) Lipid composition of human parotid salivary gland stones. J Dent Res 62: 866-869.
8. Karjalainen S, Sewon L, Soderling E, Larsson B, Johansson I, et al. (1997) Salivary cholesterol of healthy adults in relation to serum cholesterol concentration and oral health. J Dent Res 76: 1637-1643.
9. Belcourt A, Krembel J, Frank RM (1972) [Lipids of human dental plaque matrix and of filtered saliva]. Rev Odontostomatol (Paris) 19: 39-44.
10. Larsson B, Olivecrona G, Ericson T (1996) Lipids in human saliva. Arch Oral Biol 41: 105-110.
11. Slomiany BL, Witas H, Murty VL, Slomiany A, Mandel ID (1983) Association of lipids with proteins and glycoproteins in human saliva. J Dent Res 62: 24-27.
12. Hardt M, Thomas LR, Dixon SE, Newport G, Agabian N, et al. (2005) Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry 44: 2885-2899.
13. Ambatipudi K, Hagen FK, Delahunty CM, Han X, Shafi R, et al. (2010) Human Common Salivary Protein 1 (CSP-1) Promotes Binding of Streptococcus mutans to Experimental Salivary Pellicle and Glucans Formed on Hydroxyapatite Surface. J Proteome Res 9: 6605-6614.
14. Ozturk A, Famili P, Vieira AR (2010) The antimicrobial peptide DEFB1 is associated with caries. J Dent Res 89: 631-636.
15. Murty VL, Slomiany BL, Laszewicz W, Slomiany A, Petropoulou K, et al. (1985) Lipids of developing dental plaque in caries-resistant and caries-susceptible adult people. Arch Oral Biol 30: 171-175.
16. Slomiany BL, Murty VL, Aono M, Slomiany A, Mandel ID (1982) Lipid composition of human parotid and submandibular saliva from caries-resistant and caries-susceptible adults. Arch Oral Biol 27: 803-808.
17. Slomiany BL, Murty VL, Mandel ID, Sengupta S, Slomiany A (1990) Effect of lipids on the lactic acid retardation capacity of tooth enamel and cementum pellicles formed in vitro from saliva of caries-resistant and caries-susceptible human adults. Arch Oral Biol 35: 175-180.
18. Slomiany BL, Murty VL, Mandel ID, Zalesna G, Slomiany A (1989) Physico-chemical characteristics of mucus glycoproteins and lipids of the human oral mucosal mucus coat in relation to caries susceptibility. Arch Oral Biol 34: 229-237.
19. Slomiany BL, Murty VL, Slomiany A, Zielenski J, Mandel ID (1986) Mucus glycoprotein of human saliva: differences in the associated and covalently bound lipids with caries. Biochim Biophys Acta 882: 18-28.
20. Pritchard ET, Yamada JA, Cushnie JE (1971) Lipase activity of rat submandibular salivary glands. Arch Oral Biol 16: 981-983.
21. Pritchard ET, Horak H, Yamada JA (1971) Lipid synthesis in subcellular particulates isolated from rodent submandibular salivary glands. Arch Oral Biol 16: 915-928.
22. Castle JD, Jamieson JD, Palade GE (1972) Radioautographic analysis of the secretory process in the parotid acinar cell of the rabbit. J Cell Biol 53: 290-311.
23. Chiappin S, Antonelli G, Gatti R, De Palo EF (2007) Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta 383: 30-40.
24. Schachtele CF, Harlander SK, Bracke JW, Ostrum LC, Maltais JA, et al. (1978) Streptococcus mutans dextransucrase: stimulation by phospholipids from human sera and oral fluids. Infect Immun 22: 714-720.
25. Beachey EH (1981) Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. J Infect Dis 143: 325-345.

4.3 **ARTIGO 3:** Longitudinal evaluation of salivary profile from children with dental caries before and after treatment**.**

Tatiana K. S. Fidalgo[a], Liana B. Freitas-Fernandes,[a] Fabio C. L. Almeida[b], Ana P. Valente[b], Ivete P. R. Souza[a]

[a]Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Universidade Federal do Rio de Janeiro, Brazil;
[b]National Center for Nuclear Magnetic Resonance – Jiri Jonas, Medical Biochemistry Institute, Universidade Federal do Rio de Janeiro, Brazil;

Correspondence to: Dr Ivete Pomarico Ribeiro de Souza, Disciplina de Odontopediatria da FO-UFRJ, Caixa Postal: 68066 - Cidade Universitária - CCS, CEP.: 21941-971 - Rio de Janeiro – RJ – Brasil, Phone: + 55 21 25622101
E-mail: pomarico@gmail.com

ABSTRACT

Saliva is a biofluid largely used in metabolomic for assessment of local and systemic diseases. Our group was able to demonstrate salivary metabolomic signature of children with dental caries (Fidalgo et al, 2013). Thus, the aim of the current study was to investigate the changes observed for metabolites related caries caries-lesion before and after dental treatment using NMR. Saliva samples from children without caries and with dental caries before and after treatment. ^{1}H-NMR spectra were submitted to Partial Least Squared Discriminant Analysis (PLS-DA). *Streptococcus mutans* and *Lactobacillus sp* and pH were also evaluated. As expected, caries-free children presented low levels of microorganisms when comparing to children with dental caries ($p < 0.05$; Mann-Whitney test). Also, after dental treatment it was observed a reduction of microorganisms ($p < 0.05$; Wilcoxon test) and the increase of saliva pH. PLS-DA showed a clear separation of saliva from children with caries and caries-free. In addition, after dental treatment it was observed a reduction in the levels of acetate, propionate, fatty acid, butyrate, and saccharide region. PLS-DA applied on ^{1}H-NMR saliva spectra distinguished the metabolites related to dental caries before and after dental treatment.

Key-words: Saliva; Dental caries; Metabolomic profile; NMR; *Streptococcus mutans*; *Lactobacillus sp*.

1 INTRODUCTION

Saliva has been shown to be an emerging and attractive biofluid for early detection of local and systemic disorders (Aimetti et al. 2012; Bertram et al. 2009; Fidalgo et al. 2013; Takeda et al. 2009). Some studies suggestied NMR-based biomarker in saliva for systemic diseases such as cancer, diabetes mellitus, cardiovascular disease and others (Bertram et al. 2009; Cuevas-Cordoba et al. 2014; Grootveld et al. 2005; Ng et al. 2011; Sugimoto et al. 2010). However, it is important to consider the oral status when systemic condition is evaluated using saliva as biofluid. It is important to determinate the metabolite fingerprint from oral disease since these metabolites can be erroneously associated to systemic disorders (Aimetti et al. 2012; Fidalgo et al. 2013; Silwood et al. 1999).

Saliva plays important role in the maintenance of oral health though low molecular weight compounds, ions, and protein balance (Aimetti et al. 2012; Fidalgo et al. 2013; Van Nieuw Amerongen et al. 2004). Regarding oral flora, it is known that *Streptococcus mutans* can colonize oral cavity since pre-dentate periods and are acquired by caregivers, especially by mothers (Caufield et al. 1993). It was demonstrated that children with dental caries present increased counts of *Streptococcus mutans* and showed that after dental treatment of caries, these microorganisms were reduced (Parisotto et al. 2010; Tanner et al. 2011). Oral microorganisms produce organic acids, such as acetic acid, by sugar fermentation that causes falls in dental plaque pH resulting in caries (Van Houte et al. 1989).

Saliva present mechanisms to avoid this imbalance and maintenance of oral health (Rigante et al. 2008). For instance, this biofluid contain pH natural regulators systems such as bicarbonate/carbonic acid buffer and urea to control pH drop (Morou-Bermudez et al. 2011; Tayab et al. 2012). Several proteins such as statherin and proline-rich glycoproteins protect enamel from microorganism colonization as well as promote supersaturation of calcium and phosphate in the fluid phase of the dental biofilm (Garcia-Godoy et al. 2008; Tenuta et al. 2006). The sCD14 is a protein related to innate immunity and is constitutively expressed by salivary glands. It was demonstrated that children with dental caries presented absence of sCD14 when compared to caries-free ones; and after caries treatment sCD14 increased in saliva demonstrating a relationship with caries activity (Bergandi et al. 2007). Although the

large knowledge about salivary proteins and ionic content in saliva and its relationship with dental caries, is still lack of investigations on association of caries activity and low molecular weight organic metabolites (Aimetti et al. 2012; Fidalgo et al. 2013; Silwood et al. 1999).

Our group showed differences in low molecular weight salivary metabolites from children with and without caries. In our previous study we evaluate subjects with and without caries (Fidalgo et al. 2013); however it is important to elucidate if these metabolites related to dental caries were maintained levels or reduced after dental treatment as a fingerprint of disease cycle. Thus, in the current work we aimed to follow biochemical parameters when return to the oral health condition.

According to Twetman et al. (1999) and Low et al. (2007), it was necessary three months to recover homeostasis of oral microorganisms after biofilm treatment. These authors observed that after antiseptic treatment of biofilm the levels of *Streptococcus mutans* decreased and it was recovered after 3 months. Thus, in this work we investigated the metabolites related to caries activity based on NMR approach before and after treatment as well until 3 months follow-up. Partial least-squares regression discriminant analysis (PLS-DA) showed distinction of these metabolites before and after treatment as well as cariogenic microorganisms demonstrated reduced after dental treatment.

2 MATERIAL AND METHODS

2.1 Study population and patient recruitment

Diseases that compromise a large number of population present high interest in this field. In case of local diseases, early childhood caries (ECC) is recognized as a significant public health problem (Martins-Junior et al. 2013). ECC is defined as the presence of one or more decayed, missing or filled tooth surfaces in any primary tooth in a preschool-age child between birth and 71 months of age (AAPD 2011). Its consequences can affect the immediate and long-term quality of life of the child's family (Martins-Junior et al. 2013). For this reason, we decided to investigate this population with rapid progression caries.

Caries per tooth surface were diagnosed using the visual classification using the Decay-Missing-Filled Surface index (dmfs) as described by the World Health Organization (WHO, 1997). It was considered only decayed teeth and the high valued of index means high number of tooth surfaces with caries. Caries-free group was composed by children that never had any dental caries cavity. Radiographs were taken in cases of pulp involvement doubt and only manifest lesions in the primary teeth were considered. It was excluded children that presented restored surfaces and teeth with pulp involvement or with indicated extraction.

The study population consisted of 30 systemically healthy children in primary dentition with caries (n = 20; 7 female and 13 male, mean age = 2.8 years ± 0.83 and dmfs = 11.0 ± 8.5) and without dental caries (n = 10; 5 female and 5 male, mean age = 3.0 years ± 1.2 and dmfs = 0) attending the Pediatric Dentistry Clinic for regular dental care. None of the subjects had any periodontal or systemic disease nor had taken any systemic antibiotics in the 3 months prior to sample collection.

2.2 Dental treatment and saliva collection

Children with dental caries cavity had their teeth restored with composite resin (TPH, Dentisply) according with the manufacturer's instruction. Saliva samples collections were performed before treatment (n = 20), seven days (n = 20), one month (n = 19), two months (n = 18), and three months (n = 10) after dental treatment. For the control group (children without dental caries), one saliva collection was performed in one moment. Subjects that started to use antibiotics during the study were excluded as well as ones that developed systemic or oral disorder.

It was collected 1 mL of unstimulated whole saliva using a automatic pipette that was passively collected from the floor of the mouth a into a plastic universal tube and the time was set for salivary flow rate calculation. The saliva sample from all children was taken at the same time (8.00 am to 10.00 am) to avoid fluctuation in the results because cicardian saliva cycle (Dawes 1972). They were asked to refrain from oral activities for 2 h prior to saliva collection. Prior to the centrifugation, 300 µl were separated to the microbiological analysis. The remaining was centrifuged at

10,000g for 60 min at 4 °C, and the supernatants were stored at -80 °C until NMR analysis.

2.3 *Streptococcus mutans* and *Lactobacillus sp* count in saliva

Within a period of 2 hours after sampling, saliva samples were diluted to 10^0, 10^{-1}, 10^{-2}, and 10^{-3} in 0.85% NaCl sterilized. Then, 50 µl of the dilutions of saliva were platted on 10 mL Mitis salivarius agar (Difco, Detroit, USA) with bacitracin and 20% sucrose for *Streptococcus mutans* and Rogosa (Difco, Detroit, USA) for *Lactobacillus sp* and incubated in candle jars at 37°C. After 48 h, the colonies of microorganisms were counted. Colonies from patients were stored for *Streptococcus mutans* species identification though colony morphology evaluation using a stereoscopic microscope.

2.4 Nuclear magnetic resonance analysis

^{1}H-NMR spectra were acquired using a 400 MHz Advance spectrometer (Bruker Biospin, Rheinstetten, Germany). The NMR procedures were performed using a standardized protocol in accordance to the metabolomics standard iniciative (Fiehn et al. 2007). All spectra were recorded at 25^o C, with water suppression by presaturation (Piotto et al. 1992). Samples were prepared by mixing 0.54 mL of salivary supernatant, deuterium oxide (99.8 % D_2O; 0.06 mL to provide a field frequency lock) and 10 µL of solution of 4,4-dimethyl-4-silapentane-1-sulfonic acid (20 mM DSS) for chemical shift reference, δ = 0.00 ppm. The CPMG (Carr–Purcell–Meiboom–Gill) pulse sequence was used to suppress signals from proteins and other macromolecules through a T2 filter, using 1,024 scans. ^{31}P was also evaluated using CPMG pulse sequence to investigate pH of saliva samples (Nosaka et al. 1998). For standard curve, it was used phosphate solutions based in Henderson Hasselbalch equation to pH varying 0.1 from 5.8 to 7.8 and R2 = 0.99.

The ^{1}H–^{1}H total correlation (TOCSY) experiments were conducted with acquisition parameters of 256 x 2,048 points, a spectral width 12,019 Hz in each dimension and a mixing time of 70 ms. All spectra were aligned though a triplet peak in the 1.01 – 1.08 ppm region. Edge effects were evaluated by overlaying all spectra

using Topspin (Bruker Biospin, Rheinstetten, Germany). Resonance assignments were made based on Silwood et al. (2002) and the Human Metabolome database (http://www.hmdb.ca/) (Wishart et al. 2007) confirmed using TOCSY experiments. Pure compounds were added to the saliva sample such as glycine, lactate, acetate, ethanol, histidine, and tyrosine and were also analysed by CPMG (Supp. Mat. Figure 1 to 5) and TOCSY pulse sequence to confirm chemical shift.

The use of human material was approved by the proper Research Ethics Committee of Community Health Studies.

2.5 Statistical analysis

The flow rate, *Streptococcus mutans* and *Lactobacillus sp* count were tabulated and analyzed on SPSS 20.0 (SPSS Inc, IL, USA). Shapiro-Wilk normalcy test was performed and the null hypothesis was rejected ($p < 0.05$), thus it was applied nonparametric tests. Wilcoxon test was applied for assessment of paired samples (samples from subjects with dental caries and the follow-up after treatment); and Kruskal Wallis and Mann Whitney test for independent samples (samples from subjects without dental caries and with caries and the follow-up after treatment).

The metabolite data were analyzed on the statistical program AMIX (Bruker Biospin, Rheinstetten, Germany). It was chose the previous metabolites already related to dental caries. Varied bucket size was defined and the following regions were: 1.89 – 1.92 (acetate), 1.00 – 1.07 (propionate I), 2.13 – 2.20 (propionate II), 2.00 – 2.07 (ambiguous), 0.81 – 0.89 (fatty acid I), 1.21 – 1.28 (fatty acid II), 1.50 – 1.58 (butyrate), and 3.50 – 4.00 (saccharide region). Data was normalized by Pareto scaling (Ramadan et al. 2006) before applying the Partial least squares-discriminant analyses (PLS-DA) method. For evaluation of each metabolite behavior before and after dental treatment, the integral of each metabolite region, described above, was recorded.

In addition, the whole spectra were also analyzed to evaluate if other metabolite influence in caries process. Thus, each NMR spectrum was analyzed by integrating regions of bucket size of 0.03 ppm excluding the water region (4.5 - 5.5

ppm). Data was normalized by Pareto scaling (Ramadan et al. 2006) before applying the PLS-DA.

3 RESULTS

In a recent study our group identified salivary metabolites which changes are related to caries disease (Fidalgo et al. 2013). We called this metabolite group as candidate metabolites. In this study we evaluate the changes in these salivary compounds before and after restoration in order to identify a fingerprint profile of the disease cycle.

In addition, we monitored the salivary levels of *Streptococcus mutans* and *Lactobacillus sp* as an independent indicator of disease evolution.

3.1 Evaluation of salivary *Streptococcus mutans* and *Lactobacillus sp*

As expected, when cariogenic microorganisms were evaluated it was observed a higher levels of *Streptococcus mutans* (SM) and *Lactobacillus sp* (L) in children with decayed teeth surface in comparison to children that never had dental caries ($p < 0.05$; Mann Whitney test) (Figure 1 and 2). Children that had never had dental caries presented *Streptococcus mutans* levels of 6.4×10^3 CFU/ml ($\pm 1.0 \times 10^2$) and *Lactobacillus sp* of 1.0×10^0 CFU/ml ($\pm 3.0 \times 10^0$) compare to children with dental caries that have levels of 4.8×10^5 CFU/ml ($\pm 4.9 \times 10^{5)}$ and 4.2×10^3 CFU/ml ($\pm 6.0 \times 10^3$) of *Streptococcus mutans* and *Lactobacillus sp*, respectively).

After dental treatment we have performed a time-course evaluation and we observed a significant reduction ($p < 0.05$; Wilcoxon test) in *Streptococcus mutans* and *Lactobacillus sp* after 7 days (SM – 8.6×10^4 CFU/ml $\pm 2.2 \times 10^5$ and L - 1.2×10^3 CFU/ml $\pm 1.7 \times 10^3$), 1 month (SM - 5.7×10^4 CFU/ml $\pm 6.5 \times 10^4$ and L - 5.2×10^2 CFU/ml $\pm 8.5 \times 10^2$), 2 months (SM - 9.4×10^4 CFU/ml $\pm 1.3 \times 10^5$ and L - 1.0×10^3 CFU/ml $\pm 2.8 \times 10^3$), and 3 months follow-up (SM - 6.4×10^4 CFU/ml $\pm 1.2 \times 10^5$ and L - 2.7×10^2 CFU/ml $\pm 5.3 \times 10^2$). Even after three months, our study showed that after dental treatment follow-up, the levels of *Streptococcus mutans* and *Lactobacillus* count was significantly higher in children with the restoration than in children that never had dental caries ($p < 0.05$; Mann Whitney test).

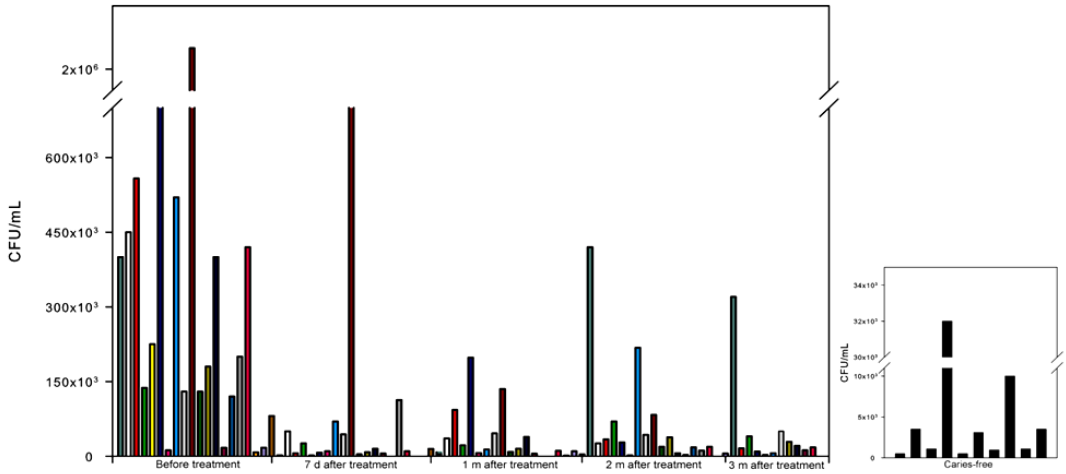


Figure 1: *Streptococcus mutans* count (CFU/mL) from each children (bars) with dental caries before and 7d, 1m, 2m, and 3m after dental treatment showing a reduction of *S. mutans* after dental treatment. The right bar chart shows reduced levels of *S. mutans* in caries-free children.

It is important to mention that the saliva flow rate was not significantly altered in caries patients, thus influencing the microorganisms count and metabolites concentration. We found a similar flow rate ($p > 0.05$) when compared the following groups: caries-free children (0.14 ml/min ± 0.04) before treatment (1.18 ml/min ± 0.09), 7 days after (0,15 ml/min ± 0,09) 1 month (0,22 ml/min ± 0,17), 2 months (0,17 ml/min ± 0,08), and 3 months (0.18 ml/min ± 0.09) after treatment.

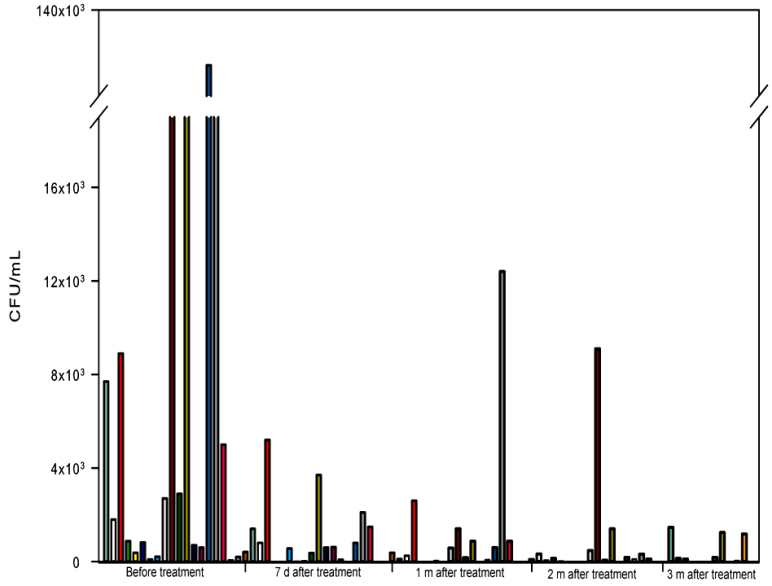


Figure 2*: Lactobacillus sp* count (CFU/mL) from each children (bars) with caries before treatment and 7d, 1m, 2m, and 3m after dental treatment showing a reduction of *Lactobacillus sp* levels. *Lactobacillus sp* in caries-free children was absent.

Figure 3 shows the 1H NMR spectra of saliva from children that never had dental caries in comparison to children with dental caries before and after dental treatment.

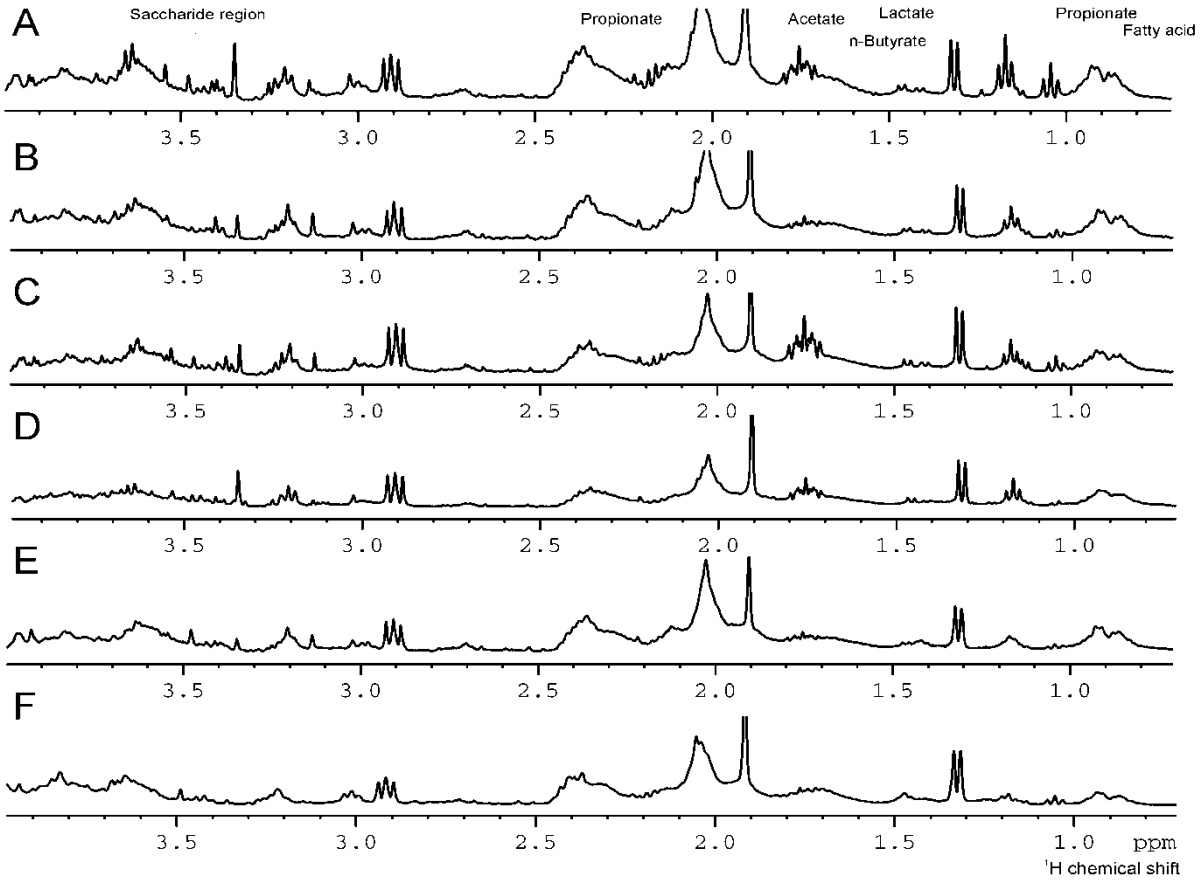


Figure 3: ^{1}H NMR saliva spectra differences among groups. A- Saliva samples from subjects with ECC , B- After 7 days, C- One month, D- Two months, E- Three months of treatment, and F- Caries-free children.

3.2 PLS-DA analysis of candidate metabolites

To evaluate metabolite changes we used a NMR approach. In order to suppress the signals macromolecules all the spectra were acquired using standard pulse sequences and a T2 filter. The *in natura* salivary samples were stable throughout the NMR acquisition period and only spectra without edge effects were included in statistical analysis.

The PLS-DA is able to explain the maximum separation between groups, it was used a dependent dichotomy variable (group) for modeling using latent variables

(Jolliffe 2002), maximizing the covariance between matrix that contain the intensities of each peak region and group. As previously described (Fidalgo et al. 2013), PLS-DA model and was able to distinguish children that never had dental caries and children with dental caries with retained 96.48% of variation (Figure 4A). When whole spectra regions were assessed was also possible distinguish children with and without caries (see Supp. Mat. Figure6).

After 3 months past dental treatment, PLS-DA showed that the candidates metabolites do not return to the normal homeostasis, i.e. similar to children that never developed the caries (Figure 4B). Fig 5 shows that after treatment, salivary metabolites delay 2 month to present a distinction in comparison to before treatment. Figure 5A and B shows that 7 days and 1 month after dental treatment was not evident the modification of salivary metabolites. The opposite can be observed in the Figure 5C and D that clearly show different profile of candidate metabolites after 2 and 3 months compared to saliva samples before dental treatment.

When we evaluated PLS-DA of all metabolites of spectra, we not found differences between control and after treatment. It is suggested that the whole spectra could hide the cluster formation and a restricted components could be responsible to caries fingerprint. Therefore, we based on these components from our previous investigation that point out low molecular weight components related to caries (Fidalgo et al. 2013).

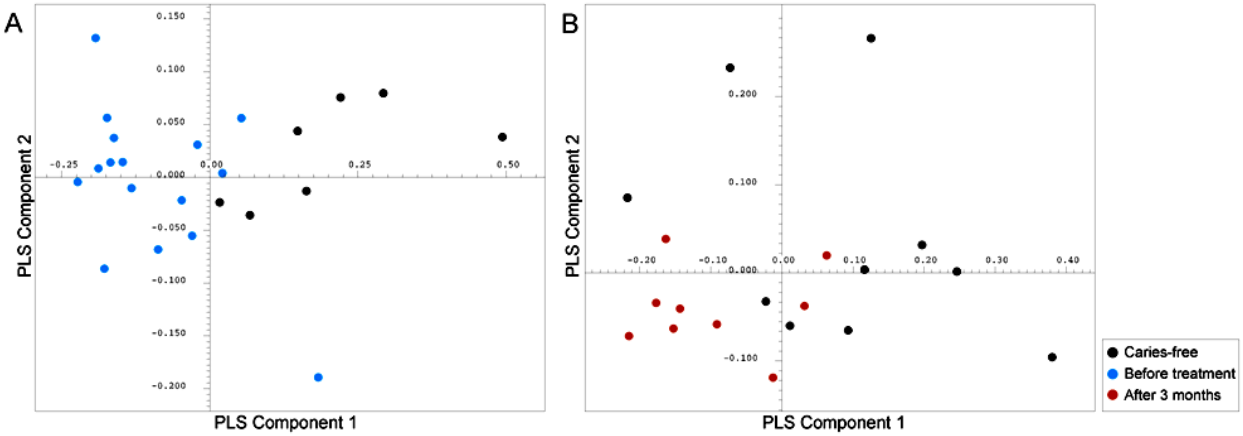


Figure 4: A- The PLS-DA retained 96.48% of variation, this model demonstrated a distinction when compared salivary samples of children that never present dental caries and children with dental caries; B- Children 3 months after dental treatment present similar profile of caries-free ones. This model retained 96.48% of the variation**.**

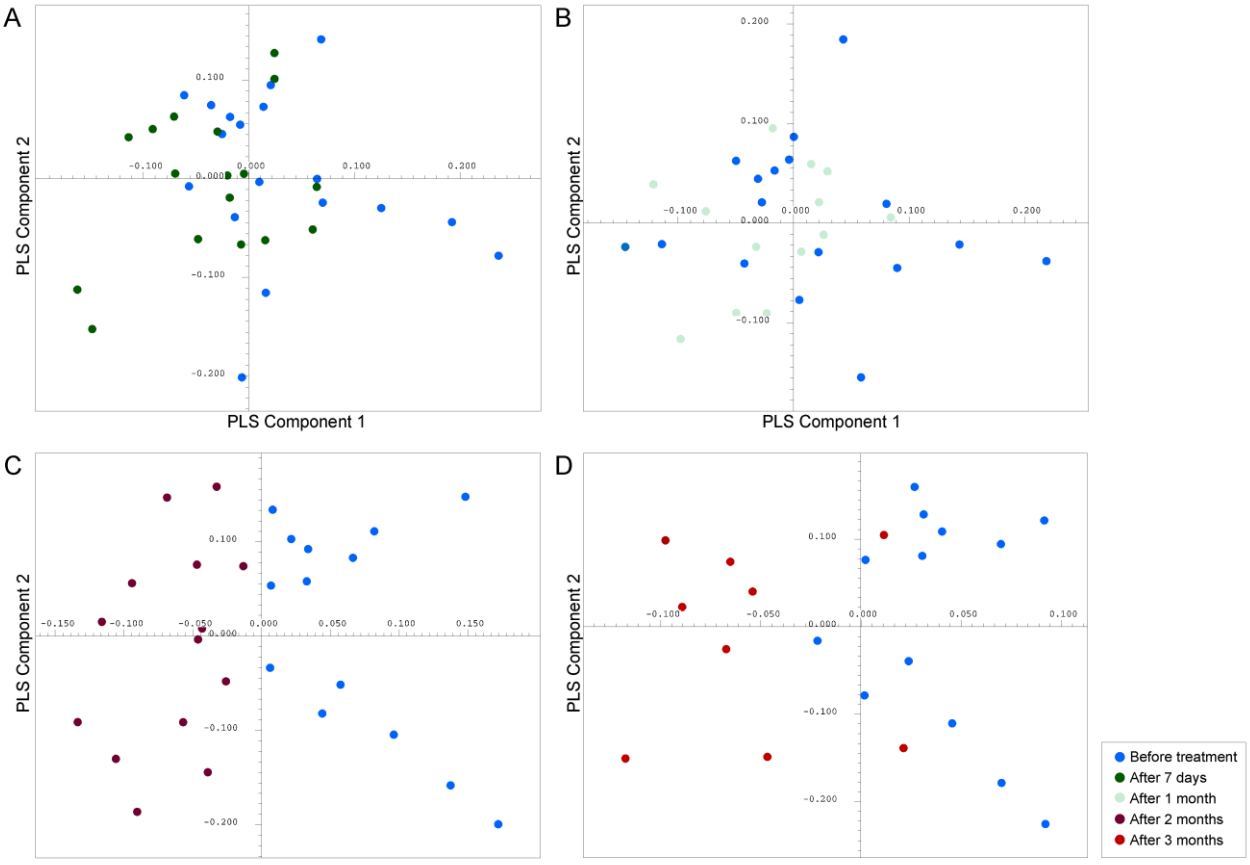


Figure 5: A- The PLS-DA showed a tendency to separation of salivary metabolites form children before and 7 days after dental treatment. B- No distinction is found between children before and 1 month after dental treatment. C, D- PLS-DA demonstrated an evident separation between children with dental caries before treatment and after 2 and 3 months, respectively

On the other hand, the analysis of whole spectra demonstrated a distinction between saliva samples from children with and without caries (Supp. Mat. Figure S-6).

3.3 Metabolites associated to dental caries

The selected metabolites acetate, n-butyrate, fatty acid, and propionate were found in higher levels in children with caries lesion. In the current work, we analyzed these metabolites before and after dental treatment and its levels after 3 months follow-up. Figure 6 shows the time-course of each metabolite that displayed significant differences on the salivary samples from the subjects with and without dental caries (Fidalgo et al. 2013).

It was found that acetate (1.92 ppm), n-butyrate (1.58 ppm), fatty acid 0.86 and 1.28 ppm) presented a descendent slope curve. Propionate (1.07 and 2.17 ppm) and saccharide region (3.50-4.00 ppm) presented a slight variation over the time, however at 3 months after dental treatment it was observed a reduction of these metabolites in comparison to the baseline. Lactate (1.32 and 4.07 ppm) is one example of metabolite that was found in constant level over the time. The ambiguous region (2.07 ppm) decreased after dental treatment.

We have also evaluated the saliva pH and it was different among groups that demonstrate an ascendant time-course. The pH median of children with dental caries at baseline (7.39) was statistically lower ($p = 0.03$; Mann-Whitney test) than children that never had dental caries (7.47). After 7 days (7.49), 1 month (7.44), 2 months (7.39), and after 3 months (7.52) of dental treatment the pH increased ($p < 0.05$; Wilcoxon test).

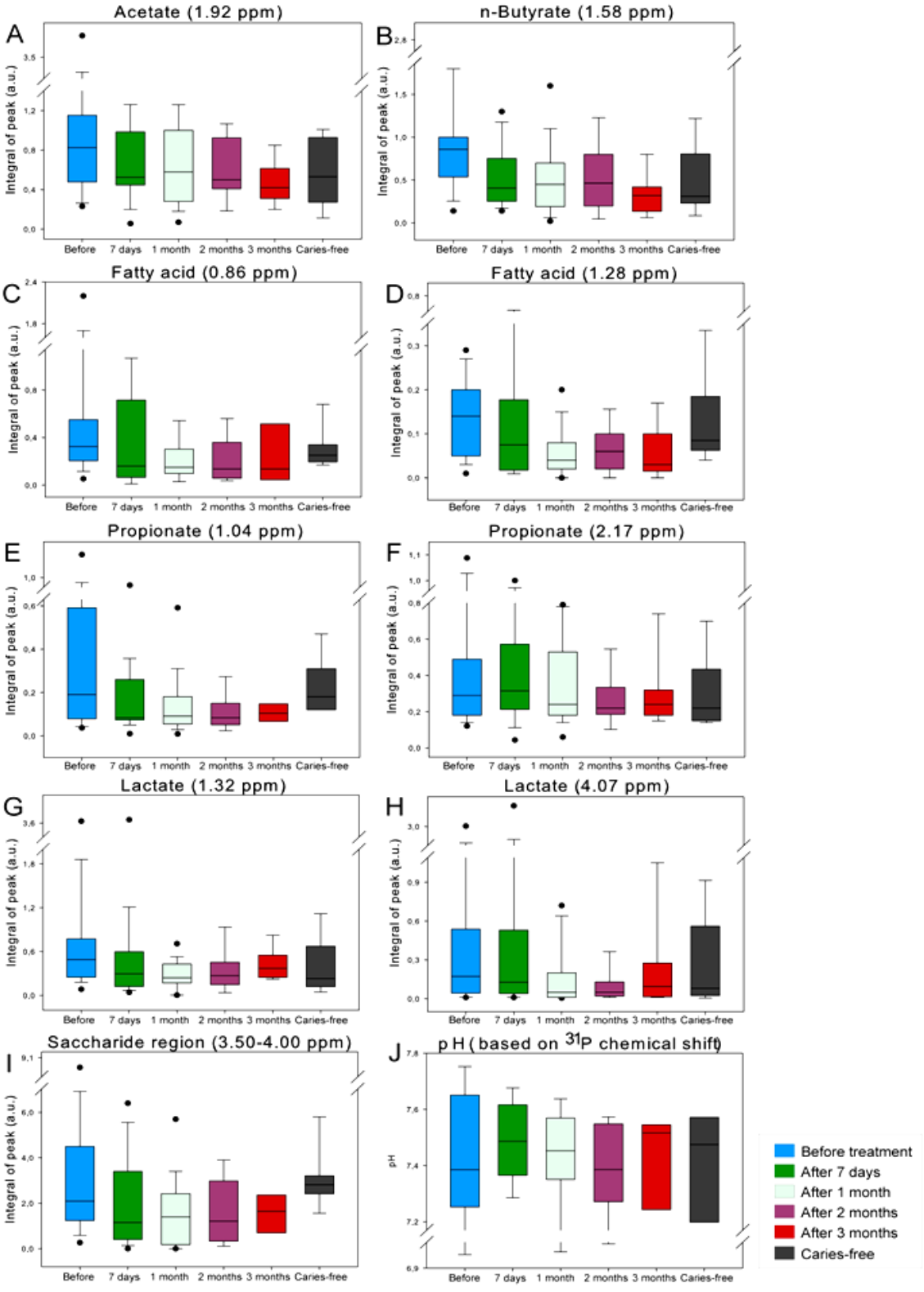


Figure 6: Representative *box plots* of candidates salivary metabolites in children with caries before and after dental treatment. Lactate is one example of unchanged metabolite. A- Acetate (1.92 ppm); B- n-Butyrate (1.58 ppm); C- Fatty acid (0.86 ppm); D- Fatty acid (1.28 ppm); E- Propionate (1.04 ppm); F- Propionate (2.17 ppm); G- Lactate (1.32 ppm); H- Lactate (4.07 ppm); I- Saccharide region (3.50-4.00 ppm); and J- pH (based on ^{31}P chemical shift).

4 DISCUSSION

We conduct the current study with clinical intervention and longitudinal evaluation in order to investigate how the candidates metabolites evolve (Fidalgo et al. 2013) after dental treatment. This is the first time that low molecular weight metabolites related to dental caries was longitudinal evaluated through metabolomic approach. Clinical studies that include treatment on metabolomics field can provide important information related to disease cycle (Puchades-Carrasco et al. 2013). The validity of metabolomics data is an important step in metabolomics field (Goodacre et al. 2007) and is provided though statistic analysis and also by confirming the metabolites findings. This study design allows validating the metabolites suggested to be related to disease, since after disorder remission the metabolites returned to lower levels trending to healthy condition. In addition, investigations that comprehend clinical treatment and longitudinal assessment over the time can provide the monitoring of treatment responses.

One remarkable difficult to conduct clinical studies is the occurrence of the dropouts and exclusions, particularly on follow-up evaluations. In the current study until two months follow-up after dental treatment the dropouts and exclusions were low. At the end of three months half of children do not remain in the study due the need to start to use antibiotics during the follow-up period and the reoccurrence of new caries lesions.

Metabolomics approach can assess information about perturbations of metabolism and establish a comprehensive metabolite fingerprint in health and disease condition (Deja et al. 2013; Fidalgo et al. 2013; Takeda et al. 2009). However, it is important to understand how metabolites supposed to be related to a specific disease behave after recovery of the health condition. Some studies have been conducted in order to validate the candidate metabolites and monitor treatment (Bertini et al. 2013; Li et al. 2013; Puchades-Carrasco et al. 2013). These studies also demonstrated that after disease remission metabolites reduced the levels of these metabolites in comparison to the baseline moment.

Acetate, butyrate, and propionate are compounds associated to bacterial metabolism which are able to decrease the pH and attract acidophilic

microorganisms such as *Streptococcus mutans* and *Lactobacillus spp* (Van Houte et al. 1989). Our results show that these organic acids are associated to the disease activity, since its reduction was observed after dental treatment. Corroborating with our findings, these metabolites were associated to caries lesion in biopsies from active lesions (Silwood et al. 1999).

As expected, the saliva pH from children with dental caries was lower in comparison to caries-free children. After dental treatment, it was observed an ascendant time-course of saliva pH. These findings are in accordance with time-course of acetate, n-butyrate, and propionate. These acids are responsible to pH falls of extracellular biofilm matrix attached to dental surface (Van Houte et al. 1989). It seems that saliva suffer less this decreasing of pH since organic acids are diluted in this biofluid and due to buffer capacity to maintain the pH nearby neutral (Morou-Bermudez et al. 2011; Tayab et al. 2012).

In the present study, a decreased saccharide concentration was observed in saliva from children after dental treatment followed by reduction of cariogenic microorganisms. *Streptococcus mutans* are naturally colonizing the mouth and its colonization increased with increasing age (Wan et al. 2003). In this context, many factors will determine dental caries lesion establishment, such as frequency of saccharide intake, oral hygiene habits, fluoride consumption, and others (Palmer et al. 2010). If this homeostasis is broken, for example with increase of sucrose permanency time in the mouth, *Streptococcus mutans* metabolize it and use for its energy requirement and result in production of organic acids (Van Houte et al. 1989). Polysaccharides that do not enter in microorganism cells may be used for the extracellular synthesis of carbohydrate polymer that allows the adhesion of Streptococcus mutans and colony growth (Mattos-Graner et al. 2000). In addition, they increase the thickness of dental plaque, resulting in enhanced rates of saccharide diffusion and acid production at deeper plaque layers nearby dental surface (Mattos-Graner et al. 2000; Van Houte et al. 1989). Lactobacillus sp is important during the caries progress process, since they are both acidogenic and aciduric and could multiply colonies in low pH of dental plaque and caries lesion irregular surface favoring their retention.

Our work demonstrated that children that never had dental caries presented a reduced number of *Streptococcus mutans* and *Lactobacillus sp* which is accordance with previous investigation (Parisotto et al. 2010; Tanner et al. 2011). In addition, children with dental caries presented higher count of these microorganisms in comparison to children after treatment and it seems that this reduction is stable during follow-up period. The adhesion and colonization of these microorganisms is modulated by roughness of hard tissue surfaces. Enamel integrity alterations leads to irregular and retentive tooth surface which enhance the colonization of these species on the tooth surfaces due to increased bacterial adherence, plaque retention and decrease in carbohydrate clearance (Li et al. 1994; Seow et al. 2000). It could be explain by the reduction of microorganisms after restoration of caries cavity.

Furthermore, the clearance of sucrose from oral cavity depends on the rate of saliva flow and spend one hour to return to its initial concentration (Sreebny et al. 1985). It is suggest that retentive surface can delay the maintenance of metabolites in contact with caries lesion, increasing the exposure time to hard tissue promoting a demineralization and sucrose with microorganisms, producing more acids.

The lipid concentrations in parotid saliva from caries-susceptible subjects are higher than those of caries-resistant subjects and this concentration is stimuli-dependent (Fidalgo et al. 2012; Fidalgo et al. 2013; Neyraud et al. 2013). The current study analyzed whole salivary samples and showed that fatty acid levels were also higher before treatment. Salivary lipids vary according to biofilm maturation and this process is accompanied by an increase of neutral and phospholipids contents (Slomiany et al. 1989). Higher salivary lipid concentration in caries subjects is associated to the increased lipid content in dental plaques and this has a considerably greater capacity to retard acid diffusion that determines the susceptibility of the tooth surface to demineralization (Slomiany et al. 1989). Also, restorations procedures do not change the risk factors, but modify the properties of oral cavity, such as roughtness surface and adhesion properties.

5 CONCLUSION

PLS-DA modeled based on ^{1}H-NMR saliva samples confirmed and validated previous candidate metabolites for caries disease. In addition, the clinical treatment provide the behavior information concerning of metabolites time-course. It was demonstrating that after dental treatment the candidate metabolites were reduced and maintained low in longitudinal evaluation.

Acknowledgments

The authors acknowledge the financial support from the following agencies: National Institute of Science and Technology of Structural Biology and Bioimaging (INCT-INBEB), CNPq, FAPERJ, FINEP, and CAPES.

REFERENCES

AAPD (2011). Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies. *Pediatr Dent*, 30(7 Suppl), 40-43.

Aimetti M, Cacciatore S, Graziano A, Tenori L (2012). Metabonomic analysis of saliva reveals generalized chronic periodontitis signature. *Metabolomics*, 8(3), 465–474.

Bergandi L, Defabianis P, Re F, Preti G, Aldieri E, Garetto S, et al. (2007). Absence of soluble CD14 in saliva of young patients with dental caries. *Eur J Oral Sci*, 115(2), 93-96.

Bertini I, Luchinat C, Miniati M, Monti S, Tenori L (2013). Phenotyping COPD by 1H NMR metabolomics of exhaled breath condensate. *Metabolomics*, *In press*(0), 0.

Bertram HC, Eggers N, Eller N (2009). Potential of human saliva for nuclear magnetic resonance-based metabolomics and for health-related biomarker identification. *Anal Chem*, 81(21), 9188-9193.

Caufield PW, Cutter GR, Dasanayake AP (1993). Initial acquisition of mutans streptococci by infants: evidence for a discrete window of infectivity. *J Dent Res*, 72(1), 37-45.

Cuevas-Cordoba B, Santiago-Garcia J (2014). Saliva: A Fluid of Study for OMICS. *Omics*,

Dawes C (1972). Circadian rhythms in human salivary flow rate and composition. *J Physiol*, 220(3), 529-545.

Deja S, Dawiskiba T, Balcerzak W, Orczyk-Pawilowicz M, Glod M, Pawelka D, et al. (2013). Follicular adenomas exhibit a unique metabolic profile. (1)H NMR studies of thyroid lesions. *PLoS One*, 8(12), e84637.

Fidalgo TKS, Abreu V, Freitas-Fernandes LB, Souza IPR, Maia LC (2012). Do salivary lipids influence dental caries susceptibility? A systematic review. *Open access scientific reports*, 1(12), 1-4.

Fidalgo TKS, Freitas-Fernandes LB, Angeli R, Muniz AMS, Gonsalves E, Santos R, et al. (2013). Salivary metabolite signatures of children with and without dental caries lesions. *Metabolomics*, 9(3), 657-666.

Fiehn O, Robertson D, Griffin J, Werf M, Nikolau B, Morrison N, et al. (2007). The metabolomics standards initiative (MSI). *Metabolomics*, 3(3), 175-178.

Garcia-Godoy F, Hicks MJ (2008). Maintaining the integrity of the enamel surface: the role of dental biofilm, saliva and preventive agents in enamel demineralization and remineralization. *J Am Dent Assoc*, 139 Suppl(25S-34S.

Goodacre R, Broadhurst D, Smilde AK, Kristal BS, Baker JD, Beger R, et al. (2007). Proposed minimum reporting standards for data analysis in metabolomics. *Metabolomics*, 3(3), 231-241.

Grootveld M, Silwood CJ (2005). 1H NMR analysis as a diagnostic probe for human saliva. *Biochem Biophys Res Commun*, 329(1), 1-5.

Jolliffe IT (2002). Principal component analysis. New York: Springer-Verlag; 2. ed. .

Law V, Seow WK (2007). A longitudinal study of 0.2% chlorhexidine gel for removal of mutans streptococci infection in preschool children. *Aust Dent J*, 52(1), 26-32.

Li L, Sui W, Che W, Li W, Chen J, Li H, et al. (2013). 1H NMR-based metabolic profiling of human serum before and after renal transplantation. *Asaio J*, 59(3), 286-293.

Li Y, Navia JM, Caufield PW (1994). Colonization by mutans streptococci in the mouths of 3- and 4-year-old Chinese children with or without enamel hypoplasia. *Arch Oral Biol*, 39(12), 1057-1062.

Martins-Junior PA, Vieira-Andrade RG, Correa-Faria P, Oliveira-Ferreira F, Marques LS, Ramos-Jorge ML (2013). Impact of early childhood caries on the oral health-related quality of life of preschool children and their parents. *Caries Res*, 47(3), 211-218.

Mattos-Graner RO, Smith DJ, King WF, Mayer MP (2000). Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children. *J Dent Res*, 79(6), 1371-1377.

Morou-Bermudez E, Elias-Boneta A, Billings RJ, Burne RA, Garcia-Rivas V, Brignoni-Nazario V, et al. (2011). Urease activity in dental plaque and saliva of children during a three-year study period and its relationship with other caries risk factors. *Arch Oral Biol*, 56(11), 1282-1289.

Neyraud E, Tremblay-Franco M, Gregoire S, Berdeaux O (2013). Relationships between the metabolome and the fatty acid composition of human saliva; effects of stimulation. *Metabolomics*, 9(1), 213-222.

Ng DJY, Pasikanti KK, Chan ECY (2011). Trend analysis of metabonomics and systematic review of metabonomics-derived cancer marker metabolites. *Metabolomics*, 7(2), 155-172.

Nosaka AY, Fukutomi S, Uemura S, Kobayashi Y, Kyogoku Y (1998). Phosphorus-31 nuclear magnetic resonance study of healthy human saliva. *Arch Oral Biol*, 43(4), 341-346.

Palmer CA, Kent R, Jr., Loo CY, Hughes CV, Stutius E, Pradhan N, et al. (2010). Diet and caries-associated bacteria in severe early childhood caries. *J Dent Res*, 89(11), 1224-1229.

Parisotto TM, Steiner-Oliveira C, Silva CM, Rodrigues LK, Nobre-dos-Santos M (2010). Early childhood caries and mutans streptococci: a systematic review. *Oral Health Prev Dent*, 8(1), 59-70.

Puchades-Carrasco L, Lecumberri R, Martinez-Lopez J, Lahuerta JJ, Mateos MV, Prosper F, et al. (2013). Multiple myeloma patients have a specific serum metabolomic profile that changes after achieving complete remission. *Clin Cancer Res*, 19(17), 4770-4779.

Ramadan Z, Jacobs D, Grigorov M, Kochhar S (2006). Metabolic profiling using principal component analysis, discriminant partial least squares, and genetic algorithms. *Talanta*, 68(5), 1683-1691.

Rigante D, Inzitari R, Carone M, Fanali C, Stabile A, Cabras T, et al. (2008). Correspondence between clinical improvement and proteomic changes of the salivary peptide complex in a child with primary Sjogren syndrome. *Rheumatol Int*, 28(8), 801-806.

Seow WK, Wan A (2000). A controlled study of the morphometric changes in the primary dentition of pre-term, very-low-birthweight children. *J Dent Res*, 79(1), 63-69.

Silwood CJ, Lynch EJ, Seddon S, Sheerin A, Claxson AW, Grootveld MC (1999). 1H-NMR analysis of microbial-derived organic acids in primary root carious lesions and saliva. *NMR Biomed*, 12(6), 345-356.

Slomiany BL, Murty VL, Mandel ID, Zalesna G, Slomiany A (1989). Physico-chemical characteristics of mucus glycoproteins and lipids of the human oral mucosal mucus coat in relation to caries susceptibility. *Arch Oral Biol*, 34(4), 229-237.

Sreebny LM, Chatterjee R, Kleinberg I (1985). Clearance of glucose and sucrose from the saliva of human subjects. *Arch Oral Biol*, 30(3), 269-274.

Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M (2010). Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics*, 6(1), 78-95.

Takeda I, Stretch C, Barnaby P, Bhatnager K, Rankin K, Fu H, et al. (2009). Understanding the human salivary metabolome. *NMR Biomed*, 22(6), 577-584.

Tanner AC, Kent RL, Jr., Holgerson PL, Hughes CV, Loo CY, Kanasi E, et al. (2011). Microbiota of severe early childhood caries before and after therapy. *J Dent Res*, 90(11), 1298-1305.

Tayab T, Rai K, Kumari AV (2012). Evaluating the physicochemical properties and inorganic elements of saliva in caries-free and caries-active children. An in vivo study. *Eur J Paediatr Dent*, 13(2), 107-112.

Tenuta LM, Del Bel Cury AA, Bortolin MC, Vogel GL, Cury JA (2006). Ca, Pi, and F in the fluid of biofilm formed under sucrose. *J Dent Res*, 85(9), 834-838.

Twetman S, Grindefjord M (1999). Mutans streptococci suppression by chlorhexidine gel in toddlers. *Am J Dent*, 12(2), 89-91.

Van Houte J, Russo J, Prostak KS (1989). Increased pH-lowering ability of Streptococcus mutans cell masses associated with extracellular glucan-rich matrix material and the mechanisms involved. *J Dent Res*, 68(3), 451-459.

Van Nieuw Amerongen A, Bolscher JG, Veerman EC (2004). Salivary proteins: protective and diagnostic value in cariology? *Caries Res*, 38(3), 247-253.

Wan AK, Seow WK, Purdie DM, Bird PS, Walsh LJ, Tudehope DI (2003). A longitudinal study of Streptococcus mutans colonization in infants after tooth eruption. *J Dent Res*, 82(7), 504-508.

Supplementary Material

Longitudinal evaluation of salivary profile from children with dental caries before and after treatment

Tatiana K. S. Fidalgo[a], Liana B. Freitas-Fernandes,[a] Fabio C. L. Almeida[b], Ana P. Valente[b] Ivete P. R. Souza[a]

[a]Department of Pediatric Dentistry and Orthodontics, School of Dentistry, universidade Federal do Rio de Janeiro, Brazil;
[b]National Center for Nuclear Magnetic Resonance – Jiri Jonas, Medical Biochemistry Institute, Federal University of Rio de Janeiro, Brazil;

pomarico@gmail.com

This document contains supporting information for the features from validation of acetate (Figure S-1), glycine (Figure S-2), lactate (Figure S-3), ethanol (Figure S-4), and saccharide region (Figure S-5); Partial Least Square Discriminant Analysis scatter plots for caries-free and caries children (Figure S-6); and boxplot of ambiguous peak (Figure S-7).

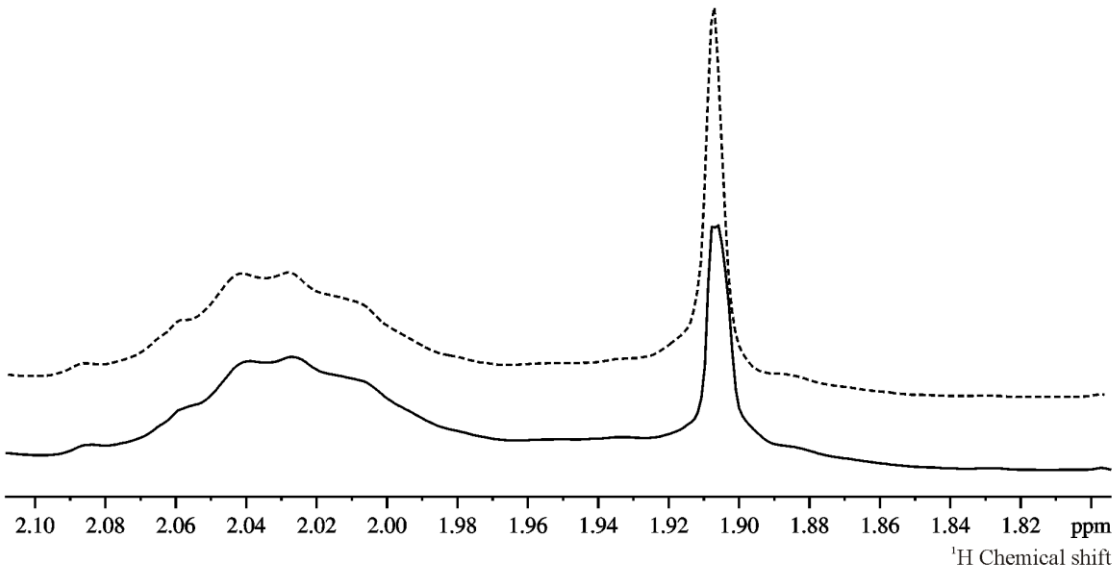


Figure S-1: Spectra showing 0.81–2.10 ppm region of high resolution ^{1}H NMR 400 MHz. Spectra of saliva samples with (dotted line) and without (filled line) acetate addition, confirming the peak.

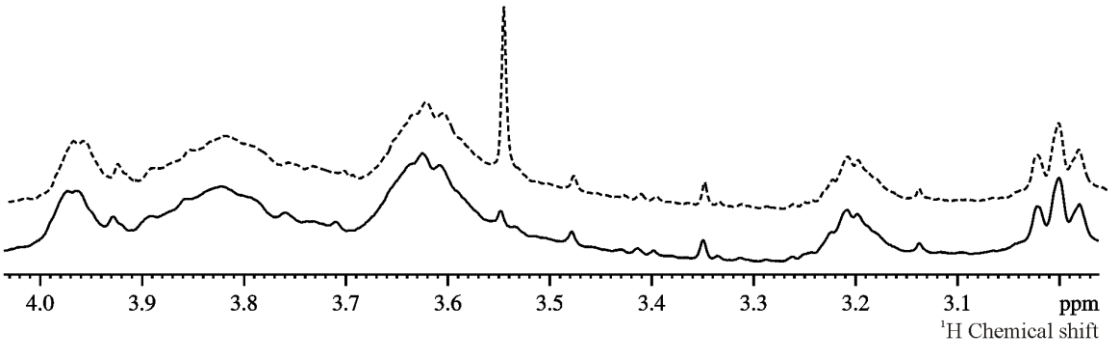


Figure S-2: Spectra showing 3.00–4.00 ppm region of high resolution ^{1}H NMR 400 MHz. Spectra of saliva samples with (dotted line) and without (filled line) glycine addition, confirming the peak.

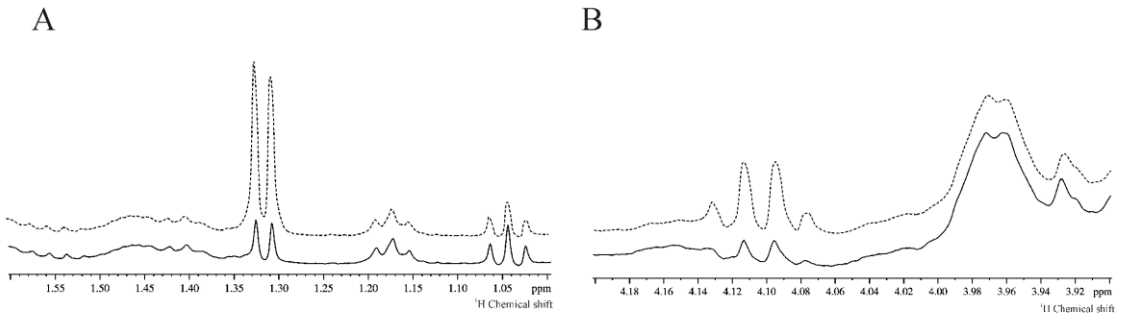


Figure S-3: Spectra of high resolution ^{1}H NMR 400 MHz. A- Spectra showing 1.00–1.60 ppm region and B- Showing 3.90-4.20 ppm region of saliva samples with (dotted line) and without (filled line) lactate addition, confirming the peak.

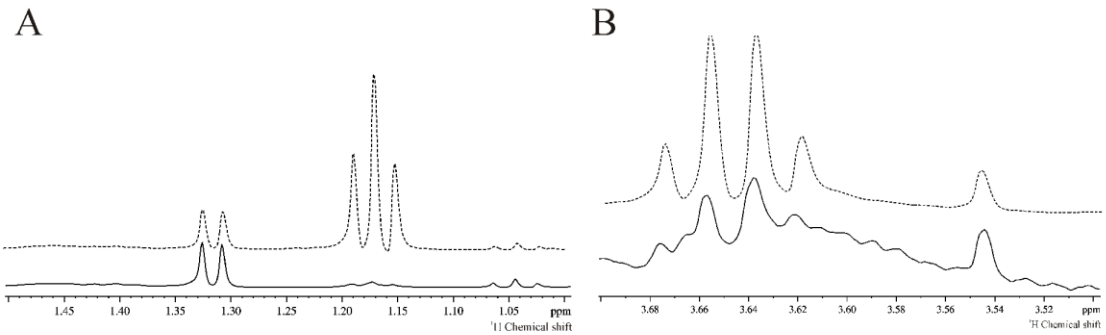


Figure S-4: Spectra of high resolution ^{1}H NMR 400 MHz. A- Spectra showing 1.00-1.50 ppm region and B- Showing 3.50-3.70 ppm region of saliva samples with (dotted line) and without (filled line) ethanol addition, confirming the peak.

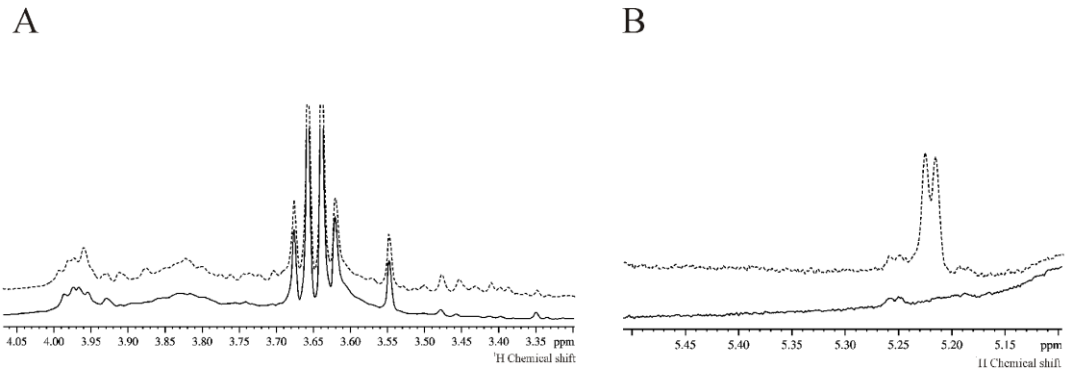


Figure S-5: Spectra of high resolution ^{1}H NMR 400 MHz. A- Spectra showing 3.30-4.05 ppm region and B- Showing 5.10-5.50 ppm region of saliva samples with (dotted line) and without (filled line) saccharide region addition, confirming the region.

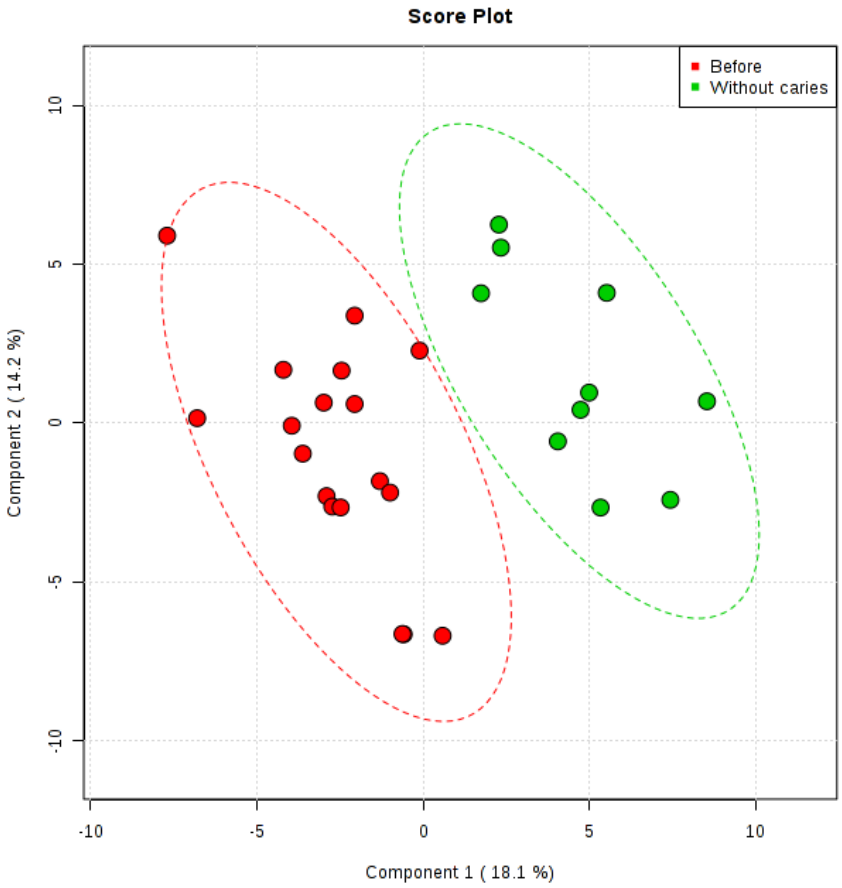


Figure S-6: Partial Least Square Discriminant Analysis model confirmed our previous finding in Fidalgo et al (2013) Metabolomics 9:657-666. The PLS-DA demonstrates the clear classification of children without caries and with caries before treatment.

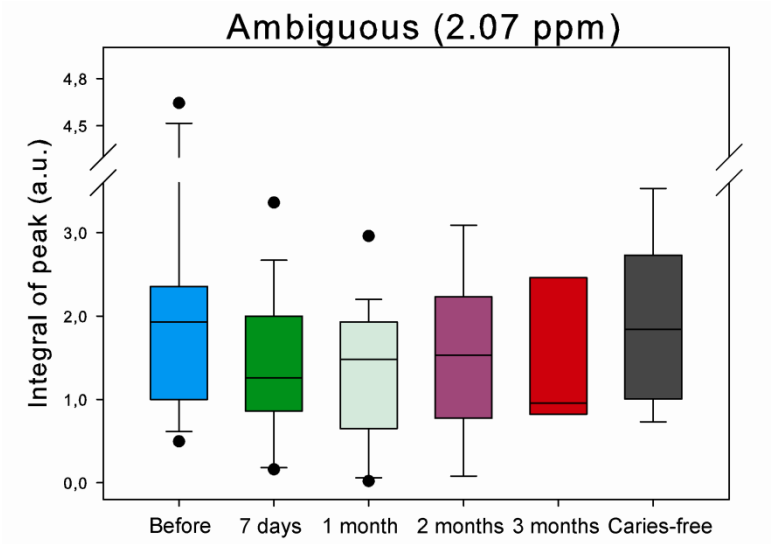


Figure S-7: *Boxplot* of ambiguous peak (2.07 ppm) from caries-free children, children with dental caries before and after dental treatment.

4.4 **ARTIGO 4:** Cluster analysis of risk factors for early childhood caries before and after dental treatment**.**

Tatiana K. S. Fidalgo[1]
Liana B. Freitas-Fernandes[1]
Isadora Passos Maciel[1]
Fabio C. L. Almeida[2]
Ana P. Valente[2]
Ivete P. R. Souza[1]

[a]Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Universidade Federal do Rio de Janeiro, Brazil;
[b]National Center for Nuclear Magnetic Resonance – Jiri Jonas, Medical Biochemistry Institute, Universidade Federal do Rio de Janeiro, Brazil;

Correspondence to: Dr Ivete Pomarico Ribeiro de Souza, Disciplina de Odontopediatria da FO-UFRJ, Caixa Postal: 68066 - Cidade Universitária - CCS, CEP.: 21941-971 - Rio de Janeiro – RJ – Brazil, Phone: + 55 21 25622101.
E-mail: pomarico@gmail.com

ABSTRACT

The aim of the study was to analyze clinical and microbiological risk factors relate with ECC before and after dental treatment. We investigated the *Streptococcus mutans* and *Lactobacillus sp* in saliva from caries-free children and ECC ones before and after dental treatment with follow-up evaluation. A questionnaire collected the demographic, dietary, hygiene, and behavioral data. Saliva samples were collected from caries-free children (n = 19) and from children with dental caries (n = 24) before and 7 days follow-up after treatment as well as 1 month, and 2 months. Caries was diagnosed using dmfs index. Saliva was prepared for *Streptococcus mutans* and *Lactobacillus sp* plate count method. After restoration of decayed teeth with composite resin, saliva samples were collected in previous mentioned follow-up periods. For statistical analysis it were applied the Chi-squared, Mann-Whitney, and Wilcoxon test with confidence interval set at 95%. PLS-DA was modeled to evaluate cluster formations. For caries group, the dmfs index mean was 11.0 ± 8.5. The prevalence of children that use nursing bottle over two years old was higher in ECC (75.0%) than in caries-free group (50.0%). No statistical difference in flow rate was observed among groups ($p > 0.05$). Caries-free children presented low levels of *Streptococcus mutans* and *Lactobacillus sp* comparing to children with ECC ($p < 0.05$; Mann-Whitney test). After dental treatment and follow-up it was also observed a significant reduction ($p < 0.05$) of *Streptococcus mutans* and *Lactobacillus sp.* Cluster analysis using PLS-DA model distinguished caries-free and ECC clusters, however after follow-up periods the cluster of treated children was not completed matched to caries-free ones. It is suggested that caries-free and ECC children present different microbial levels and clinical risk factors that influence the establishment of disease.

Key-words: Saliva; Dental caries; Children; *Streptococcus mutans*; *Lactobacillus sp*.

INTRODUCTION

Dental caries is a global public health challenge, especially among young children. Early childhood caries (ECC) is recognized as a significant public health problem in selected populations.[1] ECC is defined as the presence of one or more decayed, missing or filled tooth surfaces in any primary tooth in a preschool-age child between birth and 71 months of age. Children under 5 years of age can suffer psychological disorder as a result from oral health problems such as ECC.[2] This disorder can affect the immediate and long-term quality of life of the child and their family.[3]

Many factors will determine dental caries lesion establishment. Early acquisition of *Streptococcus mutans* (SM) associated to poor hygiene habits and high sugar intake has been strongly correlated to ECC risk and predict future caries incidence.[4-6] *Streptococcus mutans* are naturally colonizing the mouth.[7] Human mouth presents a proper ecosystem with a complex ecology of varied microbial species.[8,9] The relationship with these microorganisms should be commensal; however it is hampered due to modern diet and social behavior.[6,10] *Streptococcus mutans* have the ability to make dental plaque more porous in presence of sucrose, resulting in enhanced rates of acid diffusion nearby tooth surface.[11,12] *Lactobacillus sp* (L) is both acidogenic and aciduric being important during the caries progress process.[13]

It is well known that children with caries present higher counts of *Streptococcus mutans* and *Lactobacillus sp.*[14-16] However, few studies[9,17] are conducted to establish microorganisms count after dental treatment in ECC and post-treatment follow-up by using a broad multivariate approach. Thus, the objective of this study was to evaluate microbiological (SM and L) and clinical risk factors before and after dental treatment, as well as post-treatment follow-up. Partial least squared-discrimnant analysis (PLS-DA) is a robust method that was used to simultaneously evaluation of the different variables of each studied child.

MATERIAL AND METHODS

Study subjects

The use of human material was approved by the proper Research Ethics Committee of Community Health Studies.

The study population consisted of 43 children in primary dentition until 71 months of age with ECC (n = 24) and caries-free (n = 19) attending the Pediatric Dentistry Clinic from Federal University of Rio de Janeiro for regular dental care. None of the subjects had any periodontal or clinical evidence of any systemic disease nor had taken any systemic antibiotics in the 3 months prior to saliva sample collection.

Caries per tooth surface were diagnosed by a single calibrated examiner (Kappa = 0.98) using the visual classification using the Decay-Missing-Filled Surface (dmfs) index as described by the World Health Organization.[18] Clinical examination was performed using a dental probe, mouth mirrors, and artificial light. Radiographs were taken in cases of pulp involvement doubt and only manifest lesions in the primary teeth were considered. It was excluded children that presented restored surfaces and teeth with pulp involvement or with indicated extraction. The ECC group was composed by children with dmfs = 10.8 (decayed) ± 7.9 and caries-free group was composed by children with dmfs = 0. The caregivers were interviewed about questions concerning socio-demographics status, child's feeding practice (breast feeding, bottle feeding), dietary habits, and oral health practices.

Clinical procedures and saliva collection

Saliva samples collections were performed before treatment, seven days, one month, and two months after. For the caries-free group, saliva collection was performed in a single moment. Subjects who begin use systemic antibiotics and develop a systemical or local disorder during study periodwere excluded of the study. Patients were submitted to the 3 mL of unstimulated whole saliva collection using an automatic pipette. Saliva was passively collect from the floor of the mouth towards into a plastic universal tube for about 10 min and the salivary flow was calculated.

The saliva sample from all children were taken at the same time (8.00 am to 10.00 am) to avoid fluctuation in the results because cicardian saliva cycle.[19] They were asked to refrain from oral activities for 2 h prior to saliva collection. Prior to the centrifugation, 300 µl were separated to the microbiological analysis.

Children with ECC had their teeth restored with composite resin (TPH, Dentisply, USA) according to the manufacturer's instruction. All children were submitted to preventive measurements, such as instructions about oral hygiene and dietary habits, professional prophylaxis, and fluoride application.

Streptococcus mutans and *Lactobacillus sp* count in saliva

Streptococcus mutans and *Lactobacillus sp* were evaluated. In a maximum period of 2 hours after sampling, saliva samples were diluted to 10^0, 10^{-1}, 10^{-2}, and 10^{-3} in 0.85% NaCl sterilized solution and *Streptococcus mutans* and *Lactobacillus sp* counts were performed. For this purpose, 50 µl of the dilutions of saliva were platted on 10 mL Mitis salivarius (Difco, Detroit, USA) bacitracin 20% sucrose agar for 48 h for *Streptococcus mutans* and Rogosa (Difco, Detroit, USA) for *Lactobacillus sp* in candle jars at 37°C. After this period, the colonies of microorganisms were counted. Colonies from each patient were stored for *Streptococcus mutans* species identification though morphology evaluation using a stereoscopic microscope.

Statistical analysis

The interview answers, flow rate, *Streptococcus mutans* and *Lactobacillus sp* count were tabulated and analysed on SPSS 20.0 (SPSS Inc, IL, USA). Descriptive analysis was done for the questionnaire answers. The continuous variables such as flow rate and microorganisms count were submitted to Shapiro-Wilk normalcy test and the null hypothesis was rejected ($p < 0.05$), thus it was applied nonparametric tests. Wilcoxon test was applied for assessment of continuous paired variables; and Kruskal Wallis and Mann Whitney test for continuous independent variables. Chi-squared was applied for analysis of categorical variables. The confidence interval was set at 95%.

In addition, the Partial Least-squares Discrinmant Analysis (PLS-DA) with Metaboanalyst 2.0 (www.metaboanalyst.ca) was used for multivariate analysis of microorganisms count, flow rate, dietary and hygyne habits, and fluoride toothpaste use. PLS-DA is a multivariate linear regression model method indicated proper to identify correlations between matrices of descriptor in datasets. PLS-DA is able to analyze at many variables and plot the representative image of each subject based on input variables. Since dental caries a multifactorial disease and many variables influence in this outcome, PLS-DA model was applied to analyse this data. Methods such as PLS-DA are used for clustering groups.[20] The objective is to find a mathematical model that correctly associates all or some of the inputs with the target classes. This goal is achieved by minimizing the error between the known target and the output (model's response). It was chosen for modeling only caries-free versus ECC and caries-free versus children after 3 months follow-up. The other groups were not submitted to PLS-DA model to avoid repeated data in model, since the subjects before and after dental treatment present same questionnaire information.

RESULTS

The Table 1 shows that there were no differences in child age and gender. The group of children with ECC was composed by children with mean age = 3.3 years ± 1.7, being 9 female and 15 male. The caries-free group included children with mean age = 3.9 years ± 2.1, being 8 female and 11 male.

The ECC group was composed by 24 children and after 7 days follow-up occurred 4 drop-outs remaining 20 children. After 1 month follow-up, one children was excluded due to antibiotic need for systemic disease remaining 19 children. Finally, after 2 months follow-up, one more children were excluded due to antibiotic need remaining 18 children. Regarding the localization of decayed surfaces, it was found that superior arch presented more lesions than inferior arch ($p = 0.02$).

Table 1 shows that caries-free children that use nurse bottle were statistically higher (82.4%) than ECC children (45.8%). However, ECC number children that presented this habit until two years old was higher (75.0%) than caries-free children

(50.0%). Reported daily use of fluoride-containing toothpaste also did not differ statistically, at 66.7% children for caries-free and 70.9% ECC ones.

Table 1: Children's demographic data, localization of decayed surfaces, dietary habits, and hygiene background**.**

Parameters	Caries-free	ECC	p-value
Child age (years)	3.9 ± 2.1	3.3 ± 1.7	0.37
Gender			
Female	36.0%	44.4%	0.58
Male	64.0%	55.6%	
Decayed surface localization			
Global dmfs	0.0 ± 0.0	10.8 ± 7.9	
Superior arch	0.0 ± 0.0	8.3 ± 7.5	0.02
Inferior arch	0.0 ± 0.0	2.3 ± 2.3	
Anterior region	0.0 ± 0.0	6.3 ± 6.5	0.26
Posterior region	0.0 ± 0.0	3.8 ± 3.8	
Buccal	0.0 ± 0.0	2.4 ± 2.2	
Lingual	0.0 ± 0.0	2.0 ± 2.0	
Mesial	0.0 ± 0.0	1.9 ± 1.9	0.05
Distal	0.0 ± 0.0	1.8 ± 1.8	
Incisal/Oclusal	0.0 ± 0.0	2.4 ± 1.9	
Breastfeeding habit			
Breastfeeding	88.9%	92.0%	0.73
Exclusive breastfeeding (until 6m)	7.1%	11.1%	0.70
Nocturnal breastfeeding	83.3%	86.4%	0.79
Nocturnal hygiene	16.7%	14.3%	0.85
Nursing bottle			
Nursing bottle	82.4%	45.8%	0.02
Nursing bottle (over 2 years)	50.0%	75.0%	0.19
Nocturnal nursing bottle	66.7%	70.8%	0.77
Teeth brushing			
More than 2 times in a day	61.1%	75.0%	0.34
Fluoride toothpaste use	66.7%	70.8%	0.77
High sugar consumption	50.0%	62.5%	0.42

Table 2 shows that when cariogenic microorganisms were evaluated it was observed a higher levels of *S. mutans* and *Lactobacillus sp* in children with decayed

teeth surface in comparison to children that never had dental caries ($p < 0.01$; Mann Whitney test). Children that never had dental caries presented lowers levels of *S. mutans* and *Lactobacillus sp* compare to children with ECC ($p < 0.01$; Mann Whitney test). After 7 days, 1 month, and 2 months of dental treatment, it was observed a significant reduction in *S. mutans* and *Lactobacillus sp* ($p < 0.01$; Wilcoxon test).

Table 2: *S. mutans*, *Lactobacillus sp*, and flow rate of caries-free children and ECC before and after treatment

Groups	*S. mutans* (CFU/mL)	*S. mutans* p-value	*Lactobacillus sp* (CFU/mL)	Lactobacillus sp p-value	Flow rate (mL/min)	Flow rate p-value
ECC	2.7×10^5 ($\pm 3.8 \times 10^5$)	$< 0.01^a$	1.1×10^4 ($\pm 2.7 \times 10^4$)	$< 0.01^a$	0.179 ($\pm$ 0.9)	0.51^a
7 days follow-up	5.9×10^4 ($\pm 3.8 \times 10^5$)	$< 0.01^b$	9.1×10^2 ($\pm 1.4 \times 10^3$)	$< 0.01^b$	0.156 ($\pm$ 0.7)	0.23^b
1 month follow-up	3.4×10^4 ($\pm 1.3 \times 10^5$)	$< 0.01^c$	1.0×10^3 ($\pm 2.8 \times 10^3$)	$< 0.01^c$	0,161 ($\pm$ 0.1)	0.31^c
2 months follow-up	6.3×10^4 ($\pm 1.1 \times 10^5$)	$< 0.01^d$	6.8×10^2 ($\pm 2.1 \times 10^3$)	$< 0.01^d$	0.204 ($\pm$ 0.2)	0.57^d
Caries-free	6.5×10^3 ($\pm 1.2 \times 10^4$)	$< 0.01^e$	1.6×10^1 ($\pm 5.6 \times 10^1$)	$< 0.01^e$	0.128 ($\pm$ 0.1)	0.13^e

a = comparison between ECC and caries-free; b = comparison between ECC and 7 days follow-up; c = comparison between ECC and 1 month follow-up; d = comparison between ECC and 2 months follow-up; e = comparison between caries-free and 2 months follow-up.

Figure 1 illustrates in log10 scale that after dental treatment it was also observed a significant reduction ($p < 0.05$; Wilcoxon test) in *Streptococcus mutans* and *Lactobacillus sp*. After dental treatment, independent of the follow up, it was observed that *Streptococcus mutans* and *Lactobacillus* count was not similar to children that never had dental caries ($p < 0.05$; Mann Whitney test).

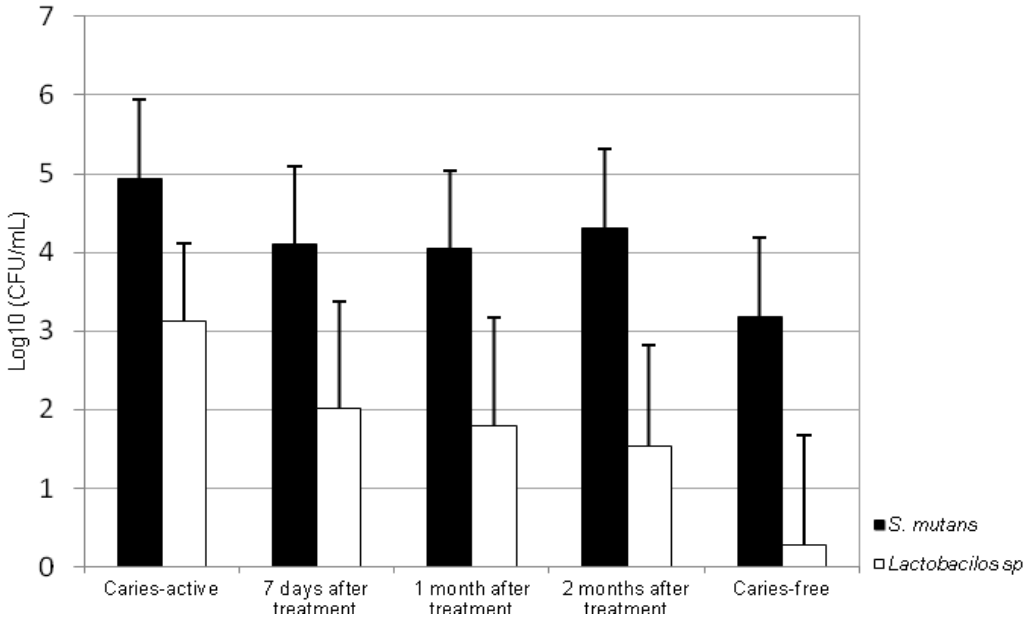


Figure 1: *Streptococcus mutans* and *Lactobacillus sp* (CFU/mL in Log10 scale) from caries-free and ECC children before and after 7 days, 1 month, and 2 months follow-up after dental treatment.

The evaluation of flow rate is important to discard the possibility of this parameter influence in the microorganism count and metabolites levels. We found a similar flow rate when comparing children caries-free that never had the disease ($p > 0.05$; Mann Whitney test). Flow rate among children before and after treatment follow-up also demonstrated to be similar ($p > 0.05$; Wilcoxon test).

Figure 2A shows that risk factors for caries included as input variables were consistent to distinguish caries-free and ECC children. PLS-DA analysis also showed that children with dental caries present higher inter-individual variability, expressed by dispersion of points. It is clearly observed two clusters separating both groups. Figure 2B shows that after 7 days follow-up of dental treatment some subjects are closer to caries-free children, but the major subjects remains far from caries-free cluster. Figures 2C and 2D showed similar cluster separation and demonstrated that after 2 and 3 months follow-up more subjects became closer to the caries-free group.

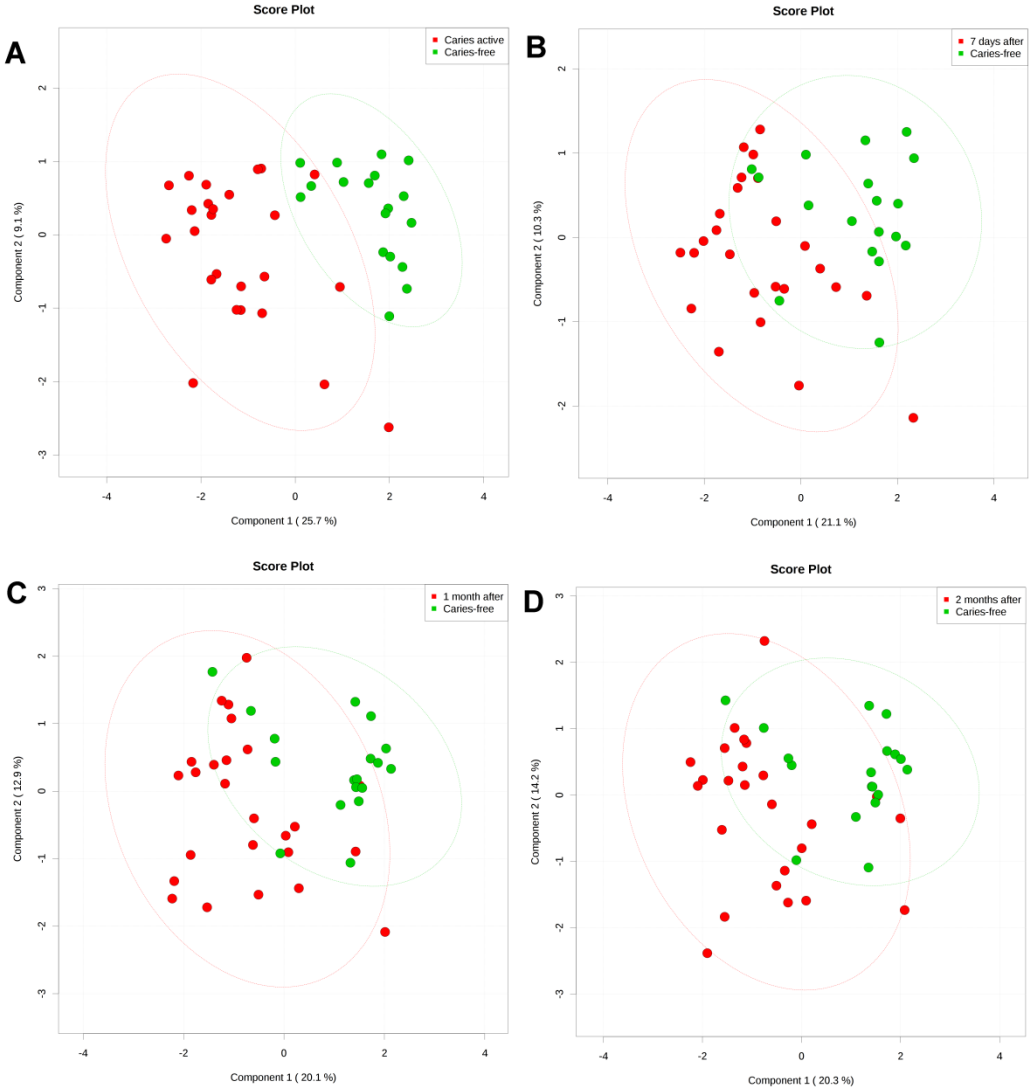


Figure 2: A- PLS-DA of caries-free children versus ECC before treatment. B- PLS-DA of caries-active children versus children after 7 days follow-up. C- PLS-DA of caries-active children versus children after 1 month follow-up. D- PLS-DA of caries-active children versus children after 2 months follow-up.

DISCUSSION

Global rates of caries have been controlled; however ECC continues to be a significant concern. This disease disproportionately affects disadvantaged populations.[21] The current study showed lower levels of *Streptococcus mutans* and *Lactobacillus sp* in caries-free children in comparison to ECC ones, corroborating with previous data.[9] In addition, we observed a reduction in these microorganisms after dental treatment and it were stable until 3 months follow-up.[13] The adhesion and colonization of these microorganisms is modulated by cavity number. Enamel

integrity alterations leads to irregular and retentive tooth surface which enhance the colonization of these species on the tooth surfaces due to increased bacterial adherence, plaque retention and decrease carbohydrate clearance.[22,23] It could explain the reduction of microorganisms after restoration of caries cavity and the difference of microorganisms counts from caries-free group. It is important to highlight that all children from ECC group do not presented any restoration at the baseline.

PLS-DA consists on a robust statistic method that can be used for multivariate analysis with large dataset,[24,25] in this case for caries risk factors. PLS-DA was successfully applied in the present study and it was possible to distinghish two clusters, from children with ECC and caries-free. Interestingly, PLS-DA also showed that after decayed surfaces restoration the microbiota did not returned to similar levels of children that never had dental caries. The higher inter-individual variability in caries active group can be explained by the range in dmft index and oral dietary and oral hygiene habits. The increased roughness of resin restoration could contribute to increase the adhesion of microorganisms and it high levels. [9] Moreover, our findings are in line with previous data. They suggest that in more aggressive caries after decayed restoration the microbiologic profile from dental plaque differ from caries-free children.[17,26]

The microorganisms evaluated are acidogenic and aciduric species and demonstrated a strong association with dental caries. The extracellular polysaccharides produced an increased porosity of the dental plaque matrix and allows the enamel demineralization by hydroxiapatite dissolution.[27] *Streptococcus mutans* are naturally colonizing the mouth and its colonization increased with increasing age.[7] In our work, it was included in caries-free and ECC group children up to 71 months and the age was similar between the groups.

Recent studies have been shown by molecular methods that other cultivable and also uncultivable species are associated with ECC.[9,28] Tunner et al. associated the microbiota after treatment to the occurrence of new lesions. It was demonstrated that after treatment children without new lesions presented reduced levels of several species in comparison to children with recurrence of new lesion, suggesting a shift in the plaque microbial complex. Children with recurrent caries presented no significant

changes in microbiota species compared to pre-treatment, suggesting maintenance of the microbiota that was responsible for new lesions. In the current work, children who began to use systemical antibiotic were excluded to the study. Antibiotics have been demonstrated to have suppressive effects on *Streptococcus mutans*. Previous studies showed that antibiotics can temporarily reduce or eliminate this microorganisms from the oral cavity.[29] Preschools children use frequently antibiotics due to high incidence of disease at this age,[30,31] it justify the drop outs. Two months follow-up was sufficient to demonstrate a stabilization of oral microbiota levels. It was confirmed by PLS-DA that showed similar cluster separation in 1 and 2 months follow-up. After 2 months the drop out began to increase due to the development of new lesions, what is high prevalent in ECC children.[9] Also, 2 months was sufitient follow-up peiod to observe a constancy in the microbiota values.

Besides oral microbiota, many other factors determine dental caries lesion establishment, such as saccharide rich diet, oral hygiene habits, fluoride consumption, and oral health behavior. Children with ECC with saccharide rich diet presented higher prevalence than caries-free ones. Our data support previous evidence of the cariogenic potential of sugar beverages.[32] The high frequency and prolonged sugar intake is important to determine the caries lesion risk.[33] More children in caries-free group used nursing bottle and both groups intakes cariogenic content. However, it was observed a prolonged use of nursing bottle over two years old in ECC children.

The clearance of sucrose from oral cavity depends on the rate of saliva flow.[34] In our study, it was not observed difference in saliva flow rate among groups. When oral homeostasis is broken, for example with increase of sucrose permanence in mouth, *Streptococcus mutans* metabolize it and use for its energy requirement and result in production of organic acids.[12] It is suggest that retentive surface prologue the maintenance of organic acids and the sugar produced by cariogenic microorganisms in contact with caries lesion. Thus, the increased exposure time of acid to hard tissue promotes a demineralization and the sucrose is metabolized by microorganisms, producing more acids.

Microorganisms from dental biofilm produce a variety of end-products that can be altered according to dietary habits. When fermentable carbohydrates are available

to biofilm, the main organic acids produced are lactic, formic, and acetic acids.[35] These acids modulate the pH drop in biofilme, resulting in demineralization hard tissue. In addition, extracellular polysaccharides produced by *Streptococcus mutans* create an environment which is advantageous for further cells attachment colony growth.[11] These extracellular polymers turn the extracellular matrix more permeable and increases the rates of more saccharide and diffusion at deeper plaque layers nearby dental surface.[11,12] The *Streptococcus mutans* express a wide range of virulence factors that are responsible for the biofilm cariogenicity. Although, saliva provides the host defense systems against these virulence factors, the balance between de- and remineralization will depend on the time of acid exposure and removal of this cariogenic biofilm.[6,36] When this process is not interrupted, the pH drops to low levels and cavity is formed. In this context, increased counts of *Lactobacillus sp* are present in the biofilm, since their it pronounced characteristic of be more acidogenic and acidophilic.[16]

It was observed in ECC group a statistically higher prevalence of decayed surfaces in superior arch than inferior arch. It can be explained by the deposition of saliva in the floor of mouth that is in direct contact with teeth from inferior arch protecting it. Saliva present an important role in basic-acid balance in the mouth and avoid pH decrease and tooth demineralization.[37] When saliva is in a neutral pH there is a super-saturated of calcium and phosphate that favors calcium deposition. Moreover, other components besides inorganic content, such as proteins, lipids, and organic low molecular weight metabolites are responsible for oral homeostasis and related dental caries.[38-40] Furthermore, the mechanical cleansing property of saliva avoid the prolonged contact of food debris and tooth surface.

ECC children, even after dental treatment, does not remained risk factors similar to caries-free children. Children that had dental caries have an increases potential to develop recurrent lesions.[9] For this especific population, preventive measures, shorter follow-up periods, and differential treatment based on oral health promotion is required to avoid caries recurrence.

CONCLUSION

The present findings showed that higher caries risk factors in ECC children. Also, after treatment there is a reduction in cariogenic microbiota levels and even after restoration of decayed surfaces, theses levels are not comparable to children who never had dental caries, suggesting a predisposing to the disease.

Acknowledgments

The authors acknowledge the financial support from the following agencies: National Institute of Science and Technology of Structural Biology and Bioimaging (INCT-INBEB), CNPq, FAPERJ, FINEP, and CAPES.

REFERENCES

1. AAPD. Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies. Pediatr Dent 2011;30(7 Suppl):40-43.

2. Moura-Leite FR, Ramos-Jorge ML, Bonanato K, Paiva SM, Vale MP, Pordeus IA. Prevalence, intensity and impact of dental pain in 5-year-old preschool children. Oral Health Prev Dent 2008;6(4):295-301.

3. Martins-Junior PA, Vieira-Andrade RG, Correa-Faria P, Oliveira-Ferreira F, Marques LS, Ramos-Jorge ML. Impact of early childhood caries on the oral health-related quality of life of preschool children and their parents. Caries Res 2013;47(3):211-218.

4. Mitrakul K, Asvanund Y, Vongsavan K. Prevalence of five biofilm-related oral streptococci species from plaque. J Clin Pediatr Dent 2011;36(2):161-166.

5. Oho T, Yamashita Y, Shimazaki Y, Kushiyama M, Koga T. Simple and rapid detection of Streptococcus mutans and Streptococcus sobrinus in human saliva by polymerase chain reaction. Oral Microbiol Immunol 2000;15(4):258-262.

6. Palmer CA, Kent R, Jr., Loo CY, Hughes CV, Stutius E, Pradhan N et al. Diet and caries-associated bacteria in severe early childhood caries. J Dent Res 2010;89(11):1224-1229.

7. Wan AK, Seow WK, Purdie DM, Bird PS, Walsh LJ, Tudehope DI. A longitudinal study of Streptococcus mutans colonization in infants after tooth eruption. J Dent Res 2003;82(7):504-508.

8. Kolenbrander PE, Palmer RJ, Jr., Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. Nat Rev Microbiol 2011;8(7):471-480.

9. Tanner AC, Kent RL, Jr., Holgerson PL, Hughes CV, Loo CY, Kanasi E et al. Microbiota of severe early childhood caries before and after therapy. J Dent Res 2011;90(11):1298-1305.

10. Linke HA, Moss SJ. Quantitation of carbohydrate sweeteners and organic acids in human oral fluid using HPLC analysis. Z Ernahrungswiss 1992;31(2):147-154.

11. Mattos-Graner RO, Smith DJ, King WF, Mayer MP. Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children. J Dent Res 2000;79(6):1371-1377.

12. Van Houte J, Russo J, Prostak KS. Increased pH-lowering ability of Streptococcus mutans cell masses associated with extracellular glucan-rich matrix material and the mechanisms involved. J Dent Res 1989;68(3):451-459.

13. Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res 2011;90(3):294-303.

14. Alaluusua S, Kleemola-Kujala E, Nystrom M, Evalahti M, Gronroos L. Caries in the primary teeth and salivary Streptococcus mutans and lactobacillus levels as indicators of caries in permanent teeth. Pediatr Dent 1987;9(2):126-130.

15. Ge Y, Caufield PW, Fisch GS, Li Y. Streptococcus mutans and Streptococcus sanguinis colonization correlated with caries experience in children. Caries Res 2008;42(6):444-448.

16. Matee MI, Mikx FH, Maselle SY, Van Palenstein Helderman WH. Mutans streptococci and lactobacilli in breast-fed children with rampant caries. Caries Res 1992;26(3):183-187.

17. Parisotto TM, King WF, Duque C, Mattos-Graner RO, Steiner-Oliveira C, Nobre-Dos-Santos M et al. Immunological and microbiologic changes during caries development in young children. Caries Res 2011;45(4):377-385.

18. World Health Organization. World Health Organization. Oral health surveys: basic methods. Geneva 1997;World Health Organization.

19. Dawes C. Circadian rhythms in human salivary flow rate and composition. J Physiol 1972;220(3):529-545.

20. Jolliffe IT. Principal component analysis. New York: Springer-Verlag; 2. ed. 2002.

21. Antunes JL, Jahn GM, de Camargo MA. Increasing inequalities in the distribution of dental caries in the Brazilian context in Finland. Community Dent Health 2005;22(2):94-100.

22. Li Y, Navia JM, Caufield PW. Colonization by mutans streptococci in the mouths of 3- and 4-year-old Chinese children with or without enamel hypoplasia. Arch Oral Biol 1994;39(12):1057-1062.

23. Seow WK, Wan A. A controlled study of the morphometric changes in the primary dentition of pre-term, very-low-birthweight children. J Dent Res 2000;79(1):63-69.

24. Ramadan Z, Jacobs D, Grigorov M, Kochhar S. Metabolic profiling using principal component analysis, discriminant partial least squares, and genetic algorithms. Talanta 2006;68(5):1683-1691.

25. Takeda I, Stretch C, Barnaby P, Bhatnager K, Rankin K, Fu H et al. Understanding the human salivary metabolome. NMR Biomed 2009.

26. Turner RJ, Sugiya H. Understanding salivary fluid and protein secretion. Oral Dis 2002;8(1):3-11.

27. De Stoppelaar JD, Van Houte J, Backer Dirks O. The relationship between extracellular polysaccharide-producing streptococci and smooth surface caries in 13-year-old children. Caries Res 1969;3(2):190-199.

28. Gross EL, Leys EJ, Gasparovich SR, Firestone ND, Schwartzbaum JA, Janies DA et al. Bacterial 16S sequence analysis of severe caries in young permanent teeth. J Clin Microbiol 2010;48(11):4121-4128.

29. Maltz M, Zickert I. Effect of penicillin on Streptococcus mutans, Streptococcus sanguis and lactobacilli in hamsters and in man. Scand J Dent Res 1982;90(3):193-199.

30. Benediktsdottir B. Upper airway infections in preschool children--frequency and risk factors. Scand J Prim Health Care 1993;11(3):197-201.

31. Torres JP, Labrana Y, Ibanez C, Kasaneva P, Farfan MJ, De la Maza V et al. Frequency and clinical outcome of respiratory viral infections and mixed viral-bacterial infections in children with cancer, fever and neutropenia. Pediatr Infect Dis J 2012;31(9):889-893.

32. Lingstrom P, van Houte J, Kashket S. Food starches and dental caries. Crit Rev Oral Biol Med 2000;11(3):366-380.

33. Moynihan P, Petersen PE. Diet, nutrition and the prevention of dental diseases. Public Health Nutr 2004;7(1A):201-226.

34. Sreebny LM, Chatterjee R, Kleinberg I. Clearance of glucose and sucrose from the saliva of human subjects. Arch Oral Biol 1985;30(3):269-274.

35. Geddes DA. Acids produced by human dental plaque metabolism in situ. Caries Res 1975;9(2):98-109.

36. Pedersen AM, Bardow A, Nauntofte B. Salivary changes and dental caries as potential oral markers of autoimmune salivary gland dysfunction in primary Sjogren's syndrome. BMC Clin Pathol 2005;5(1):4.

37. Bardow A, Moe D, Nyvad B, Nauntofte B. The buffer capacity and buffer systems of human whole saliva measured without loss of CO2. Arch Oral Biol 2000;45(1):1-12.

38. Fidalgo TKS, Abreu V, Freitas-Fernandes LB, Souza IPR, Maia LC. Do salivary lipids influence dental caries susceptibility? A systematic review. Open access scientific reports 2012;1(12):1-4.

39. Fidalgo TKS, Freitas-Fernandes LB, Angeli R, Muniz AMS, Gonsalves E, Santos R et al. Salivary metabolite signatures of children with and without dental caries lesions. Metabolomics 2013;9(3):657-666.

40. Van Nieuw Amerongen A, Bolscher JG, Veerman EC. Salivary proteins: protective and diagnostic value in cariology? Caries Res 2004;38(3):247-253.

5 DISCUSSÃO

O presente trabalho demonstrou diferenças entre componentes salivares de indivíduos com e sem cárie. Também foi demonstrado que crianças que tiveram cárie dentária, mesmo após o tratamento dental não reduzem os níveis de determinados metabólitos e da microbiota a níveis similares semelhantes às crianças que nunca tiveram a doença.

A fim de avaliar um fator de proteção do sistema imunológico que possa atuar na redução do risco à cárie dentária, foi realizada uma revisão sistemática da literatura seguida de meta-análise para investigar qual o papel da IgA-s nessa doença. A IgA-s tem a função de impedir a aderência de microrganismos cariogênicos às superfícies dentárias, além de inibição da atividade da glicosiltransferase, tem também a capacidade de neutralizar vírus e toxinas, inativar as enzimas e excluir antígeno na saliva, evitando a colonização de microrganismos cariogênicos (Smith e Mattos-Graner, 2008). Com base nos resultados da revisão sistemática e meta-analise, pode-se concluir que existe evidência moderada que aponta para uma correlação positiva de entre os níveis elevados de IgA-s e a atividade de cárie. De sete estudos incluídos na meta-análise, cinco apresentaram alta concentração de IgA-s no grupo de indivíduos com cárie e dois mostraram o oposto. Este achado demonstra que esta imunoglobulina está associada com a resposta do sistema imunológico à doença, na tentativa de eliminar os microrganismos.

Parissoto et al. (2011) encontraram uma alta concentração total do IgA-s em crianças com cárie dentária. Além disso, pré-escolares com baixas concentrações de IgA-s específica para *Streptococcus mutans* apresentavam maiores chances de desenvolver cáries. Este achado sugere que a exposição ao *Streptococcus mutans* estimula a produção de IgA-s, que desempenha um papel importante na homeostase da cavidade bucal.

Com relação aos lipídios, foi demonstrado que as concentrações desse componente na saliva de indivíduos suscetíveis à cárie são mais elevadas comparadas às de indivíduos sem cárie, corroborando com os achados prévios da literatura (Fidalgo, Freitas-Fernandes *et al.*, 2013). Tendo em vista que a associação

entre lipídeos salivares e cárie dentária ainda não está claramente estabelecida, sendo pouco difundida na literatura, objetivou-se realizar uma revisão sistemática sobre o tema. Embora alguns estudos tenham mostrado associação positiva do aumento dos níveis de lipídios com a experiência de cárie (Slomiany, Murty *et al.*, 1982; Murty, Slomiany *et al.*, 1985; Slomiany, Murty *et al.*, 1986; Slomiany, Murty *et al.*, 1989; Slomiany, Murty *et al.*, 1990; Tomita, Miyake *et al.*, 2008), ainda não há nenhuma evidência científica que suporte esta associação.

Os resultados apresentados na revisão sistemática da literatura apontaram para uma associação positiva entre cárie dentária e conteúdo lipídico salivar. Dentre as teorias para a correlação positiva entre cárie dentária e os níveis de lipídios na saliva, a mais aceita é defendida por Slomiany et al e Tomita et al (Slomiany, Murty *et al.*, 1986; Tomita, Miyake *et al.*, 2008). Essa teoria se baseia no fato de que os ácidos graxos e lipídios estão presentes no biofilme sobre a superfície dentária. A propensão à cárie ocorre através da inibição da difusão de ácidos orgânicos liberados, mantendo estes ácidos em contato com a estrutura dentária por períodos prolongados (Slomiany, Murty *et al.*, 1989).

Outras teorias também são aceitas, como os efeitos dos lipídios sobre as propriedades físico-químicas da saliva, tais como a viscosidade e solubilidade (Schachtele, Harlander *et al.*, 1978). Cita-se ainda a potencialização da atividade da glicosiltransferase, enzima associada à cariogenicidade de microrganismos bucais. Além disso, a presença de lipídios na saliva pode modificar a hidrofobicidade de microrganismos e influenciar sua adsorção sobre a estrutura dental (Beachey, 1981).

A literatura disponibiliza grande quantidade de trabalhos voltados para a caracterização da saliva de indivíduos com cárie e aponta para diferenças na concentração de íons, peptídeos, defensinas e outros tipos de proteínas (Joly, Maze *et al.*, 2004; Tao, Jurevic *et al.*, 2005; Preethi, Reshma *et al.*, 2010; Toro, Nascimento *et al.*, 2010; Hart, Corby *et al.*, 2011). Alguns autores optaram por modelos experimentais de estudo que envolvem tratamento para verificar se os componentes encontrados na saliva são uma resposta do hospedeiro à doença ou traduzem uma proteção natural do organismo (Vitorino, De Morais Guedes *et al.*, 2006; Bergandi, Defabianis *et al.*, 2007).

Neste sentido, Bergandi et al demonstraram que pacientes com cárie apresentavam ausência da proteína sCD14, quando comparados aos indivíduos sem cárie. Esta proteína está relacionada com a imunidade inata, sendo constitutivamente expressa e sintetizada pelas glândulas salivares. Foi verificado também que após o tratamento das lesões, havia um aumento de sCD14 salivar demonstrando uma relação com atividade de cárie (Bergandi, Defabianis *et al.*, 2007). No entanto, trabalhos que elucidam o perfil de componentes de baixo peso molecular nessa população ainda são escassos. Neste sentido, Fidalgo *et al* (2013) identificaram o perfil de metabólitos orgânicos de baixo peso molecular em crianças com e sem cárie. No entanto, com o modelo de estudo transversal proposto, não foi possível determinar se os componentes encontrados traduziam uma suscetibilidade à cárie ou seriam produtos provenientes da doença. A fim de elucidar esta questão, optou-se por um desenho de estudo que envolvesse intervenção e também acompanhamento longitudinal.

Ao avaliar o perfil completo de metabólitos salivares de baixo peso molecular antes e após a restauração, não foram observadas diferenças. Tampouco se observou a formação de agrupamentos quando os dados foram submetidos à análise quimiométrica por meio do método dos mínimos quadrados parciais para análise discriminante (PLS-DA). Esse fato demonstra que um número restrito de metabólitos é responsável pelas alterações após o tratamento restaurador. Uma vez que os metabólitos candidatos à biomarcadores da doença cárie foram previamente determinados (Fidalgo, Freitas-Fernandes *et al.*, 2013), se optou por avalia-los isoladamente dos demais componentes. Os dados encontrados corroboram com achados prévios, onde o PLS-DA demonstrou diferença de crianças com e sem cárie. Com os resultados encontrados foi possível validar os achados prévios em uma população distinta e com cárie de acometimento precoce.

Quando os mesmos indivíduos foram avaliados antes e após a restauração, observou-se uma redução dos níveis de todos os metabólitos candidatos à biomarcadores da cárie. Entretanto, mesmo após três meses de acompanhamento após o tratamento restaurador, os níveis desses componentes se mantinham aumentados, comparados com crianças que nunca tiveram cárie. Os achados demonstram que uma vez que a doença se instala, mesmo após o restabelecimento da saúde bucal, os metabólitos salivares sofrem modificações, mas não retornam às

concentrações iniciais. Esses resultados corroboram com os achados de Vitorino et al, que observaram que crianças com dentes restaurados possuíam diferentes concentrações de proteínas ricas em prolina (PRP) comparadas com as crianças que nunca tiveram cárie (Vitorino, De Morais Guedes *et al.*, 2006).

Não houve diferença estatística quando comparado o fluxo salivar de crianças com cárie e sem cárie; e crianças antes e após a restauração. O fluxo salivar de adultos é amplamente explorado na literatura (Humphrey e Williamson, 2001; Fenoll-Palomares, Munoz Montagud *et al.*, 2004; Torres, Nucci *et al.*, 2006; De Almeida Pdel, Gregio *et al.*, 2008), entretanto essa avaliação em crianças é mais escassa, especialmente em criaças de pouca idade (Bretz, Do Valle *et al.*, 2001).

O presente estudo excluiu crianças após 71 meses de idade, no entanto fizeram parte do mesmo crianças a partir de 24 meses de idade. Apesar do método convencional de coleta por meio da expectoração direta do fluido salivar (Navazesh e Kumar, 2008) ou pelo escoamento de saliva para o interior do tubo coletor (Bretz, Do Valle *et al.*, 2001), no presente estudo não foi possível a utilização desses métodos devido a pouca idade das crianças incluídas. Por esta razão, a coleta salivar era realizada por meio de um pipetador posicionado no soalho da cavidade bucal, sendo contabilizado o tempo necessário para completar 1 mL de saliva coletada. O presente estudo demonstrou a confiabilidade do método, uma vez que antes e após a restauração, os indivíduos mantiveram fluxos similares.

A literatura relata que crianças sem cárie apresentam maior fluxo salivar comparado com as crianças com cárie (Dawes, 1987). Entretanto, no presente estudo o fluxo salivar foi similar. Esse fato pode ser explicado pela pouca idade das crianças avaliadas em que o fluxo salivar é reduzido e aumenta até os 15 anos de idade (Andersson, 1972; Andersson, Arvidsson *et al.*, 1974; Soderling, Pienihakkinen *et al.*, 1993). Ademais, além de fatores intrínsecos como o fluxo, outros fatores extrínsecos irão determinar o estabelecimento da cárie como dieta e higiene. Nesta faixa de idade, algumas crianças podem possuir dieta rica em açúcar e higiene dental deficiente. Esses fatores associados ao menor teor mineral dos dentes decíduos (Lussi, Kohler *et al.*, 2000) são determinantes para o estabelecimento da cárie dentária. A avaliação do fluxo salivar torna-se de extrema relevância, uma vez

que a depuração de alguns compostos encontrados na saliva depende do fluxo salivar (Sreebny, Chatterjee *et al.*, 1985).

A RMN, técnica empregada no presente estudo, é capaz de fornecer informações sobre moléculas de natureza variada próxima ao seu ambiente fisiológico. Nas últimas duas décadas a RMN permitiu que pesquisadores pudessem utilizar esta técnica para o monitoramento de biofluidos para determinar e antever o estado clínico do paciente (Brindle, Antti *et al.*, 2002; Fidalgo, Freitas-Fernandes *et al.*, 2013). A RMN se destaca por ser uma técnica não-invasiva, permitir o monitoramento simultâneo de diversos componentes do biofluido e por identificar moléculas estranhas às amostras biológicas. A ressonância do fósforo (^{31}P) também é passível de estudado através da intensidade e o calculo do pH determinado por Nosaka et al (1998). A RMN é aplicada na avaliação dos metabólitos da urina, do sangue (Brindle, Antti *et al.*, 2002) e mais recentemente, da saliva (Silwood, Lynch *et al.*, 2002). Mais de 60 biomoléculas endógenas e exógenas da saliva podem ser analisadas, dentre elas as provenientes do metabolismo glandular, do fluido gengival, da dieta, de produtos relativos à saúde oral, além de produtos farmacêuticos. Diversos estudos em metabolômica têm sido realizados sugerindo biomarcadores salivares para doenças sistêmicas (Takeda, Stretch *et al.*, 2009; Sugimoto, Wong *et al.*, 2010; Cuevas-Cordoba e Santiago-Garcia, 2014). Com o crescente número de trabalhos publicados nesse campo do conhecimento utilizando a RMN como ferramente, torna-se importante avaliar o estado de saúde bucal quando doenças sistêmicas são avaliadas utilizando saliva. Por esse motivo, é de grande relevância determinar os metabólitos de doenças que afetam estruturas bucais, como demonstrou o presente estudo, uma vez que podem constituir fatores de confundimento para a determinação de doenças sistêmicas (Silwood, Lynch *et al.*, 1999; Silwood, Lynch *et al.*, 2002; Aimetti, Cacciatore *et al.*, 2012; Fidalgo, Freitas-Fernandes *et al.*, 2013).

É senso comum que os componentes salivares desempenham um papel importante para a saúde bucal e na manutenção da integridade dos tecidos dentários. No entanto, o desfecho final, "desenvolver ou não cárie", é um fenômeno complexo que envolve fatores internos do hospedeiro, como a saliva, a morfologia da superfície do dente, a saúde geral, o estado nutricional e hormonal, e uma série de fatores externos, como a dieta, a flora microbiana, a higiene bucal e a

disponibilidade de flúor (Heintze, Birkhed *et al.*, 1983). No presente estudo, pode ser observado que crianças com e sem cárie não apresentavam diferença estatística em relação à higienização e a escovação com dentifrício fluoretado. Também foi observado que um maior número de crianças sem cárie realizava amamentação artificial. Entretanto, em crianças com cárie a duração desse hábito foi maior, mesmo não havendo significância estatística, após os dois anos de idade. Sugere-se que a prolongação desse hábito favoreça o estabelecimento da cárie dentária.

O conceito de cárie dentária baseia-se no processo de sucessivas desmineralizações por meio de ácidos orgânicos produzidos pela fermentação de carboidratos metabolizados por microrganismos cariogênicos. As bactérias do biofilme produzem uma variedade de produtos finais que são modulados pela dieta (Takahashi e Nyvad, 2008). Quando carboidratos fermentáveis estão presentes, os principais ácidos orgânicos produzidos são os ácidos láctico, fórmico e acético (Geddes, 1975). Estes ácidos promovem a queda de pH do biofilme, o que resulta na desmineralização da estrutura dentária e criam um ambiente favorável ao crescimento de *Streptococcus mutans*. Além da produção de ácido, os *Streptococcus mutans* expressam uma grande variedade de fatores de virulência que são responsáveis pela cariogenicidade do biofilme adsorvido sobre o dente (Van Houte, Russo *et al.*, 1989; Liu, Yue *et al.*, 1998). Os *Lactobacillus sp* participam da progressão da lesão cariosa, por terem uma natureza mais acidofílica e acidogênica (Matee, Mikx *et al.*, 1992).

Foram observados maiores níveis de acetato em pacientes com cárie acompanhado por um aumento de microrganismo nesse grupo, evidenciando uma associação entre o acetato e essas bactérias. Observou-se maior contagem de *Streptococcus mutans* e *Lactobacillus sp* em crianças com cárie comparadas às que nunca tiveram cárie. Após a restauração, houve redução desses microrganismos e estabilidade após três meses de acompanhamento. E assim, como observado nos metabólitos de baixo peso molecular, mesmo após o tratamento a contagem ainda era superior às crianças que nunca tiveram cárie. Esses achados sugerem que medidas preventivas sejam adotadas para atender essa população específica com cárie de acomentimento precoce, assim como tempos mais curtos de acompanhamento a fim de avaliar a manutenção da saúde bucal dessas crianças.

6 CONCLUSÕES

Diante da metodologia empregada e das condições avaliadas, pode-se concluir que os componentes salivares e microbiota de indivíduos com cárie diferem daquela observada em indivíduos sem cárie.

A partir das proposições e dos resultados obtidos no presente estudo, pode-se concluir que:

- Através de uma revisão sistemática da literatura e metanálise foi possível afirmar que indivíduos com atividade de cárie apresentavam maiores níveis de IgA-s salivar comparados aos sem cárie;

- Através de uma revisão sistemática da literatura pôde-se observar que maiores quantidades de lipídeos salivares estavam associados à presença de cárie;

- Crianças com cárie de acometimento precoce apresentaram os níveis de metabólitos salivares de baixo peso molecular distintos de crianças sem cárie. Nos períodos de acompanhamento longitudinal após o tratamento dentário foi observada redução dos microrganismos e desses metabólitos. Entretanto mantiveram-se distintos daqueles observados em crianças sem cárie;

- Dentre os fatores de risco clínicos, observou-se que crianças com cárie apresentavam maior período de amamentação artificial comparado às crianças sem cárie. Com relação à microbiota, os níveis de *Streptococcus mutans* e *Lactobacillus sp* de crianças com cárie de estabelecimento precoce apresentam-se aumentados comparados aos observados em crianças sem cárie.

7 REFERÊNCIAS BIBLIOGRÁFICAS

_____. Effect of lipids on the lactic acid retardation capacity of tooth enamel and cementum pellicles formed in vitro from saliva of caries-resistant and caries-susceptible human adults. **Arch Oral Biol**, v.35, n.3, p.175-80. 1990.

_____. Mucus glycoprotein of human saliva: differences in the associated and covalently bound lipids with caries. **Biochim Biophys Acta**, v.882, n.1, Jun 3, p.18-28. 1986.

_____. Physico-chemical characteristics of mucus glycoproteins and lipids of the human oral mucosal mucus coat in relation to caries susceptibility. **Arch Oral Biol**, v.34, n.4, p.229-37. 1989.

Aapd. Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies. **Pediatr Dent**, v.30, n.7 Suppl, p.40-3. 2011.

Abrahan, R. J. e P. Loftus. In the proton and carbon-13 NMR spectroscopy - an integrates approach. **John Wiley & Sons**. 1978.

Aimetti, M., S. Cacciatore*, et al.* Metabonomic analysis of saliva reveals generalized chronic periodontitis signature. **Metabolomics**, v.8, n.3, p.465–474. 2012.

Andersson, R. The flow rate, pH and buffer effect of mixed saliva in schoolchildren. **Odontol Revy**, v.23, n.4, p.421-8. 1972.

Andersson, R., E. Arvidsson*, et al.* The flow rate, pH and buffer effect of mixed saliva in children. **J Int Assoc Dent Child**, v.5, n.1, Jul, p.5-12. 1974.

Bagherian, A. e G. Asadikaram. Comparison of some salivary characteristics between children with and without early childhood caries. **Indian J Dent Res**, v.23, n.5, Sep-Oct, p.628-32. 2012.

Baginska, J. e W. Stokowska. [Dietary habits and early childhood caries intensity among young children]. **Wiad Lek**, v.59, n.1-2, p.5-9. 2006.

Banderas-Tarabay, J. A., I. G. Zacarias-D'oleire*, et al.* Electrophoretic analysis of whole saliva and prevalence of dental caries. A study in Mexican dental students. **Arch Med Res**, v.33, n.5, Sep-Oct, p.499-505. 2002.

Beachey, E. H. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. **J Infect Dis**, v.143, n.3, Mar, p.325-45. 1981.

Bergandi, L., P. Defabianis*, et al.* Absence of soluble CD14 in saliva of young patients with dental caries. **Eur J Oral Sci**, v.115, n.2, Apr, p.93-6. 2007.

Bretz, W. A., E. V. Do Valle*, et al.* Unstimulated salivary flow rates of young children. **Oral Surg Oral Med Oral Pathol Oral Radiol Endod**, v.91, n.5, May, p.541-5. 2001.

Brindle, J. T., H. Antti*, et al.* Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. **Nat Med**, v.8, n.12, Dec, p.1439-44. 2002.

Chawda, J. G., N. Chaduvula*, et al.* Salivary SIgA and dental caries activity. **Indian Pediatr**, v.48, n.9, Sep, p.719-21. 2010.

Chopra, M., S. Jadhav*, et al.* Salivary immunoglobulin A in rheumatoid arthritis (RA) with focus on dental caries: a cross-sectional study. **Clin Rheumatol**, v.31, n.2, Feb, p.247-50. 2011.

Cuevas-Cordoba, B. e J. Santiago-Garcia. Saliva: A Fluid of Study for OMICS. **Omics**, Jan 3. 2014.

Dale, B. A., R. Tao*, et al.* Oral antimicrobial peptides and biological control of caries. **BMC Oral Health**, v.6 Suppl 1, p.S13. 2006.

Dawes, C. Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. **J Dent Res**, v.66 Spec No, Feb, p.648-53. 1987.

De Almeida Pdel, V., A. M. Gregio*, et al.* Saliva composition and functions: a comprehensive review. **J Contemp Dent Pract**, v.9, n.3, p.72-80. 2008.

Fenoll-Palomares, C., J. V. Munoz Montagud*, et al.* Unstimulated salivary flow rate, pH and buffer capacity of saliva in healthy volunteers. **Rev Esp Enferm Dig**, v.96, n.11, Nov, p.773-83. 2004.

Fernandes, F. R., A. T. Nagao*, et al.* Dental caries and salivary anti-Streptococcus mutans antibodies in IgA deficient children. **Adv Exp Med Biol**, v.371B, p.1145-8. 1995.

Fidalgo, T. K. S. **Análise do metaboloma salivar de crianças por meio da ressonância magnética nuclear**. Odontopediatria e Ortodontontia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2010. 113 p.

Fidalgo, T. K. S., L. B. Freitas-Fernandes*, et al.* Salivary metabolite signatures of children with and without dental caries lesions. **Metabolomics**, v.9, n.3, p.657-666. 2013.

Geddes, D. A. Acids produced by human dental plaque metabolism in situ. **Caries Res**, v.9, n.2, p.98-109. 1975.

Gil, V. M. S. e C. F. G. C. Geraldes. Ressonância magnética nuclear, fundamentos, métodos e aplicações. Fundação Calouste Gulbenkian, Liaboa. 1987.

Gudino, S., N. Rojas*, et al.* Colonization of mutans streptococci in Costa Rican children from a high-risk population. **J Dent Child (Chic)**, v.74, n.1, Jan-Apr, p.36-40. 2007.

Hardt, M., L. R. Thomas*, et al.* Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. **Biochemistry**, v.44, n.8, Mar 1, p.2885-99. 2005.

Hart, T. C., P. M. Corby*, et al.* Identification of microbial and proteomic biomarkers in early childhood caries. **Int J Dent**, v.2011, p.196721. 2011.

Heintze, U., D. Birkhed*, et al.* Secretion rate and buffer effect of resting and stimulated whole saliva as a function of age and sex. **Swed Dent J**, v.7, n.6, p.227-38. 1983.

Humphrey, S. P. e R. T. Williamson. A review of saliva: normal composition, flow, and function. **J Prosthet Dent**, v.85, n.2, Feb, p.162-9. 2001.

Irigoyen Camacho, M. E., L. Sanchez Perez*, et al.* Relationship between severe early childhood caries, mother's oral health and mutans streptococci in a low-income group: changes from 1996 to 2007. **J Clin Pediatr Dent**, v.33, n.3, Spring, p.241-6. 2009.

Joly, S., C. Maze*, et al.* Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. **J Clin Microbiol**, v.42, n.3, Mar, p.1024-9. 2004.

Kirtaniya, B. C., H. S. Chawla*, et al.* Natural prevalence of antibody titres to GTF of S. mutans in saliva in high and low caries active children. **J Indian Soc Pedod Prev Dent**, v.27, n.3, Jul-Sep, p.135-8. 2009.

Krasse, B. e L. Gahnberg. Available immunoglobulin A antibodies in mouth rinses and implantation of Streptococcus mutans. **Infect Immun**, v.41, n.3, Sep, p.1360-2. 1983.

Law, V., W. K. Seow*, et al.* Factors influencing oral colonization of mutans streptococci in young children. **Aust Dent J**, v.52, n.2, Jun, p.93-100; quiz 159. 2007.

Liu, L., S. Yue*, et al.* [Comparison of demineralization of different organic acid to enamel]. **Hua Xi Kou Qiang Yi Xue Za Zhi**, v.16, n.2, May, p.103-4, 113. 1998.

Lussi, A., N. Kohler*, et al.* A comparison of the erosive potential of different beverages in primary and permanent teeth using an in vitro model. **Eur J Oral Sci**, v.108, n.2, Apr, p.110-4. 2000.

Martins-Junior, P. A., R. G. Vieira-Andrade*, et al.* Impact of early childhood caries on the oral health-related quality of life of preschool children and their parents. **Caries Res**, v.47, n.3, p.211-8. 2013.

Matee, M. I., F. H. Mikx*, et al.* Mutans streptococci and lactobacilli in breast-fed children with rampant caries. **Caries Res**, v.26, n.3, p.183-7. 1992.

Mattos-Graner, R. O., D. J. Smith*, et al.* Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children. **J Dent Res**, v.79, n.6, Jun, p.1371-7. 2000.

Misra, S., J. F. Tahmassebi*, et al.* Early childhood caries--a review. **Dent Update**, v.34, n.9, Nov, p.556-8, 561-2, 564. 2007.

Murty, V. L., B. L. Slomiany*, et al.* Lipids of developing dental plaque in caries-resistant and caries-susceptible adult people. **Arch Oral Biol**, v.30, n.2, p.171-5. 1985.

Navazesh, M. e S. K. Kumar. Measuring salivary flow: challenges and opportunities. **J Am Dent Assoc**, v.139 Suppl, May, p.35S-40S. 2008.

Nieuw Amerongen, A. V., C. H. Oderkerk*, et al.* Role of mucins from human whole saliva in the protection of tooth enamel against demineralization in vitro. **Caries Res**, v.21, n.4, p.297-309. 1987.

Nobre Dos Santos, M., L. Melo Dos Santos*, et al.* Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. **Caries Res**, v.36, n.5, Sep-Oct, p.347-52. 2002.

Nogueira, R. D., W. F. King*, et al.* Mutans streptococcal infection induces salivary antibody to virulence proteins and associated functional domains. **Infect Immun**, v.76, n.8, Aug, p.3606-13. 2008.

Olsson, J. e M. Svanberg. Oral implantation in man of Streptococcus mutans in relation to salivary IgA activity. **Scand J Dent Res**, v.99, n.6, Dec, p.489-97. 1991.

Omar, O. M., N. M. Khattab*, et al.* Glucosyltransferase B, immunoglobulin a, and caries experience among a group of Egyptian preschool children. **J Dent Child (Chic)**, v.79, n.2, May-Aug, p.63-8. 2012.

Orstavik, D. e P. Brandtzaeg. Secretion of parotid IgA in relation to gingival inflammation and dental caries experience in man. **Arch Oral Biol**, v.20, n.11, Nov, p.701-4. 1975.

Parisotto, T. M., W. F. King*, et al.* Immunological and microbiologic changes during caries development in young children. **Caries Res**, v.45, n.4, p.377-85. 2011.

Peretz, B. e G. Gluck. Early childhood caries (ECC): a preventive-conservative treatment mode during a 12-month period. **J Clin Pediatr Dent**, v.30, n.3, Spring, p.191-4. 2006.

Preethi, B. P., D. Reshma*, et al.* Evaluation of Flow Rate, pH, Buffering Capacity, Calcium, Total Proteins and Total Antioxidant Capacity Levels of Saliva in Caries Free and Caries Active Children: An In Vivo Study. **Indian J Clin Biochem**, v.25, n.4, Oct, p.425-8. 2010.

Ranadheer, E., U. A. Nayak*, et al.* The relationship between salivary IgA levels and dental caries in children. **J Indian Soc Pedod Prev Dent**, v.29, n.2, Apr-Jun, p.106-12. 2011.

Rose, P. T., R. L. Gregory*, et al.* IgA antibodies to Streptococcus mutans in caries-resistant and -susceptible children. **Pediatr Dent**, v.16, n.4, Jul-Aug, p.272-5. 1994.

Rosenblatt, A. e P. Zarzar. The prevalence of early childhood caries in 12- to 36-month-old children in Recife, Brazil. **ASDC J Dent Child**, v.69, n.3, Sep-Dec, p.319-24, 236. 2002.

Sanders, J. K. M. e B. K. Hunter. Modern NMR spectroscopy - A guide for chemists. **Oxford University Press, Oxford**. 1994.

Schachtele, C. F., S. K. Harlander*, et al.* Streptococcus mutans dextransucrase: stimulation by phospholipids from human sera and oral fluids. **Infect Immun**, v.22, n.3, Dec, p.714-20. 1978.

Schroth, R. J., D. J. Brothwell*, et al.* Caregiver knowledge and attitudes of preschool oral health and early childhood caries (ECC). **Int J Circumpolar Health**, v.66, n.2, Apr, p.153-67. 2007.

Shifa, S., M. S. Muthu*, et al.* Quantitative assessment of IgA levels in the unstimulated whole saliva of caries-free and caries-active children. **J Indian Soc Pedod Prev Den**t, v.26, n.4, Dec, p.158-61. 2008.

Silwood, C. J., E. J. Lynch*, et al.* 1H-NMR analysis of microbial-derived organic acids in primary root carious lesions and saliva. **NMR Biomed**, v.12, n.6, Oct, p.345-56. 1999.

Silwood, C. J., E. Lynch*, et al.* 1H and (13)C NMR spectroscopic analysis of human saliva. **J Dent Res**, v.81, n.6, Jun, p.422-7. 2002.

Slomiany, B. L., V. L. Murty*, et al.* Lipid composition of human parotid and submandibular saliva from caries-resistant and caries-susceptible adults. **Arch Oral Biol**, v.27, n.10, p.803-8. 1982.

Smith, D. J. e R. O. Mattos-Graner. Secretory immunity following mutans streptococcal infection or immunization. **Curr Top Microbiol Immunol**, v.319, p.131-56. 2008.

Soderling, E., K. Pienihakkinen*, et al.* Salivary flow rate, buffer effect, sodium, and amylase in adolescents: a longitudinal study. **Scand J Dent Res**, v.101, n.2, Apr, p.98-102. 1993.

Sreebny, L. M., R. Chatterjee*, et al.* Clearance of glucose and sucrose from the saliva of human subjects. **Arch Oral Biol**, v.30, n.3, p.269-74. 1985.

Sugimoto, M., D. T. Wong*, et al.* Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. **Metabolomics**, v.6, n.1, Mar, p.78-95. 2010.

Svec, P., I. Sedlacek*, et al.* Lactobacillus spp. associated with early childhood caries. **Folia Microbiol (Praha)**, v.54, n.1, p.53-8. 2009.

Takahashi, N. e B. Nyvad. Caries ecology revisited: microbial dynamics and the caries process. **Caries Res**, v.42, n.6, p.409-18. 2008.

Takeda, I., C. Stretch*, et al.* Understanding the human salivary metabolome. **NMR Biomed**, v.22, n.6, Jul, p.577-84. 2009.

Tao, R., R. J. Jurevic*, et al.* Salivary antimicrobial peptide expression and dental caries experience in children. **Antimicrob Agents Chemother**, v.49, n.9, Sep, p.3883-8. 2005.

Thaweboon, S., B. Thaweboon*, et al.* Salivary secretory IgA, pH, flow rates, mutans streptococci and Candida in children with rampant caries. **Southeast Asian J Trop Med Public Health**, v.39, n.5, Sep, p.893-9. 2008.

Tomita, Y., N. Miyake*, et al.* Lipids in human parotid saliva with regard to caries experience. **J Oleo Sci**, v.57, n.2, p.115-21. 2008.

Toro, E., M. M. Nascimento*, et al.* The effect of sucrose on plaque and saliva urease levels in vivo. **Arch Oral Biol**, v.55, n.3, Mar, p.249-54. 2010.

Torres, S. R., M. Nucci*, et al.* Variations of salivary flow rates in Brazilian school children. **Braz Oral Res**, v.20, n.1, Jan-Mar, p.8-12. 2006.

Touger-Decker, R. e C. Van Loveren. Sugars and dental caries. **Am J Clin Nutr,** v.78, n.4, Oct, p.881S-892S. 2003.

Valaitis, R., R. Hesch*, et al.* A systematic review of the relationship between breastfeeding and early childhood caries. **Can J Public Health**, v.91, n.6, Nov-Dec, p.411-7. 2000.

Van Houte, J., J. Russo*, et al.* Increased pH-lowering ability of Streptococcus mutans cell masses associated with extracellular glucan-rich matrix material and the mechanisms involved. **J Dent Res**, v.68, n.3, Mar, p.451-9. 1989.

Vitorino, R., S. De Morais Guedes*, et al.* Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility. **Eur J Oral Sci**, v.114, n.2, Apr, p.147-53. 2006.

World Health Organization. World Health Organization. Oral health surveys: basic methods. **Geneva**, v.World Health Organization. 1997.

Wyne, A. H. Early childhood caries. A review. **Indian J Dent Res**, v.7, n.1, Jan-Mar, p.7-15. 1996.

8 ANEXOS

ANEXO 1

UNIVERSIDADE FEDERAL DO RIO DE JANEIRO
UFRJ

INSTITUTO DE PUERICULTURA E PEDIATRIA MARTAGÃO GESTEIRA
COMITÊ DE ÉTICA EM PESQUISA

MEMORANDO DE APROVAÇÃO

O projeto "Análise da saliva total de crianças de 5-12 anos de idade através da Espectroscopia de Ressonância Magnética Nuclear", de número 23/07-CEP/IPPMG, de responsabilidade da Dra. Raquel dos Santos Pinheiro, foi analisado por este Comitê e aprovado nesta data.

Rio de Janeiro, 14 de dezembro de 2007

Ricardo Hugo da Silva e Oliveira
Coordenador do CEP/IPPMG

ANEXO 2

PARECER Nº130/2009
PROCESSO Nº66/2009

Projeto de Pesquisa: Análise IN Vitro dos metabólicos salivares de crianças com lesões cariosas.

Pesquisador: Tatiana Kelly da Silva Fidalgo

O Comitê de Ética em Pesquisa, tendo em vista o que dispõe a Resolução 196/96 do Conselho Nacional de Saúde, resolve APROVAR o presente projeto.

Informamos que o CEP está à disposição do pesquisador para quaisquer esclarecimento ou orientação que se façam necessários no decorrer da pesquisa.

Lembramos que o pesquisador deverá apresentar relatório da pesquisa no prazo de um ano a partir desta data.

Cidade Universitária, 03 de dezembro de 2009.

Marisa Palácios
Coordenadora CEP/NESC

Instituto de Estudos de Saúde Coletiva-CCS/UFRJ
Praça Jorge Machado Moreira, 100 - Cidade Universitária
Ilha do Fundão – Rio de Janeiro RJ
CEP: 21.941-598 - Rio de Janeiro –
Tel (021) 2598 9328 Tel/Fax (021) 2270 0097
e-mail: cep@nesc.ufrj.br

ANEXO 3

Salivary metabolite signatures of children with and without dental caries lesions

Tatiana K. S. Fidalgo[a], Liana B. Freitas-Fernandes,[a] Renata Angeli,[b] Adriane M. S. Muniz,[c,e] Elicardo Gonsalves,[d] Raquel Santos[a], Jurandir Nadal[e], Fabio C. L. Almeida[b], Ana P. Valente[b] Ivete P. R. Souza[a]

[a]Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Brazil;
[b]National Center for Nuclear Magnetic Resonance – Jiri Jonas, Medical Biochemistry Institute, Federal University of Rio de Janeiro, Brazil;
[c]Physical Education College of Brazilian Army, EsEFEx, Janeiro, Brazil;
[d]School of Physics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;
[e]Biomedical Engineering Program, COPPE, Federal University of Rio de Janeiro, Brazil.

Correspondence to: Dr Ivete Pomarico Ribeiro de Souza, Disciplina de Odontopediatria da FO-UFRJ, Caixa Postal: 68066 - Cidade Universitária - CCS, CEP.: 21941-971 - Rio de Janeiro – RJ – Brazil, Phone: + 55 21 25622101
E-mail: pomarico@superig.com.br

Metabolomics
DOI 10.1007/s11306-012-0484-7

ORIGINAL ARTICLE

Salivary metabolite signatures of children with and without dental caries lesions

Tatiana K. S. Fidalgo · Liana B. Freitas-Fernandes · Renata Angeli · Adriane M. S. Muniz · Elicardo Gonsalves · Raquel Santos · Jurandir Nadal · Fabio C. L. Almeida · Ana P. Valente · Ivete P. R. Souza

Received: 20 August 2012 / Accepted: 20 November 2012

Abstract A metabolomic approach was used to analyze endogenous metabolites and to correlate with a specific biological state. The analysis of salivary metabolites is a growing area of investigation with potential for basic and clinical applications. Analyses of children's saliva in different dentitions and with or without caries could potentially reveal a specific profile related to oral disease risk. Nuclear Magnetic Resonance (NMR) is well suited for mixture analysis followed by Principal Component Analysis combined with Linear Regression (PCA-LR) statistics and was used to identify differences in the salivary metabolites. The classificatory analysis was performed using PCA-LR based on 1,000 cross-validation bootstrap runs from both classifiers in order to increase the data information from a small sample size. The PCA-LR presented a statistically good classificatory performance for children with and without caries with an accuracy of 90.11 % ($P < 0.001$), 89.61 % sensitivity ($P < 0.001$), and 90.82 % specificity ($P < 0.001$). Children with caries lesions presented higher levels of several metabolites, including lactate, fatty acid, acetate and n-butyrate. Saliva from subjects with different dentition stages was also analyzed. Although the salivary samples were poorly classified, permanent dentition presented increased levels of acetate, saccharides and propionate. The NMR data and PCA-LR were able to classify saliva from children with or without caries, with performance indexes comparable to the partial least-squares regression discriminant analysis (PLS-DA) results also performed. Our data also showed similar salivary metabolite profiles for healthy subjects despite the differences in their oral hygiene habits, socioeconomic status and food intake.

Keywords Children · Saliva · Dental caries · Metabolomic profile · NMR

Electronic supplementary material The online version of this article (doi:10.1007/s11306-012-0484-7) contains supplementary material, which is available to authorized users.

T. K. S. Fidalgo · L. B. Freitas-Fernandes · R. Santos · I. P. R. Souza (✉)
Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21941-913, Brazil
e-mail: pomarico@gmail.com

R. Angeli · F. C. L. Almeida · A. P. Valente
National Center for Nuclear Magnetic Resonance—Jiri Jonas, Medical Biochemistry Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

A. M. S. Muniz
Physical Education College of Brazilian Army, EsEFEx, Rio de Janeiro, Brazil

A. M. S. Muniz · J. Nadal
Biomedical Engineering Program, COPPE, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

E. Gonsalves
School of Physics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

1 Introduction

Among the biofluids, saliva is likely the easiest biofluid to collect and is very informative with regard to biological status. Saliva composition presents a potential source of novel diagnostic markers for both systemic and oral diseases because most components found in the blood are also present in saliva (Grootveld and Silwood 2005; Pfaffe et al. 2011; Ryan et al. 2011; Zhang et al. 2012). Salivary metabolite signatures have been identified for different

Published online: 05 December 2012

diseases such as oral, breast and pancreatic cancer and autoimmune, cardiovascular and metabolic diseases, for example, diabetes mellitus (Al-Tarawneh et al. 2011; Madsen et al. 2010; Streckfus et al. 2006; Sugimoto et al. 2010; Takeda et al. 2009; Tiziani et al. 2009).

Ideally, more sensitive and specific markers will identify early stages of diseases. Integrated platforms have been used to provide fast and reproducible analyses. Despite significant developments in analytical technologies for biofluid analyses, the identification of biomarkers remains a challenge (Bergandi et al. 2007; Hardt et al. 2005). Difficulties arise from the fact that biofluids are complex mixtures and that metabolomics is based on the analysis of a large number of variables in comparison to the number of samples. The use of multivariate data analysis techniques and chemometrics is a commonly employed strategy to obtain reliable results (Bollard et al. 2005; Wei et al. 2011).

Analysis can be unsupervised or supervised (Bereton 2006; Goodacre et al. 2004). In an "unsupervised" chemometric analysis, the system is provided a set of inputs and then clusters the metabolite data into groups. For a multivariate analysis, this optimization procedure is typically a dimensionality reduction, which means that a large body of metabolite data is summarized by a few parameters with a minimal loss of information (Goodacre et al. 2004). In the "supervised" analysis, the desired responses associated with each input are known. The goal is to find a mathematical model that correctly associates all or some of the inputs with the target classes. This goal is achieved by minimizing the error between the known target and the output (model's response). Methods such as principal component analysis (PCA) and partial least-squares regression (PLS) discriminant analysis (PLS-DA) are widely used in metabolomics for clustering (Jolliffe 2002; Madsen et al. 2010). In addition, some supervised approaches allow the identification of which metabolites are the most important for the group key separation (Bertram et al. 2009; Bollard et al. 2005; Favretto et al. 2012; Jolliffe 2002; Kochhar et al. 2006; Lindon et al. 2001; Madsen et al. 2010; Tiziani et al. 2009; Wei et al. 2011).

Because of the number of variables in comparison with the number of samples, these methods can lead to overestimations of success (Westerhuis et al. 2008), resulting in a possible overfitting of the classifier model. Therefore, a rigorous cross-validation approach to classify a group of subjects should be performed (Bertram et al. 2009; Liu et al. 2010; Martin et al. 2009; Walsh et al. 2006). In this study, we used 1,000 bootstrap runs for cross-validation.

Nuclear magnetic resonance (NMR) is well suited for mixture studies and has been applied with success to metabolite studies with biofluids with little or no sample preparation steps. Metabolites from adult saliva have been previously analyzed using NMR and classified using only PLS-DA and PCA score plots (Bertram et al. 2009; Takeda et al. 2009).

In this work, we investigated whether the salivary metabolite composition changes during developmental processes, from the first set of teeth, called primary dentition, going though a transition period with both primary and permanent teeth are present (mixed dentition) until the last primary tooth is lost reaching permanent dentition. Moreover, we analyzed the metabolites of children with caries to identify makers for caries activity.

NMR data were evaluated using PLS-DA and PCA and logistic regression (LR) as a linear classifier for discriminating between the salivary NMR patterns of subjects with caries lesions and those that were caries lesion-free as well as among healthy children with primary, mixed and permanent dentitions. For the performance evaluations, the overall accuracy (ACC), the area under the receiver operating characteristic curve (AUC), the sensitivity and the specificity from both classifiers were compared using 1,000 cross-validation bootstrap runs to increase the amount of information from a small sample size. The main purpose of our study was to derive boundaries of physiological normality and oral disease by using metabolic profiles analyzed by NMR.

2 Materials and methods

2.1 Sample collection and preparation

Sixty-five systemically healthy children attending the Pediatric Dentistry Clinic for regular dental care were recruited for the study. None of the subjects had any periodontal or systemic disease nor had taken any systemic antibiotics or used anti-bacterial toothpaste in the 3 months prior to sample collection. Saliva was analyzed from children in all stages of teeth development: from the presence of the first set of teeth, called primary dentition, going through a transition period in which both primary and permanent teeth are present (mixed dentition) and the last permanent dentition.

We also analyzed saliva from children with caries. To evaluate dental caries prevalence the Decay-Missing-Filled Teeth index (DMFT) was used "dmft" for primary teeth, "DMFT" for permanent teeth and "dmft/DMFT" for mixed dentition (in accordance to the World Health Organization). This index is based on in-field clinical examination of individuals by using a probe, mirror and cotton rolls, and simply counts the number of decayed, missing (due to caries only) and restored teeth.

Children with dental caries in mixed dentition ($n = 15$; mean age $= 7.23 \pm 2.01$, 6 female and 9 male; dmft $= 0.33$ and DMFT $= 5.40$) and age-matched children without dental

caries (n = 18; mean age = 7.94 ± 2.09, 9 female and 9 male; dmft = 0.00 and DMFT = 0.00) were recruited (see Supplementary Material Table S-1).

The group of orally healthy children with different dentitions consisted of children with dmft/DMFT = 0, and the composition according to dentition stage as following: primary (n = 15; mean age = 4.27 ± 1.27, 11 female and 4 male), mixed (n = 18; mean age = 7.94 ± 2.09, 9 female and 9 male) and permanent (n = 17; mean age = 10.88 ± 1.05, 9 female and 8 male) dentition.

Patients were required to expectorate 3 mL of unstimulated whole saliva into a plastic universal tube for about 5 min at ~10 a.m. They were asked to refrain from oral activities for 2 h prior to saliva collection. All samples were centrifuged at 10,000*g* for 60 min at 4 °C, and the supernatants were stored at −80 °C until NMR analysis.

The use of human material was approved by the proper Research Ethics Committee of Community Health Studies.

2.2 NMR measurements

NMR spectra were acquired using a 400 MHz Advance spectrometer (Bruker Biospin, Rheinstetten, Germany). All spectra were recorded at 25 °C, with water suppression by presaturation (Piotto et al. 1992). Samples were prepared by mixing 0.45 mL of salivary supernatant, deuterium oxide (99.8 % D_2O; 0.05 mL to provide a field frequency lock) and 500 µM solution of sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TSP) (30 µl solution of 5.0 mM TSP) for chemical shift reference, δ = 0.00 ppm. The CPMG (Carr–Purcell–Meiboom–Gill) pulse sequence was used to suppress signals from proteins and other macromolecules through a T_2 filter, using 1,024 scans. ^1H–^1H total correlation (TOCSY) experiments were conducted with acquisition parameters of 256 × 2,048 points, a spectral width 12,019 Hz in each dimension and a mixing time of 70 ms.

After spectra acquisition, edge effects were evaluated by overlaying all spectra using Topspin (Bruker Biospin, Rheinstetten, Germany), representative spectra showing 0.85–1.50 ppm region are illustrated in Supplementary Material Fig. S-1. Resonance assignments were made based on Silwood et al. (2002) and the Human Metabolome database (http://www.hmdb.ca/) (Wishart et al. 2007) confirmed using TOCSY.

2.3 Statistical analysis

The metabolite data were analyzed on the statistical program AMIX (Bruker Biospin, Rheinstetten, Germany). Each NMR spectrum was analyzed by integrating regions of bucket size of 0.01 ppm without the water region (4.5–5.5 ppm). Data was normalized by Pareto scaling (Ramadan et al. 2006) before applying the PLS-DA and PCA methods.

2.4 Dental caries assessment

The datasets of caries-lesion and caries-free subjects were stored in a matrix **E** [33 × 906], with row representing subjects (15 caries-lesion and 18 caries-free), and columns the chemical shifts (906 buckets).

2.5 Oral healthy children in different dentitions

To improve statistics results the comparison was performed in pairs. The three healthy dentition stages were analyzed by three combinations and each combination was stored in different matrices: (1) primary and mixed [33 × 906]; (2) primary and permanent [32 × 906]; and (3) mixed and permanent dentitions [35 × 906].

2.6 PLS-DA

This method explains the maximum separation between two defined class samples in the data matrix **E**, where a dependent dichotomy variable *y* is modeled using latent variables (Jolliffe 2002), maximizing the covariance between matrix **E** and *y*. For dental caries assessment *y* was set to 1 and 0 to the subjects with and without dental caries, respectively. For healthy dentition stage assessment *y* was related to paired combinations as already described.

2.7 PCA and logistic regression

PCA was applied to the covariance matrices of each matrix **E** studied (Jolliffe 2002). The scree plot test was applied to select the relevant PCs for the analysis (Jolliffe 2002), and corresponding PC scores were used as the initial input variables for LR (PCA-LR). LR estimates the probability of a dichotomous outcome event being related to a set of explanatory variables (Schumacher et al. 1996).

The stepwise approach was used to select the input variables by the Akaike information criterion (AIC), followed by χ^2 (Chi squared) test to contrast with a full model including all PC scores selected by the scree plot or with subsets of variables close to the final model. The final selected PC was used to analyze the epochs with higher loading factor values, identifying the metabolite differences between comparisons.

2.8 Performance comparisons evaluation

The models' performances were assessed using the area under the receiver operating characteristic curve (AUC),

accuracy (ACC), sensitivity, and specificity indexes. The classifier performances were estimated over 1,000 bootstrap samples, as a resampling technique (Sahiner et al. 2008), using the set of samples not included in the respective bootstrap.

Comparisons between PLS-DA and PCA-LR performance indexes classifiers models for dental caries assessments and healthy dentition stage evaluations were performed using paired Student t test, with $P < 0.05$. Therefore, the results of class predictions values (AUC, accuracy, sensitivity, and specificity) after PLS-DA and PCA-LR were compared as an input variable to do the t test. All signal processing procedures and statistical tests were executed in Matlab R2010b (The Mathworks, USA).

3 Results

We used NMR for metabolite analyses, and all the spectra were acquired using standard pulse sequences, including a T_2 filter to suppress the signals from proteins and other macromolecules. The whole salivary samples were stable throughout the NMR acquisition period.

The resonances that corresponded to the salivary components were assigned on the basis of the chemical shift reports available from Silwood (Silwood et al. 2002) and the Human Metabolome database (http://www.hmdb.ca/) (Wishart et al. 2007). Assignments were confirmed through an analysis of the TOCSY spectrum.

3.1 Dental caries assessment

To evaluate whether our methodology was capable of distinguishing among specific individual oral conditions, we compared the results from subjects with and subjects without caries lesions. We decided to investigate subjects with mixed dentitions because they were the largest recruited group. Figure 1 shows the saliva ^{1}H NMR spectra for the caries lesion-free (a) and caries lesion (b) subjects.

To quantify the differences, each spectrum was analyzed using AMIX. The spectral intensity variation was recorded, and the metabolites in the spectra were assigned. All spectra were carefully calibrated to avoid peak shifts. We analyzed all spectra manually to identify possible differences that would interfere with our results. After that the intensities were extracted and statistically analyzed. Actually, saliva spectra display very small differences in chemical shift between samples.

The NMR data analysis aimed to find a particular profile for each group, resulting in a qualitative analysis of the samples. As described below, the salivary metabolite intensity data could be used to observe differences.

3.2 Comparison between PCA-LR and PLS-DA

The PCA and PLS-DA scatter plot were initially performed demonstrating a visual cluster formation for assessment of dental caries (Figs. S-2 and S-3, respectively).

The NMR data were analyzed using an unsupervised method (PCA) and a supervised (PLS-DA). The models

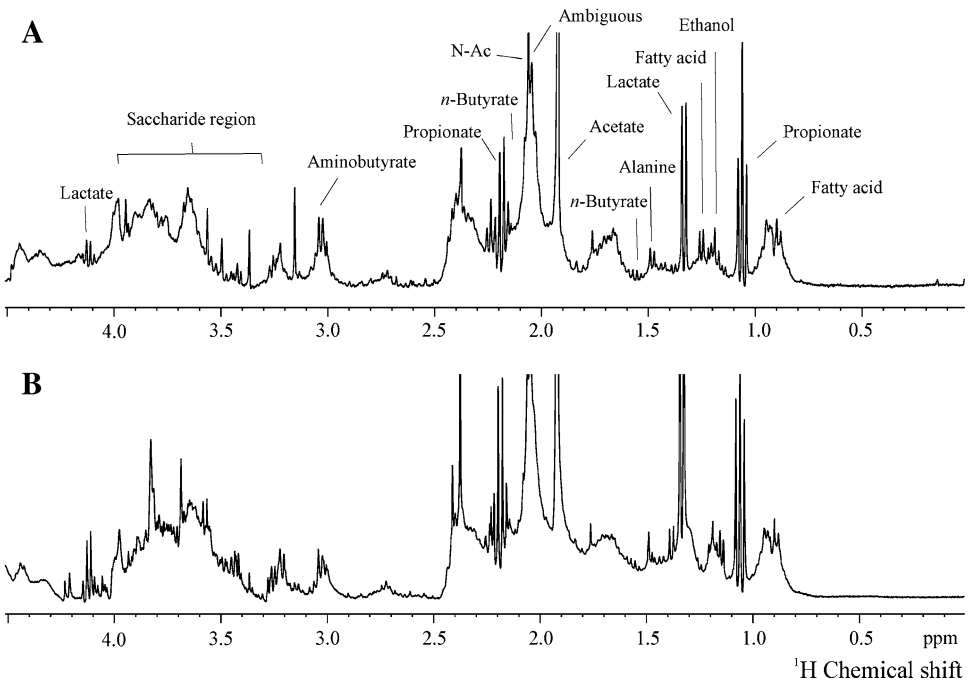


Fig. 1 Representative ^{1}H NMR spectra of whole saliva from (**a**) caries-lesion free and (**b**) caries-lesion subjects and the media of spectra demonstrating metabolites assignment of 0.00–4.50 ppm region

performances resulted from the 1,000 bootstrap samples indicated that both models presented a high performance. Table 1 shows the performances indexes of the classifications developed with PLS-DA and PCA-LR methods for children saliva with and without caries. The introduction of PCA with logistic regression presented an improved AUC, accuracy, and specificity compared to PLS-DA.

Figure 2 presents the cross validation obtained using 1,000 bootstrap samples for both models. Subject #5 was classified more than 90 % as a caries-free subject in both models, despite that no clear difference in the spectrum was observed, such as line width or intense peaks or clinical condition that would assign it as an outlier. Overall, the PLS-DA model produced more false negative subjects compared to PCA-LR.

Table 1 Performances indexes of the classifications performed with PLS-DA and PCA-LR methods for salivary metabolite from children with and without caries

Parameters	PLS-DA (%)	PCA-LR (%)	*P value*
AUC	85.88 ± 9.76	99.55 ± 6.19	<0.001
Accuracy	85.38 ± 9.78	90.11 ± 6.97	<0.001
Sensitivity	92.81 ± 11.15	89.61 ± 12.29	<0.001
Specificity	78.94 ± 13.98	90.82 ± 11.10	<0.001

Overall, the subjects were correctly classified, and only two subjects (#22 and #32) produced false positive results. These results indicate that the salivary metabolite analysis was able to classify the subjects with and without caries.

The PLS-DA model retained eight scores in the analysis, which accounted for 74.97 % of the total variation. Analyses using PCA and linear regression retained 10 PCs in the analysis, which accounted for 86.13 % of the total variation. The stepwise approach based on logistical regression selected the PC2, PC4 and PC5 as the final input variable model. Therefore, those loading factors were used to identify which metabolites presented marked differences in the NMR spectrum (values far from zero) between the caries lesion and caries lesion-free subjects. Caries lesion subjects presented a reduction in levels of phenylalanine, propionate and saccharide region and increases of lactate, fatty acid, acetate, butyrate and an ambiguous component (Fig. 3, see Supplementary Material Table S-2). Figure 3 is a summary of the assignment performed from the salivary samples that were determined using the identification of the Human Metabolomic Data Base and confirmed by TOCSY experiments. Box plots illustrate the amount of each metabolite that displayed significant differences between the salivary samples from the children with and without dental caries. It is also possible to observe the metabolite changes in the salivary samples, and each graphic also presents the variation among the individuals.

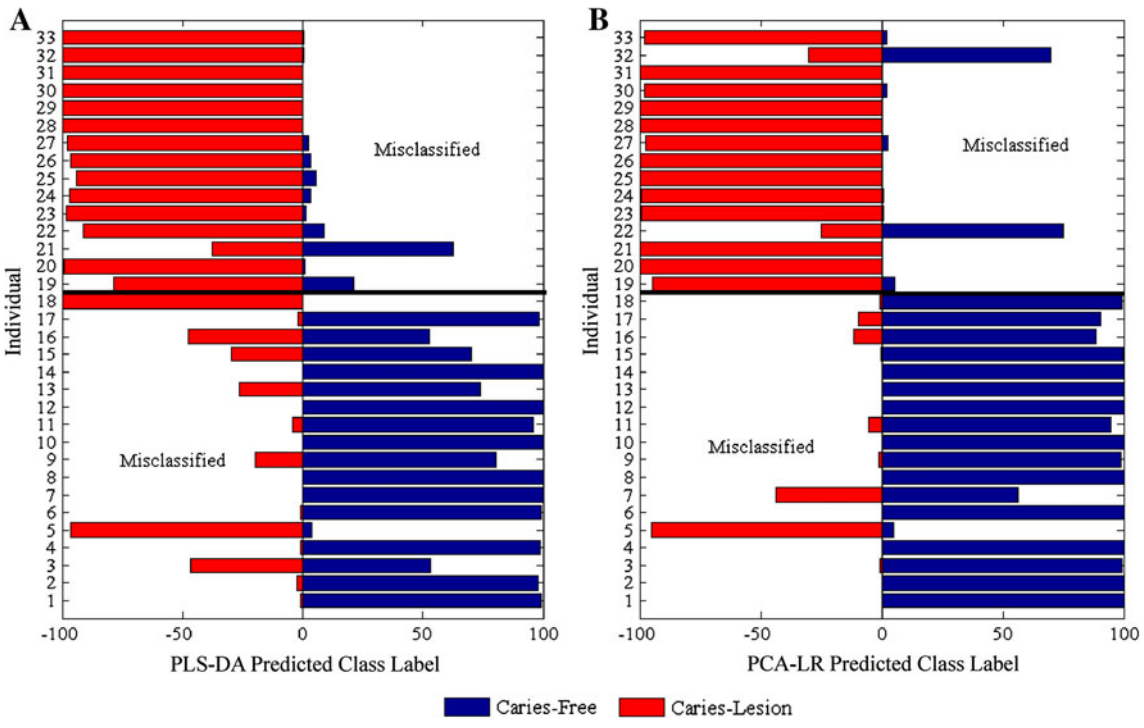


Fig. 2 Cross validation approach using 1,000 bootstrap for each model. Individuals 1–18 are caries free and 19–33 with caries and they are represented by a strap. **a** The predicted class label obtained using PLS-DA and **b** using PCA-LR. Each individual was classified as with caries (*red* strap) or without (*blue* strap)

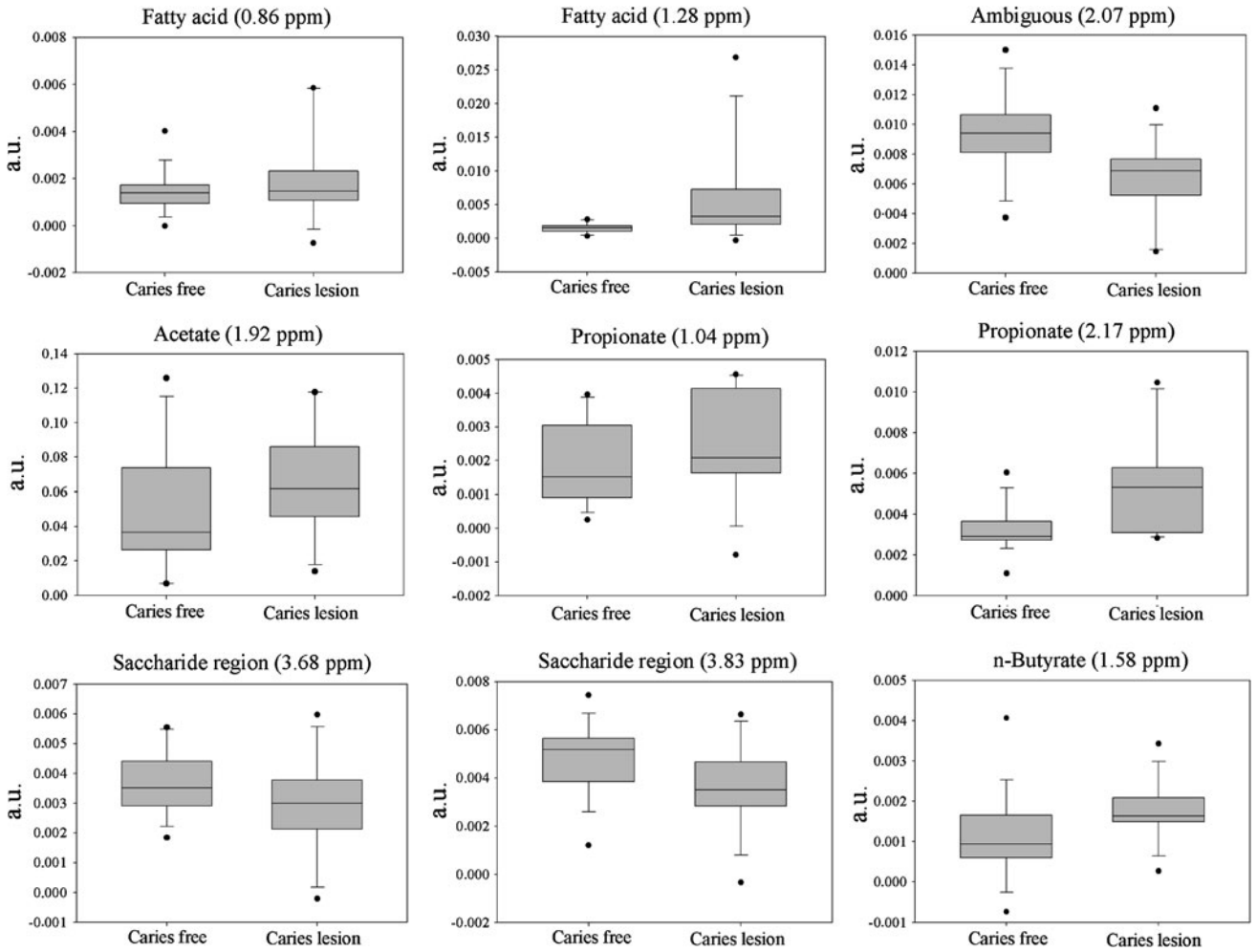


Fig. 3 Representative *box plots* of salivary metabolites markers candidates in children saliva with caries identified by PCA-LR

Moreover, the false negative classification did not correlate with the number of caries or decayed, missing or restored teeth evaluated by dmft/DMFT index (see Supplementary Material Table S-1).

3.3 Oral health of the children with different dentitions

Figure 4 shows the 400 MHz 1D ^{1}H NMR spectra for the children's salivary samples and is divided by dentition group, that is, primary (Fig. 4a), mixed (Fig. 4b), and permanent (Fig. 4c).

The PCA and PLS-DA scatter plot were initially performed demonstrating no visual cluster formation when dentitions stages were assessed (see Supplementary Material Figs. S-4 and S-5, respectively).

The PLS-DA model retained six scores for the primary and mixed comparison (accounting for 53.33 % of the total data variation), eight scores for the primary and permanent comparison (60.45 % of the total data variation) and six scores for the mixed and permanent comparison (50.24 % of the total data variation). When compared to the subjects with primary dentition, the salivary samples from the subjects with mixed dentitions presented increased levels of lysine, saccharide region and ethanol. When discriminating between primary and permanent dentitions, the latter presented higher levels of butyrate, ambiguous, lysine, saccharide region, phenylalanine, and propionate. Finally, for the comparison between mixed and permanent dentitions, the subjects with permanent dentitions presented increased levels of acetate, ambiguous, saccharide region, propionate, and lactate (Fig. 5 and Supplementary Material Table S-4). Figure 5 shows the box plots for each metabolite that displayed significant differences among the salivary samples from the subjects with different dentitions.

In the PCA method combined with LR, the PCA retained 10 PCs in the analysis for all healthy-dentition comparisons, which accounted for 81.22 % (comparison 1), 82.07 % (comparison 2) and 82.22 % (comparison 3) of

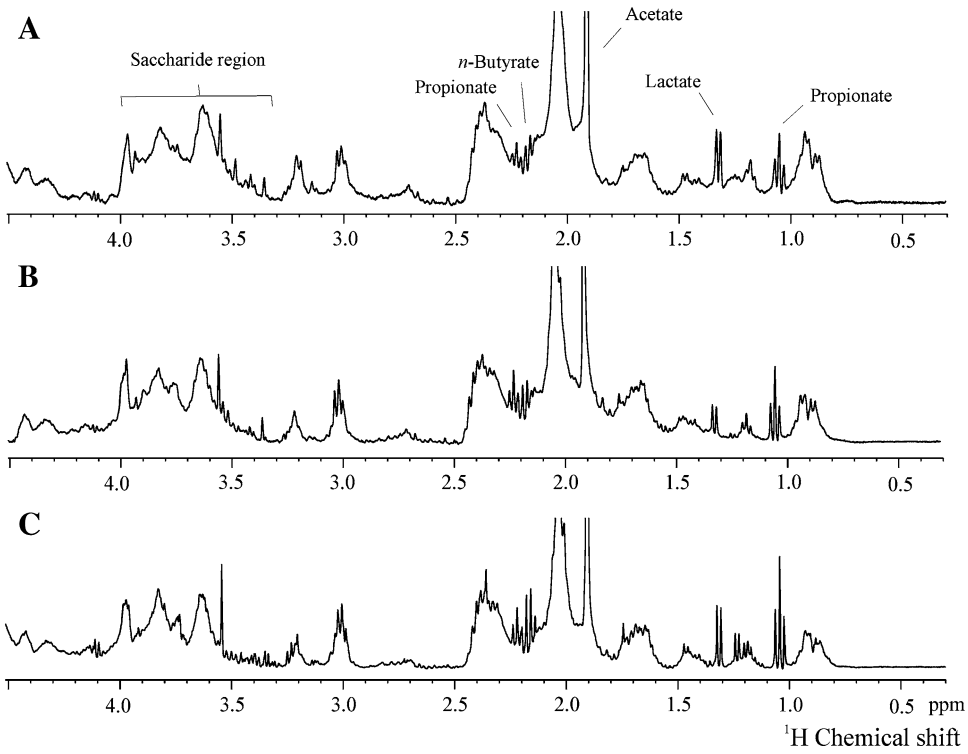


Fig. 4 Representative ^{1}H NMR spectrum of child saliva samples in the 0–4.5 ppm regions. **a** primary dentition; **b** mixed dentition; **c** permanent dentition

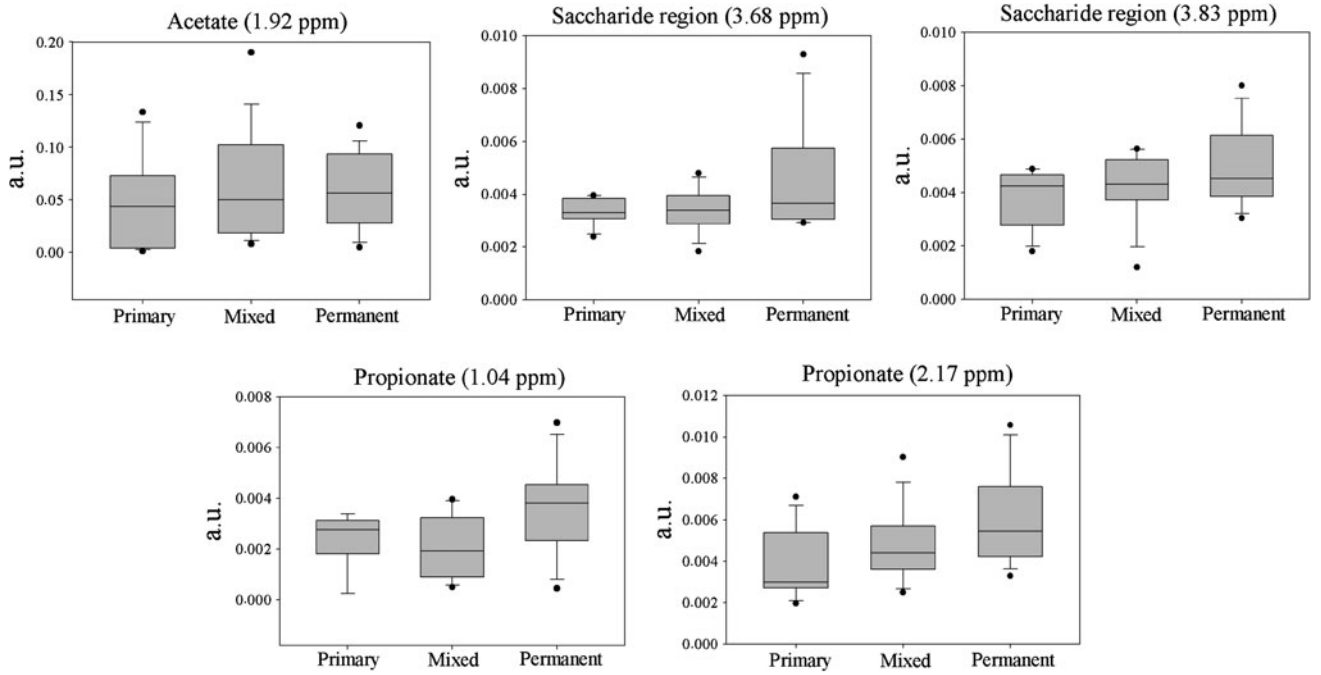


Fig. 5 *Box plots* for each metabolite that displayed significant differences among the salivary samples from the subjects with different dentitions using PCA-LR method

the total variation. As the final input variable model, the stepwise approach on logistical regression selected the PC1, PC2, PC3, PC4 and PC5 for comparison 1; PC1, PC5, PC7, PC8 for comparison 2 and PC5, PC6 and PC9 for comparison 3. Therefore, these loading factors were used to identify which metabolites presented differences in the NMR spectrum between each comparison.

When using PCA-LR and comparing the salivary samples from the subjects with primary and mixed dentitions, the mixed dentition subjects had increased levels of only

the amount of acetate. For the comparison between the subjects with primary and permanent dentitions, the latter presented increased levels of acetate, saccharide region and propionate. When discriminating between the subjects with mixed and permanent dentitions, the salivary samples from the subjects with permanent dentitions presented increased levels of acetate and propionate. Supplementary Material Table S-3 displays the comparisons among dentitions using PLS-DA and PCA-LR and the differences among them.

The PLS-DA and PCA-LR methods presented lower performances for dentition stage assessment (Supplementary Material Table S-3) compared to the prior dental caries assessment (Table 1). Logistic regression presented higher AUC and ACC compared to PLS-DA model for all the healthy subject comparisons. The specificities for comparisons 1 and 2 were not different for the models. Moreover, the sensitivity on combination 3 was not significantly different even with a higher PCA-LR value.

The PLS-DA class predictions from the healthy salivary samples among the different dentition stages presented more misclassified subjects compared to PCA-LR (Supplementary Material Fig. S-6).

4 Discussion

To evaluate the impact of age-related differences, we divided the subjects into primary, mixed and permanent dentition groups. Our hypothesis was that hormonal variations would alter the metabolite compositions within the saliva of the differently aged children. We obtained a very similar profile for all the healthy children investigated, and only acetate, saccharides and propionate were identified at larger concentrations in the saliva of the children with permanent dentitions, as compared to the children with primary dentitions (Silwood et al. 1999).

This same methodology was used to evaluate salivary samples from children with and without dental caries and was able to identify a profile for the caries condition in which there was a reduction of phenylalanine, propionate and saccharides as well as an increase in lactate, fatty acid, acetate and butyrate (Fig. 3 and Supplementary Material Table S-2). Both PLS-DA and PCA-LR produced the same profile for the caries and healthy groups (Supplementary Material Table S-2 and Table S-3).

The metabolite profile patterns from the healthy children could be helpful in identifying metabolic fingerprints for assessments of specific diseases in subsequent studies. The salivary metabolite profiles from the orally healthy children displayed a similar pattern, demonstrating that the normal profile was independent of normal physiological development, oral hygiene habits, socioeconomic status and food intake. However, more differences were observed within the primary dentition group than in the permanent dentition group and will be discussed later.

Metabolomics can assess perturbations in biological systems that are caused by diseases and can lead to treatments. For example, metabolomics is currently being employed to diagnose cancers by analyzing body fluids, which is an improved diagnostic tool that has facilitated screenings for such classes of diseases, and these types of diagnoses have been able to accurately predict the disease profiles of the affected individuals (Favretto et al. 2012; Liu et al. 2010; Walsh et al. 2006).

Sugimoto et al. (2010) demonstrated that specific markers for oral, breast and pancreatic cancer could be found in the saliva. These authors used mass spectrometry to analyze 215 individuals and 57 metabolites, and this combined dataset represents a cancer-specific signature in the saliva. Among the identified markers, they found several amino acids, such as leucine, tryptophan, and phenylalanine, as well as aminobutyric acid, and taurine. Wei et al. (2011) obtained similar results using NMR for the salivary metabolome detection of oral squamous cell carcinoma (Wei et al. 2011). In addition, Takeda et al. (2009) found that salivary metabolites contained significantly higher amounts of propionate, lactate and taurine in males.

Silwood et al. (2002) reported GABA in adult saliva. GABA has been detected in the cerebrospinal fluid of young children and is an inhibitory neurotransmitter found in the nervous systems. Alterations of GABA levels are correlated with some degenerative diseases (Kuroda et al. 1982).

According to Tomita et al. (2008), the lipid concentrations in parotid saliva from caries-susceptible subjects are higher than those of caries-resistant subjects. The current study analyzed whole salivary samples and found that fatty acid levels were also higher in caries lesions individuals. This variation in lipid levels and fatty acid composition may be associated with caries development. The presence of lipids on the salivary pellicle of tooth surfaces accentuates caries development through the inhibition of acid diffusion (Slomiany et al. 1989). Neyraud et al. (2012) found higher levels of fatty acid in stimulated saliva in comparison to rest saliva.

Lactate, acetate and n-butyrate have also been found in larger quantities in caries subjects. These compounds are formed by bacterial metabolism and reduce the pH and increase the porosity of the dental plaque matrix (van Houte 1994). Takahashi et al. (2010) obtained similar results from studies on supragingival plaque and oral bacteria. Also, our results show a clear relationship between organic acids in the saliva from subjects with caries and the ones observed in biopsies from active lesions (Silwood et al. 1999).

In the present study, a decreased saccharide concentration was related to caries subjects, allowing for speculation

that this substrate is used for bacterial energetic metabolism in caries lesions subjects as a result of a likely higher oral colonization of cariogenic microorganisms.

Several studies have evaluated the correlation between salivary metabolites and caries occurrence; however, no one study has demonstrated how a profile of salivary components relates to caries occurrence. PCA-LR allowed for the obtainment of a profile of salivary components. The aim of our study was to use NMR metabolomics to identify metabolites that might change in the salivary samples of a group of patients with a certain disease and to detect metabolites that are altered throughout the progression of the disease. These results might be a useful tool for better understanding saliva composition and its impact on oral cavity integrity.

In addition to disease assessment, it is important to recognize the differences in salivary metabolite composition in healthy subjects and the changes that might occur from childhood through the pre-pubertal period in association with normal physiological development (Di Luigi et al. 2006). Even though they did not find a data distinction with regard to salivary metabolites in different ages, Kochhar et al. (2006) evaluated three biofluids from young (18–29 years) and older (>46 years) adults and found a difference in the plasma and urine concentrations of metabolites, such as amino acids, lipids, and citrate. The present study indicates slight differences in salivary metabolites among dentition stages, which may be related to both physiological and social behavioral changes during the pre-pubertal period. The higher concentrations of microbial metabolites, such as propionate, acetate, and sugar, in the permanent dentition subjects' saliva may be related to the eruption of permanent teeth as well as to increased contact surface areas and sites for bacterial adhesion. Knowing the metabolite profile patterns of healthy subjects could be a helpful foundation for further assessments of specific diseases.

5 Conclusions

NMR and PLS-DA/PCA-LR displayed similar pattern profiles for the salivary metabolites of orally healthy children, which was independent of oral hygiene habits, socioeconomic status and food intake. Further differences were observed between salivary samples from individuals with primary rather than permanent dentitions. Finally, we found that these methods were able to create a metabolic profile that could distinguish between subjects with caries and those that were free of caries lesion.

Acknowledgments The authors acknowledge the financial support from the following agencies: National Institute of Science and Technology of Structural Biology and Bioimaging (INCT-INBEB), CNPq, FAPERJ, FINEP, and CAPES. We also acknowledge the Bruker (Bruker Biospin, Rheinstetten, Germany) for providing a demo license of AMIX program during the analysis of the data.

Conflict of interest None.

References

Al-Tarawneh, S. K., Border, M. B., Dibble, C. F., & Bencharit, S. (2011). Defining salivary biomarkers using mass spectrometry-based proteomics: A systematic review. *OMICS: A Journal of Integrative Biology, 15*(6), 353–361.

Bereton, R. G. (2006). Consequences of sample size, variable selection, and model validation and optimisation, for predicting classification ability from analytical data. *Trac-Trends in Analytical Chemistry, 25*(11), 1103–1111.

Bergandi, L., Defabianis, P., Re, F., Preti, G., Aldieri, E., Garetto, S., et al. (2007). Absence of soluble CD14 in saliva of young patients with dental caries. *European Journal of Oral Sciences, 115*(2), 93–96.

Bertram, H. C., Eggers, N., & Eller, N. (2009). Potential of human saliva for nuclear magnetic resonance-based metabolomics and for health-related biomarker identification. *Analytical Chemistry, 81*(21), 9188–9193.

Bollard, M. E., Stanley, E. G., Lindon, J. C., Nicholson, J. K., & Holmes, E. (2005). NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. *NMR in Biomedicine, 18*(3), 143–162.

Di Luigi, L., Baldari, C., Gallotta, M. C., Perroni, F., Romanelli, F., Lenzi, A., et al. (2006). Salivary steroids at rest and after a training load in young male athletes: Relationship with chronological age and pubertal development. *International Journal of Sports Medicine, 27*(9), 709–717.

Favretto, D., Cosmi, E., Ragazzi, E., Visentin, S., Tucci, M., Fais, P., et al. (2012). Cord blood metabolomic profiling in intrauterine growth restriction. *Analytical and Bioanalytical Chemistry, 402*(3), 1109–1121.

Goodacre, R., Vaidyanathan, S., Dunn, W. B., Harrigan, G. G., & Kell, D. B. (2004). Metabolomics by numbers: Acquiring and understanding global metabolite data. *Trends in Biotechnology, 22*(5), 245–252.

Grootveld, M., & Silwood, C. J. (2005). 1H NMR analysis as a diagnostic probe for human saliva. *Biochemical and Biophysical Research Communications, 329*(1), 1–5.

Hardt, M., Thomas, L. R., Dixon, S. E., Newport, G., Agabian, N., Prakobphol, A., et al. (2005). Toward defining the human parotid gland salivary proteome and peptidome: Identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. *Biochemistry, 44*(8), 2885–2899.

Jolliffe, I. T. (2002). *Principal component analysis* (2nd ed.). New York: Springer.

Kochhar, S., Jacobs, D. M., Ramadan, Z., Berruex, F., Fuerholz, A., & Fay, L. B. (2006). Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabonomics. *Analytical Biochemistry, 352*(2), 274–281.

Kuroda, H., Ogawa, N., Yamawaki, Y., Nukina, I., Ofuji, T., Yamamoto, M., et al. (1982). Cerebrospinal fluid GABA levels in various neurological and psychiatric diseases. *Journal of Neurology, Neurosurgery and Psychiatry, 45*(3), 257–260.

Lindon, J. C., Holmes, E., & Nicholson, J. K. (2001). Pattern recognition methods and applications in biomedical magnetic resonance. *Progress in NMR Spectroscopy, 39*(1), 1–10.

Liu, L., Aa, J., Wang, G., Yan, B., Zhang, Y., Wang, X., et al. (2010). Differences in metabolite profile between blood plasma and serum. *Analytical Biochemistry, 406*(2), 105–112.

Madsen, R., Lundstedt, T., & Trygg, J. (2010). Chemometrics in metabolomics—a review in human disease diagnosis. *Analytica Chimica Acta, 659*(1–2), 23–33.

Martin, F. P., Rezzi, S., Pere-Trepat, E., Kamlage, B., Collino, S., Leibold, E., et al. (2009). Metabolic effects of dark chocolate consumption on energy, gut microbiota, and stress-related metabolism in free-living subjects. *Journal of Proteome Research, 8*(12), 5568–5579.

Neyraud E, Tremblay-Franco M, Gregoire S, Berdeaux O, Canlet C. (2012). Relationships between the metabolome and the fatty acid composition of human saliva; effects of stimulation. *Metabolomics*. doi:10.1007/s11306-012-0440-6.

Pfaffe, T., Cooper-White, J., Beyerlein, P., Kostner, K., & Punyadeera, C. (2011). Diagnostic potential of saliva: current state and future applications. *Clinical Chemistry, 57*(5), 675–687.

Piotto, M., Saudek, V., & Sklenar, V. (1992). Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *Journal of Biomolecular NMR, 2*(6), 661–665.

Ramadan, Z., Jacobs, D., Grigorov, M., & Kochhar, S. (2006). Metabolic profiling using principal component analysis, discriminant partial least squares, and genetic algorithms. *Talanta, 68*(5), 1683–1691.

Ryan, D., Robards, K., Prenzler, P. D., & Kendall, M. (2011). Recent and potential developments in the analysis of urine: A review. *Analytica Chimica Acta, 684*(1–2), 8–20.

Sahiner, B., Chan, H. P., & Hadjiiski, L. (2008). Classifier performance prediction for computer-aided diagnosis using a limited dataset. *Medical Physics, 35*(4), 1559–1570.

Schumacher, M., Robner, R., & Wemer, V. (1996). Neural networks and logistic regression part I. *Computational Statistics and Data Analysis, 21*(1), 661–682.

Silwood, C. J., Lynch, E., Claxson, A. W., & Grootveld, M. C. (2002). 1H and (13)C NMR spectroscopic analysis of human saliva. *Journal of Dental Research, 81*(6), 422–427.

Silwood, C. J., Lynch, E. J., Seddon, S., Sheerin, A., Claxson, A. W., & Grootveld, M. C. (1999). 1H-NMR analysis of microbial-derived organic acids in primary root carious lesions and saliva. *NMR in Biomedicine, 12*(6), 345–356.

Slomiany, B. L., Murty, V. L., Mandel, I. D., Zalesna, G., & Slomiany, A. (1989). Physico-chemical characteristics of mucus glycoproteins and lipids of the human oral mucosal mucus coat in relation to caries susceptibility. *Archives of Oral Biology, 34*(4), 229–237.

Streckfus, C. F., Bigler, L. R., & Zwick, M. (2006). The use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to detect putative breast cancer markers in saliva: A feasibility study. *Journal of Oral Pathology and Medicine, 35*(5), 292–300.

Sugimoto, M., Wong, D. T., Hirayama, A., Soga, T., & Tomita, M. (2010). Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics, 6*(1), 78–95.

Takahashi, N., Washio, J., & Mayanagi, G. (2010). Metabolomics of supragingival plaque and oral bacteria. *Journal of Dental Research, 89*(12), 1383–1388.

Takeda, I., Stretch, C., Barnaby, P., Bhatnager, K., Rankin, K., Fu, H., et al. (2009). Understanding the human salivary metabolome. *NMR in Biomedicine, 22*(6), 577–584.

Tiziani, S., Lopes, V., & Gunther, U. L. (2009). Early stage diagnosis of oral cancer using 1H NMR-based metabolomics. *Neoplasia (New york, N.Y), 11*(3), 269–276. 264p is following 269.

Tomita, Y., Miyake, N., & Yamanaka, S. (2008). Lipids in human parotid saliva with regard to caries experience. *Journal of Oleo Science, 57*(2), 115–121.

van Houte, J. (1994). Role of micro-organisms in caries etiology. *Journal of Dental Research, 73*(3), 672–681.

Walsh, M. C., Brennan, L., Malthouse, J. P., Roche, H. M., & Gibney, M. J. (2006). Effect of acute dietary standardization on the urinary, plasma, and salivary metabolomic profiles of healthy humans. *The American Journal of Clinical Nutrition, 84*(3), 531–539.

Wei, J., Xie, G., Zhou, Z., Shi, P., Qiu, Y., Zheng, X., et al. (2011). Salivary metabolite signatures of oral cancer and leukoplakia. *International Journal of Cancer, 129*(9), 2207–2217.

Westerhuis, J. A., Hoefsloot, H. C. J., Smit, S., Vis, D. J., Smilde, A. K., van Velzen, E. J. J., et al. (2008). Assessments of PLSDA cross validation. *Metabolomics, 4*(1), 81–89.

Wishart, D. S., Tzur, D., Knox, C., Eisner, R., Guo, A. C., Young, N., et al. (2007). HMDB: The human metabolome database. *Nucleic acids research, 35*(Database issue), D521–D526.

Zhang, A., Sun, H., Wang, P., Han, Y., & Wang, X. (2012). Recent and potential developments of biofluid analyses in metabolomics. *Journal of Proteomics, 75*(4), 1079–1088.

9 APÊNDICE

APÊNDICE 1

FACULDADE DE ODONTOLOGIA
DEPARTAMENTO DE ODONTOPEDIATRIA E ORTODONTIA
DISCIPLINA DE ODONTOPEDIATRIA

Anamnese e exame clínico

Paciente Nº: _____ Aluno responsável pelo tratamento:_____
Nome: _____ Data: ____/____/____
Endereço: _____
Cep: _____ Cidade: _____ Telefones _____/_____
Nascimento: ____/____/____ Idade: _____ Sexo: _____

1 - Tem alguma alteração sistêmica?
( ) Não ( ) Sim Caso sim, qual? _____

2 – Usa medicamentos continuamente?
( ) Não ( ) Sim Caso sim, qual? _____
Quando foi a última vez que tomou antibiótico ou anti-histamínico? _____

3 – Amamentação no peito?
( ) Não ( ) Sim Caso sim, até quando? _____
Exlusiva?
( ) Não ( ) Sim Caso sim, até quando? _____
Mamava de madrugada?
( ) Não ( ) Sim Caso sim, limpava? ( ) Não ( ) Sim
5 – Mamou no peito exclusivamente?
( ) Não ( ) Sim Caso sim, até quantos anos? _____

6 – Amamentação artificial (mamadeira)?
( ) Não ( ) Sim Caso sim, até quantos anos? _____
Intervalo entre as mamadas: _____
Conteúdo da mamadeira: _____
Toma mamadeira para dormir?
( ) Não ( ) Sim Caso sim, até quantos anos? _____
Toma mamadeira dormindo?
( ) Não ( ) Sim Caso sim, até quantos anos? _____

7 – escova os dentes? ( ) Não ( ) Sim
8 - Quem escova os dentes? ( ) A criança ( ) O responsável
8 – Quantas vezes ao dia? _____ Horários: _____
9 – Usa pasta com flúor? ( ) Não ( ) Sim Qual pasta: _____
10 – Come muito doce? ( ) Não ( ) Sim

Ficha de exame de cárie dentária

	55	54	53	52	51	61	62	63	64	65
Vestibular										
Palatina										
Mesial										
Distal										
Oclusal										

	85	84	83	82	81	71	72	73	74	75
Vestibular										
Palatina										
Mesial										
Distal										
Oclusal										

C	**E**	**Ei**	**O**	**Ceod**

- **CONDIÇÃO DENTAL DECÍDUO:** A=Hígido; B=Cariado; C=Restaurado com cárie; D=Restaurado sem cárie; E=Perdido por cárie; F=Perdido por outras razões; G=Selante; H= Apoio de ponte ou coroa; K=Não erupcionado; L=Dente excluído; T=Trauma (fratura).

Parâmetros bioquímicos e microbiológicos

- **Volume coletado durante 10 minutos (fluxo)**

  Antes: _____ 1 semana: _____

  1 Mês: _____ 2 Meses: _____

  3 Meses: _____ 6 Meses: _____

- **pH**

  Antes: _____ 1 semana: _____

  1 Mês: _____ 2 Meses: _____

  3 Meses: _____ 6 Meses: _____

- ***Lactobacilos***

Antes: _____ 1 semana: _____
1 Mês: _____ 2 Meses: _____
3 Meses: _____ 6 Meses: _____

- ***S. mutans***

Antes: _____ 1 semana: _____
1 Mês: _____ 2 Meses: _____
3 Meses: _____ 6 Meses: _____

- **Outros**

_____
_____
_____
_____
_____
_____
_____
_____
_____

APÊNDICE 2

FACULDADE DE ODONTOLOGIA
DEPARTAMENTO DE ODONTOPEDIATRIA E ORTODONTIA
DISCIPLINA DE ODONTOPEDIATRIA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Prezado responsável,

Será realizado um estudo na Odontopediatria da Faculdade de Odontologia da UFRJ, com o objetivo de analisar a saliva de seu filho através de Ressonância Nuclear Magnética e por eletroforese. Nesta pesquisa será pedido ao seu filho(a) que cuspa em um "potinho de plástico", e será realizado exame clínico bucal, o que não causará nenhum desconforto a ele (a).

Sua participação é voluntária e, caso não queira participar, sua recusa não causará nenhum prejuízo ao tratamento odontológico da criança nesta instituição. O pesquisador responsável (Prof[a] Dr[a] Ivete Pomarico Ribeiro de Souza) poderá ser acessado para esclarecimento de eventuais dúvidas, a qualquer momento, pelos telefones (21) 2562-2101, ramal 6. O responsável poderá solicitar a saída do paciente deste estudo em qualquer momento, assim como a própria criança e, neste caso, os responsáveis pelo projeto se comprometem a não utilizar as informações obtidas. Os dados individuais dos participantes serão mantidos sob sigilo, sendo manipulados apenas pelos responsáveis pela pesquisa e arquivados por um período de 5 anos. Entretanto os resultados, em sua totalidade, serão publicados em literatura científica especializada, estando também disponíveis para consulta na Biblioteca da Disciplina de Odontopediatria da FO/UFRJ localizada no anexo da Disciplina no 3º andar do Hospital Universitário Clementino Fraga Filho ou na Biblioteca Central do Centro de Ciências da Saúde (CCS/UFRJ). Caso você tenha dificuldade em entrar em contato com o pesquisador responsável, comunique o fato à Comissão de Ética em Pesquisa do Instituto de Estudos em Saúde Coletiva pelo telefone (21) 2598-9293 ou pelo e-mail cep@nesc.ufrj.br.

Atenciosamente,

_____

Prof[a] Dr[a] Ivete Pomarico Ribeiro de Souza
Professora Titular da Disciplina de Odontopediatria – FO/UFRJ
Professora Orientadora da pesquisa

Eu, _____, identidade n.º_____, responsável pelo menor _____, certifico que lendo as informações acima concordo com o que foi exposto, e autorizo a doação da saliva para este estudo.

RJ, _____ de _____ de 2012.

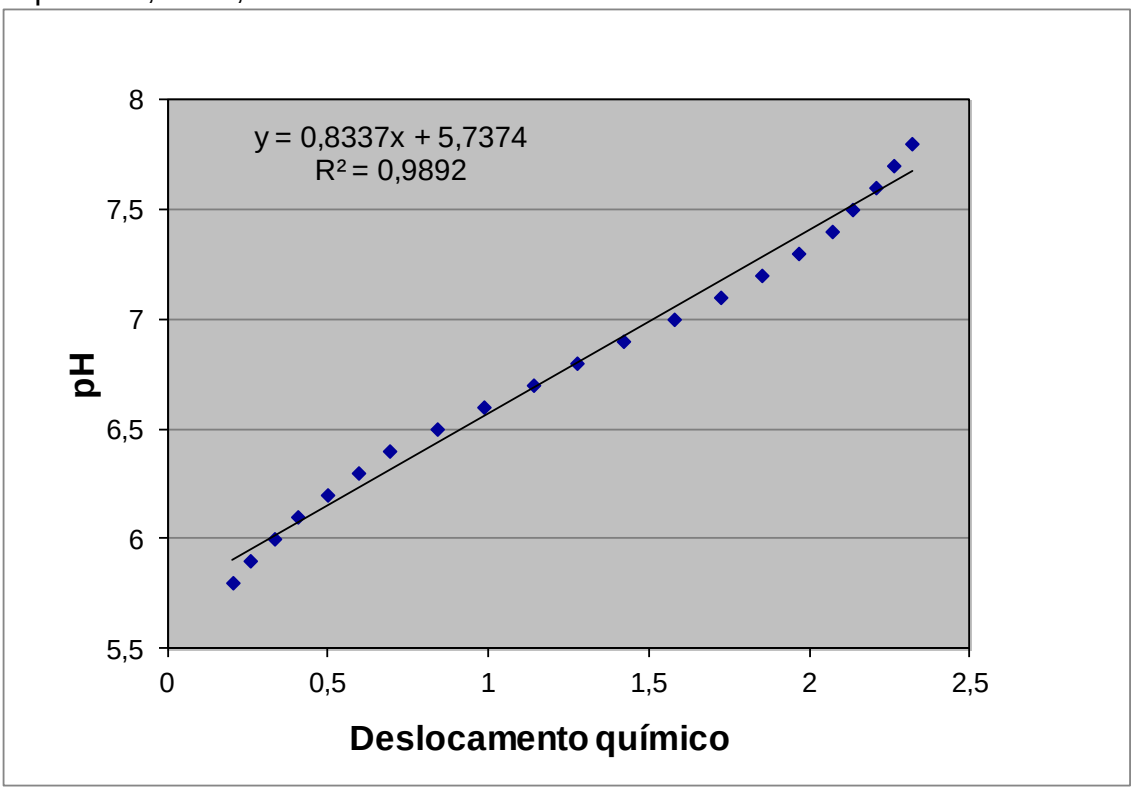
_____

Assinatura do responsável

APÊNDICE 3

Curva padrão e equação da reta de pH baseada no deslocamento químico do ^{31}P com variação de 0,1. Valor de R^2 = 0,99, demonstrando confiabilidade da equação da reta.

- pH de 5,8 a 7,8.



APÊNDICE 4

Conforme previamente descrito por Fidalgo (Fidalgo, 2010):

“A RMN é um fenômeno que pode ser observado em qualquer isótopo que apresente números quânticos de spin, como por exemplo o ^{1}H, ^{13}C e o ^{15}N que possuem número de spin I=½, podendo assumir dois estados quânticos magnéticos distintos, a saber +½ ou -½ (Abrahan e Loftus, 1978; Gil e Geraldes, 1987). As transições entre os estados de energia podem ocorrer por emissão, ou absorção de radiação eletromagnética de frequência. Um núcleo interage com uma radiação eletromagnética na qual a frequência depende efetivamente do campo aplicado e da natureza do núcleo. Para que ocorra o fenômeno de RMN é necessário perturbar o sistema através da aplicação de um pulso de radiofrequência, perpendicular ao campo magnético estático. Quando um núcleo, ou partícula absorve uma energia de radiofrequência o vetor de magnetização será rotacionado, distante do seu estado de equilíbrio (Sanders e Hunter, 1994).

Após a excitação, a amostra voltará gradualmente ao seu estado de equilíbrio inicial, através de uma série de processos chamados de relaxação. Durante o intervalo de tempo entre cada pulso, um sinal de radiofrequência, no domínio do tempo, chamado de sinal de FID é emitido pelos núcleos à medida que eles relaxam e retornam ao seu estado de menor energia (m = + ½) (Abrahan e Loftus, 1978).

Ao avaliar uma molécula, os núcleos de hidrogênio localizam-se em regiões de densidade eletrônica maior do que em outros, Dessa forma alguns prótons tendem a absorver energia em campos magnéticos de intensidades ligeiramente diferentes, resultando em sinais de RMN em diferentes regiões do espectro, resultando em diferentes deslocamentos químicos (Gil e Geraldes, 1987). Porém, a intensidade do campo em que a absorção ocorre depende sensivelmente das ligações químicas vizinhas de cada próton, por modificarem de forma diferente o campo magnético.

Para um determinado campo magnético externo, um próton que está fortemente protegido pelos elétrons não pode absorver a mesma energia que um próton de baixa proteção. Um próton protegido ou blindado absorverá energia num campo externo de maior intensidade, frequências mais elevadas. Desta forma será então necessário um campo externo mais intenso para compensar o efeito do pequeno campo induzido (Sanders e Hunter, 1994).

O grau de proteção do próton pelos elétrons adjacentes dependerá da densidade eletrônica em torno desse próton, e esta depende da presença de grupos vizinhos eletronegativos. A proximidade dos prótons desses grupos influenciará diretamente no seu grau de blindagem (proteção). Quanto mais próximo destes grupos, menos blindado estará o próton. O próton do hidrogênio é o mais desblindado, portanto este elemento é o que mais sofre influência do campo magnético, sendo vantajosa a avaliação deste elemento, além do fato de estar em abundância na natureza (99,98%).

Em um espectro de RMN, os sinais podem resultar em picos únicos ou singletos, mas podem resultar em dupletos, tripletos, quadripletos e etc. Esta apresentação dos picos está relacionada com o chamado acoplamento escalar ou spin-spin. Este fenômeno ocorre quando núcleos de diferentes ambientes eletrônicos estão próximos entre si. Os deslocamentos químicos são medidos na escala horizontal do espectro, em Hertz (Hz), e normalmente exprimidos em partes por milhão (ppm), pois os deslocamentos associados são muito pequenos quando comparados com a intensidade do campo magnético externo. Quanto mais para esquerda se localiza o sinal, menor é o campo magnético sobre o núcleo (Gil e Geraldes, 1987; Sanders e Hunter, 1994).”