Salivary BPIFA1 (SPLUNC1) and BPIFA2 (SPLUNC2 A) are modified by head and neck cancer radiotherapy

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Abstract

Objective: To determine the effects of radiotherapy on salivary BPIFA expression and to investigate the role of BPIFA in the development of known radiotherapy side effects.

Materials and Methods: Unstimulated whole-mouth saliva was collected from 45 cancer patients (1 week before treatment, during the treatment, and 1 week after completion of radiotherapy) and from 20 controls. BPIFA1 and BPIFA2 expression was detected by western blotting and analyzed along with clinicopathologic data and side effects from the radiotherapy.

Results: A facial radiation field was associated with lower salivary flow during and after radiotherapy and correlated with side effects, mainly mucositis. Salivary BPIFA1 expression levels were similar between the control group and the patient group before treatment. On the other hand, BPIFA2 levels were higher in the patient group before treatment compared with the control group. BPIFA concentration was modified by radiotherapy as BPIFA1 levels increased ($P = .0081$) and BPIFA2 decreased ($P < .0001$). Higher levels of BPIFA1 were associated with the presence of mucositis ($P = .0363$) and its severity ($P = .0500$).

Conclusions: The present study found that levels of BPIFA1 and glycosylated forms of BPIFA2 are affected by radiotherapy, suggesting that these proteins may play a role in the oral microenvironment in irradiated patients with head and neck cancer.

Statement of Clinical Relevance: *The present study which showed that BPIFA proteins expression was modified in patients who underwent radiotherapy may contribute to better understanding the etiology and could be useful to improve the management of its side effects of radiotherapy.*

Head and neck cancer represents around 4% of all malignant tumors in humans and is usually diagnosed at advanced stages.[1] Radiotherapy is commonly used for the treatment of head and neck cancer, often in association with surgery and/or chemotherapy.[2] Conventional head and neck radiotherapy generally involves high doses (60 gray [Gy] or higher) in fractionated daily doses, as determined by the diagnosis and clinical stage.[3] All tissues involved in the radiation field are affected, which results in both acute and chronic side effects.[4; 5; 6; 7] The severity of these complications is related to multiple factors, such as the volume of irradiated tissue, radiation dose, and individual patient factors, including poor oral hygiene, professional dental care, smoking, alcohol intake, and immune health.[4; 5] The most common acute reactions are mucositis, dysgeusia, dermatitis, and candidiasis, which are reversible. Xerostomia is often observed at an early stage of treatment; however, it can also develop into a chronic complication alongside radiation-related caries and osteoradionecrosis.[3; 4; 5; 6; 7; 8; 9] Head and neck radiotherapy may modify oral defensive mechanisms, particularly by decreasing salivary flow and altering saliva composition.[10; 11]

The palate, lung and nasal epithelium clone (PLUNC) was first described in the nasal epithelium, trachea, and bronchus of mouse embryos,[12] and subsequently a family of human equivalents was described.[13] The human genes are located on chromosome 20 q11.2, close to genes encoding the lipopolysaccharide-binding protein and bactericidal or permeability-increasing protein (BPI), which play important roles in the innate immune response to gram-negative bacteria.[13; 14; 15] PLUNCs can be subdivided in two groups, short (SPLUNC) and long (LPLUNC) proteins, based on their predicted structure being homologous to either one or both domains of BPI.[16; 17] Recently, PLUNC family members have been included in the BPI fold–containing superfamily, leading to a new nomenclature whereby SPLUNC proteins now have the designation BPIFA, and LPLUNC proteins have the designation BPIFB.[18; 19]

A number of studies have demonstrated the expression of BPIFA proteins in saliva,[14; 20; 21] salivary glands and salivary gland tumours,[14; 16; 22; 23; 24] and other neoplasms.[25; 26; 27; 28; 29; 30] Variations in BPIF protein expression have also been reported in healthy patients and in a number of inflammatory and infectious diseases.[14; 20; 31] Each family member has a selective expression profile in the upper airways and oral cavity tissues and fluids.[18; 32] The specific function of these proteins is still not well defined, but evidence exists for their participation in host innate immunity with antimicrobial and anti-inflammatory effects.[14; 16; 17; 31] The anti-inflammatory function has been associated with the regulation of macrophagic activity,[15] particularly the cellular response to lipopolysaccharide.[16] However, to date, there is no convincing evidence that BPIF proteins exert direct killing activity; they are more likely to be bacteriostatic, promoting agglutination of bacteria and modulating cytokine production.[33]

Saliva sampling offers some advantages in comparison with other sampling procedures, as it is minimally invasive, can identify local or systemic effects of radiotherapy, and can be used to predict toxicity or prognosis.[34; 35] The antimicrobial and anti-inflammatory properties of BPIFA proteins and the participation of microorganisms and the immune response in the etiology of oral adverse effects of radiotherapy suggest that a better understanding of the role of these proteins in the oral microenvironment is necessary. Therefore, the aim of this study was to test the hypothesis that radiotherapy can modify salivary BPIFA expression and

that the resulting modifications are associated with the development of the acute and debilitating side effects of radiotherapy, namely, mucositis.

Materials and Methods

The study was approved by the Ethics Committee for Human Studies, Piracicaba Dental School, Brazil (protocol number: 142/2010). Written informed consent was obtained from all patients entered in the study.

Patients and clinical features

A longitudinal case control clinical study was performed with a study group (n = 45) that consisted of consecutive patients receiving radiotherapy for head and neck cancer in the Oncology Centre, and a control group (n = 20) of normal, healthy volunteers. Of the patients who received radiotherapy, 22 underwent concomitant chemotherapy, which was given in a regimen of 1 day per week with cisplatin and 5-fluorouracil. Clinicopathologic data, such as gender, age, tumor size, and location, were collected retrospectively from the patients' charts.

Radiotherapy

No patients had previously been treated for any form of cancer. Conformal radiotherapy was performed with the linear accelerator Varian Clinac 600 C (Palo Alto, CA). Patients were grouped according to radiation field because of the compromise of salivary glands; those who received radiation in the facial region (radiation field involving facial region, RFIFR) and those who did not receive radiation in facial region. The therapy was given 5 days a week in daily doses of 180 to 200 centigrays, with a total dose of radiation ranged from 63 to 78 Gy. All the patients included in the RFIFR group received radiation doses above 60 Gy in the salivary glands.

Clinical evaluation

All patients received preradiotherapy orientation and dental treatment at the Oral Diagnosis Clinic (Piracicaba Dental School, University of Campinas) before the first radiation session. Subsequent examinations were performed weekly during the treatment and also 1 week following treatment. The side effects, which were scored in the weekly evaluations, included hyposalivation, mucositis, dermatitis, and dysgeusia. The severity of mucositis was determined by using the World Health Organization oral toxicity scale (four grades of severity).[36] For statistical analysis, mucositis grade was simplified to mild mucositis (grades 1 and 2) and severe mucositis (grades 3 and 4).[37]

Collection of saliva samples

Unstimulated whole-mouth saliva was collected in the morning, between 9 and 11 a.m. The patients abstained from eating, drinking, and tooth brushing for at least 1 hour before sample collection. Five minutes before collection, the patients rinsed their mouths with

water. Each patient let the naturally produced saliva drain into a sterile glass cup, without any stimulation, for 5 minutes. The saliva flow rate (mL/min) was measured immediately after saliva collection. Saliva samples were immediately placed on ice (0°C), transported to the laboratory, and then centrifuged (14,000 rpm for 6 minutes at 18°C). The supernatants were stored at −80°C for later use. Three collections were programmed: 1 week before treatment, between the 15th and 17th sessions (week 4 of a 6- to 7-week program), and 1 week after completion of the radiotherapy course. Salivary flow was considered normal when it was 0.3 mL/min or greater, and hyposalivation if it was less than 0.3 mL/min.[38]

Western blot

Total protein concentration was measured using a protein assay (Bradford reagent, Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Under reducing conditions, 10 µg of protein per sample was resolved on a 12% sodium dodecyl sulfate polyacrylamide gel and transferred onto a nitrocellulose membrane. Ponceau S staining confirmed the effectiveness of the transfer and loading quality. Membranes were incubated overnight at 4°C with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for blocking. After three washes of 15 minutes each in TBST, the membranes were incubated with primary antibody, diluted in blocking solution, for 2 hours. The antibody concentrations used were 1:500 for BPIFA2 and 1:250 for BPIFA1. Membranes were washed three times in TBST and incubated with a relevant horse radish peroxide–conjugated secondary antibody for 1 hour. The antibody-binding activity was detected by using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). The intensity of each band was measured with the software Gel Analyzer 2010 (Lazar Software, developed by Dr. Istvan Lazar, Debrecen, Hungary), obtaining arbitrary densitometry units (*adu*) for quantification and comparison.

Statistical analysis

The groups were compared by using the coefficient of kurtosis, the coefficient of asymmetry, and the Shapiro-Wilk test to determine distribution. Groups normally distributed were compared by analysis of variance (ANOVA), and those with a non-normal distribution were compared by using the Wilcoxon rank sum test. The association between nominal variables was tested with the use of the Pearson chi-square. Densitometry values were divided in two groups: above the median and below the median. A linear regression model of BPIFA, with gender (as a categorical variable) and age, were performed. ANOVA based on a generalized linear mixed model with repeated measures was applied to test the effect of time and chemotherapy on BPIFA expression. A significance level of 5% (0.05) was used in all tests. Statistical calculations were performed with the SAS System 9.3 (SAS Institute Inc. The SAS System, Cary, NC, 2010) and graphs constructed with GraphPad Prism 5 (Graphpad Software Inc. La Jolla, CA, 2007).

Results

Clinicopathologic findings

Sixty-five patients, with a mean age of 58.2 years (SD of 10.2) in the patient group and 55.8 years (SD of 8.6) in the control group, were included in the study. Descriptive comparisons showed that the characteristics of the patient group were similar to 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 clinicopathologic data of the population are provided in Tables I and II.

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. Clinicopathologic 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 tumor		
Squamous cell carcinoma	41	91.11
Salivary gland tumor	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

	n	Percentage
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 not involving facial region.

Salivary flow

A reduction in salivary flow rates during and after radiotherapy was observed, and this was more pronounced in RFIFR patients (Figure 1). There was no significant difference in the salivary flow of the control group (mean = 0.702 mL/min) compared with that of the first collection from the patient group (mean = 0.912 mL/min), (*P* = .1438, ANOVA).

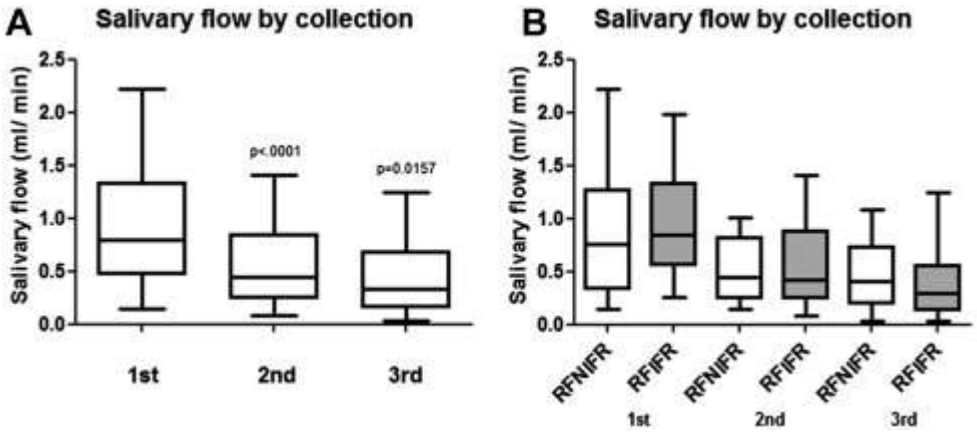

Fig. 1. Salivary flow is decreasing during the radiotherapy. The first collection was taken 1 week before treatment. The second collection was taken between the 15th to 17th sessions. The third collection was taken 1 week after the radiotherapy.

A statistically significant association was found between salivary flow and phase of sample collection (*P* < .0001), with significant differences being observed between the first and second collection (*P* < .0001), the first and third collection (*P* < .0001) and the second and third collection (*P* = .0157). No association between salivary flow and chemotherapy was observed.

Side effects associated with radiation field and chemotherapy

A statistically significant association between radiation field and the presence (*P* = .0110) and severity (*P* = .0143) of mucositis during radiotherapy was found. Dysgeusia and radiation field also showed a statistically significant association (*P* = .0076). There was no

correlation between hyposalivation or dermatitis and the radiation field. Detailed information regarding the secondary effects of radiotherapy is described in Table III. Three patients from the study group died as a result of their cancer after the first collection and four patients after the second collection. The addition of chemotherapy to the treatment regime did not correlate with the presence and/or severity of mucositis or any other side effect.

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

	n	Percentage	P value	RFIFR	RFNIFR	P value
Mucositis*			.0136			.0110
Yes	29	69.05		21	8	
No	13	30.95		4	9	
Mucositis severity*			.3951			.0143
Absent	13	30.95		4	9	
Mild	18	42.86		11	7	
Severe	11	26.19		10	1	
Dysgeusia*			<.0001			.0076
Yes	35	83.33		24	11	
No	7	16.67		1	6	
Dermatitis*			<.0001			1.0000
Yes	42	100.00		25	17	
No	0	00.00		0	0	
Hyposalivation†			<.0001			.0801
No	13	34.21		5	8	
Yes	10	26.32		6	4	

N, number; *RFIFR*, radiation field involving facial region; *RFNIFR*, radiation field not involving facial region.

*Data obtained without three patients who died from cancer after the first collection.
†Data obtained after the third collection, excluding three patients who died from cancer after the first collection and four patients who died after the second collection.

BPIFA expression in control and study groups

There was no correlation between age, gender, or clinical stage and BPIFA1 and BPIFA2 expression.

BPIFA1 levels were variable in the saliva of the control group, ranging from 0 to 6909 *adu* (Figure 2, A). BPIFA2 levels were also variable in this group, ranging from 0 to 12958 *adu* for the glycosylated form, 3 to 30181 *adu* for the nonglycosylated form and 303 to 37281 *adu* for total BPIFA2 (Figure 2, B). BPIFA1 and BPIFA2 levels in control samples were compared with those in the initial collection from the study group. No statistically significant difference in BPIFA1 expression was found between the controls and the patients at the beginning of the study (P = .1903, Kruskal-Wallis test); however, a significant difference was apparent between the patients and the controls in terms of the expression of total BPIFA2 (P = .0016, ANOVA) and its glycosylated form (P < .0001, ANOVA) (Table IV).

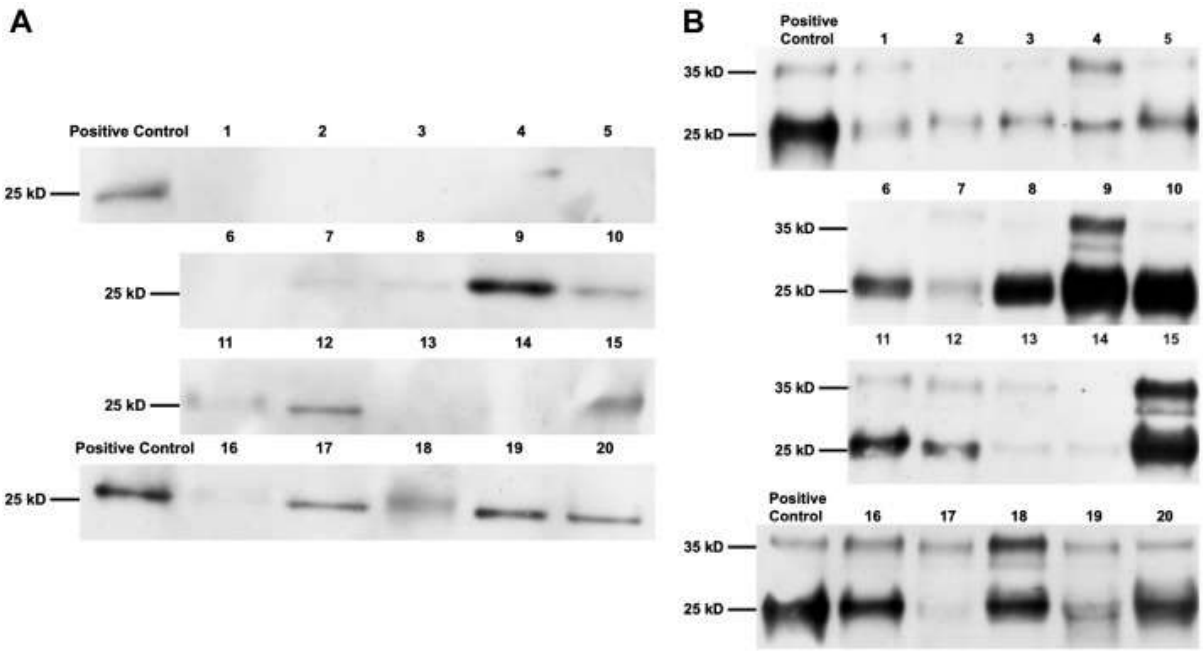

Fig. 2. **A**, Western blot showing the variability of salivary BPIFA1 expression in the 20 patients in the control group. A healthy subject that expressed positively BPIFA1 was used as positive control of western blot. **B**, Western blot showing the variability of salivary BPIFA2 A expression in the 20 patients in the control group. A healthy subject who expressed BPIFA2 A positively was used as positive control for the western blot.

Table IV. Case control analysis of BPIFA protein expression

	n	Mean	SD	P value
BPIFA1				.1903
Study	45	1622.24	3684.26	
Control	20	1114.95	1682.54	
BPIFA2 A glycosylated form				<.0001
Study	45	10270.84	6486.71	
Control	20	2176.30	3500.50	
BPIFA2 A non-glycosilated form				.1777
Study	45	5632.45	5542.22	
Control	20	9753.95	9994.50	
Total BPIFA2 A				.0016
Study	45	16788.46	11895.12	
Control	20	11930.25	12155.20	

N, number; *SD*, standard deviation.

BPIFA proteins and mucositis

A statistically significant association was observed in the third (posttreatment) sample between increased levels of BPIFA1 and the presence (*P* = .0363) and severity (*P* = .0500) of

mucositis; no such association was observed in samples collected during treatment (P = .7175). There was no correlation between BPIFA2 expression and the development of mucositis (Table V).

Table V. Mucositis and BPIFA expression

	BPIFA1 (2nd collection)			BPIFA1 (3rd collection)			BPIFA2 (2nd collection)			BPIFA2 (3rd collection)		
	AM	**BM**	**P value**	**AM**	**BM**	**P value**	**AM**	**BM**	**P value**	**AM**	**BM**	**P value**
Mucositis*			.7175			.0363*			0.2777			1.0000
Yes	13	12		16	10		11	14		13	13	
No	5	6		3	9		7	4		6	6	
Mucositis severity*			.9242			.0500*			0.4921			.4655
Absent	5	6		3	9		7	4		6	6	
Mild	8	7		12	5		6	9		10	7	
Severe	5	5		4	5		5	5		3	6	

N, number; *AM*, above the median; *BM*, below the median.

*Data obtained without seven patients that died due to cancer after the third collection.

BPIFA proteins associated with phase of sample collection or chemotherapy

Statistically significant differences in the levels of BPIFA1 between patients were found at each time point (P = .0081), and these differences were also significant between the first and second samples (P = .0070) and the first and third samples (P = .0048) (Figure 3). Levels of the glycosylated form of BPIFA2 also varied between patients at each collection point (P < .0001), and, again, the differences were statistically significant between the first and second samples (P < .0001) and the first and third samples (P < .0001) (Figure 4). Interestingly, there was no significant difference in the levels of the nonglycosylated form of BPIFA2, and only a trend toward lower levels of total BPIFA2 was evident; however, there was considerable variability in expression levels between patients. No correlation between BPIFA levels and chemotherapy was found at any point across the period of study.

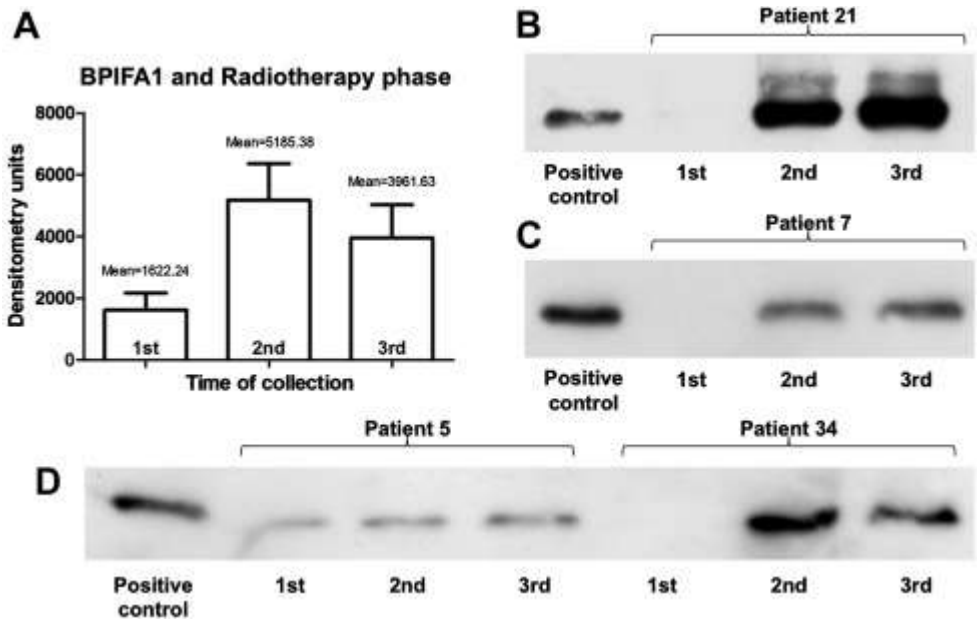

Fig. 3. BPIFA1 expression showing higher levels in the second and third collections (**A**), showing difference statistically significant between the phases of sample collection (*P* = .0081).* Expression pattern of salivary BPIFA1 (**B**, **C**, and **D**). A healthy subject who expressed BPIFA1 positively was used as positive control for the western blot.

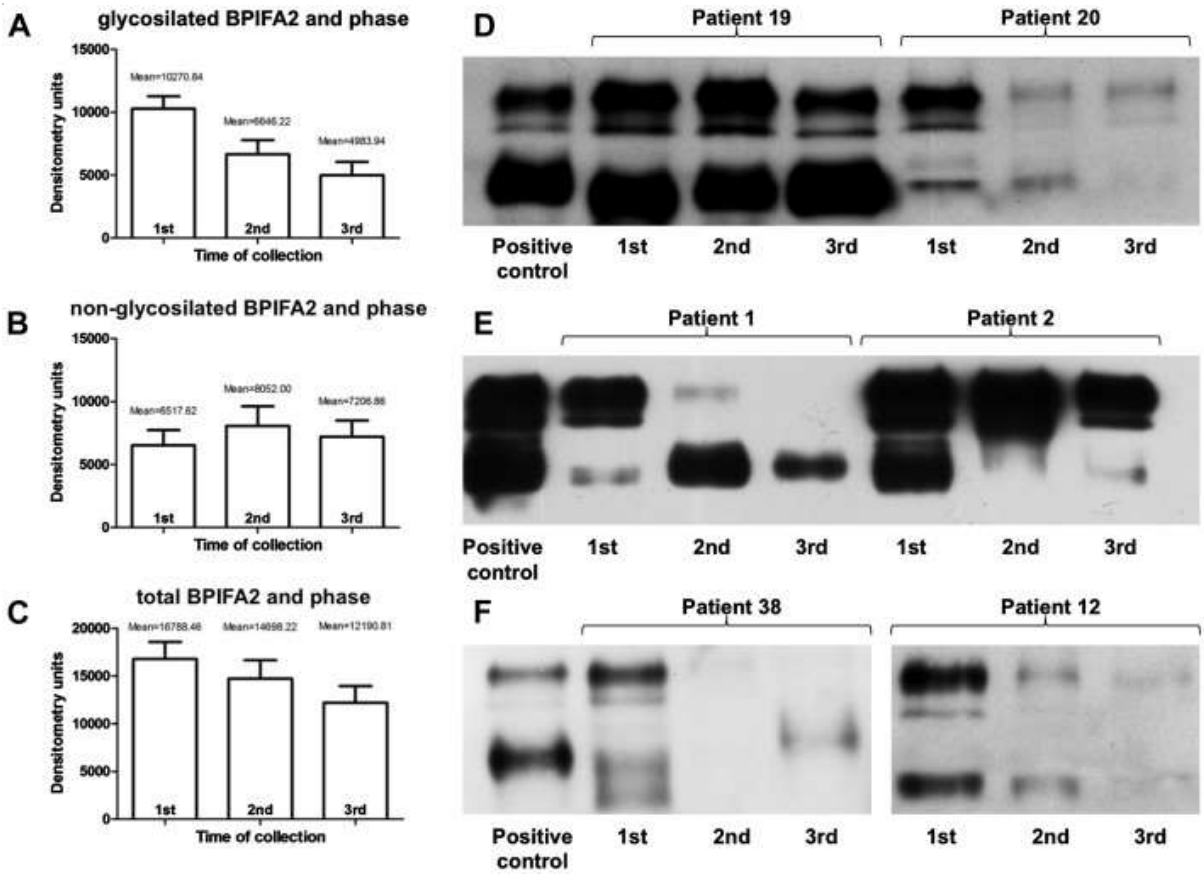

Fig. 4. Glycosylated form of BPIFA2 A expression showed association with phase of sample collection (*P* < .0001)* presenting lower levels in the second and third collections (**A**). Levels of nonglycosylated form of BPIFA2 A (**B**) and total BPIFA2 A (**C**) were not modified by the radiotherapy. Expression pattern of salivary BPIFA2 A (**D**, **E**, **F**). A healthy subject who expressed BPIFA2 A positively was used as positive control for the western blot.

Discussion

In the present study, we noticed decreased mean levels of glycosylated BPIFA2 and increased mean levels of BPIFA1, which suggested that radiotherapy can modify the salivary BPIFA concentrations of these putative host defense proteins. Radiation-related salivary gland hypofunction results in reduced salivary flow, which can also manifest as xerostomia (defined as the subjective perception of dry mouth) and in modifications of the chemical composition of saliva.[39] In our study, we observed a decrease in salivary flow during radiotherapy. Initially, salivary flow levels were very similar between the control group and the patient group, confirming that any later changes in flow rate would be due to the radiotherapy treatment. A reduction in salivary flow can have a significant impact on quality of life, with minimal recovery after completion of the course of therapy.[40] Alterations in biochemical composition of saliva, including viscosity, pH, protein, and electrolyte concentrations, as well as modifications in microflora, showing a predisposition to *Candida* proliferation with an increased number of non-*albicans* species, have been recorded. [35; 41; 42 ; 43] It has been hypothesized that changes in the normal oral microflora, particularly gram-negative bacteria, are linked to the etiology and severity of mucositis [44; 45 ; 46]; however, this hypothesis remains controversial. [47; 48 ; 49]

It has been reported that the nasal polyps of patients with chronic sinusitis have decreased numbers of glands and that this is associated with defects in the production and release of innate defense molecules, such as BPIFA1 and BPIFB2.[50] Interestingly, in the present study, lower concentrations of BPIFA2 were observed in the final sample (collected 1 week after completion of treatment), which suggested an association with increased acini damage and salivary gland hypofunction due to the radiation. An inverse relationship between salivary flow rates and *C. albicans* counts in saliva has previously been reported, with the suggestion that salivary gland hypofunction is an increased risk factor for the development of candidiasis. [39; 41 ; 51]

BPIFA proteins are differentially expressed in normal salivary glands and salivary gland tumors with BPIFA1 being expressed in mucous acini and BPIFA2 in serous acini.[24] This difference in expression suggests that each one occupies an individual niche and potentially has individual functions.[18 ; 20] Irradiation appears to have a greater effect on the function of parotid glands because serous acini are more susceptible to permanent radiotherapy-induced damage than are mucous acini,[43] resulting in thicker saliva. As a serous salivary gland, the parotid gland only produces one of the BPIF proteins, BPIFA2, and thus we might have expected to see a greater effect of radiation therapy on BPIFA2 levels.[14 ; 24] Our results are consistent with this in that there was decreased expression of the glycosylated form of BPIFA2 and a trend toward decreased total BPIFA2 but increased expression of BPIFA1 over the time of the study.[5]

Salivary proteins, even if present at only relatively low concentrations, provide antimicrobial defense, as they are able to act in at least an additive manner if not synergistically.[33] It seems reasonable to assume that the damage caused by radiation therapy will affect the expression levels of a number of salivary proteins, including a number of innate defense proteins. Several salivary proteins concentrations were studied in irradiated patients,

showing that radiation can modify the oral microenvironment and may be useful in predicting toxicity or tumor response.[10; 34 ; 52]

The radiation field is the main risk factor associated with side effects, and we observed a statistically significant correlation between RFIFR and mucositis and dysgeusia. Recovery of function from the symptoms of dysgeusia, hyposalivation, mucositis, dermatitis, and candidiasis was not considered in our study but could be contemplated in future research. We suggest that the expression of BPIFA proteins is also affected by the radiation field because lower levels of BPIFA2, which is exclusively produced in the mouth, mainly by the parotid gland, are observed compared with BPIFA1.

Variations of BPIFA expression in normal saliva samples have previously been reported,[14 ; 20] and similar differences in expression were found in this study. No correlation with age or gender has been reported or was found in this study. Kohlgraf et al.[20] suggested that the variability in salivary BPIFA1 concentration could be associated with periodontal health; no such measurement was, however, made in their study. All patients included in our study received preradiotherapy dental treatment, and a significant number of the patients were edentulous; therefore, we suggest that periodontal health would not have influenced BPIFA concentrations in the present study; many other factors could be involved.

In the pretreatment patients' samples, BPIFA1 and BPIFA2 (total and glycosylated) levels were higher than in the control group, but only the differences in BPIFA2 were statistically significant. An inflammatory response to the tumor, leading to a higher expression of defense molecules in saliva, may be the reason for this elevated BPIFA1 expression, which has also been previously described in a range of carcinomas, including lung cancer and head and neck squamous cell carcinomas.[53 ; 54] Concentrations of BPIFA1 are also increased in chronic obstructive pulmonary disease and in smokers;[55] most of our patients with head and neck cancer were smokers, and this could further explain the higher BPIFA1 levels in our study group, as the control patients were not smokers. No statistical difference was seen in the levels of nonglycosylated BPIFA2 between the patient group and the control group; however, there was a trend toward higher values in the control group. The relevance of this is not clear and should be investigated in future studies.

BPIFA1 levels increased throughout the course of treatment, and this increase was maintained 1 week after completion; expression levels correlated with the presence and severity of mucositis. Previously published data have indicated an increase in anti-inflammatory mediators in saliva in response to radiation-induced damage and resulting mucositis.[56 ; 57] Recently, it was proposed that BPIFA1 might play a role in suppressing allergic inflammation and that decreasing BPIFA1 expression could result in a higher inflammatory response.[50] Direct damage of mucosal epithelial cells by radiation results in the development of mucositis, which is associated with increased inflammation. The increasing levels of BPIFA1 during and after the radiotherapy may form part of the immune response to this inflammatory stimulus. Although changes in BPIFA values could play a causal role in the oral side effects of radiation, they could be merely present without having any relationship whatsoever.

Many salivary proteins undergo complex posttranslational modification, including glycosylation, and it is assumed that this has a major effect on their function.[58] BPIFA2 is a heavily N-glycosylated protein,[14] and on a western blot, the two upper bands correspond to the glycosylated form of BPIFA2, whereas the lower band represents the nonglycosylated form. In the present study, we observed a change in the glycosylation pattern of BPIFA2, as the patients with cancer expressed higher levels of the glycosylated form of BPIFA2 and lower levels of the nonglycosylated form compared with controls. These results suggest that the increased levels of the glycosylated form of the protein may be associated with an immune response to the cancer environment in the oral cavity. Decreased levels of glycosylated BPIFA2 were observed during the radiotherapy, which can be associated with the radiation-induced damage and potentially decreased immune response in the patients.

The expression pattern of BPIFA2 (the human ortholog of rodent parotid secretory protein [PSP])[59] is more restricted than that of other family members, as it is only found in the oral cavity.[19] Other PSP-related proteins, for example, bsp30, have been described in the saliva of cows and cattle and are highly expressed in parotid glands.[59; 60 ; 61] We observed decreasing levels of BPIFA2 during and after radiotherapy, which might have serious consequences for the oral health of irradiated patients, as previous studies with PSP-related proteins have demonstrated a potential role in host defense through antibacterial[62; 63; 64 ; 65] and anticandidal activity,[66] as well as anti-inflammatory effects.[67] Lower levels of BPIFA2 might be associated with secondary radiotherapy effects, so further studies are needed to evaluate antimicrobial activity against human *Candida* species and oral bacteria and thus fully clarify the function of BPIFA proteins in the homeostasis of the oral microenvironment.

Changes in BPIFA1 expression and of the glycosylated form of BPIFA2 are associated with the phase of sample collection and must be considered an important part of the modifications in salivary composition induced by radiotherapy. As the time span of this study was relatively short, the changes observed may be associated with the acute side effects of radiation. Further studies with a longer patient follow-up time would allow us to determine whether the changes in protein expression are associated with late effects, such as a radiation-related caries and osteoradionecrosis. Previous studies have reported that radiotherapy can lead to alterations in the chemical composition of saliva, but whether this allows the development of a more pathogenic or cariogenic microflora, particularly in relation to levels of *Streptococcus mutans* and *Lactobacillus* species, remains controversial.[68 ; 69] As mentioned previously, reduced BPIFA expression has been linked to bacterial colonization in patients with chronic sinusitis with nasal polyps, which suggests that reduced BPIFA2 expression might lead to a reduced immune response.[70] The increase in BPIFA1 expression across the time of this study suggests that BPIFA2 protein, which is exclusively produced in the oral cavity, is of greater importance in oral homeostasis. We were unable to show any association between infection and alteration in BPIFA levels, but further microbiologic research must be carried out to study the interaction of BPIFA proteins with the cariogenic microflora associated with radiation-related caries and the *Candida* species associated with oral candidiasis.

Conclusions

This is the first study to show an alteration in salivary BPIFA proteins in irradiated patients with head and neck cancer, suggesting that these proteins may play an important role in maintaining the oral microenvironment. Further information about alterations in salivary BPIFA and other salivary protein levels could be useful in the development of better artificial saliva, which would improve the quality of life of irradiated patients with head and neck cancer and other patients suffering from hyposalivation.

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