
WILFREDO ALEJANDRO GONZALEZ ARRIAGADA

# "EXPRESSION OF SPLUNC1 (BPIFA1) AND SPLUNC2A (BPIFA2A) IN SALIVA OF PATIENTS UNDERGOING RADIOTHERAPY"

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**FACULDADE DE ODONTOLOGIA E PIRACICABA**

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Thesis presented to the Piracicaba Dental School of the State University of Campinas to obtain the Ph.D. grade in Stomatopathology, in the Stomathology Area.

Tese à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Doutor em Estomatopatologia, na área de Estomatologia.

Orientador: Professor Dr. Márcio Ajudarte Lopes

Este exemplar corresponde à versão final da tese defendida pelo aluno Wilfredo Alejandro Gonzalez Arriagada e orientada pelo Prof. Dr. Márcio Ajudarte Lopes.

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## RESUMO

**OBJETIVO:** A radioterapia causa alterações na composição salivar e as proteínas PLUNC participam na resposta imune inata da cavidade oral. O objetivo desse estudo foi verificar se a radioterapia é capaz de modificar a expressão de PLUNC salivar e se essas proteínas estão associadas com os efeitos colaterais.

**MATERIAIS E MÉTODOS:** Foi coletada saliva não estimulada de 65 voluntários (45 pacientes com câncer e 20 controles). No grupo de estudo a coleta foi realizada uma semana antes do início da radioterapia, no meio do tratamento e uma semana após o término. A expressão de SPLUNC1 e SPLUNC2A foi detectada por western blot e foi analisada com os dados clínico-patológicos e efeitos colaterais.

**RESULTADOS:** Foi notada uma redução do fluxo salivar durante e após o término da radioterapia, sendo mais acentuada nos pacientes que foram submetidos a radioterapia envolvendo a região facial. O campo de radiação facial foi correlacionado com os efeitos colaterais, principalmente com a presença (p=0,0110) e intensidade (p=0,0143) de mucosite. SPLUNC1 e SPLUNC2A foram detectadas na saliva dos pacientes sem tratamento em concentrações variáveis. O grupo de estudo mostrou níveis de SPLUNC2A significantemente maiores que o grupo controle, enquanto SPLUNC1 não mostrou diferenças. A concentração de PLUNC foi modificada pela radioterapia, observando diminuição dos níveis de SPLUNC2A na sua forma glicosilada (p<,0001) e aumento dos níveis de SPLUNC1 (p=0,0081) na segunda e terceira coletas. A única associação entre efeitos colaterais da radioterapia e PLUNC foi a presença (p=0,0363) e intensidade (p=0,0500) da mucosite com maiores níveis SPLUNC1.

**CONCLUSÕES:** O presente estudo reportou que os níveis de SPLUNC1 e SPLUNC2A glicosilada são afetados pela radioterapia, sugerindo que essas proteínas podem ter importância no microambiente oral dos pacientes irradiados na cabeça e pescoço



Palavras-chave: Western blot, PLUNC, mucosite, candidose, efeitos colaterais.

## ABSTRACT

**OBJECTIVE:** Radiotherapy causes alteration in saliva composition and PLUNC proteins participate in innate immunity of the oral cavity. The aim of this study was to verify if radiotherapy is able to modify the salivary PLUNC expression and if they are associated with radiotherapy side-effects.

**MATERIALS AND METHODS:** Unstimulated whole-mouth saliva of 65 voluntaries (45 cancer patients and 20 controls) was collected. In the study group the collection was performed one week before the beginning of radiotherapy, in the middle of the treatment and one week after finishing. SPLUNC1 and SPLUNC2A expression were detected by western blotting and was analyzed with clinicopathological data and radiotherapy side-effects.

**RESULTS:** Reduction of salivary flow rates was observed during and after conclusion of radiotherapy, being more accentuated in patients who underwent radiotherapy involving the facial region. Facial radiation field was correlated with collateral effects, mainly with the presence ($p=0.0110$) and severity ($p=0.0143$) of mucositis. SPLUNC1 and SPLUNC2A were detected in saliva of patients without treatment in variable concentrations. The study group showed levels of SPLUNC2A significantly higher than the control group, while SPLUNC1 did not show differences. Concentration of PLUNC was modified by radiotherapy, observing decreasing of glycosilated form of SPLUNC2A levels ($p<.0001$) and increasing of SPLUNC1 levels ($p=0.0081$) in the second and third collections. The only association between collateral effects of radiotherapy and PLUNC was the presence of mucositis ($p=0.0363$) and its severity ($p=0.0500$) with higher levels of SPLUNC1.

**CONCLUSIONS:** The present study reported that levels of SPLUNC1 and glycosilated SPLUNC2A are affected by the radiotherapy, suggesting that these proteins may have importance in the oral microenvironment of irradiated head and neck cancer patients.



Keywords: Western blotting, PLUNC, mucositis, candidiasis, adverse effects.

**SUMÁRIO**

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*Ele nunca saberá onde sua influência*
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*(Henry Brooks Adam, 1838-1918)*

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"Não me imponha o que você sabe;
quero explorar o desconhecido,
e ser a origem das minhas próprias descobertas.."

(Humberto Maturana, Biólogo chileno, Prece do Estudante)

## INTRODUÇÃO

O câncer de cabeça e pescoço é uma das malignidades de maior incidência no ser humano. Apesar de inúmeros avanços em campanhas de conscientização e prevenção, estas taxas não têm diminuído nas últimas décadas. Além disso, infelizmente o diagnóstico é feito tardiamente na maioria das vezes. O tratamento de um modo geral é realizado principalmente através de cirurgia, radioterapia e quimioterapia, podendo ser usados isoladamente ou combinados.

A radioterapia é um dos tratamentos mais usados no tratamento de câncer de cabeça e pescoço e com significantes benefícios. Porém, causa diversos efeitos colaterais na região onde é aplicada. Esses efeitos colaterais podem ser temporários ou permanentes, e podem complicar a correta realização do tratamento, diminuindo chances de cura e aumentando os custos. Consequentemente, pode diminuir a qualidade de vida do paciente durante e após o tratamento, gerando novos desafios.

Os efeitos colaterais mais frequentemente associados à radioterapia na região de cabeça e pescoço são dermatite, mucosite, disfagia, perda de paladar, candidose, trismo, cárie de radiação, xerostomia e osteorradionecrose. A diminuição do fluxo salivar é uma complicação que começa na segunda semana de tratamento e se mantém após o fim do mesmo, podendo persistir de forma permanente. A literatura também relata mudanças na qualidade da saliva, adicionalmente à diminuição da quantidade. Como a saliva cumpre um papel fundamental na alimentação, fonação e imunidade da cavidade oral do paciente, estudos dos componentes salivares e das estratégias de proteção das glândulas salivares tornam se importantes para o tratamento dos pacientes.

As proteínas PLUNC foram recentemente descritas na saliva e participam na imunidade inata do hospedeiro, possuindo propriedades antimicrobianas e antiinflamatórias, mas se desconhece qual é o papel específico destas proteínas no microambiente oral. Sendo assim, o presente estudo se propõe testar a hipótese de que a radioterapia é capaz de alterar os níveis de PLUNC na saliva e que essas mudanças estariam associadas a efeitos colaterais agudos do tratamento, principalmente xerostomia, candidose e mucosite.

## Câncer de cabeça e pescoço

O câncer de cabeça e pescoço corresponde a 3,3% dos casos de neoplasias malignas no ser humano (Siegel et al., 2014), sendo seu diagnóstico na maioria das vezes feito em estádios avançados. Conseqüentemente, o tratamento geralmente é cirúrgico complementado com radioterapia e/ou quimioterapia, ou simplesmente radioterapia com quimioterapia adjuvante. Esse tratamento é em muitos dos casos mutilante ou paliativo, afetando a qualidade de vida e tempo de sobrevida do paciente.

No Brasil, segundo dados do Instituto Nacional de Câncer (INCA) e Ministério da Saúde, excluindo o câncer de pele não melanoma, o câncer de boca ocupa o quinto lugar entre os tipos de câncer mais incidentes no sexo masculino e o sétimo lugar no sexo feminino, enquanto que o câncer de laringe tem uma incidência menor em ambos os gêneros (Instituto Nacional de Cancer, 2009).

O perfil clássico do paciente com câncer de cabeça e pescoço é um homem, acima de 40 anos, fumante e etilista, que geralmente tem uma má higiene oral. No entanto, nas últimas décadas a incidência nos grupos que estão fora daquele perfil tem tido um aumento, dessa forma se nota um crescimento na incidência em mulheres e em jovens não fumantes e não etilistas (Santos-Silva et al., 2011). O tabaco continua sendo o principal fator de risco para câncer de boca e laringe, e o álcool aumenta ainda mais o risco quando associado com tabagismo. Porém outros fatores como o vírus papiloma humano tem sido associados à maior incidência, principalmente em jovens (Santos-Silva et al., 2011).

O câncer de boca e laringe é uma doença silenciosa, que causa dor e manifestações clínicas evidentes em estágios avançados da doença. Apesar das campanhas de prevenção e de promoção da saúde oral, as taxas de incidência não têm baixado drasticamente em décadas, sendo necessário aprimorar essas estratégias em busca de um diagnóstico precoce para evitar as sequelas da doença e o mesmo tratamento pelo diagnóstico tardio (Gonzalez-Arriagada et al., 2013). Por outro lado, a radioterapia tem um papel fundamental no tratamento desses pacientes e é indicada na grande maioria deles.

## Radioterapia

A maioria dos pacientes com tumores de cabeça e pescoço são submetidos à radioterapia como tratamento único ou em conjunto com cirurgia e/ou quimioterapia, Nestas situações, altas doses de radiação são utilizadas, afetando vários locais da cavidade oral, maxila, mandíbula e glândulas salivares (Bernier and Bentzen, 2006; Jham and da Silva Freire, 2006; Rubira et al., 2007). Em geral, a radioterapia convencional de cabeça e pescoço envolve doses fracionadas, com um total ao redor de 4000-7000 cGy, e está limitada pela proximidade de tecidos radio-sensíveis como medula espinal, cérebro e glândulas parótidas (Bourhis et al., 2005).

Apesar dos benefícios do tratamento por radiação, ele pode causar diversos efeitos colaterais que podem ser agudos ou tardios (González Arriagada et al., 2010; Specht, 2002; Vissink et al., 2003). Dessa forma, há intentos para melhorar a eficácia da radioterapia e diminuir os efeitos colaterais. Sendo assim, tem sido desenvolvido novas técnicas de radioterapia de intensidade modulada (IMRT), combinação da radioterapia com radiosensibilizadores ou drogas citotóxicas, e novas terapias molecular alvo dirigida (Begg et al., 2011; Bernier and Bentzen, 2006; Bourhis et al., 2005). A radioterapia tem como objetivo destruir as células malignas, mas todos os tecidos envolvidos no campo recebem radiação, causando complicações (Andrews and Griffiths, 2001; Bernier and Bentzen, 2006; Chambers et al., 2004; Nicolatou-Galitis et al., 2003; Ohrn et al., 2001; Sciubba and Goldenberg, 2006; Silva et al., 2009; Specht, 2002; Vissink et al., 2003). Essas complicações podem ser diminuídas ou controladas, através de uma avaliação odontológica e é acompanhado por dentista antes, durante e depois do tratamento radioterápico (González Arriagada et al., 2010; Jham and da Silva Freire, 2006; Schiodt and Hermund, 2002; Specht, 2002).

## Efeitos colaterais da radioterapia

O tratamento de radioterapia tem efeitos colaterais de curto e longo prazo (Bennenbroek et al., 2003). Esses efeitos dependem da dose e fracionamento da radiação,

volume de tecido irradiado, tipo de radiação e duração da terapia. Se estes efeitos são muito incapacitantes, os pacientes podem interromper o tratamento influenciando nas expectativas de resolução da doença e encarecendo os custos do tratamento. Os fatores individuais dos pacientes como má higiene oral, condição dos tecidos orais, hábitos de fumo e álcool, sistema imunológico, condições gerais de saúde e seguimento pelo cirurgião dentista, estão associados com a intensidade e aparecimento desses efeitos (Rubira et al., 2007; Specht, 2002; Vissink et al., 2003).

As complicações agudas ocorrem durante o tratamento, podendo ser reversíveis ou irreversíveis, tais como xerostomia, mucosite, perda do paladar, candidose e dermatite (Andrews and Griffiths, 2001; Bennenbroek et al., 2003; González Arriagada et al., 2010; Guchelaar et al., 1997; Ohrn et al., 2001; Sciubba and Goldenberg, 2006). As complicações tardias, como cáries de radiação e osteorradionecrose, são normalmente irreversíveis, e diminuem consideravelmente a qualidade de vida do paciente (Jham and da Silva Freire, 2006). Xerostomia, também é considerada um efeito colateral tardio e geralmente se mantêm como um efeito permanente (Bourhis et al., 2005).

**Saliva e radioterapia**

A saliva como método de diagnóstico e monitorização das doenças e tratamentos apresenta vantagens quando comparada com os procedimentos comumente usados nos laboratórios que envolvem análise de componentes celulares e químicos do sangue. A coleta de saliva total é um método simples e não invasivo, sem a necessidade de equipamento sofisticado e que pode fornecer muitas informações do que está acontecendo com o paciente (Zhang, 2013).

A saliva pode ser definida como saliva glandular-específica ou saliva total. A saliva glandular específica pode ser coletada diretamente dos dutos de saída das glândulas salivares e é mais utilizada para a detecção de alterações glandulares específicas. A saliva total é mais utilizada para estudar influências sistêmicas, por ser uma mistura de fluidos orais e incluir secreções de todas as glândulas salivares maiores e menores, além de vários outros componentes como fluido crevicular gengival, expectorado bronquial e secreções

nasais, derivados do soro e sangue por feridas orais, bactérias e produtos bacterianos, vírus e fungos, células epiteliais descamadas, outros componentes celulares e restos de alimentos (Kaufman and Lamster, 2002). A coleta da saliva pode ser estimulada e não estimulada.

As glândulas salivares possuem dois tipos de ácinos, serosos e mucosos, sendo que cada glândula salivar produz um tipo de saliva de acordo com os ácinos que constituem seu parênquima. Dessa forma, a glândula parótida é uma glândula eminentemente serosa, a glândula submandibular, sublingual e algumas glândulas menores são mistas, e existem glândulas salivares mucosas.

Diversos estudos têm associado à alteração da quantidade e qualidade da saliva com a radioterapia. A hipossalivação e sensação de boca seca são largamente descritos na literatura, no entanto as mudanças na composição salivar são discutidas. Estudos nesta linha relacionam à radiação com mudanças na microbiota oral detectada na saliva, electrólitos, imunoglobulinas, enzimas e outras proteínas que participam na defesa imune do hospedeiro. Peptídeos catiônicos salivares e outras proteínas salivares, como, lisozima, BPI, PLUNC, amilase salivar, cistatinas, proteínas ricas em prolina, mucinas e peroxidases são as principais responsáveis pela imunidade inata (Fabian et al., 2008).

## PLUNC

### *Definição e Descrição da família PLUNC*

PLUNC (acrônimo de *palate, lung and nasal epithelium clone*) é uma família de proteínas descoberta no epitélio nasal do embrião de rato (Weston et al., 1999), mas que também foi identificada em outras espécies (Leclair, 2003; LeClair et al., 2001; Sung et al., 2002). O isolamento e a caracterização do PLUNC no humano foram reportados por Bingle et al. (2000) na traquéia, vias aéreas superiores, epitélio nasofaríngeo e glândula salivar (Bingle and Bingle, 2000). As proteínas dessa família exibem uma homologia estrutural com a BPI (*bactericidal/permeability-increasing protein*), uma proteína que participa na resposta imune inata contra as bactérias, tendo sido sugerida a participação delas na resposta imune.

A família PLUNC é composta por dez proteínas divididas em dois grupos segundo o tamanho e estão codificadas por genes localizados no cromossomo 20q11.2. Um grupo é denominado SPLUNC (*short PLUNC*), as quais contêm um domínio único relacionado ao domínio N-terminal da BPI, e inclui as proteínas SPLUNC1, SPLUNC2, SPLUNC3 e SPLUNC4 (BASE, *breast cancer and salivary expressed*). O segundo grupo é denominado LPLUNC (*long PLUNC*), e possui dois domínios BPI, N- e C-terminal, incluindo as proteínas LPLUNC1, LPLUNC2, LPLUNC3, LPLUNC4, LPLUNC5 e LPLUNC6 (Bingle and Craven, 2002; Bingle and Craven, 2004; Bingle et al., 2004; Egland et al., 2003). A expressão da proteína SPLUNC5 parece estar restrita ao epitélio interpapilar da superfície dorsal da língua dos ratos (LeClair et al., 2004). Atualmente as proteínas PLUNC/PSP/BSP30/SMGB foram classificadas dentro da superfamília *BPI fold-containing* (Bingle et al., 2011), as quais são codificadas por genes no cromossomo 20. Dessa forma, todos os membros da família foram renomeados usando a sigla BPIF. Assim, as SPLUNC passam a ser chamadas BPIFA e as LPLUNC passam a ser BPIFB.

SPLUNC2 apresenta-se como uma forma glicosilada e não glicosilada, e ambas 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 (Bingle and Bingle, 2011).

***Funções das proteínas PLUNC***

Atualmente existe evidência científica disponível a respeito da função desempenhada pelas proteínas PLUNC nos mecanismos de defesa imune inata do hospedeiro (Bingle and Bingle, 2000; Bingle and Craven, 2003). Esta informação é baseada nas semelhanças estruturais com outras proteínas como a BPI, PSP (*parotid secretory protein*) e outras proteínas que participam na defesa do hospedeiro contra bactérias gram negativas. Tem sido sugerido que são proteínas secretadas e que agem bloqueando a junção de LBP (*Lipopolysaccharide-binding protein*) ao LPS (*Lipopolysaccharide*) e impedindo a ativação dos macrófagos pelo LPS, assim como um papel regulador antiinflamatório na resposta ao LPS (Bingle and Gorr, 2004; Bingle et al., 2004; Levy, 2000; Levy et al., 2003;

Weiss, 2003; Wheeler et al., 2003). Além da semelhança estrutural, apresentam expressão similar em sítios de epitélio oral, nasofaríngeo e respiratório. Por esses motivos, os autores têm descrito todo esse grupo de proteínas como formando parte de uma superfamília (Andrault et al., 2003; Ball et al., 2003; Beamer, 2003; Bingle and Bingle, 2000; Bingle and Craven, 2002; Bingle and Gorr, 2004; Bourhis et al., 2005; Hou et al., 2004; Larsen et al., 2005; Leclair, 2003; Sung et al., 2002).

A cavidade oral está continuamente exposta a diversos organismos potencialmente patogênicos, sendo a saliva é importante na manutenção da saúde oral (Amerongen and Veerman, 2002; Bingle and Gorr, 2004). Esta capacidade protetora da saliva se deve, ao menos parcialmente, à presença na sua composição de diversas proteínas e peptídeos com atividade antimicrobiana e antiinflamatória que evitam infecções recorrentes e toleram as populações microbianas residentes (Bingle and 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 cáries em pacientes com um fluxo salivar diminuído como pacientes submetidos à radioterapia na região de cabeça e pescoço ou diagnosticados com síndrome de Sjögren (Dirix et al., 2006; Jonsson et al., 2002). A expressão das proteínas PLUNC já foi demonstrada na saliva, o que sugere uma eventual participação delas na resposta imune inata da cavidade oral (Bingle and Gorr, 2004).

A literatura mostra que a SPLUNC1 é uma proteína imuno-defesiva inata que pode se ligar ao LPS e, por conseguinte, neutralizar a endotoxina através de seu domínio BPI (Ghafouri et al., 2003; Zhou et al., 2006). A capacidade do PLUNC se ligar ao LPS lhe permite afetar o crescimento bacteriano (Geetha et al., 2005; Wu et al., 2009; Zhou et al., 2006). Por outro lado, outros estudos demonstraram que a SPLUNC1 e SPLUNC2, possuem atividade antibactericida para diversas bactérias gram negativas, particularmente a *Pseudomona aeruginosa* (Geetha et al., 2003; Gorr et al., 2008; Zhou et al., 2008). Zhou et al. (2008) também propuseram uma ação antiviral do SPLUNC1 contra o Vírus Epstein Barr (EBV), visto que aumentaria a ruptura e apoptose dos linfócitos infectados com EBV, regulando a expressão das proteínas de membrana do EBV e sugerindo que pode inibir o potencial oncogênico do EBV no epitélio respiratório.

A existência de diferentes proteínas dentro desta família sugere que elas podem possuir diferenças na atividade biológica ou que elas podem ser seletivamente efetivas para o controle de determinados patógenos, moléculas de LPS, ou outras moléculas antigênicas (Bingle and Gorr, 2004).

***Expressão das proteínas PLUNC***

As proteínas da família PLUNC têm sido identificadas em diferentes fluidos no ser humano tais como a saliva, o fluido nasal, as secreções traqueo-bronquiais e as pulmonares. Os sítios de expressão dessas proteínas incluem, principalmente, glândulas salivares e epitélio respiratório nasal, traqueal ou bronquial, apresentando uma expressão seletiva de cada proteína em cada região anatômica, o que está associado às peculiaridades dependentes da diversa flora microbiana que habita cada uma destas regiões (Andrault et al., 2003; Bingle and Craven, 2002; Ghafouri et al., 2003; Kim et al., 2006; Leclair, 2003). SPLUNC1, SPLUNC2 e LPUNC1 foram identificados na saliva por diferentes pesquisadores, sugerindo que são produzidas pelas glândulas salivares (Campos et al., 2004; Ghafouri et al., 2003; Ramachandran et al., 2006; Vitorino et al., 2004).

Níveis aumentados de PLUNC foram relatados em pacientes fumantes e expostos a irritantes químicos, assim como no escarro de pacientes com doença respiratória obstrutiva crônica (Ghafouri et al., 2002; Di et al., 2003). Bingle et al. (2007) reportaram um aumento na expressão de SPLUNC1 nas vias aéreas menores dos pulmões em pacientes com fibrose cística, o que pode ser interpretado como uma resposta defensiva do epitélio ao componente infeccioso (Bingle et al., 2007). Por outro lado, foi descrita a expressão de PLUNC nos grânulos específicos de neutrófilos humanos (Bartlett et al., 2008) e mastócitos (Gonzalez-Arriagada et al., 2012), células importantes na resposta imune do hospedeiro. Chu et al. (2007) estudaram a função e regulação da SPLUNC1 na infecção por *Mycoplasma pneumoniae* e na inflamação alérgica (asma), concluindo que participa na defesa imune contra a infecção nas vias aéreas e inibe a produção epitelial de IL-8. Eles também reportaram que a inflamação alérgica (com uma maior expressão de IL-13) diminui

significativamente a expressão de SPLUNC1, o que em parte contribuiria para a natureza persistente das infecções bacterianas em pacientes alérgicos.

Diversos estudos relataram a expressão das proteínas PLUNC nas glândulas salivares e saliva por imunoistoquímica, Western blot e outras técnicas (Bingle et al., 2011; Gonzalez-Arriagada et al., 2012; Kohlgraf et al., 2012). A expressão de SPLUNC2 também foi identificada em queratinócitos gengivais e seria regulado por fatores humorais e bactérias, nos sugerindo um papel na defesa gengival (Shiba et al., 2005). Wu et al. (2009) relataram uma diminuição de SPLUNC2 na saliva de pacientes com periodontite agressiva generalizada. Essa diminuição poderia ser o resultado da resposta imune inibitória induzida pela bactéria que causa a periodontite o que sugere que as glândulas salivares estão envolvidas no processo de resposta imune na doença periodontal (Wu et al., 2009)

A expressão de membros da família PLUNC também foi demostrada em fetos humanos de 12 a 25 semanas de gestação em glândulas salivares maiores e menores, concluindo que a expressão de SPLUNC1 tem início na vida intra-uterina, quando as glândulas salivares estão em um estágio de morfodiferenciação avançado com marcação nos plugs de mucina dos ductos estriados e no citoplasma dos ácinos mucosos. Pela negatividade para SPLUNC2, os autores sugeriram que a expressão dessa proteína tem início na vida pós-natal (Alves, 2010).

Bingle et al. (2009) observaram que SPLUNC2 apresenta expressão predominantemente nos ácinos serosos de glândulas salivares maiores, nos ductos das glândulas salivares maiores e menores, assim como nos túbulos sero-mucosos de glândulas salivares menores da mucosa oral, região posterior da língua e tonsila. A expressão de SPLUNC1 foi relatada nos ácinos mucosos de glândulas salivares menores (Bingle et al., 2009).

A proteína SPLUNC1 (LUNX) tem sido confirmada como um marcador diagnóstico em câncer de pulmão, especialmente no caso de micrometástase de câncer de células não pequenas, adenocarcinoma, carcinoma mucoepidermóide e carcinoma brônquio-alveolar (Benlloch et al., 2009; Bingle et al., 2005; Cheng et al., 2008; Iwao et al., 2001; Kim et al., 2007; Li et al., 2005; Mitas et al., 2003). Também foi identificada a expressão de PLUNC em carcinoma nasofaríngeo e câncer gástrico (He et al., 2005; Sentani et al., 2008; Yasui et

al., 2009; Zhang et al., 2003). SPLUNC4 pode ser usado em provas diagnósticas para câncer de mama, pois é expresso em linhas celulares malignas em uma grande proporção de tecidos de câncer de mama primários, sendo que em tecidos normais só é expresso em glândulas salivares (Egland et al., 2003).

Vargas et al. (2008) e González-Arriagada et al. (2012) mostraram a expressão das proteínas PLUNC (SPLUNC1, SPLUNC2, LPLUNC1 e LPLUNC2) em tumores malignos e benignos de glândula salivar, sugerindo a utilidade diagnóstica no caso do carcinoma mucoepidermóide de alto grau pela expressão em plugs de mucina, células intermediárias e mucosas.

Portanto, como as proteínas PLUNC participam da imunidade inata e têm funções antibacterianas e antiinflamatórias, o objetivo do estudo desenvolvido foi verificar se a radioterapia é capaz de modificar a expressão de SPLUNC1 e SPLUNC2 na saliva e se essas proteínas estão associadas com os efeitos adversos associados ao tratamento de radiação na região de cabeça e pescoço.

CAPITULO 1

Artigo submetido para publicação no periódico (Oral Diseases)

**Salivary SPLUNC1 and SPLUNC2A is Modified by Head and Neck Cancer Radiotherapy**

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**ABSTRACT**

**OBJECTIVE:** Head and neck radiotherapy is associated with adverse effects, affecting saliva composition and oral defensive mechanisms. PLUNC proteins participate in innate immunity with anti-bacterial and anti-inflammatory functions. The aim of the study was to verify if salivary PLUNC expression can be modified by radiotherapy and its association with side-effects.

**MATERIALS AND METHODS:** Unstimulated whole-mouth saliva of 65 voluntaries (45 cancer patients and 20 controls) was collected. SPLUNC1 and SPLUNC2A expression were analyzed by western blotting and was compared with clinicopathological data and radiotherapy side-effects.

**RESULTS:** Facial radiation field was associated with a more accentuated salivary flow reduction during and after radiotherapy and was correlated with side-effects, mainly mucositis. Salivary PLUNC in control patients were detected in variable concentrations. Study group showed SPLUNC2A levels significantly lower than control group, while SPLUNC1 did not show differences. PLUNC concentration was modified by radiotherapy, observing decreasing of glycosilated SPLUNC2A ($p<.0001$) and increasing of SPLUNC1 ($p=0.0081$). SPLUNC1 was associated with the presence of mucositis ($p=0.0363$) and its severity ($p=0.0500$).

**CONCLUSIONS:** The present study reported that levels of SPLUNC1 and glycosilated SPLUNC2A are affected by the radiotherapy, suggesting that these proteins may have importance in the oral microenvironment of irradiated head and neck cancer patients.

**Keywords:** Western blotting, PLUNC, SPLUNC1, SPLUNC2, mucositis, candidiasis, xerostomia, adverse effects.

## INTRODUCTION

Head and neck cancer represents about 3% of all malignant tumours in humans and is usually diagnosed at advanced stages (Greenlee et al., 2000). Radiotherapy is one of the most used treatment for head and neck cancer, and it is frequently associated with surgery and/or chemotherapy (Bhide et al., 2012). Conventional head and neck radiotherapy generally involves high doses of about 60 Gy or higher in fractionated daily doses determined by the diagnosis and clinical stage (Bourhis et al., 2005). The tissues involved in the radiation field are all affected, causing acute and chronic side-effects (Specht, 2002, Vissink et al., 2003, Sciubba & Goldenberg, 2006, González Arriagada et al., 2010). These complications are associated to multiple factors such as volume of irradiated tissue, radiation dose, and individual patient factors, including poor oral hygiene, smoking, alcoholism, immune system, and professional dental care (Specht, 2002, Vissink et al., 2003). The most common acute reactions are mucositis, dysgeusia, dermatitis and candidiasis, which are reversible. Xerostomia is observed in an early stage of treatment. However, such as radiation-related caries and osteoradionecrosis, it is also considered a chronic complication (Ohrn et al., 2001, Specht, 2002, Vissink et al., 2003, Bourhis et al., 2005, Sciubba & Goldenberg, 2006, Jham et al., 2008, González Arriagada et al., 2010). Radiotherapy in head and neck may modify oral defensive mechanisms, particularly by decreasing salivary flow rate and altering saliva composition, such as increasing levels of pathogenic microorganisms (Vuotila et al., 2002, Grotz et al., 2003).

PLUNC (palate, lung and nasal epithelium clone) protein was described in the nasal epithelium, trachea and bronchus of the mouse embryo (Weston et al., 1999), and later isolated in humans. Its genes are located in chromosome 20q11.2, in close proximity to genes encoding the lipopolysaccharide-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI), which act in the innate immune response to gram-negative bacteria (Bingle & Bingle, 2000, Bingle & Gorr, 2004, Bingle et al., 2009). These proteins are divided in two groups according to their length, short PLUNC (SPLUNC) and long PLUNC (LPLUNC) (Bingle & Craven, 2002, Bingle & Craven, 2004). Recently, the PLUNC family members were included in the BPI fold-containing

superfamily, leading to a new nomenclature by which the proteins are renamed as BPIF, thus SPLUNC have the designation BPIFA and LPLUNC have the designation BPIFB (Bingle et al., 2011, Bingle & Bingle, 2011).

Several studies have demonstrated the expression of PLUNC in saliva (Vitorino et al., 2004, Ramachandran et al., 2006, Kohlgraf et al., 2012), salivary glands and salivary gland tumours (Bingle & Craven, 2002, Vargas et al., 2008, Bingle et al., 2009, da Silva et al., 2011, Gonzalez-Arriagada et al., 2012), and other neoplasms (Iwao et al., 2001, Zhang et al., 2003, Bingle et al., 2005, He et al., 2005, Cheng et al., 2008, Sentani et al., 2008). Each family member has a selective expression in upper airways and oral cavity tissues and fluids (Leclair, 2003, Bingle & Bingle, 2011). Variations in concentrations of PLUNC values in oral and upper airways fluids have been reported in healthy and unhealthy patients (Ghafouri et al., 2002, Di et al., 2003, Bingle et al., 2009, Kohlgraf et al., 2012). The specific function of these proteins is still not well defined, but exist evidence of its participation in the host innate immunity with antimicrobial and anti-inflammatory effects (Bingle & Craven, 2002, Bingle & Craven, 2004, Chu et al., 2007, Bingle et al., 2009). The anti-inflammatory function is based on the regulation of macrophagic activity (Bingle & Gorr, 2004).

Given the antimicrobial and anti-inflammatory properties of PLUNC proteins and the participation of microorganisms and inflammation in the aetiology of oral adverse effects of radiotherapy, a better understanding of the role of these proteins in the oral microenvironment is necessary. Then, the aim of this study was to test the hypothesis if radiotherapy is able to modify the salivary PLUNC expression and if there is any association between these modifications with acute collateral effects of radiotherapy, mainly mucositis and candidiasis.

## MATERIALS AND METHODS

The study was approved by the Ethics Committee for Human Studies, Piracicaba Dental School (protocol number: 142/2010). A written informed consent was obtained from all the voluntaries of the study.

**Patients and clinical features**

A longitudinal case-control clinical study was performed with a study group (n=45) that consisted in consecutive patients that were evaluated to receive radiotherapy in head and neck region for cancer treatment in the Oncology Centre, and a control group (n=20). Clinicopathological data such as gender, age, tumour size and location were collected retrospectively from the patients' charts.

**Radiotherapy**

All the patients included in the case group were not previously treated of another cancer. Conformational radiotherapy was performed with the linear accelerator Varian Clinac 600C (Palo Alto, CA, USA). According to radiation field, patients were grouped in those that received radiation in the facial region (radiation field involving facial region, RFIFR) and those that did not received radiation in facial region (radiation field non involving facial region, RFNIFR). The total dose of radiation was up to 60Gy in all the patients included in the study fractionated in daily doses.

**Clinical evaluation**

Before starting the radiotherapy, all patients received pre-radiotherapy orientation and dental treatment at the Oral Diagnosis Clinic. Subsequent examinations were performed every week during the radiation therapy, from the first week until a week after the treatment. The side-effects considered in the weekly evaluation were xerostomia, mucositis, candidiasis, dermatitis and dysgeusia. Severity of mucositis was determined using the World Health Organization (WHO) oral toxicity scale (four grades of severity). For statistical analysis, mucositis grade was simplified as mild mucositis (grades 1 and 2) and severe mucositis (grades 3 and 4).

**Collection of saliva samples**

Unstimulated whole-mouth saliva was collected in the morning (between 9 and 11 am). The patients were oriented to abstain for eating, drinking and tooth brushing for at least 1 h before sample collection. Five minutes before the collection the subjects rinsed the mouth with water. Each patient let the naturally produced saliva drain into a sterile glass cup, without any stimulation for 5 min. The saliva flow rate (ml/min) was measured immediately after saliva collection. Saliva samples were immediately placed on ice (4ºC), transported to the laboratory, and was centrifuged (14,000 rpm for 6 min) at 18°C. The supernatants were stored in recipients of 2 ml at -70°C for later use. Three collections were programmed; before, during and after the radiotherapy. Salivary flow was considered normal when it was greater than 0.5 ml/min, mild hyposalivation when it was between 0.3 and 0.5 ml/min, and severe hyposalivation if it was less than 0.3 ml/min.

**Western blot**

Quantification of total protein of supernatants was performed using the spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Samples were mixed with dithiothreitol (DTT) in loading buffer. An amount of 10 µg of protein per well was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%). The proteins were transferred onto a nitrocellulose membrane and a Ponceau stain was done to confirm the effectiveness of transference. All membranes were incubated overnight at 4ºC with skim milk with 5% Tris-buffered saline and Tween 20 (TBST) for blocking. After three washes of 15 min each, the membranes were incubated with primary antibody for 2 hours with blocking solution. The concentrations used for the antibodies were 1:500 for SPLUNC2A and 1:250 for SPLUNC1. Then, membranes were washed three times and were incubated with secondary antibody for 1 hour. The detection was performed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The rabbit anti-human SPLUNC1 and SPLUNC2A monoclonal antibodies were supplied by Dr. Lynne Bingle (Oral and Maxillofacial Pathology, School of Clinical Dentistry, University of Sheffield, Sheffield, UK).

Densitometry of intensity of each band was measured using the software Gel Analyzer 2010 (Lazar Software, developed by Dr. Istvan Lazar, Debrecen, Hungary), obtaining arbitrary densitometry units (adu) for measuring and comparison.

### Statistical analysis

Descriptive analysis based on contingency tables and basic statistics described the sample characteristics. To compare independent groups, the coefficient of kurtosis, the coefficient of asymmetry and the Shapiro-Wilk test were applied in the residuals. Residuals normally distributed were compared by ANOVA and which were non-normally distributed was compared by Wilcoxon rank sum test. Association between nominal variables was tested by Pearson qui-square. In this analysis densitometry values were divided in two groups: over the median and under the median. Linear regression model of SPLUNC in function of gender as a dummy variable and age were adjusted. Analysis of variance based on generalized linear mixed model with repeated measures was applied to test the effect of time and QT in the SPLUNC. The significance level of 5% (0.05) was used in all tests. Statistics were calculated using the SAS System 9.3 (SAS Institute Inc. The SAS System, Cary, NC; USA, 2010) and graphs constructed with GraphPad Prism 5 (Graphpad Software Inc. La Jolla, CA; USA, 2007).

## RESULTS

### Clinicopathological findings

Sixty-five patients were analyzed with a mean age of 58.2 years (SD of 10.2) for the control group and 55.8 years (SD 8.6) for the study group. The descriptive information showed that the characteristics of the study group are similar with the known profile of head and neck cancer in developing countries, being more common in males, over 40 years (with a peak incidence in the sixth decade) and mainly diagnosed in advanced stages. Demographic and clinicopathological data of the population is listed in the Table I and Table II.

**Salivary flow**

The salivary flow variation of the study group is showed in the Figure 1. We observed a reduction in the salivary flow rates during and after radiotherapy, accentuated in the patients that were irradiated in a field involving the facial region. The salivary flow of control group (mean=0.702 ml/min) was compared with the salivary flow in the first collection of the study group (mean=0.912 ml/min) and did not show significant difference ($p=0.1438$, ANOVA).

Correlation statistically significant was showed between salivary flow and phase of sample collection ($p<.0001$). The correlation was observed in the first ($p<.0001$), second ($p<.0001$) and third time ($p=0.0157$) of sample collection. There was no correlation between salivary flow values with chemotherapy ($p=0.2830$) or chemotherapy associated with phase of collection sample ($p=0.1373$).

**Collateral effects associated to radiation field and chemotherapy**

Statistically significant correlation between radiation field and, presence of mucositis during radiotherapy ($p=0.0110$) and its severity ($p=0.0143$) was found (Fig. 2). Dysgeusia and radiation field also showed correlation statistically significant ($p=0.0076$). Candidiasis and radiation field were not statistically significant correlated ($p=0.0650$), even though a higher number of patients irradiated in the facial region presented candidiasis. Xerostomia, hyposalivation and dermatitis did not show correlation with radiation field. Detailed information of secondary effects of radiotherapy is described in Table III. Three patients of the study group died because the cancer after the first collection and four patients after the second collection. Chemotherapy associated to radiotherapy was not correlated with the presence and severity of mucositis, candidiasis or any other collateral effect.

**PLUNC proteins associated to age, gender and clinical stage**

The results did not show correlation of SPLUNC1 and SPLUNC2A with age, gender or clinical stage.

**PLUNC expression in control and study groups**

SPLUNC1 densitometry values were variable in saliva and ranged from 0 to 6909 adu (Fig.3). Densitometry values for SPLUNC2A were also variable between the voluntaries of the control group and ranged from 0 to 12958 adu for glycosilated SPLUNC2A, from 3 to 30181 adu for non-glycosilated SPLUNC2A and from 303 to 37281 adu for total SPLUNC2A (Fig.4). SPLUNC1 and SPLUNC2A of control samples were compared with first collection of the study group. SPLUNC1 did not show significant statistical difference between the groups ($p=0.1903$, Kruskal-Wallis test). Statistical analysis showed significant difference between study and control group for total SPLUNC2A ($p=0.0016$, ANOVA) and glycosilated SPLUNC2A ($p<.0001$, ANOVA). However, for non-glycosilated SPLUNC2A no difference was observed (Table IV).

**PLUNC proteins and collateral effects of radiotherapy**

Correlation statistically significant was observed between SPLUNC1 and the presence ($p=0.0363$) and severity ($p=0.0500$) of mucositis in the third collection, but no correlation was observed in the first ($p=0.8586$) and second ($p=0.7175$) collection. Candidiasis did not show correlation with SPLUNC1. Mucositis and candidiasis did not show correlation with total, glycosilated and non-glycosilated SPLUNC2A.

**PLUNC proteins associated with phase of sample collection or chemotherapy**

The results showed correlation statistically significant between SPLUNC1 and phase of sample collection ($p=0.0081$). The correlation was observed in the first ($p=0.0070$) and second ($p=0.0048$) time of sample collection (Fig. 5). There was no correlation with chemotherapy ($p=0.6924$). Glycosilated SPLUNC2A also showed correlation statistically

significant with phase of sample collection (p<.0001). The correlation was observed in the first (p<.0001) and second (p<.0001) time of sample collection (Fig. 6). There was no correlation with chemotherapy (p=0.7496). Total and non-glycosilated SPLUNC2A did not show correlation phase of sample collection or chemotherapy.

## DISCUSSION

PLUNC has been described in human saliva and other fluids (Vitorino et al., 2004, Bingle et al., 2009), and variations of these proteins were reported in healthy subjects and several diseases (Chu et al., 2007, Bingle et al., 2009, Kohlgraf et al., 2012). They are one of the defence proteins present in saliva and have a function in the host innate immunity. These proteins became interesting molecules to study in oral diseases because the high concentrations in saliva and salivary glands, and their structural similarities to BPI-like proteins, suggesting antimicrobial and anti-inflammatory functions. The antimicrobial effects are associated to the regulation of cellular response to LPS (Bingle & Craven, 2002, Bingle & Craven, 2004), however they seems not to exert direct killing activity, but are likely to be bacteriostatic, promoting agglutination of bacteria and modulating cytokine production (Fabian et al., 2012).

Based on its LPS binding ability, it was speculated that PLUNC proteins may affect microorganisms, including the previously reported action against Mycoplasma and Pseudomonas (Ghafouri et al., 2003, Geetha et al., 2005, Seshadri et al., 2012).The anti-inflammatory function is due to the interaction with macrophages (Bingle & Gorr, 2004).

Saliva offers some advantages in comparison with blood or other samples from cancer patients, because it is a minimally invasive procedure, it that can identify local or systemic effects of therapy, and also can be used to predict toxicity or prognosis (Tiwari, 2011, Citrin et al., 2012). Then, based on PLUNC properties and because radiotherapy side-effects are associated with inflammatory and microbial factors, the aim of this study was to evaluate the association of acute side-effects of radiotherapy and variations of SPLUNC1 and SPLUNC2A levels induced by radiation.

Two proteins of the PLUNC family were analyzed in the present study, SPLUNC1 and SPLUNC2A. Different expression in normal salivary glands and in tumours was reported for these proteins. In normal salivary glands, SPLUNC1 is expressed in mucous acini while SPLUNC2A is expressed in serous acini, suggesting that they are specifically produced for these acini (Gonzalez-Arriagada et al., 2012). The difference in expression between different PLUNC proteins suggests that each one occupies an individual niche and has individual functions (Bingle & Bingle, 2011, Kohlgraf et al., 2012). Some authors suggested that, in irradiated patients, the function of parotid glands is more affected than the other salivary glands, because serous acini are more susceptible to permanent radio-induced damage in comparison with mucous acini (Almstahl & Wikstrom, 2003). Parotid gland is a serous salivary gland and only express SPLUNC2, suggesting that, in radiation, SPLUNC2 will be more affected than SPLUNC1, that is one of the major products of the minor salivary glands of the oral cavity (Bingle et al., 2009, Gonzalez-Arriagada et al., 2012). Our results were coincident with this suggestion showing decreased expression in total and glycosilated SPLUNC2A and increased expression of SPLUNC1 in patients after beginning of the radiotherapy. This is associated with the production of thicker saliva in these patients.

Radiation-related salivary gland hypofunction derives in a reduced salivary flow that can also result in xerostomia (defined as the subjective perception of dry mouth) and modifications of the chemical composition of saliva (Hopcraft & Tan, 2010). In the present study, we observed a decreased salivary flow during radiotherapy. However, no worseness was observed in patients who also received chemotherapy, suggesting that this secondary effect is mainly dependent of radiotherapy. The control group did not show difference statistically significant in salivary flow levels when compared to study group, indicating that production of saliva was normal before the therapy and was affected during the treatment. The reduction of salivary flow is an effect that heavily affects the quality of life and with minimal recovery after the therapy (Lal et al., 2010). The alterations of biochemical composition includes viscosity, pH, proteins and electrolyte concentrations, and modifications in microflora, showing a predisposition to Candida proliferation with an increased number of non-albicans species (Almstahl & Wikstrom, 2003, Tiwana et al.,

2011, Tiwari, 2011, Karbach et al., 2012). These changes in normal oral microflora and gram-negative bacteria were associated with mucositis aetiology and severity (Sonis, 1998, Sonis, 2009, Gaetti-Jardim et al., 2011). However, this hypothesis is still controversial in the literature (Wijers et al., 2001, El-Sayed et al., 2002, Stokman et al., 2003).

It has been reported that a decrease in number of glands is associated to defects in production and release of innate defence molecules as SPLUNC1 and LPLUNC2 in nasal polyps of patients with chronic sinusitis (Seshadri et al., 2012). Interestingly, in the current study lowest media of PLUNC concentrations were observed in the third sample collection, suggesting the association with higher rates of salivary gland hypofunction and acini damage related to radiation. The literature reported an inverse relationship between salivary flow rates and Candida albicans counts in saliva, proposing that patients with hypofunction of salivary glands are at greater risk of developing candidiasis (Soysa et al., 2004, Hopcraft & Tan, 2010, Karbach et al., 2012). Our results suggest that salivary flow and radiation field are more important factors for candidiasis than changes in levels of PLUNC.

Salivary proteins provide antimicrobial defence, even if they are present in concentrations that are lower than efficient, because they act together at the time to lead an efficient elimination of the target (Fabian et al., 2012). For this reason it is probably that the chemical impairment related to radiation therapy of salivary composition affect several protein concentrations and not only one of them. Vuotila et al. (2002) studied the association of radiation effects with salivary MMPs concentration, because its role in mucosal ulceration and dental caries cavitation. These authors did not find correlation of levels of MMP-8 and MMP-9 with radiation-induced lesions (Vuotila et al., 2002). Recently it was suggested that cytokines, which are involved in inflammation and wound healing, may be elevated in tumour and normal tissues following irradiation and that pro-inflammatory cytokine expression in these tissues may predict for toxicity or tumour control (Citrin et al., 2012). Also it was reported lower levels of MUC5B glycoprotein in irradiated patients (Dijkema et al., 2012). In the present study we noticed decreased mean levels of SPLUNC2A and increased mean levels of SPLUNC1 in irradiated patients, suggesting that radiotherapy is able to modify the salivary PLUNC concentrations.

Radiation field is the main factor associated to collateral effects. We observed statistically significant correlation of the radiation field involving facial region with mucositis and dysgeusia. Candidiasis also showed a higher incidence in patients with radiation involving the facial region, but it was not statistically significant. The recuperation after the treatment of dysgeusia, xerostomia, hyposalivation, mucositis, dermatitis and candidiasis were not considered in our study, but could be contemplated in further research. Coadjuvant chemotherapy did not show significant association with collateral effects, but is probably associated with the severity of these effects.

Variations of PLUNC expression in healthy and unhealthy patients were reported (Kohlgraf et al., 2012). In our results, we find a wide range in SPLUNC1 and SPLUNC2A expression. The variability in salivary SPLUNC1 between different subjects was also reported by Kohlgraf et al. (2012) and Bingle et al. (2009) also reported the variability in SPLUNC2A expression (Bingle et al., 2009, Kohlgraf et al., 2012). We did not found association of this variability with age or gender. Kohlgraf et al. (2012), although they did not measure periodontal disease, suggested that the wide range of concentration of salivary SPLUNC1 could be associated with the periodontal health. All the patients included in our study received pre-radiotherapy dental treatment and a high number of the patients were toothless, thus we observed that periodontal health was not a factor that induced the variability in PLUNC concentrations, suggesting that other factors could be involved, such as genetic polymorphism, that originate the variability of salivary PLUNC expression.

SPLUNC1 and SPLUNC2A (total and glycosilated) values were higher in study group than control group. This can be associated to inflammatory response against the tumour, leading to a higher expression of defence molecules in saliva. Diverse carcinomas exhibit elevated SPLUNC1 mRNA expression, such as non-small cell lung carcinomas, adenocarcinomas, bronchoalveolar carcinomas and oral squamous cell carcinomas (Kohlgraf et al., 2012). Concentrations of SPLUNC1 are increased in chronic obstructive pulmonary disease and in smokers (Ghafouri et al., 2002), which can be another factor to explain the higher levels in the study group, because most of head and neck cancer patients are smokers. Non-glycosilated SPLUNC2A did not show statistically significant difference between study and control group, however showed higher values in control group. This is an immature isoform

of SPLUNC2 and could be more expressed in control group because the lower inflammatory stimuli in patients with a healthy oral mucosa and without tumour.

SPLUNC1 has selective inhibition of microorganisms, reducing levels of Mycoplasma pneumonia and the production of its cytokines (IL-8 production). Additionally, an up-regulation of SPLUNC1 expression after an infection could be critical to an efficient clearance of an invading pathogen (Chu et al., 2007). SPLUNC1 showed increasing levels during and after the radiotherapy and a significant correlation with mucositis and its severity in the third collection, which can be explained by the presence of anti-inflammatory mediators in saliva when mucositis and other inflammatory stimuli associated to secondary effects of radiotherapy are present. Chu et al. (2007) reported decreasing levels of SPLUNC1 associated to IL-13 in the upper airways and that SPLUNC1 binds to bacterial lipoproteins preventing the linking to their receptor (e.g., TLR-2) on the cell surface, leading to dampened cell activation (Chu et al., 2007). Could be interesting, in future studies with saliva, to consider the levels of IL-13 or TLR-2, for a better understanding of mechanisms of regulation of oral PLUNC proteins. Recently it was reported increased susceptibility to Pseudomonas aeruginosa in SPLUNC1 knockout mice, supporting the function of the protein against bacteria (Liu et al., 2013). Recent study suggested that SPLUNC1 is able to suppress the allergic inflammation, speculating that the increased inflammation seen in polyps may be related in part to decreasing SPLUNC1 expression (Seshadri et al., 2012). Mucositis is originated by a direct damage of epithelial cells of mucosa by radiation, which is associated with a great inflammatory process. The increasing levels of SPLUNC1 during and after the radiotherapy could be explained as a response of the immune system to this inflammatory stimulus. Decreased SPLUNC1, is also theoretically associated with decreased ability to clear pathogens and deregulation of ionic balance (Seshadri et al., 2012). We observed higher levels in SPLUNC1, although we do not know which oral pathogens are susceptible to SPLUNC1 action. In addition, candidiasis was not correlated with SPLUNC1.

Many salivary proteins undergo complex post-translational modification, including glycosylation and it is assumed that it has a major effect on their function (Helmerhorst & Oppenheim, 2007). SPLUNC2 is a heavily glycosylated protein and its western blot

expression shows three bands, identifying the expression of three different isoforms associated to the N-glycosylation (Bingle et al., 2009, Gonzalez-Arriagada et al., 2012). The two upper bands correspond to non-glycosilated SPLUNC2A, an immature form of the protein, and the lower band represents the glycosilated protein, that means a mature form of the protein. This distinction is important to understand the results associated to inflammatory process, where mature forms of these proteins are required.

The expression pattern of SPLUNC2 is more restricted than the other family members, and is only found in the oral cavity (Bingle et al., 2011). SPLUNC2 was demonstrated as the human orthologue of rodent PSP (Bingle et al., 2004). Further PSP-related proteins, termed bsp30, have been described in saliva of cow and other mammalian, and are highly expressed in parotid gland (Wheeler et al., 2002, Bingle et al., 2004, Wheeler et al., 2007). We observed decreasing levels of SPLUNC2 during and after the radiotherapy, which can have consequences in the oral microenvironment of irradiated patients. Studies with PSP-related proteins support the host defence function of these proteins, showing that it binds to bacterial membranes (Robinson et al., 1997) and has anti-candidal activity (Khovidhunkit et al., 2005). Bovine bsp30 proteins exhibit growth-suppression activity against Pseudomonas aeruginosa and Streptococcus pneumonia (Haigh et al., 2008). SPLUNC2 has exhibited growth-suppression effects on bacteria (Geetha et al., 2003) and peptides derived from SPLUNC2 can agglutinate bacteria (Gorr et al., 2008) and have anti-inflammatory effects (Geetha et al., 2005). Studies in vitro have showed that recombinant PSP inhibit the growth of Candida albicans in culture (Khovidhunkit et al., 2005). Although lower levels of SPLUNC2A can be associated with secondary radiotherapy effects, in the current study no statistically correlationation with candida was observed. Therefore, further studies evaluating the antimicrobial effect against human candida sp and oral bacteria are needed to clarify the function of PLUNC proteins in the oral microenvironment homeostasis.

Changes in glycosilated SPLUNC2A and SPLUNC1 levels associated to the phase of sample collection were showed. These alterations during the radiotherapy must be considered as part of the modifications in salivary chemical composition induced by radiotherapy. These changes can be associated with acute side-effects of radiation. Further studies may contemplate the patients for longer follow-up to recognize if the alterations are

associated with late-effects such as a radiation-related caries and osteoradionecrosis. English-language literature reported the effect of radiotherapy to modify the chemical composition of saliva, but is still controversial about its influence to generate a more pathogenic or cariogenic microflora, considering levels of Streptococcus mutans and Lactobacillus species (Epstein et al., 1996, Epstein et al., 1998). Further studies about salivary PLUNC variations and its interaction with cariogenic microflora could account for part of the etiopathogenesis of radiation-related caries. Reduced PLUNC expression is associated with bacterial colonization in patients with chronic sinusitis with nasal polyps, which suggest that patients with reduced PLUNC expression might have immune defect in defeating bacterial infection (Tsou et al., 2013), which can explain a higher incidence of opportunistic infections in radiotherapy. We suggest that oral opportunistic infections can be associated lower SPLUNC2A levels.

In conclusion, this is first study that showed alteration in salivary PLUNC proteins in irradiated head and neck cancer patients, suggesting that these proteins may have importance in the oral microenvironment and can be indirectly associated with the acute or late side-effects of the radiotherapy.

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**TABLES**

**Table I.** Demographic data of the population included in the study.

| | Control group (n=20) | | Study group (n=45) | |
|---|---|---|---|---|
| | **n** | **%** | **n** | **%** |
| **Gender** | | | | |
| Male | 17 | 85.00 | 38 | 84.44 |
| Female | 3 | 15.00 | 7 | 15.55 |
| **Age** | | | | |
| 40-49 | 4 | 20.00 | 8 | 17.77 |
| 50-59 | 10 | 50.00 | 20 | 44.44 |
| 60-69 | 5 | 25.00 | 8 | 17.77 |
| >70 | 1 | 5.00 | 9 | 20.00 |
| **Mean age (SD)** | 58.2 (10.2) | | 55.8 (8.6) | |

n=number

**Table II.** Clinicopathological data of the population included in the study group.

| | n | Percentage |
|---|---|---|
| **Localization** | | |
| Oral cavity | 17 | 26.15 |
| Oropharynx | 4 | 6.15 |
| Salivary glands | 2 | 3.08 |
| Hypopharynx | 2 | 3.08 |
| Larynx | 15 | 23.08 |
| Unknown primary | 5 | 7.69 |
| **Type of primary tumour** | | |
| Squamous cell carcinoma | 41 | 91.11 |
| Salivary gland tumour | 2 | 4.44 |
| Other | 2 | 4.44 |
| **Radiation field** | | |
| RFIFR | 27 | 60.00 |
| RFNIFR | 18 | 40.00 |
| **Clinical stage** | | |
| I | 7 | 15.56 |
| II | 4 | 8.89 |
| III | 10 | 22.22 |
| IV | 24 | 53.33 |
| **Chemotherapy** | | |
| Yes | 22 | 48.89 |
| No | 23 | 51.11 |
| **Surgery** | | |
| Yes | 13 | 28.89 |
| No | 32 | 71.11 |

n=number; RFIFR=radiation field involving facial region; RFNIFR=radiation field non-involving facial region.

**Table III.** Secondary effects associated to radiotherapy of the patients included in the study group.

| | n | Percentage | p value | RFIFR | RFNIFR | p value |
|---|---|---|---|---|---|---|
| **Mucositis*** | | | 0.0136 | | | **0.0110** |
| Yes | 29 | 69.05 | | 21 | 8 | |
| No | 13 | 30.95 | | 4 | 9 | |
| **Mucositis severity*** | | | 0.3951 | | | **0.0112** |
| Absent | 13 | 30.95 | | 4 | 9 | |
| Mild | 18 | 42.86 | | 11 | 7 | |
| Severe | 11 | 26.19 | | 10 | 1 | |
| **Candidiasis*** | | | 0.2170 | | | 0.0650 |
| Yes | 17 | 40.48 | | 13 | 4 | |
| No | 25 | 59.52 | | 12 | 13 | |
| **Xerostomia*** | | | <.0001 | | | 0.1580 |
| Yes | 36 | 85.71 | | 23 | 13 | |
| No | 6 | 14.29 | | 2 | 4 | |
| **Dysgeusia*** | | | <.0001 | | | **0.0076** |
| Yes | 35 | 83.33 | | 24 | 11 | |
| No | 7 | 16.67 | | 1 | 6 | |
| **Dermatitis*** | | | . | | | . |
| Yes | 42 | 100.00 | | 25 | 17 | |
| No | 0 | 00.00 | | 0 | 0 | |
| **Hyposalivation**** | | | <.0001 | | | 0.1739 |
| No | 13 | 34.21 | | 5 | 8 | |
| Mild | 10 | 26.32 | | 6 | 4 | |
| Severe | 15 | 39.47 | | 11 | 4 | |

n=number; RFIFR=radiation field involving facial region; RFNIFR=radiation field non-involving facial region.

* Data obtained without three patients that died because the cancer after the first collection.

** Data obtained without three patients that died because the cancer after the first collection and four patients after the second collection.

**Table IV.** Case-control analysis of PLUNC proteins expression.

| | n | Mean | SD | p value |
|---|---|---|---|---|
| **SPLUNC1** | | | | 0.1903 |
| Study | 45 | 1622.24 | 3684.26 | |
| Control | 20 | 1114.95 | 1682.54 | |
| **Glycosilated SPLUNC2A** | | | | **<.0001** |
| Study | 45 | 10270.84 | 6486.71 | |
| Control | 20 | 2176.30 | 3500.50 | |
| **Non-glycosilated SPLUNC2A** | | | | 0.1777 |
| Study | 45 | 5632.45 | 5542.22 | |
| Control | 20 | 9753.95 | 9994.50 | |
| **Total SPLUNC2A** | | | | **0.0016** |
| Study | 45 | 16788.46 | 11895.12 | |
| Control | 20 | 11930.25 | 12155.20 | |

n=number.

**FIGURES AND LEGENDS**

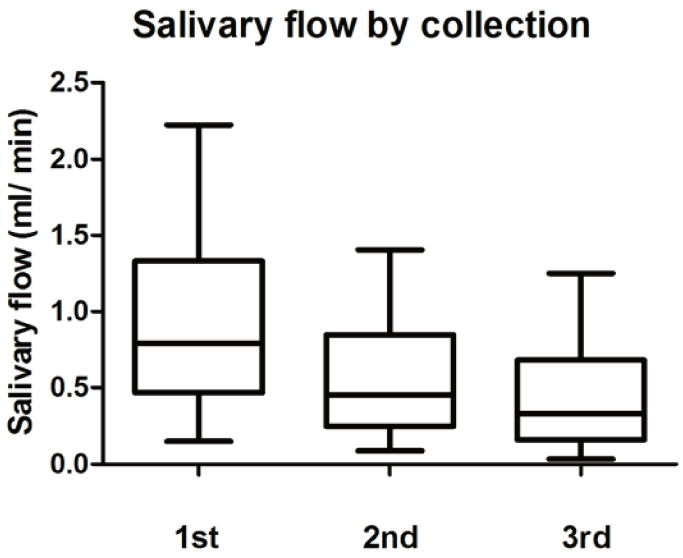


**Figure 1.** Salivary flow is decreasing during the radiotherapy.

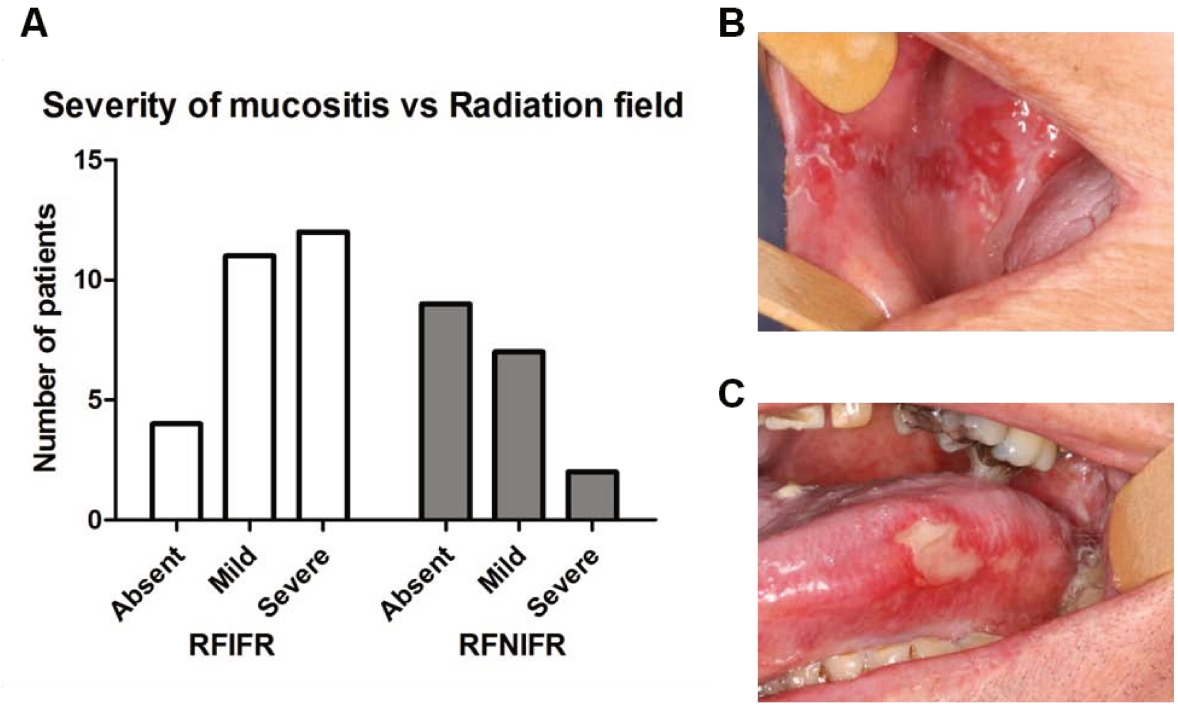


**Figure 2.** A) More cases of severe mucositis in observed in RFIFR (radiation field involving facial region), B) Mild grade of mucositis (grade II) and C) Severe grade of mucositis (grade III) observed in a patient irradiated in the facial region.

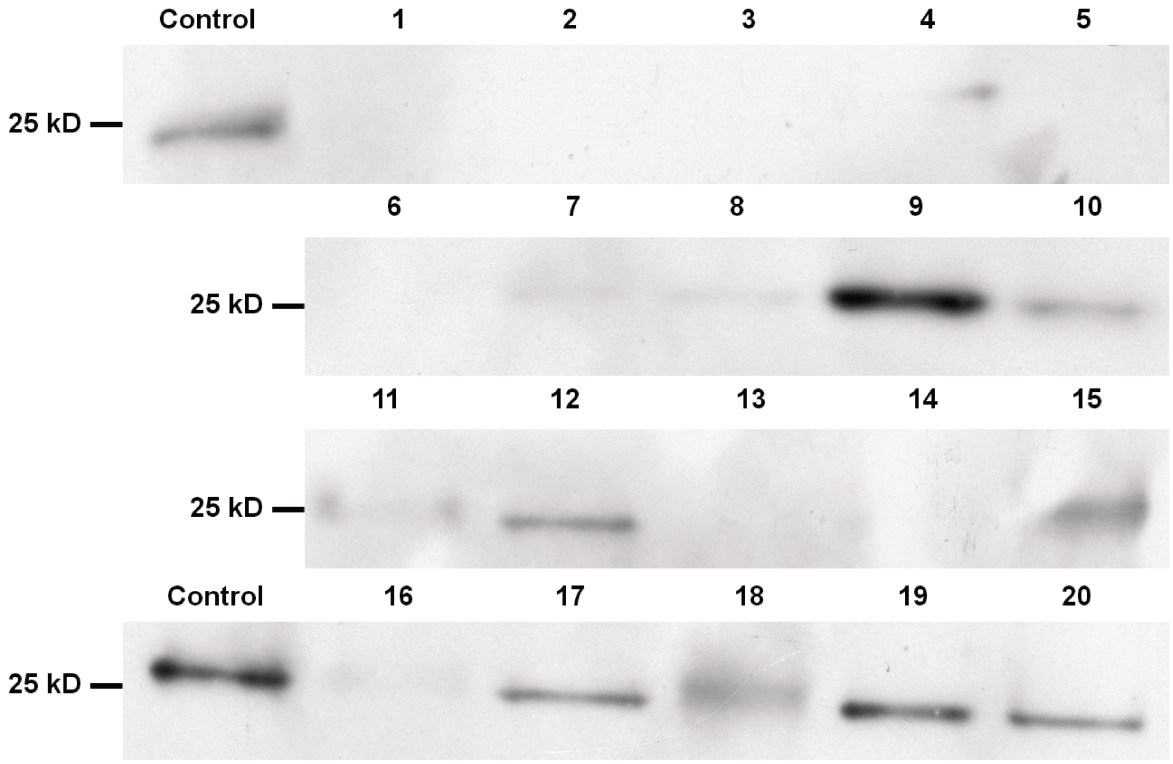


**Figure 3.** Western blot showing great variability of salivary SPLUNC1 expression in twenty subjects of the control group. A healthy subject that expressed positively SPLUNC1 was used as internal control.

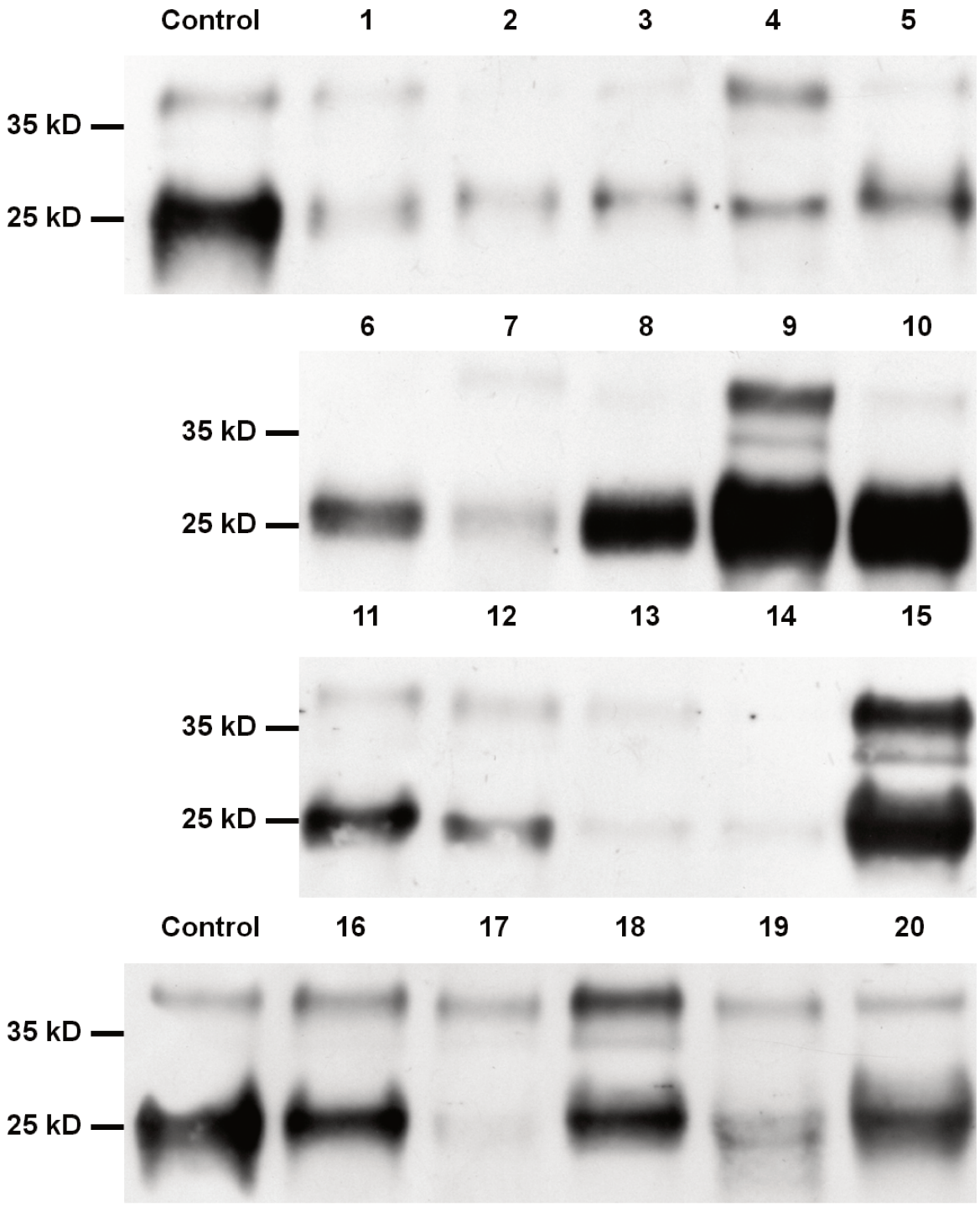


**Figure 4.** Western blot showing great variability of salivary SPLUNC2A expression in twenty subjects of the control group. A healthy subject that expressed positively SPLUNC2A was used as internal control.

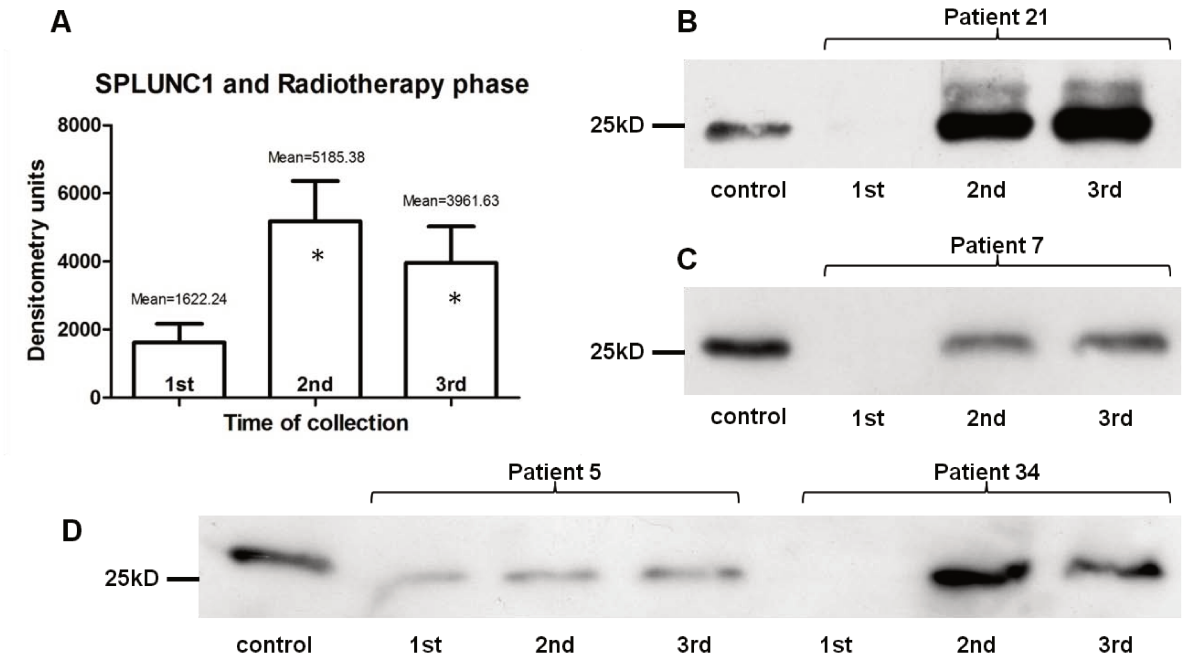


**Figure 5.** SPLUNC1 expression showing higher levels in the second and third collections (A). Pattern of salivary SPLUNC1 (B, C, D). A healthy subject that expressed positively SPLUNC1 was used as internal control.

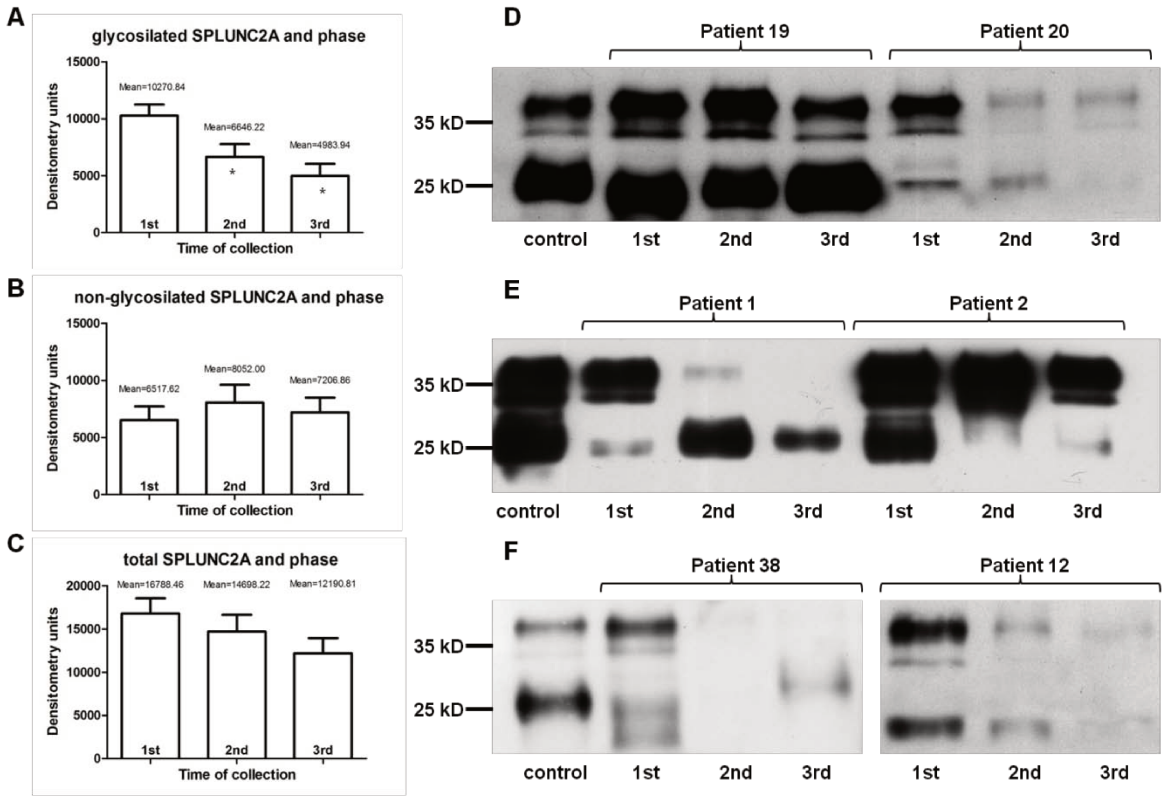


**Figure 6.** Glycosilated SPLUNC2A expression (A) and total SPLUNC2A expression (C) presenting lower levels in the second and third collections; and non-glycosilated SPLUNC2A expression were not modified by the radiotherapy (B). Pattern of salivary SPLUNC2A (D, E, F). A healthy subject that expressed positively SPLUNC2A was used as internal control.

## CONCLUSÕES

1. A redução do fluxo salivar é mais acentuada nos pacientes cujo campo de radiação inclui a região facial. Os pacientes irradiados exclusivamente na região do pescoço apresentam uma redução menos intensa do fluxo salivar.
2. Os valores de SPLUNC1 e SPLUNC2A são variáveis em indivíduos saudáveis, mas as causas dessas variações são desconhecidas.
3. Os valores SPLUNC2A nos pacientes do grupo controle foram maiores que no grupo estudo, enquanto que os valores de SPLUNC1 não mostraram diferencia significativa entre grupos. Uma mudança no padrão de maturação da proteína SPLUNC2A também foi observada com uma maior produção de proteína glicosilada nos pacientes com câncer quando comparados com os pacientes sem doença. Sugerimos que estaria associado à resposta inflamatória contra o tumor, levando a uma expressão aumentada de moléculas defensivas da saliva.
4. Os valores de SPLUNC1 e SPLUNC2A totais foram afetados pela radioterapia. SPLUNC1 apresentou aumento e SPLUNC2A apresentou diminuição dos níveis, principalmente na sua forma glicosilada. Esses resultados estariam associados com a mudança da consistência salivar, sugerindo que ácinos serosos são mais susceptíveis à radiação.
5. A mucosite foi o único efeito colateral associado com o aumento dos níveis de SPLUNC1 salivar.
6. SPLUNC1 e SPLUNC2A podem ter importância no microambiente oral dos pacientes irradiados na cabeça e pescoço, no entanto novos estudos são necessários para elucidar as funções dessas proteínas na cavidade oral.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

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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
CEP/FOP/UNICAMP

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

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