LARA MARIA ALENCAR RAMOS INNOCENTINI

“SPLUNC EXPRESSION IN SALIVA OF PATIENTS UNDERGOING CHEMOTHERAPY”

“EXPRESSÃO DE SPLUNC NA SALIVA DE PACIENTES SUBMETIDOS À QUIMIOTERAPIA”

PIRACICABA

2013

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA E PIRACICABA

LARA MARIA ALENCAR RAMOS INNOCENTINI

"SPLUNC EXPRESSION IN SALIVA OF PATIENTS UNDERGOING CHEMOTHERAPY"

Orientador: Professor Dr. Márcio Ajudarte Lopes

"EXPRESSÃO DE SPLUNC NA SALIVA DE PACIENTES SUBMETIDOS À QUIMIOTERAPIA"

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Estomatopatologia da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Doutora em Estomatopatologia - Área de concentração em Patologia.

Doctorate thesis presented to the Stomatopathology Postgraduation Programme of the Piracicaba Dental School of the State University of Campinas to obtain the Ph.D. grade in Stomatopathology - Area of Concentration in Pathology.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE
DEFENDIDA PELA ALUNA LARA MARIA ALENCAR RAMOS INNOCENTINI
E ORIENTADA PELO PROF. DR. MÁRCIO AJUDARTE LOPES
Assinatura do Orientador

_____

PIRACICABA

2013

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

R147e

Ramos, Lara Maria Alencar, 1985-
Expressão de SPLUNC na saliva de pacientes submetidos à quimioterapia / Lara Maria Alencar Ramos Innocentini. – Piracicaba, SP : [s.n.], 2013.

Orientador: Márcio Ajudarte Lopes.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Saliva. 2. Quimioterapia. 3. Mucosite. I. Lopes, Márcio Ajudarte,1967-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: SPLUNC expression in saliva of patients undergoing chemotherapy
Palavras-chave em inglês:
Saliva
Chemotherapy
Mucositis
Área de concentração: Patologia
Titulação: Doutora em Estomatopatologia
Banca examinadora:
Márcio Ajudarte Lopes [Orientador]
Luis Carlos Spolidório
Sérgio Vitorino Cardoso
Pablo Agustin Vargas
Maria Elvira Pizzigatti Corrêa
Data de defesa: 09-08-2013
Programa de Pós-Graduação: Estomatopatologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 09 de Agosto de 2013, considerou a candidata LARA MARIA ALENCAR RAMOS INNOCENTINI aprovada.

Prof. Dr. MARCIO AJUDARTE LOPES

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Prof. Dr. PABLO AGUSTIN VARGAS

Profa. Dra. MARIA ELVIRA PIZZIGATTI CORRÊA

DEDICATÓRIA

Aos meus pais, Adelson e Ana, por sempre me mostrarem exemplos de boa convivência, respeito e amor ao próximo. Por me ensinarem como agir com responsabilidade diante dos desafios pessoais e profissionais, e como lidar com as dificuldades do cotidiano, sendo forte e perseverante perante os obstáculos.

Ao meu irmão Eduardo, pelo companheirismo sempre dispensado, pelas palavras de admiração, pelo amor e consideração. Pelos anos de parceria e compartilhamento dos mesmos princípios morais, muito fielmente transmitidos pela convivência diária com nossos sempre dedicados pais.

Ao meu querido marido Rafael, especialmente pelo apoio imprescindível nas minhas escolhas, pelo incentivo dado para que eu melhorasse minha formação. E principalmente pela paciência e demonstrações diárias de amor e carinho, sabendo sempre as palavras certas a serem ditas para aquietar meus momentos de angústia.

.

AGRADECIMENTOS ESPECIAIS

Primeiramente a Deus pela possibilidade de realizar todas as atividades previstas nessa importante etapa de minha vida.

Aos amigos de todo os momentos do curso Wilfredo Alejandro González Arriagada e José Ribamar Sabino Bezerra Júnior, por sempre estarem comigo nos momentos difíceis e por comemorarem comigo as vitórias.

Às minhas colegas do laboratório de biologia molecular, Elizabete Bagordakis, Ana Camila Pereira Messeti, Priscila Campioni Rodrigues, Isadora Luana Flores por todo apoio nos momentos de dúvidas e pelos ensinamentos.

Especialmente às amigas, Rose Mara Ortega, Débora Campanella Bastos, Luciana Yamamoto, Sibele Nascimento de Aquino e Andreia Bufalino que com toda paciência me acompanharam e me ensinaram nos primeiros contatos com a Biologia Molecular.

Aos meus colegas de curso Katya Pulido Diaz, Marcondes Sena Filho, Marisol Martinez Martinez, Sabrina Nogueira de Moraes, Victor Hugo Toral, Lucas Novaes Teixeira, Alicia Rumayor Piña, Ana Lucia Noronha, Patrícia do Socorro Queiroz Feio, Rogério de Oliveira Gondak, Marianne de Vasconcelos Carvalho, Bruno Augusto Benevenuto de Andrade pelos agradáveis momentos de convivência.

À Andreia Aparecida Silva por todo auxílio no decorrer do curso, pelo companheirismo e pela imensurável contribuição para a realização desse trabalho.

À família Innocentini por terem me acolhido como mais uma filha. Especialmente em nome do meu sogro Lourenço Innocentini Neto e Cristina Helena da Costa Pereira Innocentini.

Às minhas queridas cunhadas Fabiana Innocentini Margiotti e Mariana Innocentini Gonçalves pelo companheirismo de sempre e pela amizade de verdadeiras irmãs.

Ao meu cunhado Guilherme da Costa Pereira Innocentini e sua namorada Emilie Tarbagayre pelo carinho e atenção sempre.

Aos meus concunhados Cristian Margiotti e Joaquim Gonçalves, pela amizade e pelo carinho de sempre.

À minha amada sobrinha Beatriz Innocentini Margiotti pela doçura natural e ao meu pequeno afilhado Lucas Innocentini Margiotti por toda alegria que trouxe às nossas vidas.

Aos meus avós, tios e primos, por todos bons momentos passados juntos, e pela base sólida que sempre me ofereceram.

Aos meus padrinhos Júnior Coimbra e Laudecy Coimbra por todo o carinho e apoio durante toda minha vida.

Aos funcionários do OROCENTRO, Rogério Elias, Aparecida Campeão, Daniele, Maria Aparecida e Elizabete por toda dedicação.

Aos amigos de OROCENTRO, Felipe Paiva, Camila Borges, Karina Moraes Faria e Rodrigo Neves Silva pelo companheirismo nos momentos de trabalho e pelos bons momentos de descontração.

Aos meus amigos do período do mestrado, Marília Ferreira Andrade, Marco Túlio Brazão Silva, Mirna Scalon Cordeiro e Polliane Morais Carvalho pelos bons momentos vividos e pela amizade de sempre.

Aos novos colegas de curso, Fernanda dos Santos Moreira, Harim Tavares dos Santos, Nathalia Caroline de Souza Lima, Juscelino de Freitas Jardim, Estêvão Azevedo Melo, Renato Assis Machado e Wagner Gomes da Silva.

Aos inesquecíveis colegas, Ana Carolina Prado Ribeiro, Fernanda Viviane Mariano, Marco Aurélio Carvalho Andrade, Adriele Gouvêa, Carolina Cavalcanti Bitu, e Mário Romañach.

Aos pacientes que participam da pesquisa, pela bondade, paciência e por todo aprendizado de vida que me passaram.

AGRADECIMENTOS

Ao meu orientador Professor Doutor Márcio Ajudarte Lopes pela atenção dispensada e pelo apoio em todo o curso, sempre oferecendo os melhores direcionamentos nos momentos decisivos do doutorado. Agradeço pela transmissão de conhecimentos práticos e teóricos que farão parte da minha atuação daqui em diante, assim como pelo notável enriquecimento da minha formação crítica.

Aos professores Oslei Paes de Almeida, Edgar Graner e Jacks Jorge por todo ensinamento transmitido e pela sempre agradável convivência.

Ao professor Pablo Agustin Vargas por proporcionar a aproximação com o conhecimento que é foco dessa tese, principalmente por ter iniciado o contato com a Universidade de Sheffield (Inglaterra) abrindo as portas para uma rica interação conosco.

Ao coordenador do curso durante os dois primeiros anos da minha formação o Professor Doutor Ricardo Della Coletta que auxiliou na execução da etapa de biologia molecular realizada em minha pesquisa e sempre se mostrou prestativo perante minhas dúvidas.

Ao Professor Doutor Alan Roger dos Santos Silva que contribuiu de forma imensamente enriquecedora na minha formação clínica durante o período de atuação no OROCENTRO. E por sempre dispensar uma atenção especial em todos os momentos nos quais foi requisitado tanto de forma profissional como pessoal.

À Professora Doutora Maria Elvira Pizzigatti Corrêa pelo apoio conjunto nesse trabalho oferecendo oportunidade que a aluna de pós-doutorado Andreia Silva coletasse casos para obtermos o número de pacientes previstos no presente estudo. Assim como pela atenção dispensada na análise e aprimoramento dos dados desse trabalho.

À Professora Doutora Lynne Bingle pela colaboração no desenvolvimento deste estudo ao ceder os anticorpos e por compartilhar sua experiência profissional, assim como por me aceitar como orientada em período de estágio. Ao Professor Doutor Paul Speight atualmente diretor da Faculdade de Odontologia e professor do Departamento de Patologia

Oral e Maxilofacial da Universidade de Sheffield (Inglaterra) por me aceitar como aluna estagiária em seu Departamento.

A todos os amigos profissionais da área que contribuíram para a execução deste trabalho, especialmente a Andreia Aparecida Silva, Wilfredo Alejandro Arriagada e José Ribamar Sabino Júnior.

Aos funcionários do Centro de Oncologia do Hospital dos Fornecedores de Cana, às técnicas em enfermagem Janaína, Luciana, e à secretária Raimunda. À gestora geral do hospital Ana Lúcia Galvão. Ao médico hematologista André Gervatoski Lourenço e à médica radioterapeuta Ana Paula.

À FAPESP (Fundação de Apoio à Pesquisa do Estado de São Paulo) pelos imprescindíveis auxílios concedidos na forma de bolsa de doutorado (Proc. 2011/00711-9).

Aos funcionários vinculados ao Programa de Pós-Graduação em Estomatopatologia da Faculdade de Odontologia de Piracicaba – UNICAMP, pelo apoio e boa vontade no decorrer do curso. No laboratório de patologia às técnicas Geovania e Fabiana e os técnicos João e Adriano. No setor administrativo à secretária Luana.

À Faculdade de Odontologia da Universidade Federal de Uberlândia (FOUFU) e aos membros do Departamento de Patologia Bucal, especialmente ao meu orientador do mestrado o Professor Doutor Sérgio Vitorino Cardoso e ao Professor Doutor Adriano Mota Loyola, por todo o ensinamento durante os períodos de minha graduação e mestrado.

“As coisas mais maravilhosas que podemos experimentar são as misteriosas. Elas são a origem de toda verdadeira arte e ciência. Aquela para quem essa sensação é um estranho, aquele que não mais consegue parar para admirar e extasiar-se em veneração, é como se estivesse morto: seus olhos estão fechados."
(Albert Einstein)

RESUMO

A quimioterapia é um dos tratamentos utilizados para a cura do câncer, e têm como princípio o uso de drogas que alteram o metabolismo das células neoplásicas levando a morte celular. Apesar dos benefícios do tratamento quimioterápico, as drogas também afetam células normais e geram efeitos colaterais que prejudicam diretamente a qualidade de vida dos pacientes. Tais efeitos na cavidade oral podem ser classificados como agudos (mucosite, hipossalivação, candidose, herpes) ocorrem durante o tratamento, e tardios (hipossalivação) ocorrem após a conclusão do tratamento. A mucosite é o efeito colateral agudo mais importante, e causa principalmente dor e disfagia. Além da alteração na quantidade da saliva os quimioterápicos também alteram os componentes da mesma, predispondo os tecidos orais a alguns efeitos secundários. As proteínas SPLUNC (short palate, lung and nasal epithelium clone) foram identificadas em glândulas salivares, saliva, fluídos das vias aéreas e outros tecidos, com uma possível participação no sistema imune inato dessas regiões. O objetivo desse estudo foi avaliar a correlação entre a expressão das proteínas SPLUNC1 e SPLUNC2A com a presença de efeitos colaterais como mucosite e hipossalivação durante a quimioterapia, assim como a quantificação dessas proteínas antes, durante e após o tratamento. Foram realizadas três coletas de saliva total em 20 pacientes saudáveis, 20 pacientes submetidos à quimioterapia convencional e em 15 pacientes submetidos a regimes de condicionamento prévios ao transplante autólogo de células tronco hematopoiéticas e as proteínas foram avaliadas por meio de western blotting. Foi observada uma significativa mudança na qualidade da saliva durante a quimioterapia, caracterizada pelo aumento na expressão de SPLUNC1, mas não foi comprovado que essa variação esteja associada ao agravamento da mucosite e hipossalivação.

Palavras-chave: SPLUNC, saliva, quimioterapia, mucosite, hipossalivação.

ABSTRACT

Chemotherapy is one of the treatments used to cure cancer, and have as a principle the use of drugs that alter the metabolism of neoplastic cells leading to cell death. Despite of the benefits of chemotherapy, the drugs also affect normal cells and cause side effects that directly affect the quality of life of patients. Such effects in the oral cavity can be classified as acute (mucositis, hyposalivation, candidosis, herpes) occur during the treatment, and late (hyposalivation) occur after completion of treatment. Mucositis is the most important acute side effect, and mainly causes pain and dysphagia. Besides the change in the amount of saliva, chemotherapy also alter the saliva composition, predisposing oral tissues to some collateral effects. Proteins SPLUNC (short palate, lung and nasal epithelium clone) were identified in salivary glands, saliva, fluid from the airways and other tissues, with a possible role in the innate immune system in these regions. The aim of this study was to evaluate the correlation between the expression of proteins SPLUNC1 and SPLUNC2A with the presence of side effects such as mucositis and hyposalivation during chemotherapy, as well as the quantification of these proteins before, during and after treatment. Were performed three collections of whole saliva in 20 healthy subjects, 20 patients undergoing conventional chemotherapy and 15 patients undergoing conditioning regimens prior to autologous hematopoietic stem cell transplantation and proteins were evaluated by western blotting. We observed a significant change in the quality of saliva during chemotherapy, characterized by increased expression of SPLUNC1, but it was not observed any association with worsening of hyposalivation and mucositis.

Keywords: SPLUNC, saliva, chemotherapy, mucositis, hyposalivation.

SUMÁRIO

INTRODUÇÃO

As neoplasias são a segunda causa de mortes por doença no mundo, e cerca de 70% dos pacientes doentes receberão quimioterapia anti-neoplásica no decorrer do tratamento (Barnes et al., 2005). Dependendo do tipo, da dosagem e da frequência de utilização dos agentes quimioterápicos, importantes complicações bucais podem surgir (Sonis, 2004).

Como a quimioterapia não diferencia as células cancerosas que se dividem rapidamente das células normais com alta capacidade de replicação, as células epiteliais orais que se incluem nesse grupo são diretamente afetadas (Childers et al., 1993). Além disso, a boca abriga muitos microorganismos, que em um hospedeiro mielossuprimido, podem resultar em uma série de infecções secundárias (Mendonça et al., 2005). De acordo com relatos da literatura, cerca de 40% dos pacientes oncológicos submetidos ao tratamento quimioterápico apresentam complicações orais decorrentes de toxicidade direta ou indireta, como mucosite, xerostomia e infecções fúngicas ou virais (Harris, 2006).

O transplante de células tronco hematopoiéticas (TCTH) é uma estratégia terapêutica empregada no tratamento de alguns tumores sólidos, neoplasias hematológicas e desordens não malignas, particularmente doenças autoimunes. E os regimes de condicionamento para o TCTH consistem na administração de quimioterápico e/ou radioterapia em altas doses (mieloablativo) ou com doses reduzidas (não-mieloablativo) antecedendo a infusão das células tronco hematopoiéticas. Têm como principais objetivos, a imunossupressão do doador e erradicação ou diminuição da doença residual de base, o que permite a pega das células infundidas (Copelan, 2006).

Uma neutropenia intensa e prolongada dos regimes de condicionamento predispõe os pacientes a altos riscos de infecções bacterianas e fúngicas (Domingo-Gonzalez & Moore, 2013). Assim, o ideal para os pacientes oncológicos é que sejam examinados pelo cirurgião-dentista antes do início do tratamento oncológico proposto, visando principalmente eliminar possíveis focos de infecção na boca. Tendo em vista que alguns episódios sépticos em pacientes neutropênicos estão relacionados com a microbiota

da cavidade bucal e esta intervenção odontológica precoce diminui a ocorrência e a gravidade das complicações bucais decorrentes da terapia anti-neoplásica (Sonis et al., 2001).

A negligência nesses cuidados preventivos pode resultar em desconforto e dor intensa no local, nutrição deficiente, atrasos na administração ou limitações de dosagens nos tratamentos indicados, aumentando o tempo de hospitalização e os custos, e uma possível interrupção do tratamento com decorrente prejuízo do mesmo (Ferreti et al., 1990).

Mucosite

A mucosite é uma resposta inflamatória da mucosa oral decorrente da ação das drogas anti-neoplásica e/ou radiação ionizante, sendo caracterizada por hiperemia, edema, ulceração, dor, sensação de queimação, e algumas vezes hemorragia e infecção secundária, causando muito desconforto ao paciente (Bensadoun et al., 2001; Balakirev et al., 2001). O sistema de gradação da mucosite oral segundo a Organização Mundial de Saúde (OMS) define que, grau 0 não possui mucosite oral, grau 1 existe dor e eritema na mucosa, no grau 2 existe ulceração, mas o indivíduo está apto a ingerir sólidos, no grau 3 existem extensas ulcerações e o indivíduo está apto a ingerir apenas líquido e grau 4 as extensas ulcerações impossibilitam a alimentação.

O eritema e as eventuais ulcerações que se desenvolvem na mucosite são decorrentes do dano ao epitélio e conjuntivo da mucosa com morte celular mediada por uma complexa série de eventos celulares e moleculares. Didaticamente a patofisiologia da mucosite foi caracterizada por Sonis et al. (2007) em cinco fases: iniciação, resposta ao dano primário, sinalização e amplificação, ulceração e cicatrização.

As consequências dessa condição incluem a redução na dose dos quimioterápicos, pausas no tratamento de radiação, cessação da terapia anti-neoplásica, dependência de nutrição parenteral, administração de opióides e hospitalização causando consequentemente maior morbidade. (Sonis, 2004; Migliorati et al., 2006).

Em pacientes submetidos a regimes de altas doses de quimioterapia para o TCTH, principalmente os que receberão transplante alogênico uma incidência alta (75-100%) apresentam mucosite, ocasionando prognósticos desfavoráveis devido a suscetibilidade de bacteremias nesses pacientes (Pico et al., 1998; Kashiwazaki et al., 2012).

Até o presente momento algumas propostas de terapia alternativa foram testadas para o tratamento da mucosite oral, como as prostaglandinas E_2 (Labar et al., 1993), vitamina E (Borek, 2004), hidroclorido de benzidamina (Karavana Hizarcioğlu et al., 2011), todavia a melhor alternativa terapêutica ainda não está bem definida.

A crioterapia que consiste em mastigar suavemente pequenos cubos de gelo, é uma das técnicas usadas para prevenção da mucosite induzida por quimioterápicos de rápida metabolização (Yokomizo et al., 2004). Embasada no estímulo a vasoconstricção local no momento da infusão da droga, com consequente redução do fluxo sanguíneo na mucosa oral o do efeito citotóxico da droga. Possui as vantagens de ser simples, econômica e normalmente bem tolerada pelos pacientes (Migliorati et al., 2006).

O tratamento com o fator recombinante de crescimento de queratinócitos, palifemina, demonstrou reduzir a duração e a gravidade da mucosite oral depois de ciclos de quimioterapia em pacientes com neoplasias malignas hematológicas, em pacientes com câncer colorretal metastático tratados pelo regime quimioterápico de fluorouracil (Spielberger et al., 2004; Rosen et al., 2006; Barasch et al., 2009; Sonis, 2009) e principalmente em pacientes submetidos a altas doses de quimioterapia para transplante de células tronco hematopoiéticas (Abidi et al., 2013).

O laser de baixa potência tem demonstrado elevada eficácia na prevenção e no tratamento da mucosite radio e quimio induzida, reduzindo picos de dor e diminuindo o tempo para cicatrização (Villar et al., 2009). Pode-se considerar também que o laser associado com agentes de ação em todo o trato gastrointestinal sujeito a mucosite, pode ser mais efetivo levando em consideração a qualidade de vida dos pacientes como um todo (Jaguar et al., 2007).

Os estudos têm demonstrado que a aplicação de laser protela o tempo de início, atenua o pico de intensidade, e diminui a duração da mucosite oral (Schubert et al., 2007; Kuhn et al., 2009).

Baseado em evidência disponível na literatura, a aplicação de laser de baixa potência para a prevenção de mucosite quimioinduzida parece ser seguro e eficiente, se convertendo em uma importante ferramenta coadjuvante no tratamento dos pacientes com doenças onco hematológicas e tumores sólidos (Schubert et al., 2007; Antunes et al., 2007; Arora et al., 2008; Genot-Klarstersky et al., 2008; Villar et al., 2009).

Em pacientes submetidos ao condicionamento para transplante de células tronco hematopoiéticas, a laserterapia resultou numa considerável redução na incidência de mucosite grave e na intensidade de dor, e por consequência a necessidade de nutrição parenteral e uso de opióides também foi reduzida, sendo uma importante terapia para redução dos custos com os efeitos adversos do tratamento (Bezinelli et al., 2013).

O recomendação atualmente preconizado pelo Grupo de Estudo em Mucosite da MASCC (Multinational Association of Supportive Care in Cancer), consiste na aplicação do comprimento de onda de 650 nm, potência de 40 mW, e cada centímetro quadrado tratados com o tempo necessário para uma dose de energia no tecido de 2 J/cm^2 (2 s/ponto) para a prevenção da mucosite oral em pacientes adultos recebendo transplante de células tronco hematopoiéticas, condicionados com altas doses de quimioterapia, com ou sem irradiação total do corpo (Migliorati et al., 2013).

Recentemente, em uma revisão de literatura, suplemento de zinco administrado oralmente foi sugerido para prevenção de mucosite oral em pacientes submetidos à radioquimioterapia, enquanto que a glutamina intravenosa foi contraindicada para prevenção da mucosite em pacientes submetidos a altas doses de quimioterapia pré transplante de células tronco hematopoiéticas (Yarom et al., 2013).

Saliva e quimioterapia

A saliva total é um fluído recolhido da cavidade oral produzido pelas glândulas salivares maiores (parótida, sublingual, submandibular) e menores, e é obtida pela mistura

com microorganismos orais, secreções nasais e bronquiais, refluxo gastrointestinal, células descamadas e constituintes do sangue após a sua libertação na boca (Chiappin et al., 2007; Fabian et al., 2008).

A saliva é constituída por 98 % de água e 2 % de outros componentes, como eletrólitos (Na, K, Ca, Mg, carbonatos de hidrogênio, fosfatos), muco com mucopolissacarídeos e glicoproteínas, substâncias antissépticas (peróxido de hidrogênio, imunoglobulina A – IgA) e vários enzimas (α-amilase, lisozimas, lípase lingual) (Pink et al., 2009). Alguns destes componentes como os hormônios são provenientes dos capilares sanguíneos, que passam via junções intercelulares dos ductos, e pelo plasma sanguíneo (via fluido crevicular ou diretamente da mucosa oral) (Chiappin et al., 2007).

Cada glândula salivar secreta um tipo de saliva diferente, sendo que as glândulas parótidas e von Ebner secretam um fluído seroso, rico em amilase, proteínas ricas em prolina e fosfoproteínas. Por outro lado, as glândulas Blandin-Nuhm secretam fluído mucoso, rico em mucinas e as glândulas sublingual e submandibular produzem fluidos sero-mucosos (Chiappin et al., 2007; Pink et al., 2009).

A variação quantitativa e qualitativa da saliva depende principalmente do olfato, do paladar, da mastigação, do estado psicológico e emocional, de drogas, da idade e da higiene oral do paciente entre outros (Chiappin et al., 2007). Com relação ao proteoma presente na saliva, diversos estudos identificaram mais de 1000 proteínas, algumas também presentes no plasma (Yan et al., 2009).

Um dos principais alvos de estudo atualmente são as inúmeras proteínas de defesa presentes na saliva. Algumas destas proteínas de defesa, tal como as imunoglobulinas em ativação imunológica inata e adquirida (Fabian et al., 2008). Peptídeos catiônicos salivares e outras proteínas salivares, como, lisozima, BPI, proteínas semelhantes à BPI e PLUNC, amilase salivar, cistatinas, proteínas ricas em prolina, mucinas e peroxidases são as principais responsáveis pela imunidade inata (Fabian et al., 2008). Atualmente vários estudos têm proposto a substituição da avaliação de componentes do plasma pela análise da saliva, por representar um método mais fácil, barato e menos invasivo para o paciente (Zhang et al, 2013). Dessa forma, o entendimento das alterações nas glândulas salivares é de extrema importância.

Conceitualmente a hipossalivação se refere a uma redução mensurável e determinada por métodos pré-estabelecidos da quantidade de saliva (Kopittke et al., 2005). Enquanto que a xerostomia é sensação de boca seca relatada pelo paciente, sendo avaliada assim de forma subjetiva. Dessa forma, não raramente é difícil determinar se a queixa, ou avaliação subjetiva da xerostomia realmente reflete um comprometimento funcional da glândula salivar (Bagesund et al., 2000). Alguns pacientes podem, por exemplo, apresentar o fluxo salivar normal, ou seja, não possuir hipossalivação, mas relatam a sensação de boca seca devido à composição mais mucosa ou viscosa da saliva.

Dentre os sintomas mais relatados pelos pacientes submetidos à quimioterapia, a xerostomia fica atrás somente da mucosite. É predominante no gênero feminino e entre 41e 90 anos de idade (Hespanhol et al., 2010). É importante avaliar o fluxo salivar antes do tratamento quimioterápico, visto que os pacientes já podem apresentar diminuição da produção de saliva devido a uma série de outras condições como idade e uso de medicamentos (McCarthy et al., 1998; Scully, 2003).

No regime de quimioterapia os danos às glândulas salivares estão relacionados à ação dos quimioterápicos induzindo a dilatação ductal em glândulas salivares menores e a degeneração acinar, principalmente em pacientes submetidos a um período maior que três semanas de quimioterapia, podendo variar de acordo com o protocolo estabelecido (Baum et al., 1985). Ocorre também porque os pacientes apresentam concentrações dos agentes quimioterápicos na saliva, o que resulta numa toxicidade às glândulas salivares, sendo um dos efeitos colaterais mais comuns associados a algumas medicações (Epstein et al., 2002; Scully, 2003) .

A disfunção das glândulas salivares também é uma das complicações orais que podem aparecer após o transplante de células tronco hematopoiéticas como resultado direto e indireto da toxicidade do regime de condicionamento pré-transplante, afetando principalmente as glândulas parótidas. Esses danos são em geral irreversíveis quando o paciente recebe irradiação total do corpo associada à quimioterapia (Chaushu et al., 1995).

Como já está bem estabelecido, os pacientes submetidos ao transplante autólogo apresentaram menos hipossalivação e uma melhor recuperação funcional glandular após três meses do transplante, que os pacientes submetidos ao transplante de

células tronco hematopoiéticas alogênico (HSCT) (Dens et al., 1996). Isto ocorre devido aos diferentes regimes de condicionamento utilizado em cada modalidade, onde principalmente há uma alteração significativa no fluxo salivar de pacientes submetidos ao TMO alogênico devido à complementação do regime com irradiação total do corpo (Laaksonen et al., 2011).

Tratamentos recomendados para amenizar os sintomas de boca seca presentes nos pacientes submetidos à quimioterapia são, o uso diário de creme dental fluoretado, enxaguatório bucal sem álcool e gomas de mascar sem açúcar a base de sorbitol, assim como o géis para equilíbrio do pH oral e substitutos da saliva a base de carboximetilcelulose (Ship et al., 2007; Hahnel et al., 2009). Agentes colinérgicos como a pilocarpina e a cevimelina também são utilizados, e demonstram bons resultados na estimulação glandular (Berk, 2008). Outras práticas alternativas como acupuntura, hipnose, laserterapia, e oxigenação hiperbárica sugerem uma melhora na hipossalivação e na xerostomia (Garcia et al., 2009; Schiff et al., 2009; Simões et al., 2010; Forner et al., 2011).

PLUNC

PLUNC (palate, lung and nasal epithelium clone) é uma família de proteínas que foi primeiramente descrita no epitélio nasal de embriões de camundongos e traqueia/brônquios de camundongos adultos (Weston et al., 1999). Posteriormente, outros estudos mostraram também sua expressão em palato, septo nasal, pulmão, entre outros tecidos de rato, camundongo, porco, vaca e humanos (Weston et al., 1999; LeClair et al., 2001; Sung et al., 2002; LeClair, 2003a). A família PLUNC compreende nove genes localizados em único lócus no cromossomo 20q11.2. (Bingle CD et al., 2011). A função ainda não é bem definida, mas essa proteína pode ter um papel na morfogênese das estruturas faciais, olfação e participação na defesa inata do hospedeiro em boca, nariz e pulmões. Comumente foi relatada como associada a outras proteínas como a PSP (parotid secretory protein), BPI (bactericidal/permeability increasing protein), LBP (lipopolysaccharide-binding protein), SMGB (submandibular gland protein B), PLTP (phospholipid transfer protein), CETP (cholesteryl ester transfer protein) e outras proteínas

secretoras do epitélio nasofaringeal e de glândulas salivares. LBP e BPI são proteínas antagônicas; a primeira é uma proteína pró-inflamatória, e a segunda tem uma função anti-inflamatória, mantendo uma regulação da resposta inflamatória desencadeada pelo LPS bacteriano (Bingle & Bingle, 2000; Bingle & Craven, 2002; Sung et al., 2002; Ball et al., 2003; Beamer, 2003; Andrault et al., 2003; LeClair, 2003b; Bingle & Craven, 2004; Hou et al., 2004; Larsen et al., 2005).

A função defensiva da PLUNC é suportada pela homologia estrutural com proteínas que participam na defesa do hospedeiro contra bactérias Gram-negativas como a BPI, o que permite sugerir que se trata de proteínas secretadas e que agem bloqueando a junção de LBP ao LPS, e impedindo a ativação dos macrófagos pelo LPS, tal como a BPI, tendo papel regulador anti-inflamatório na resposta ao LPS (Levy et al., 2003; Weiss, 2003; Wheeler et al., 2003; Bingle et al., 2004; Bingle & Gorr, 2004). Zhou et al. (2006) reportaram que a SPLUNC1 é uma proteína imuno-defensiva inata que pode se ligar ao LPS e neutralizar a endotoxina através de seu domínio BPI (Zhou et al., 2006).

A saliva e os fluidos da superfície das vias aéreas expressam PLUNC além de outros fatores antibacterianos e têm uma reconhecida importância da saúde oral e respiratória (Martin, 2000; Amerongen & Veerman. 2002; Bingle & Gorr, 2004). A participação da saliva na prevenção de infecções tem sido demonstrada em estudos que associam um aumento das infecções orais como candidose e de cáries em pacientes com fluxo salivar diminuído, por exemplo, pós-radioterapia e devido à síndrome de Sjögren (Jonsson et al., 2002; Dirix et al., 2006).

A família é composta por dez proteínas divididas em dois grupos baseados em seu tamanho (Bingle et al., 2000). Um grupo denominado “proteínas curtas”, as quais contêm um domínio único relacionado ao domínio N-terminal da BPI, que inclui SPLUNC1, SPLUNC2, SPLUNC3 e BASE; e um segundo grupo denominado “proteínas longas”, que possui os dois domínios BPI, que compreende LPLUNC1, LPLUNC2, LPLUNC3, LPLUNC4, LPLUNC5 e LPLUNC6 (Bingle & Craven, 2002; Egland et al., 2003; Bingle & Craven, 2004; Bingle et al., 2004). Por último, foi descrita a proteína SPLUNC5 em epitéilo interpapilar na superfície dorsal da língua de ratos (LeClair, 2004).

Algumas proteínas da família PLUNC têm sido identificadas em diferentes fluidos no ser humano, tais como, saliva, fluido nasal e secreções traqueo-bronquiais e pulmonares; e os sítios de expressão dessas proteínas incluem glândulas salivares maiores e epitélio respiratório nasal, traqueal ou bronquial, entre outros tecidos (LeClair et al., 2001; Bingle & Craven, 2002; Andrault et al., 2003; Kim et al., 2006). SPLUNC1, SPLUNC2 e LPLUNC1 têm sido identificadas em saliva por diferentes pesquisadores sugerindo que são produzidas pelas glândulas salivares (Ghafouri et al., 2003; Campos et al., 2004; Vitorino et al., 2004; Ramachandran et al., 2006; Vargas et al., 2008). A expressão de PLUNC também foi reportada em grânulos específicos de neutrófilos humanos, células importantes da resposta imune do hospedeiro, que também podem liberar essas proteínas ao serem estimulados. Embora esse estudo não tenha conseguido demonstrar a atividade antibacteriana do PLUNC, o armazenamento e a secreção por uma célula fagocítica, suporta a hipótese de que essa proteína participa na reposta inflamatória e contribui na defesa do hospedeiro (Bartlett et al., 2008).

SPLUNC1 tem sido localizada, nas células epiteliais das vias aéreas superiores, na superfície do epitélio que cobre as vias aéreas principais, e nas secreções nasais, mas a expressão mais significante dessa proteína tem sido observada em células mucosas e ductos de glândulas submucosas do trato respiratório. Uma expressão intensa também foi detectada em glândulas menores do nariz, seios paranasais, região posterior da língua e tonsilas, sugerindo a presença nas secreções mucosas desses tecidos (Bingle et al., 2005). Essa proteína foi confirmada como um marcador diagnóstico em câncer de pulmão, no caso de micrometástase de câncer de células não pequenas, adenocarcinoma, carcinoma mucoepidermóide e carcinoma brônquio-alveolar (Bingle et al., 2005; Kim et al., 2007).

Também foi identificada em carcinoma nasofaríngeo, câncer de mama e câncer gástrico (Egland et al., 2003; He et al., 2005; Sentani et al., 2008; Yasui et al., 2009). Bingle et al. (2007) reportaram um incremento na expressão de SPLUNC1 nas vias aéreas menores dos pulmões em pacientes com fibrose cística, que pode ser interpretado como uma resposta defensiva do epitélio ao componente infeccioso nessa doença. Chu et al., (2007) estudaram a função e regulação da SPLUNC1 na infecção por *Mycoplasma pneumoniae* e na inflamação alérgica (asma). Baseados nos resultados concluíram que a

SPLUNC participa na defesa imune contra a infecção nas vias aéreas e inibe a produção epitelial de IL-8, mas que a inflamação alérgica (com uma maior expressão de IL-13) diminui significativamente a expressão de SPLUNC1, o que em parte contribuiria na natureza persistente das infecções bacterianas em pacientes alérgicos.

Recentemente, Liu et al., (2013) avaliaram a importância da atividade surfactante de SPLUNC1 nas secreções epiteliais das vias aéreas e confirmaram a importância dessa proteína na manutenção da baixa tensão superficial dos fluidos dessa região. Demonstrando assim que em camundongos sem o gene SPLUNC1 existe um aumento de infecções por *Klebsiella pneumoniae,* devido à falta de prevenção na formação do biofilme bacteriano. Além da ação antimicrobiana direta, e propriedades surfactantes que impedem a formação do biofilme, SPLUNC1 também possui atividade quimiotática que induz inflamação aguda na presença de corpos estranhos, como sugerido por Di et al., (2013) que mostraram a ação pró-inflamatória aguda e anti fibrose pulmonar de SPLUNC1 contra partículas inaladas pulmonares.

Em geral os trabalhos encontrados na literatura sugerem que alterações inflamatórias reduzem a expressão de SPLUNC1, como no estudo de pacientes com líquen plano que quando comparados com controles normais apresentaram menores níveis de SPLUNC1 (Yang et al., 2006) e estudos relacionados que avaliaram indivíduos expostos à poluentes do meio industrial, indivíduos fumantes ou que sofriam de alergias, todos mostraram uma redução da produção de SPLUNC1 em fluídos nasais (Ghafouri et al., 2002; Ghafouri et al., 2003; Ghafouri et al., 2006). Em outros estudos, realizados em pacientes diabéticos, resultados distintos foram mostrados, esses pacientes diabéticos tipo 2 apresentaram aumento da produção de SPLUNC1 no estudo de Rao et al., (2009), e uma redução significativa no estudo de Border et al.(2012).

Atualmente um consenso para sistematização da nomenclatura das proteínas PLUNC/PSP/BSP30/SMGB as classificou como uma subfamília da superfamília BPI fold-containing (Bingle CD et al., 2011). Essa nova nomenclatura para PLUNC foi discutida baseada no conhecimento de que essas proteínas são codificadas por genes de um único lócus no cromossomo 20q11 humanos e esses locos conservados são encontrados em todos os mamíferos. Devido à crescente complexidade desta família de genes assim como

nomenclaturas conflitantes, todos os membros da família foram renomeados usando a sigla BPIF seguida de número. Assim, os membros da família que contêm um único domínio como SPLUNC passam a ser chamados BPIFA e LPLUNC passa a ser BPIFB (Bingle CD et al. 2011).

Estudos recentes demonstram que SPLUNC1 (BPIFA1) e LPLUNC1 (BPIFB1) apresentam localização distinta e são apenas parcialmente sobrepostas. SPLUNC1 (BPIFA1), por exemplo, é altamente expressa no epitélio respiratório e glândulas de Bowman das vias nasais, enquanto LPLUNC1 (BPIFB1) está presente em um pequeno subconjunto de células caliciformes nas vias nasais e faringe. LPLUNC1 (BPIFB1) também está presente nas glândulas serosas, na região proximal da língua onde está co-localizada com o membro da família específico das glândulas salivares, SPLUNC2 (BPIFA2E) e também nas glândulas do palato mole (Musa et al., 2012).

SPLUNC2 foi primeiramente identificada na saliva a partir das glândulas parótidas e submandibular/sublingual, sendo diferencialmente secretada pelas três glândulas, o que pode justificar os diferentes padrões de glicosilação presentes na saliva total (Bingle L et al., 2011). SPLUNC2/PSP humana é expressa em glândulas salivares, saliva, células epiteliais gengivais e como componente da película adquirida (Geetha et al., 2003; Shiba et al., 2005; Siqueira et al., 2010). Nas células epiteliais de gengiva, a expressão de SPLUNC2 é regulada pela bactéria Gram-negativa *Porphyromonas gingivalis* e pela citocina pro-inflamatória TNFα (Shiba et al., 2005). Na glândula parótida humana SPLUNC2 é expressa em ambas as células epiteliais acinar e ductal (Geetha et al., 2003).

Gorr et al., (2008) analisaram o peptídeo GL-13 presente na SPLUNC2 que têm ação de aglutinar as bactérias Gram-negativas *Pseudomonas aeruginosa* e *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, e Gram-positiva *Streptococcus gordonii*. Formas variantes de GL-13 revelaram que a aglutinação também depende do número de grupos amina no peptídeo, e inibem a adesão de bactérias às superfícies de plástico e impede a propagação da infecção por *P. aeruginosa* em um modelo de folha de alface, sugerindo que a GL-13 é ativo *in vivo*. Além disso, a aglutinação GL-13 aumenta a fagocitose de *P. aeruginosa* por macrófagos. Sugerindo que GL-13 representa uma classe de peptídeos

antimicrobianos, que não matam bactérias diretamente, mas em vez disso reduzem a aderência de bactérias e ajudam a promover a aglutinação das mesmas.

A proteína existe como uma forma N-glicosilada e não glicosilada, e ambos parecem induzir aglutinação de bactérias, uma importante função antibacteriana das proteínas salivares, e também se ligam a lipopolissacarídeos sugerindo que a proteína pode desempenhar um papel anti-inflamatório. Gorr et al., (2011) analisaram dois peptídeos baseados na sequência de SPLUNC2. Os peptídeos GL13K e GL13NH2, onde o primeiro parece ligar-se a endotoxinas, e o GL13NH2 aglutina bactérias e aumenta o "clearance" dos macrófagos, sendo sugerido assim o desenvolvimento de novos peptídeos com ação antimicrobiana e anti-inflamatória. Recentemente, foi relatada uma função complementar a esse peptídeo de inibição da secreção estimulada de lipopolissacarídeos e fator de necrose tumoral pelos macrófagos (Abdolhosseini et al., 2012).

Já os níveis de SPLUNC2 na saliva não têm sido estudados extensivamente em indivíduos com morbidades, mas existem alguns exemplos de variações da sua produção. Por exemplo, verifica-se que SPLUNC2 é reduzida em saliva de pacientes com periodontite (Wu et al., 2009). Outro estudo recente mostrou uma significativa diferença na expressão de SPLUNC2 em pacientes com HIV também infectados por CMV (citomegalovírus) ou com micobactérias, onde um particular aumento de SPLUNC2 foi notado nos ácinos serosos em torno dessas lesões na glândula parótida (da Silva et al., 2011). Mais uma vez, a importância funcional desta proteína ainda não está clara, e trabalhos futuros são necessários para determinar se as diferenças na expressão tecidual que resultam em níveis salivares alteradas da proteína.

Bingle et al. (2009) estudaram a expressão da SPLUNC2 em glândulas salivares maiores e menores normais pela produção de anticorpos contra SPLUNC2 correspondentes a dois epítopos, um na porção interna correspondente ao SPLUNC2A e outro correspondente à extremidade C, SPLUNC2B. No mesmo estudo, foi mostrado que SPLUNC2 é expressa predominantemente em células serosas de glândulas salivares maiores, assim como em túbulos sero-mucosos de glândulas salivares menores na mucosa oral, região posterior da língua e tonsila, contudo SPLUCN2B não é expressa nas estruturas

semilunares de glândulas salivares mistas. A expressão dessas proteínas nesses sítios de defesa do hospedeiro suporta a teoria de que a SPLUNC agiria na defesa imune inata.

Considerando que efeitos colaterais relacionados à terapia de altas doses de quimioterapia prévia ao TCTH e à quimioterapia convencional, como a mucosite oral e hipossalivação, podem ser parcialmente mediadas por alterações na composição e quantidade de saliva. O objetivo deste estudo foi analisar a expressão de SPLUNC em pacientes submetidos à TCTH autólogo e a doses padrão de quimioterapia, avaliando sua correlação com a presença e intensidade dos efeitos colaterais e a sua variação durante e após o tratamento.

CAPITULO 1

Artigo submetido para publicação no periódico (Archives of Oral Biology)

SPLUNC expression in saliva of patients undergoing autologous haematopoietic stem cell transplantation

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ABSTRACT

Objective: The aim of this study was to analyse flow rate and SPLUNC2A and SPLUNC1 expression in patients who underwent high-dose chemotherapy (HDT) followed by autologous haematopoietic stem cell transplantation (autologous HSCT). **Design:** A sample of autologous HSCT patients was analysed. Unstimulated saliva was collected for 5 min before HDT, and on D+7 and D+21, and SPLUNC expression was analysed by Western blotting. The data were statistically analysed one-way ANOVA, Fisher's test, Wilcoxon

test, Mann-Whitney test, P<0.05). **Results:** Fifteen patients were included in this study: mean age 45 ± 14 years, 9 men and 6 women. Densitometry of protein bands showed a higher level of SPLUNC2A after transplant (D+7) compared with before HDT; however, it was not statistically significant (P=0.58), and on D+21 SPLUNC2A levels were reduced, being very similar to the pre-HDT levels. SPLUNC1 was also increased after transplant (on D+7), and showed an important reduction on D+21, where the comparison between D+7 and D+21 showed statistical significance (P=0.02), indicating an important variation in SPLUNC1 production after autologous HSCT therapy. In addition, salivary flow rate was decreased after autologous HSCT (0.40 ± 0.33) compared with before HDT (0.47 ± 0.31), and remained reduced on D+21 (0.37 ± 0.20). However, these differences were not statistically significant (P=0.30). **Conclusions:** The present study, despite the small sample, shows that patients who underwent HDT for autologous HSCT may have changes in SPLUNC2A and SPLUNC1 expression and in salivary flow rate.
Key words: SPLUNC1, SPLUNC2A, autologous haematopoietic stem cell transplantation HSCT, high-dose therapy, mucositis

INTRODUCTION

High-dose therapy (HDT) followed by autologous haematopoietic stem cells transplantation (autologous HSCT) is generally the treatment of choice for patients who do not respond to other therapeutic options and have good clinical condition for this procedure (1). In global use of transplantation, autologous HSCT continues to be more highly implemented than allogenic transplants (2). Common indications for autologous HSCT are malignancies such as plasma cell myeloma, non-Hodgkin lymphoma, Hodgkin lymphoma, acute myeloid leukaemia, neuroblastoma, Ewing sarcoma, other rare cancers of childhood, germ-cell tumours, and non-malignant disorders, particularly autoimmune diseases (3).

HDT is associated with several side-effects, such as oral and gastrointestinal mucositis, dry mouth, changes in taste and alterations in saliva quantity and components (4). Oral mucositis (OM) is the most important complaint among these effects, and occurs

with clinically significant frequency and severity in patients who receive conditioning therapy for autologous HSCT (5).

Lately, the term syndrome was suggested to describe a set of signs and symptoms associated with HDT, including disruption of mucosal epithelial barrier structure, erythema, edema, ulcerations, pseudomembrane formation, bleeding, and reduced saliva. These alterations may cause pain and a significant impact on patients' quality of life (6). Currently, researchers are improving the knowledge about aspects of mucositis pathobiology, relating to targeted therapy-induced toxicity, toxicity clustering and the investigation of genetic polymorphisms, aiming to predict toxicity risk for individual interventions (7).

A new nomenclature was recently proposed for PLUNC/PSP/BSP30/SMGB proteins classified now as a subfamily of the BPI fold-containing superfamily, a family of putative innate immune proteins that are expressed predominantly in the oral cavity and airways (8).

The SPLUNC1 (BPIFA1) protein is a clone found in the minor glands of the nose, sinus, posterior tongue, and tonsil (9), which suggests an association between SPLUNC1 production and mucous cells. These data were further confirmed by the pattern of expression in major salivary glands, where the protein was only produced in glands of a mixed or mainly mucous cell type (submandibular and sublingual), but not by the serous cells of the parotid gland (10). However in some minor glands, SPLUNC1 staining overlapped with other isoforms: SPLUNC2 and LPLUNC1 (11, 12).

SPLUNC2 is a restricted protein member in the PLUNC subfamily; it is only found in serous cells in the major salivary glands, and in the sero-mucus tubules of the minor glands in the oral mucosa, posterior tongue, and tonsil (11, 13, 14). Moreover, just a few studies have been performed analysing SPLUNC2 in disease subjects, which showed reduced SPLUNC2 in the saliva of patients with periodontitis (15), and a significant difference in the expression of SPLUNC2 in human immunodeficiency virus patients who were infected with cytomegalovirus or with mycobacteria (16).

It is possible that the underlying mechanism of HDT-related OM may be partly mediated by alterations in the composition and quantity of saliva. Therefore, the aim of this

study was to analyse SPLUNC expression in patients undergoing autologous HSCT and its correlation with the side-effects of high-dose chemotherapy, mainly mucositis and hyposalivation.

MATERIALS AND METHODS

Human subjects

This prospective study was approved by the local Ethics Committee of the State University of Campinas (Approval No. 142/2010). Written and verbal explanations of the study design were provided to the patients before written informed consent was obtained. Patients with previous history of radiotherapy for head and neck cancer were excluded from this study. Demographic characteristics of the 15 autologous HSCT patients are listed in **Table 1**.

Systemic supportive care consisted of broad-spectrum antibiotics in addition to antifungal and antiviral prophylaxis, which began 1 week before the conditioning regimen. The broad-spectrum antibiotics were changed when the fever was present.

Oral examination

Oral examination was performed by dentists at the Dental Service prior to auto-HSCT and consisted of a physical examination and orthopantomographic and periapical radiographs. Acute dental infections were treated prior auto-HPCT by the Dental Ambulatory Service. In this phase, the patient diagnosis was important because patients with multiple myeloma undergoing bisphosphonate treatment received special care to avoid bisphosphonate-related osteonecrosis of the jaw. Clinical assessment was performed daily, starting on first day of HDT and ending at patient discharge. In addition, a visual analogue scale (VAS) was shown to the patients to evaluate the pain. The evaluation included a subjective assessment of dryness of the mouth, difficulty in swallowing, burning sensation and changes in taste. The physical examination was performed by two dentists, establishing

the severity of mucositis according to the World Health Organization WHO oral toxicity scale (17).

Characterisation of xerostomia and hyposalivation

Xerostomia was considered as a patient complaint of dry mouth and hyposalivation was defined as a salivary flow rate of lower than 0.3 ml/min of output saliva. The procedure consisted of a 5-min salivary expectoration into a sterile, previously weighed glass cup. Patients were asked not to swallow during the collection period. Salivary flow rates were calculated based on the saliva collected per min and converted to ml/min (18).

Saliva collection

Whole unstimulated saliva was collected at three different time points: (i) 1 day before HDT; (ii) on day 7 post transplantation when severe collateral effects were expected; and (iii) 21 days post transplantation, the time point when patients are recovering. For this study, subjects were asked to rinse the mouth prior to saliva collection. All samples were collected before noon and always at 1 hour after the previous meal, snack, or consumption of beverages. After collection, the saliva was centrifuged at 14,000 rpm for 6 min at 18°C and supernatants were transferred to new vials. Supernatants were stored at -80°C with ethylenediamine tetraacetic acid (EDTA) (0.5M) and phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors until the assay was performed.

Western blotting

The total protein content in the clear supernatant was measured using a Bradford assay with bovine serum albumin as a standard. Total protein quantification was performed by spectrophotometry of each sample. Samples were mixed with dithiothreitol (DTT) in loading buffer and heated, and 4 µg of protein per well was analysed by sodium

dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE, 12%). After the proteins were transferred onto a nitrocellulose membrane, to confirm the effectiveness of the transference, a Ponceau stain was used and pictures were taken to verify that the same quantity of sample had been applied in each well. All membranes were incubated overnight at 4ºC with skim milk with 5% Tris-buffered saline and Tween 20 (TBST). After four washes of 15 min each, the blots were incubated with primary antibodies against SPLUNC2A (1:500) and SPLUNC1 (1:200), for 2 hours with blocking solution. After four washes, they were incubated with secondary antibody for 1 hour and detection was performed using enhanced chemiluminescence (ECL) from Amersham Biosciences, Inc. The rabbit anti-human SPLUNC monoclonal antibody (isoforms SPLUNC2A and SPLUNC1) used in Western blotting was generously provided by Dr. Lynne Bingle, Sheffield, UK. Relative band intensity was measured using the Gel Analyzer 2010 software and corrected for background using an equivalent area of blank film. All samples were normalised using the total protein concentration of each sample.

Statistical analysis

All data were analysed using SAS (SAS Institute Inc. The SAS System, release 9.3. SAS Institute Inc., Cary:NC, 2010). All graphics were made using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Non-parametric tests including Kruskal-Wallis test, Wilcoxon two-sided test, Mann-Whitney test and parametric test as T-test were performed. $P<0.05$ was taken to indicate significance when comparing the groups. Correlation studies were performed using Cramer's V test, Tukey and Fisher's Exact tests.

RESULTS

In the first collection (pre-HDT), 33% (5/15) of conditioned patients had an unstimulated salivary secretion rate of less than or equal to 0.3 ml per min and the second collection (D+7) was 40% (6/15). In addition, comparison of salivary gland secretion in the entire cohort between pre-HDT (0.47 ± 0.30 ml per min), D+7 (0.40 ± 0.23 ml per min),

and D+21 (0.37 ± 0.20) was not statistically significant (P=0.54). Xerostomia was present in 60% (9/15).

OM was present in 73% (11/15), and the overall incidence of OM grades 3 and 4 was 33% (8/18). Among the patients receiving cluster prophylaxis therapy for mucositis, just two patients presented mucositis classified as grade 2, both was diagnosed with multiple myeloma that underwent HDT with melphalan (200 mg/m^2), and **Figure 1** is showing one of them.

Densitometry demonstrated significant variation in SPLUNC1 expression between the three time points (P=0.04), with SPLUC1 increased on D+7 and reduced on D+21; D+7 *vs* D+21 (P=0.02). However, no significant difference was found between pre-HDT and D+7 (P=0.09) (**Figure 2A**). Western blotting showed higher levels of SPLUNC1 production on D+7 in four subjects (**Figure 2 B, C**). However no association between mucositis and hyposalivation was observed (**Table 2**).

Total SPLUNC2A levels were increased on D+7, but no significant differences were observed (P=0.58) (**Figure 3 A, B**). Comparison pre-HDT *vs* D+7 (P=0.26), D+7 *vs* D+21 (P=0.58), and three time points comparison for N-glycosylated bands (P=0.35) and non-glycosylated bands (P=0.46) (**Figure 3 C, D**).

Analysis of the association between SPLUNC2A total levels and the presence of mucositis and hyposalivation showed higher levels of SPLUNC2A (>9828 µg/µl) than SPLUNC2A (<9828 µg/µl) in patients with mucositis on D+7 and hyposalivation (low flow rate) (**Figure 4** and **Figure 5**). Total protein measurement by Bradford protein assay showed no significant difference between the three time points (P=0.58); there was also no significant difference between pre-HDT *vs* D+7 or D+7 *vs* D+21.

The incidence of side-effects was higher on day 7 post transplantation compared with the 'pre-HDT' assessment. Dry mouth sensation was reported by 60% of patients and change in taste by 93%. None of these alterations were related in the pre-HDT assessment. Pain analysis by VAS showed that 6 patients (40%) did not report pain, 3 patients reported pain between 3 and 5 (20%), and the other 6 had pain between 6 and 8 (40%).

On day 21 post transplantation, all patients reported a significant improvement in oral discomfort. The severity of oral pain was reduced and SPLUNC1 secretion level was significantly lower than on D+7 (**Figure 2**).

DISCUSSION

The present analysis is a clinical and laboratory assay, intending to understand if changes in salivary proteins related to innate immune capacities might have a correlation with mucositis and hyposalivation caused by high dose chemotherapy. Nevertheless, a cluster of factors in the development of these side-effects have to be considered.

Several studies have shown that SPLUNC levels are reduced on study group when compared with healthy control subjects (19-23). In contrast, SPLUNC expression showed elevated levels in the nasal respiratory epithelium of rats after olfactory bulbectomy, and PLUNC mRNA levels were elevated in the lungs of patients with chronic obstructive pulmonary disease (24, 25). The results suggested that SPLUNC1 increased expression in the present study is a proportion of mucous saliva presenting on D+7, because saliva was noticeably stickier in this period where 60% of patients related xerostomia.

The preservation of salivary gland function is quite important to keep oral health and macrobiotic balance (26, 27). In this study, whole saliva was collected, as it is a major determinant of the environment on all the oral surfaces. It is well known that saliva plays an important role in physico-chemical as well as immune defence of the oral and mucosal surfaces, via both direct antimicrobial action and agglutination or surface exclusion of microbes. Moreover, the protein selected in our study suggests a close relationship with host immunity variation in post autologous HSCT patients and protein levels, suggesting a higher protein level during the pancytopenia period.

A considerable reason for this can be a complex host immunity reaction that occurs after HSCT, wherever granulocyte macrophage colony-stimulating factor (GM-CSF) is an important cytokine for regulating innate immune cells, particularly macrophages that produce this cytokine. A study performed in lung disease has shown that GM-CSF is

required for effective clearance of pathogens but it is produced by alveolar epithelial cells in the lung (3).

Additionally, it is important to consider that some suppliers doses of GM-CSF are administrated in patients during the pancytopenia period after autologous HSCT, which may imply alterations that can explain the upregulation of some components of the innate immune system, such as the BPI fold-containing family. It is well established that severe mucositis during the period of neutropenia is a cause of septicaemia in HSCT, so we consider this problem the main reason for patient saliva analyses, because increased SPLUNC protein might predict some factors for the development of infection (systemic or local) in transplant patients (28).

In the present study, all patients underwent high-dose melphalan, at a dose of 200 mg/m^2. The incidence of severe OM is generally higher (98%) after a regimen of total body irradiation, etoposide, and CY (29), whereas chemotherapy-only conditioning regimens, such as high-dose melphalan (HDM) or BCNU-etoposide-cytarabine-melphalan have a lower incidence of severe OM of 46% and 42%, respectively (30). Our patients received chemotherapy-only conditioning regimens and showed a lower percentage value (33%) of severe mucositis.

Oral health is directly influenced by an adequate salivary gland function (31). Patients receiving a bone marrow transplant may present alterations in salivary flow, altered buffer capacity, and microorganisms, resulting in increased risk of caries (31). These findings indicate that transplant recipients can contribute to a higher caries risk and oral complications such as mucositis during the early post transplant period (32, 33). Another factor that might contribute to the low incidence of oral severe mucositis in our study was previous dental care in all patients.

SPLUNC2 N-glycosylation was demonstrated by PNGse F treatment used to remove N-linked sugars. It resulted in the reduction of three bands to one band of approximately 25 kDa; however, no differences in function have been shown for the isoforms (11, 34, 35). Comparison of these bands separately at the three time points showed that there was not significant change in these isoforms, but a significant variation was observed in these two forms between the second and third collections.

In conclusion, the increased SPLUNC1 and SPLUNC2 in patients who underwent HDT for autologous HSCT may be related to changes in saliva composition. However, this is the first study to analyse the levels of these proteins in saliva; other studies that include a larger number of patients are necessary.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGMENTS

This work was supported by grants from Sao Paulo Research Foundation process 2011/00711-9, and Lara M. A. Ramos is supported by the same institution.

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TABLES

Table 1. Clinical features of autologous HSCT (n = 15).

Variable	N
Age mean (range)	47.6 (23-68)
Male/female	9/6
Underlying disease	
NHL	4
HL	3
MM	8
High dose therapy	
BEAM	5
BEAM-like	2
Melphalan	8
Disease status after transplantation	
VGPR	5
PR	10
NE	0

Abbreviations: NHL = non-Hodgkin lymphoma; HL = Hodgkin lymphoma; MM = multiple myeloma; BEAM = armustine, etoposide, cytarabine, and melphalan; BEAM-like = lomustine, etoposide, cytarabine and melphalan; VGPR = very good partial response; PR = partial response; NE = not evaluable (having a non-secretory disease with non signficant bone marrow involvement).

Table 2. Evaluation of salivary levels of SPLUNC1 associated with mucositis and hyposalivation at collection 2.

Variable	Presence	**SPLUNC1 ratio - n (%)**		Fisher's test P	Cramer's V
		Present	Absent		
Mucositis	Yes	1 (20.00)	8 (53.33)	0.3189	0.2901
	No	4 (80.00)	7 (46.67)		
Hyposalivation	Yes	2 (40.00)	4 (26.67)	0.6126	0.1260
	No	3 (60.00)	11(73.33)		
		SPLUNC1 total – n (%)			
		Present	Absent		
Mucositis	Yes	8 (80.00)	3 (60.00)	0.5604	0.2132
	No	2 (20.00)	2 (40.00)		
Hyposalivation	Yes	4 (40.00)	2 (40.00)	1.0000	0.0000
	No	6 (60.00)	3 (60.00)		

P<0.05 – statistical significance

FIGURES AND LEGENDS

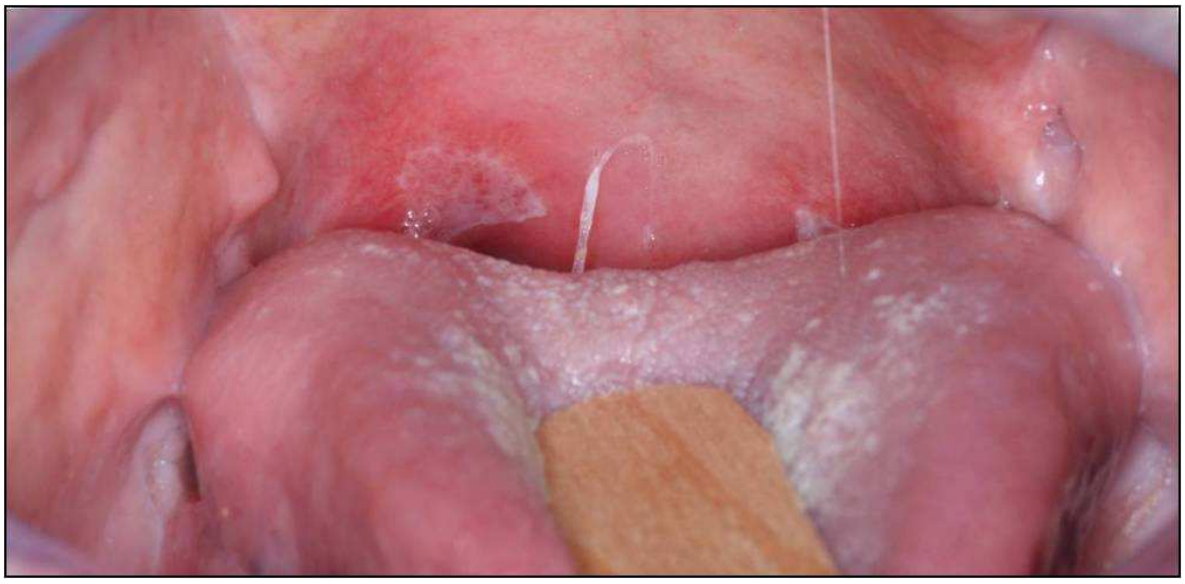

Figure 1. Oral mucositis grade 2 on the soft palate at D+7 in a patient that underwent high dose of melphalan (200 mg/m^2), this view shows as well as the appearance of thickness saliva in this period.

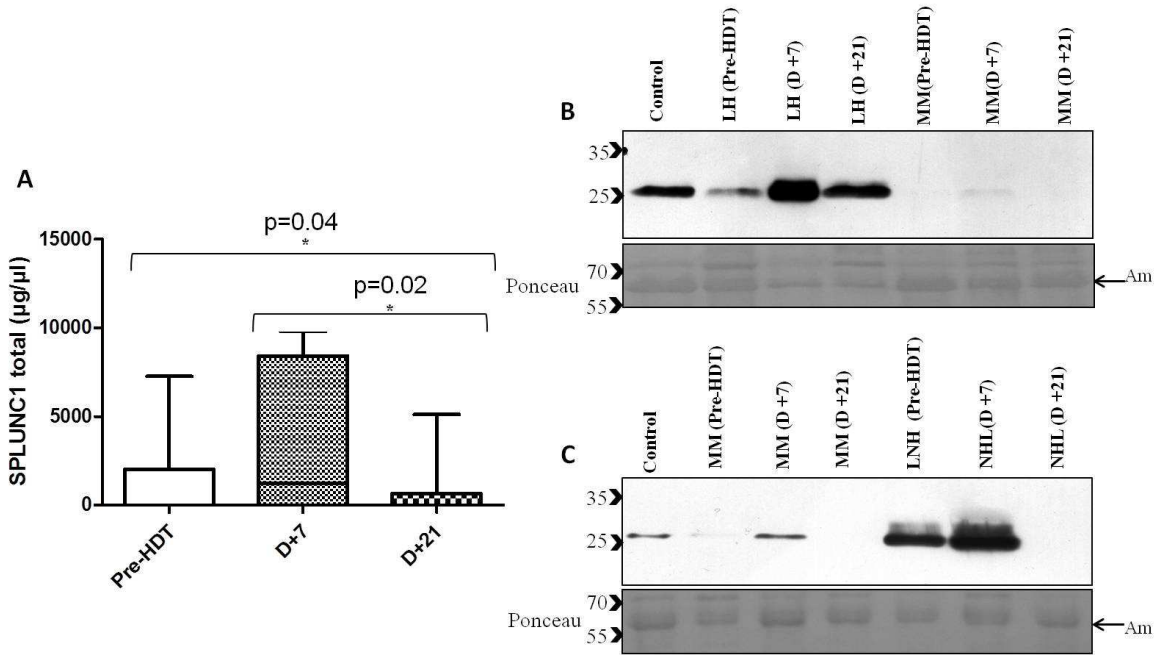

Figure 2. SPLUNC1 concentration in pre high-dose therapy (pre-HDT) *vs* D+7 *vs* D+21 samples (P=0.04), pre-HDT *vs* D+7 (P=0.09) and D+7 *vs* D+21 (P=0.02) (A). *P<0.05 indicates statistical significance. Western blotting showing differential expression of SPLUNC1 in three time points comparison from saliva of patients with non-Hodgkin lymphoma (NHL), Hodgkin lymphoma (HL) and multiple myeloma (MM) (B) and (C). The positions of molecular mass markers (kDa) are shown for the stained gel and the positions of amylase (Am) in Ponceau S coloration.

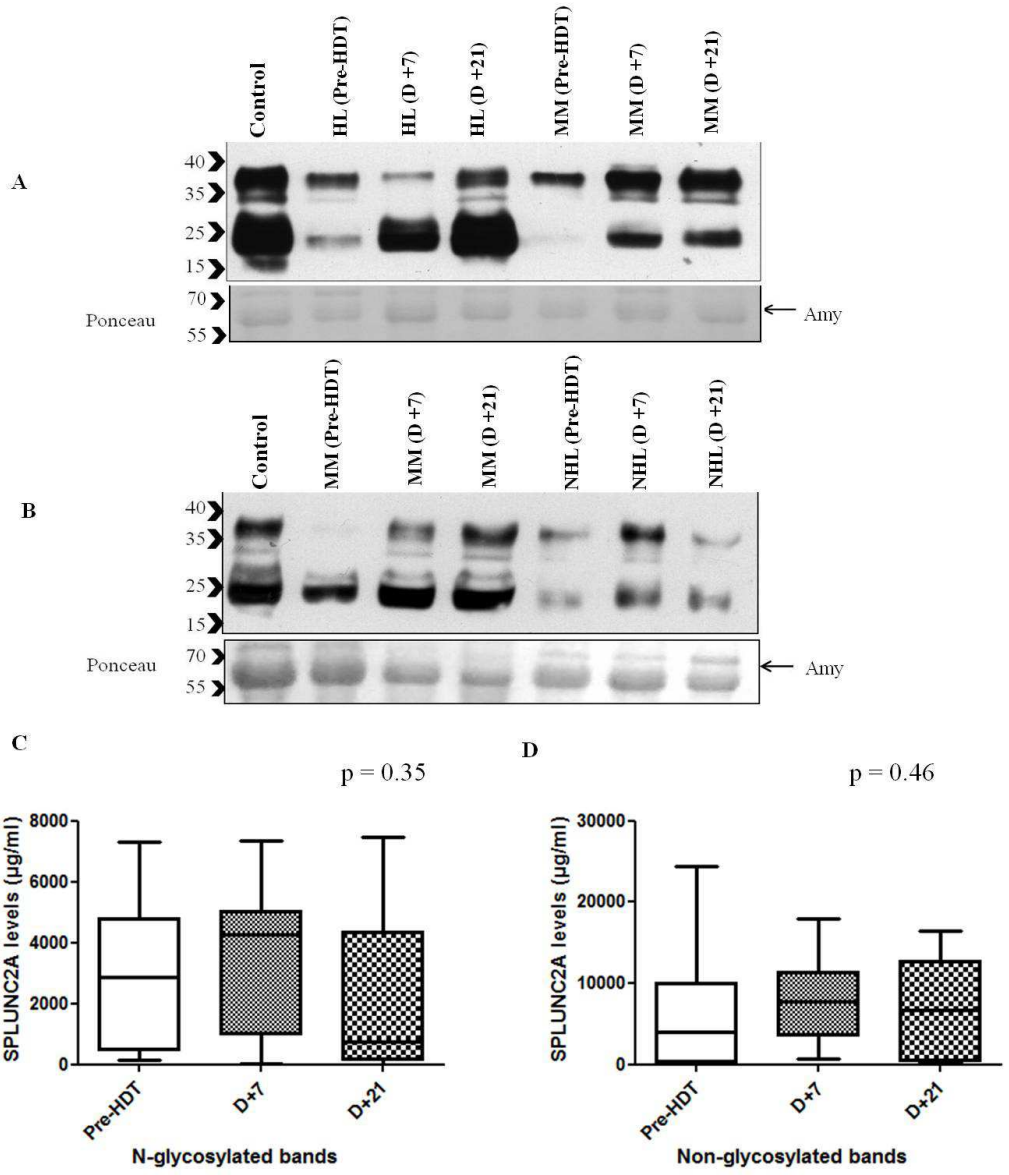

Figure 3. Differential expression of SPLUNC2A, showing three time points comparison, (pre-HDT), (D+7) and (D+21) in saliva of patients with non-Hodgkin lymphoma (NHL), Hodgkin lymphoma (HL) and multiple myeloma (MM) (A) and (B). The positions of molecular mass markers (kDa) are shown for the stained gel (arrowheads) and the positions of amylase (Amy) in Ponceau coloration (arrows). N-glycosylated and non-glycosylated bands showed a discrete increase at D+7, comparison pre-HDT *vs* D+7 (P=0.26), D+7 *vs* D+21 (P=0.58), three time points comparison for N-glycosylated bands (P=0.35) (C) and non-glycosylated bands (P=0.46) (D).

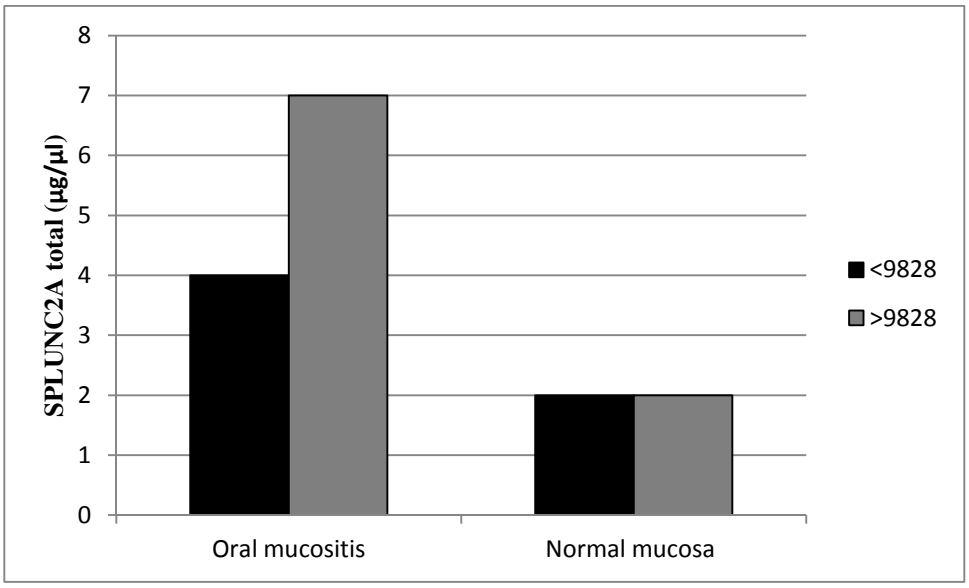

Figure 4. Oral mucositis results in increased SPLUNC2A. Salivary SPLUNC2 post-HDT samples (D+7). P = 1.0000. Fisher's Exact Test.

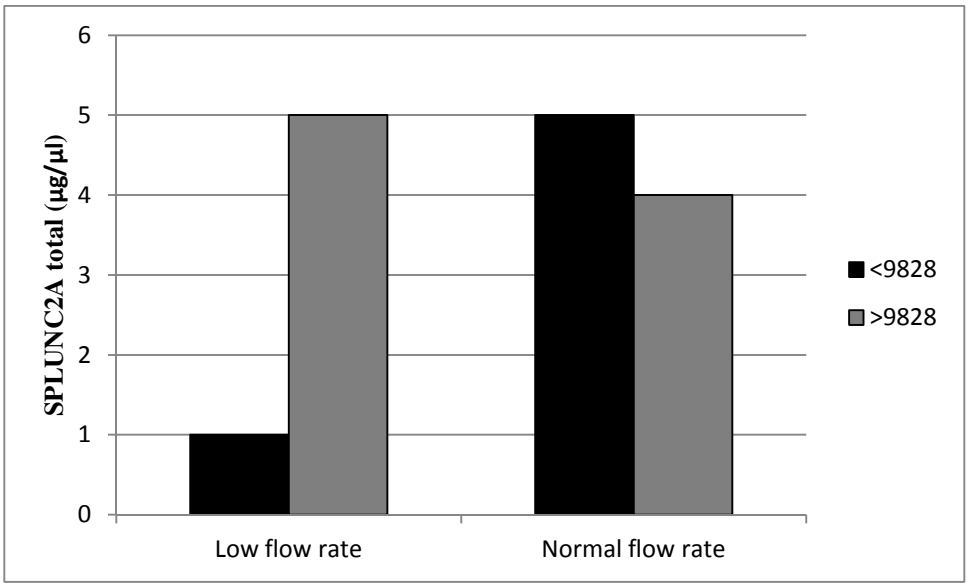

Figure 5. Hyposalivation results in increased SPLUNC2A. Salivary SPLUNC2 post-HDT samples (D+7). P=0.2867. Fisher's Exact Test.

CAPÍTULO 2

Artigo nas normas do periódico *Immunological Investigations.*

SPLUNC expression in saliva of patients receiving standard dose chemotherapy

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ABSTRACT

Objective: The aim of this study was to analyze SPLUNC2A and SPLUNC1 expression in patients who underwent chemotherapy regarding their therapy's side effects. **Materials and Methods:** This study was performed with whole unstimulated saliva collected for 5 min before, during, and after chemotherapy. SPLUNC expression was evaluated by western blotting. The data were statistically analyzed (ANOVA, paired T test, and unpaired tests, Mann-Whitney and Kruskal-Wallis, $p<0.05$). **Results:** Forty patients were included in this

study, 20 chemotherapy patients and 20 ages matched healthy controls (mean age 46 ± 14). Densitometry of protein bands of the chemotherapy group showed SPLUNC2A decreased after chemotherapy administration, but was not statistically significant (p=0.24), and SPLUNC1 increased after chemotherapy, without statistical significance either (p=0.82). On the other hand, comparison with healthy controls and the first collection of the chemotherapy group, showed that salivary flow rate (p=0.007) and SPLUNC1 (p=0.002) were higher in healthy subjects and SPLUNC2A levels was smaller (p=0.04). **Conclusion:** The present study, despite the small sample, describes recent findings in patients who underwent chemotherapy showing changes in SPLUNC expression and in salivary flow rate.

Keywords: SPLUNC1, SPLUNC2A, chemotherapy, hyposalivation, mucositis.

INTRODUCTION

Chemotherapy (CT) might cause heavy toxicity triggering oral side effects such as, mucositis, hyposalivation/xerostomia, dysphagia and infections leading considerable morbidity (Scully, 2003). Oral mucositis (OM) is one of the most common and debilitating complication in patients undergoing chemotherapy, and has been reported affecting many patients depending of the drug and frequency (Raber-Durlacher et al., 2010). The most common chemotherapeutics drugs that induce mucositis are antimetabolites such as, 5-fluorouracil and methotrexate (Kostler et al., 2001).

PLUNC (palate lung and nasal epithelium clone)/PSP (parotid secretory protein) are proteins currently classified as a subfamily of the BPI fold-containing superfamily (Bingle et al., 2011). SPLUNC2, the human homologue of mouse parotid secretory protein (PSP), was shown to suppress the growth of *Pseudomonas aeruginosa* (Geetha et al., 2003). Peptides derived from PSP inhibited the secretion of TNFα from a macrophage cell line (Geetha et al., 2005) and promoted the agglutination of Gram-positive and Gram-negative bacteria (Gorr et al., 2008). SPLUNC2 is restricting expressed in oral cavity produced by serous cells in major salivary glands and sero-mucous ducts in minor salivary glands from posterior portion of the tongue and tonsil (Bingle et al., 2009).

SPLUNC1 is other member of secretory protein that has a larger expression in human mainly in nasal, oropharyngeal, lung epithelia, and may also be expressed in Na^+ absorbing tissues, including the stomach, colon and kidney (Garcia-Caballero et al., 2009). Both proteins SPLUNC1 and SPLUNC2A are related as putative innate immunity proteins, with bactericidal and anti-inflammatory activities (Gorr et al., 2011; Musa et al., 2012).

Saliva has been recognized as an excellent diagnostic medium since it has an abundance of proteins, offers an easy, inexpensive, safe, and non-invasive approach and can be helpful for detection of several biomarkers (Zhang et al., 2013). It is known that the composition and quantity of saliva influence the oral health, so the objective of this study evaluated salivary components that belong to innate immune system and flow rate intending to understand the influence of these factors during chemotherapy.

MATERIAL AND PATIENTS

Human subjects

This is a case-control study that included subjects referred for evaluation at Oncology Center at Chemotherapy Department and healthy controls referred for Oral Medicine Service at Dental School of the State University of Campinas from 2011 to 2013. The study was approved by the local Ethics Committee of the State University of Campinas (Approval No. 142/2010). Saliva was collected once from healthy males and females' age matched controls, and from subjects submitted to chemotherapy at three times point: (1) before Chemotherapy, (2) after 6 days of the first chemotherapy cycle, and (3) after 20 days of last chemotherapy cycle. Written informed consent was obtained. Patients under 18 years old and with previous history of radiotherapy for head and neck cancer was excluded of this study. Clinical-demographic features such as, age, sex and diagnosis of the chemotherapy's patients was showed (**Table 1**).

Saliva samples

The measurement of unstimulated salivary flow rate (USSFR) consisted of a 5 min of whole salivary expectoration into a sterile previously weighted glass cup. Saliva was collected from healthy subjects once during the morning period. Patients were asked not to swallow during the collection period, to rinse the mouth prior to saliva collection, all samples were collected before the noon as well, and always one hour after previous meal, snack, or consumption of beverages. Salivary flow rates were calculated based of saliva collected per minute and converted to ml/min (Davies et al., 2002). A USSFR value under 0.3 ml/min was considered low or hyposalivation (Márton et al., 2008). After collection, saliva was centrifuged at 14.000 rpm for 6 min at 18°C and supernatants were transferred to new vials. Supernatants were stored at - 80°C with EDTA (0,5M) and PMSF for protease inhibition.

Western Blot

The total protein content in the clear supernatant was measured using a Bradford assay with bovine serum albumin as a standard. The total protein quantification was performed by spectrophotometer to each sample, it was mixed to DTT in loading and heating sample, and applied 4 µg of protein per well, with SDS polyacrylamide gel electrophoresis (SDS-PAGE 12%), after transference was performed to nitrocellulose membrane and to confirm the effectiveness of transference a Ponceau S coloration was used and pictures were took to verify the same quantity of sample applied in each well. All membranes were incubated overnight to 4ºC with skim milk 5% with TBST (Tris-Buffered Saline and Tween 20). After 4 washes of 15 minutes each, the samples were incubate with primary antibodies SPLUNC2A (1:500) and SPLUNC1 (1:200), two hours with blocking solution and after 4 washes again, and were incubate with the secondary antibody for one hour and it was detected with enhanced chemiluminescence - ECL (Amersham Biosciences, Inc.). SPLUNC rabbit anti-human monoclonal antibody (isoforms SPLUNC2A and SPLUNC1) used in western blotting was generously provided by Dr

Lynne Bingle, Sheffield, UK. Bands intensity was measured using the Gel Analyzer 2010 software and corrected for background using an equivalent area of blank film, all samples were normalized using total protein of each sample (Kohlgraf et al., 2012).

Statistical analysis

All data were analyzed using the SAS (SAS Institute Inc. The SAS System, release 9.3. SAS Institute Inc., Cary:NC, 2010). Non-parametric tests including Wilcoxon, Kruskal–Wallis, and Mann-Whitney tests were performed for comparison between control and chemotherapy groups. $P<0.05$ was accepted to indicate significance when comparing the groups.

RESULTS

Pre (CT) and 6 days pos (CT) assessment

Clinical and laboratory features of oral side effects

Oral mucositis (OM), from degree 1 to 3, was recorded in 8 patients (40%) (10% with mucositis grade 1, 25% with mucositis grade 2 and 5% with mucositis grade 3). Mucositis-related signs were initially observed on days 2–6 (median; day 4) and were most severe on day 3 after chemotherapy and the maximum value attribute to visual analogical scale (VAS) of pain was 7.

Dry mouth sensation was reported by 8 (40%) of the patients before the therapy and by 12 (60%) of the patients after chemotherapy. Oral infections (candidosis or herpes), change in taste, and difficulty to swallow were reported in 15%, 35%, and 30% of patients, respectively, compared with none pre-CT.

Salivary flow rate

Control *vs* CT group – Salivary flow rate comparison between healthy controls and the first collection of the CT patients, showed higher salivary flow rate in control group (mean salivary flow rate was 0.72 *vs* 0.39 ml/min, p=0.007).

There was no statistically significant change in the salivary flow rate between collection before and 6 days after CT (mean salivary flow rate was 0.39 vs 0.49 ml/min, respectively). The majority of the saliva samples collected pre-CT was clear and flow and the samples collected on day 6 after CT were unclear and sticky.

Salivary composition

Control *vs* CT group – An increased total SPLUNC2A and glycosylated SPLUNC2A were observed contrasting with a decreased SPLUNC1 total and SPLUNC1 ratio in CT group, statistically significant (as show at **Table 2** and **Figure 3**).

Compositional alterations were noted in the saliva samples obtained pre-CT vs post-CT. Considering SPLUNC pre-CT (means of total SPLUNC2A = 16595.250 µg/µl; total SPLUNC1 = 535.400 µg/µl; Total protein = 1.122 µg/µl), and post-CT (means of total SPLUNC2A = 12685.800 µg/µl; SPLUNC1 = 610.850 µg/µl; Total protein = 1.350 µg/µl), these data showed a slight reduction in SPLUNC2A and a slight increase in SPLUNC1. There were no significant differences in the levels of total protein, SPLUNC2A, SPLUNC1 pre and post CT (**Table 3).** All patients that complained of dry mouth have total protein levels higher 1,0 µg/µl.

Day 20 after last chemotherapy cycle

Clinical and salivary composition assessment

A third saliva sample was collected from ten patients 20 days after last chemotherapy cycle. The majority of patients reported a significant improvement in oral discomfort 20 days post last cycle. The severity of oral pain significantly decreased,

however, dry mouth sensation and abnormal taste sensation remained common. SPLUNC2A levels returned to normal values (means of ratio SPLUNC2A = 0.62 µg/µl; SPLUNC1 = 0.0 µg/µl; Total protein = 0.96 µg/µl), but were still lower than those measured pre-CT.

DISCUSSION

CT potentially induces compositional changes in saliva slightly increasing salivary sodium and chloride concentrations as well as decreasing inorganic phosphate concentrations in even though lower or unchanged flow rates, that ductal modification mechanisms are affected. Evaluating unstimulated whole saliva (UWS) and stimulated whole saliva (SWS), the total protein analysis in UWS showed secretory IgA decreased in response to CT (Kohlgraf et al., 2012). Our results show that chemotherapy toxicity will affect the quantity and quality of saliva probably through changes in the various pathways to the salivary glands or effects on acinar cells translated in SPLUNC expression during chemotherapy.

The reason of these proteins SPLUNC2 and SPLUNC1 have been selected and quantified, was because the first protein represents serous secretion and second one mucous secretion of saliva (Bingle et al., 2009). SPLUNC2 is generally expressed in major salivary glands and in some specific serous cells of minor glands (Geetha et al., 2005). This pattern had already shown by immunohistochemistry, in which only mixed or mainly mucous cell type major salivary glands produce SPLUNC1 (submandibular and sublingual), but not the serous cells of the parotid gland (Vargas et al., 2008). Confirming previous observations SPLUNC1 was a major protein product of the minor salivary glands of the oral cavity (Bingle et al., 2005).

Parasympathetic stimulation produces copious saliva of low protein concentration while sympathetic stimulation produces little saliva but of high protein concentration and may thus give a sensation of dryness (Carlson, 2000; Pink et al., 2009). In accordance with this knowledge we investigated the correlation between dry mouth

complaint and higher total protein level up to 1,0 µg/µl, and all patients in this group have xerostomia.

SPLUNC2 antibodies were generated, with properties to recognize two epitopes, SPLUNC2A recognises an epitope that is found internally within the protein, and SPLUNC2B, recognises an epitope at the extreme C-terminus of the protein, however this last isoform is more restricted and SLUNC2B does not detect protein within the serous demilunes of the glands as SPLUNC2A (Bingle et al., 2009). The SPLUNC2A choice was justified because more widely expression related to location of SPLUNC2A was described, although in whole saliva SPLUNC2B stain is stronger.

Regardless the glycosylation pattern usually attribute important changes in protein function, SPLUNC2 showed in previous study that both glycosylated and unglycosylated forms were captured by LPS and could be eluted with a non-ionic detergent, presenting though the same functions (Ramachandran et al., 2008); (Gorr et al., 2011). This present study did not found any difference between these two forms expression at three times point comparison, but non-glycosylated form was higher in all moments analyzed.

Mucositis can vary considerably in accordance with drug and dose regimen with severe cases often causing significant infection, pain, and loss of function. Defined as common and treatment-limiting side effect of cancer therapy (Sonis, 2009), a severe oral mucositis can lead the patient to interrupt or discontinue cancer therapy and thus may have an impact on cure of the primary disease. The literature supports the use of some agents, such as cryotherapy for stomatotoxic chemotherapy during bolus fluorouracil, biologic response modifiers and growth factors (Migliorati et al., 2006; Sonis, 2009). We based our choice of second collection and oral evaluation in the knowledge of the chemotherapy-induced mucositis usually develops within 4–7 days after initiation of treatment and peaks within 2 weeks (Raber-Durlacher et al., 2010). However more investigations about topical and systemic agents probably present in saliva might improve mucositis healing.

SPLUNC 1 was propose as an alternative therapy for cystic fibrosis in lungs because his function was related as well as an autocrine inhibitor of epithelial sodium channel (ENaC) and a specific peptide was suggested for this disease (Garcia-Caballero et al., 2009; Hobbs et al., 2012). Even as SPLUNC1 might be administrated in asthma

patients with a Th2 phenotype for attenuating the excessive airway inflammation associated with (or without) bacterial infection (Garcia-Caballero et al., 2009). We decided to investigate SPLUNC variation in patients receiving chemotherapy aiming to understand if its presence or absence may improve oral patient conditions.

Assuming that oral mucositis induced by chemotherapy has a start point in releasing of several inflammatory cytokines such as, TNF-α, IL-1β, and IL-6 and the direct effect of chemotherapeutic drugs in basal epithelium and connective tissue (Scully et al., 2006), and after the oral microbial environment. Probably SPLUNC has not involvement in primary etiology of OM, because despite the course of mucositis is influenced by oral environment, neither changes in saliva nor the microorganisms were consider relevant as a primary factor (Sonis, 2009).

Regarding increasing Gram-negative organisms during OM ulceration and reestablishment of normal bacterial proportions as a requirement for spontaneous ulcer resolution (Sonis, 2009), SPLUNC2 with Gram-negative agglutination and anti-inflammatory function might help to reduce the mucositis time. SPLUNC2A protein might have an indirect influence in oral mucositis mechanism or maybe avoid the development of mucositis hampering the TNF-α release as proven in previous study (Gorr et al., 2011). Although it is contradictory because a study did not demonstrated influence with SPLUNC secretion from nasal epithelial cells and proinflammatory mediators (Kim et al., 2006). Additionally some authors suggested the use of the anti-inflammatory agents directed at pro-inflammatory cytokine inhibition have shown promise for reducing mucositis severity in animal models; however, results are not uniformly positive (Haagen et al., 2009; Melo et al., 2008).

In summary, we have shown an increase SPLUNC1 and decreased SPLUNC2A in saliva of patients undergoing chemotherapy. It is unclear the reason of this differential production, so we suggest that SPLUNC proteins during the therapy were altered because of the saliva quality that became more mucous after chemotherapy. The nature of our studies does not allow us to determine if the increased or decreased SPLUNC excretion is associated with disease severity, and the development of quantitative assays might highlight if the of this protein role is a potential biomarker for oral healthy.

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TABLES

Table 1. Distribution of patients according to age, gender, diagnosis and salivary flow rate.

Parameter	**N= 20 (%)**
Age	
< 45 years	7 (35)
≥ 45 years	13 (65)
Gender	
Male	5 (25)
Female	15 (75)
Diagnosis	
Hematologic malignances	5 (25)
Solid Tumors	15 (75)
Salivary flow rate (collection 2)	
> 0.3 ml/min	14 (70)
< 0.3 ml/min	6 (30)

Table 2. Protein changes in human SPLUNC from chemotherapy patients and controls.

Variable	Group	Mean	Std Dev	P
SPLUNC2A Glycosylated	Controls	953.650	1215.622	0.0107[b]
	Chemotherapy	4817.189	6532.402	
SPLUNC2A Non-Glycosylated	Controls	8457.500	6087.157	0.4311
	Chemotherapy	10116.019	8477.099	
SPLUNC2A ratio[a]	Controls	15626.122	21079.946	0.1481
	Chemotherapy	25840.764	30073.995	
SPLUNC2A Total	Controls	9411.150	6259.156	0.0495 [b]
	Chemotherapy	14933.208	30073.995	
SPLUNC1 Total	Controls	1327.400	1825.467	0.0021[b]
	Chemotherapy	449.510	1062.192	
SPLUNC1 ratio[a]	Controls	1830.085	2858.931	0.0028[b]
	Chemotherapy	632.450	1471.083	
Total Protein	Controls	1.104	0.656	0.4664
	Chemotherapy	1.182	0.995	

a - Ratio means that protein levels were normalized after densitometry by total protein stain intensity of each sample. b - Significant difference $p<0.05$ compared to controls. Std Dev – standard deviation. Wilcoxon two- Sample test.

Table 3. Three times comparison for each variable at chemotherapy group.

Variable	Group	Mean	Std Dev	F test P value	Tukey's test
SPLUNC2A Glycosylated	Before	5012.100	5773.372	0.8836	A
	After	4245.100	5099.700		A
	20 days	5397.462	9486.127		A
SPLUNC2A Non-Glycosylated	Before	11583.150	8337.703	0.0340*	A
	After	8440.700	8145.264		A
	20 days	10436.308	9375.837		A
SPLUNC2A ratio[a]	Before	30961.102	34175.881	0.1524	A
	After	18949.563	25955.469		A
	20 days	29382.488	29299.942		A
SPLUNC2A Total	Before	16595.250	12469.556	0.2481	A
	After	12685.800	10131.533		A
	20 days	15833.769	16620.721		A
SPLUNC1 Total	Before	535.400	1194.517	0.8200	-
	After	610.850	1179.009		-
	20 days	0.000	0.000		-
SPLUNC1 ratio[a]	Before	843.744	1653.166	-	-
	After	737.381	1605.925		-
	20 days	0.000	0.000		-
Total Protein	Before	1.122	0.828	0.4664	A
	After	1.350	1.294		A
	20 days	0.966	0.539		A

a - Ratio means that protein levels were normalized after densitometry by total protein stain intensity of each sample. * Significant difference $p<0.05$. Std Dev – standard deviation. F test and Tukey's test.

FIGURES AND LEGENDS

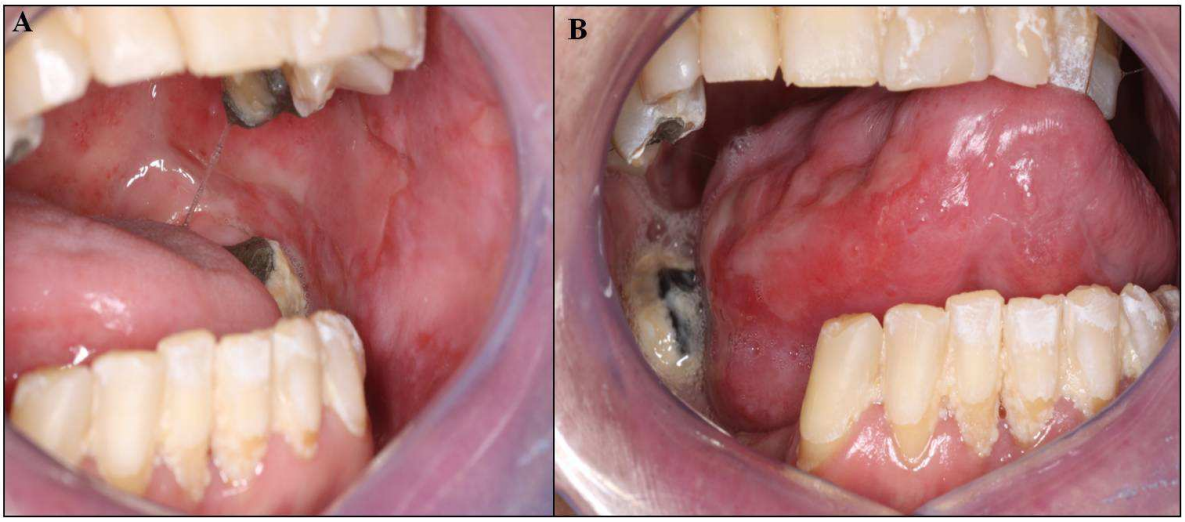

Figure 1. (A) Extensive ulceration covered by pseudo-membrane on the left buccal mucosa, (B) Similar alteration on the tongue. The patient was unable to eat solid foods and oral mucositis was graded 3 on WHO scale. This presentation was detected at 1 week after 5-fluorouracil bolus.

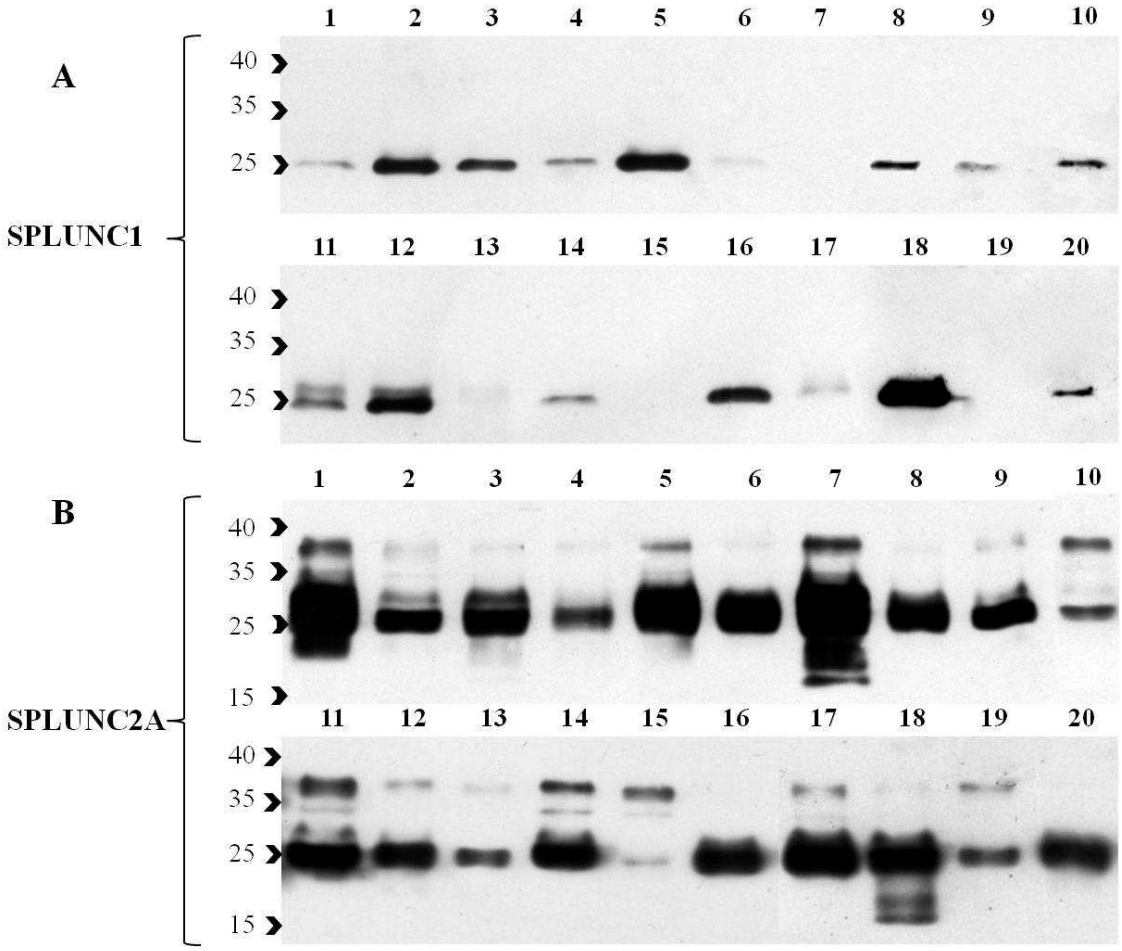

Figure 2. Western blots comparing SPLUNC1 and SPLUNC2A in human saliva from 20 systemically healthy subjects. There were bands indicative of SPLUNC1 expression in almost all samples (A) and SPLUNC2A expression in all saliva supernatants (B).

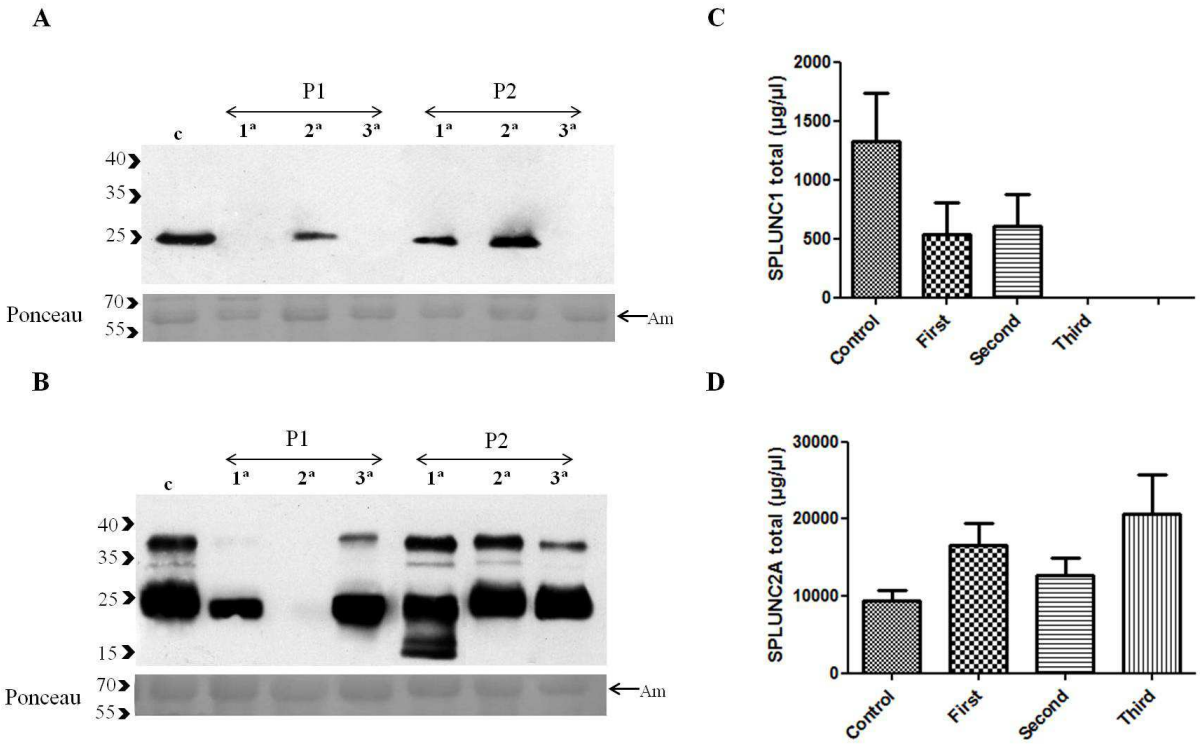

Figure 3. Western blotting comparing SPLUNC1 and SPLUNC2A in saliva of 2 patients (P1) and (P2), before (1ª), during (2ª) and after (3ª) chemotherapy and a control positive donor (c). There were bands indicative of an increased SPLUNC1during chemotherapy (A) e (C) and reduction or absence of SPLUNC2A and in saliva supernatants during treatment (B) e (D). Below each gel Ponceau S stain showing 68 kDa bands corresponding to α-amylase (Am) protein.

CONCLUSÃO

_ Os pacientes submetidos à quimioterapia convencional e os pacientes submetidos ao condicionamento quimioterápico prévio ao TCTH autólogo já apresentavam o fluxo salivar reduzido na primeira coleta de saliva, efeito que pode estar relacionado à condição basal apresentada pelos pacientes do primeiro grupo e a regimes quimioterápicos anteriores ao transplante no segundo grupo.

_ O aumento da proteína SPLUNC1 na saliva após a quimioterapia convencional e após sete dias do TCTH autólogo pode sugerir uma alteração na qualidade da saliva que se tornou mais mucosa após estas terapias.

_ O aumento da expressão de SPLUNC2A após o transplante e redução após a quimioterapia convencional mostra que outros estudos são necessários para melhor entendimento da função desta proteína.

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Estudo clínico laboratorial de pacientes que receberão radioterapia e quimioterapia"**, protocolo nº 142/2010, dos pesquisadores Lara Maria Alencar Ramos Innocentini, Marcio Ajudarte Lopes e Wilfredo Alejandro González Arriagada, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 24/12/2010.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Clinical laboratory study of patients who will receive radiotherapy and chemotherapy"**, register number 142/2010, of Lara Maria Alencar Ramos Innocentini, Marcio Ajudarte Lopes and Wilfredo Alejandro González Arriagada, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 12/24/2010.

Prof. Dr. Pablo Agustin Vargas
Secretário
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Prof. Dr. Jacks Jorge Junior
Coordenador
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Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.