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Salivary BPIFA proteins are altered in patients undergoing hematopoietic cell transplantation

BPIFA expression in HCT patients

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ABSTRACT

Objective: The aim of this study was to evaluate the expression of BPIFA proteins in the saliva and salivary glands of hematopoietic cell transplant (HCT) patients. **Material and Methods:** This longitudinal study included patients who had undergone autologous HCT (auto-HCT) and allogeneic HCT (allo-HCT) and unstimulated saliva were collected at three timepoints, with a fourth collection at oral chronic graft versus host disease (cGVHD) onset. BPIFA expression was analysed by Western blotting in saliva and immunostaining in the minor salivary glands of cGVHD patients. **Results:** Auto-HCT patients showed increased levels of BPIFA1 (P = 0.021) and BPIFA2 at D+7 (P = 0.040), whereas allo-HCT group demonstrated decreased expression of BPIFA2 at D+8 (P = 0.002) and at D+80 (P = 0.001) and a significant association between BPIFA2 low levels and hyposalivation was observed (P = 0.02). BPIFA2 was significantly lower in the cGVHD patients when compared to baseline (P = 0.04). **Conclusions:** The results of this study show distinct pattern of expression of BPIF proteins in both auto-HCT and allo-HCT recipients with decreased levels of BPIFA2 during hyposalivation and cGVHD. Further studies are necessary to elucidate these proteins mechanisms and their clinical implications in these groups of patients.

KEYWORDS: BPIFA, conditioning regimen, hematopoietic cell transplantation, saliva, salivary glands

1 INTRODUCTION

Hematopoietic cell transplantation (HCT) is the treatment of choice for a number of malignant and non-malignant diseases involving the hematopoietic system and for some solid tumours (Gratwohl et al., 2011; Passweg et al., 2012). The treatment is, however, often associated with several oral side-effects including oral mucositis, oral infections, alterations in taste and in saliva quantity and composition (Sonis et al., 2001; Sonis et al., 2004; Passweg et al., 2012; Uutela et al., 2019). Graft-versus-host disease (GVHD) is a common complication of allogenic HCT (allo-HCT), affecting multiple organ systems and causing significant morbidity and mortality (Lee, Vogelsang, & Flowers, 2003; Kuten-Shorrer, Woo, & Treister, 2014; Presland, 2017). The oral cavity is frequently affected, and chief complaints tend to be related to pain associated with ulcers, xerostomia and occasionally taste alterations (Kuten-Shorrer et al., 2014). A review on acute and chronic GVHD proteomics suggested saliva as a potential source of prognostic and predictive biomarkers (Presland, 2017).

The BPI fold-containing superfamily (BPIF) represents a group of hydrophobic, lipid-binding proteins predominantly found in the upper respiratory tract, nasal cavity, ears and oral cavity (L. Bingle & Bingle, 2011a). They have been shown to have some actions against gram-negative bacteria (Liu, Zhang, Wu, French, & He, 2016; Lukinskiene et al., 2011). BPIFA1 protein is expressed in the major salivary glands and in the minor glands of the nose, sinus, posterior tongue and tonsil (L. Bingle et al., 2005), being produced in mucous cell type (Vargas, Speight, Bingle, Barrett, & Bingle, 2008). BPIFA2 is expressed by the serous cells of the major and minor salivary glands (Biron et al., 2000; Hopcraft & Tan, 2010; L. Bingle & Bingle, 2011b). Changes in BPIFA1 protein levels have previously been shown to be associated with disease severity in chronic obstructive pulmonary disease (COPD) and in patients with oral mucositis following radiotherapy for head and neck cancer, (González-Arriagada et al., 2015; De Smet, Seys & Verhamme, 2017) and BPIFA1 expression has been proposed as a potential tool for the detection of lung cancer (Sun et al., 2018). Increased levels of BPIFA2 have been found in acute kidney injury following analysis in urine and blood and thus proposed as a potential biomarker for early diagnosis (Kota et al., 2017).

The underlying mechanisms of oral side-effects during and after HCT are not well established, but may be partly mediated by alterations in the composition and quantity of saliva secreted into the oral cavity. The aim of this study, therefore, was to analyse the expression of BPIFA proteins in the saliva and in the salivary glands of patients who were undergoing HCT and to associate their expression levels with side-effects related to the treatment, which are mainly mucositis, hyposalivation and GVHD.

2 MATERIALS AND METHODS

2.1 Patients

This was a prospective non-randomized, observational study enrolling patients who undergoing HCT at Bone Marrow Transplantation Unit of the Clinical Hospital from University of Campinas (HC-UNICAMP) and at the

Oncology Centre of the Sugarcane Suppliers Hospital (CEON-HFCP) from 2010 to 2013. The clinical study was performed with consecutive patients undergoing allogeneic HCT (allo-HCT) and autologous HCT (auto-HCT). The Institutional Review Board of both centres approved this study and informed consent was obtained from all individual participants included in the study (#142/2010 and #06/2011), and that the study was performed in accordance with the Declaration of Helsinki. Inclusion criteria were patients undergoing first allogeneic or autologous HCT and older than 18 years old. Exclusion criteria was positive history of radiotherapy for head and neck cancer.

Sixty-five patients were enrolled in this study, 52% were male and all patients were aged between 20 and 68 years. Other demographic and clinicopathologic data of both the allo-HCT (n = 50) and auto-HCT (n = 15) participants are provided in (**Table 1).**

2.2 Oral examination

All patients were submitted to oral examination performed by trained dentists prior to transplantation, consisting of physical examination and orthopantomographic image evaluation. Dental and periodontal infections were treated prior to HCT by the Dental Ambulatory Service. Clinical assessment was performed daily starting on the first day of conditioning regimen and ending at patients' discharge. The physical examination was performed by two calibrated dentists in order to establish the severity of oral mucositis according to the World Health Organization (WHO) oral toxicity scale (WHO, 1979) which was classified as absent (Grade = 0), mild (Grade 1 and Grade 2) and severe (Grade 3 and Grade 4).

2.3 Unstimulated saliva collection

Unstimulated saliva was collected from patients undergoing auto-HCT at three different timepoints: pre-conditioning regimen (pre-CR), on day 7 (D+7) post transplantation and 21 days (D+21) post transplantation. Unstimulated saliva was also collected, at 3 different timepoints, pre-CR, at D+8 and at D+80 after transplantation, from patients undergoing allo-HCT, and from 8 patients who developed cGVHD. These time points were selected considering first time the basal saliva condition before the treatment, second time, the worst moment of oral side effects during HCT and third time after expected recovery of therapy and when applicable, a fourth collection for late side effects as cGVHD. All samples were collected before noon and always at least 1 hour after any meal, snack or consumption of beverages. To prevent degradation of the proteins, ethylenediamine tetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF), at final concentrations of 2 mM, were immediately added to the collected saliva and stored on ice. All samples were then centrifuged at 13,000 rpm for 5 minutes at 4°C to remove debris. The supernatant of each sample (upper 2/3) was stored in new vials at -70°C prior to analysis.

Unstimulated salivary secretion rates (USSR) of < 0.5 ml/min were considered as hyposalivation. The procedure to measure salivary flow consisted of a 5-min salivary expectoration into a sterile, previously weighed, glass cup. Patients were asked not to swallow during the collection period. USSR was calculated based on the saliva collected per min and converted to ml/min (Hopcraft & Tan, 2010).

For auto-HCT all 15 patients had saliva collected in three time points and statistical analysis of protein levels were performed for all samples. Nevertheless, fifty patients undergoing allo-HCT were enrolled at the pre-CR period,

three patients died before the second collection point (n=47) and further eleven died before the third collection point (n=36), saliva was collected from all patients and statistical analysis was performed for all samples.

2.4 Western blotting (WB)

The total protein content in the clear supernatant was measured using a Bradford assay (Bradford reagent, Sigma Aldrich, St. Louis, MO) with BSA as a standard and quantification by spectrophotometry (Spectronic – GenesisTM 2, EUA). Samples were mixed with dithiothreitol (DTT) in loading buffer and heated, and 4 µg of protein per well was analysed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE, 12%). After the proteins were transferred onto a nitrocellulose membrane, to confirm the effectiveness of the transference, a Ponceau stain was used and pictures were taken to verify that the same quantity of sample had been applied in each well. All membranes were incubated overnight at 4ºC with skim milk with 5% Tris-buffered saline and Tween 20 (TBST). After four washes of 15 min each, the blots were incubated with primary antibodies against SPLUNC2A (1:500) and SPLUNC1 (1:200), for 2 hours with blocking solution. After four washes, they were incubated with secondary antibody for 1 hour and detection was performed using enhanced chemiluminescence (ECL) from Amersham Biosciences, Inc. The rabbit anti-human SPLUNC monoclonal antibody (isoforms SPLUNC2A and SPLUNC1) used in Western blotting was generously provided by Dr. Lynne Bingle, Sheffield, UK (González-Arriagada et al., 2015). Densitometric analysis of the proteins was performed with the aid of the *Molecular Analyst* software (Bio Rad, EUA). All samples were normalized using the total protein concentration of each sample and a positive control subject was used in each gel to perform all westerns blotting.

2.5 Immunohistochemistry (IHC)

Biopsy samples of the minor salivary glands were taken from patients with oral cGVHD for haematoxylin and eosin (H.E.) stain and immunostaining. Immunohistochemistry was carried out as previously described (L. Bingle et al., 2005; Vargas et al., 2008), using custom made, affinity-purified, anti-peptide BPIFA1 and BPIFA2 antibodies from Eurogentec, with full characterization as previously described (L. Bingle et al., 2009). All slides were digitalized using an Aperio Scan Scope CS Scane (Aperio, Vista, CA, USA) and visualized with Image Scope software (Aperio, Vista, CA, USA) using pixels in a 22.148µm².

2.6 Statistical analysis

Patients were included by convenience when eligible for inclusion criteria. All data were analysed using SAS (SAS Institute Inc., *SAS/STAT® User's Guide, Version 9*, Cary, NC: SAS Institute Inc., 2003). Nominal variables were tested with a Fisher's Exact test. Normal and non-normal distributed groups were compared by analysis of variance (ANOVA) and with Wilcoxon rank sum test, respectively. A parametric t-Student test was performed to compare continuous variables in different periods. ANOVA based on a generalized linear mixed model with repeated measures was applied to test the effect of pre-CR for HCT on BPIFA expression. A significance level of 5% (0.05) was considered in all tests. All graphics were made using R Development Core Team (Vienna, Austria, 2016).

3 RESULTS

3.1 Salivary assessment - Autologous transplant patients

BPIFA proteins associated with phase of sample collection. A significant variation in the salivary BPIFA1 expression was seen among the three timepoints ($P = 0.04$) in 15 patients evaluated. The expression was significantly greater at D+7 than D+21 ($P = 0.02$), however, no significant difference was found between pre-CR and D+7 ($P = 0.09$) (**Figure 2a**). Expression of the glycosylated form of BPIFA2 was significantly increased at D+7 in comparison to D+21 ($P = 0.04$) (**Figure 2b**). There was no significant difference between pre-CR and D+7 ($P = 0.39$) or pre-CR and D+21 ($P = 0.21$).

3.1.1 BPIFA proteins associated with side effects

Mucositis x BPIFA. Mucositis was present in 11/15 (73%) of patients at D+7, 6/15 (40%) presented with mild and 5/15 (33%) with severe mucositis. The incidence of side-effects was higher on day 7 post transplantation and by day 21 post transplantation all patients reported a significant improvement in oral discomfort. BPIFA1 expression was significantly higher on D+7 than pre-CR (**Figure 2a**), however, no association was seen with the mucositis score ($P = 0.16$) (**Table 2**). There was also no significant difference between the expression of glycosylated (35 kDa) ($P = 0.39$), non-glycosylated (25 kDa) ($P = 0.66$) total BPIFA2 ($P = 0.57$) and mucositis (**Table 2**).

Hyposalivation x BPIFA. Eight (53%) out of 15 pre-CR patients, 11/15 (73%) at D+7 and 10/15 (67%) at D+21 showed signs of hyposalivation as measured by USSR. The median was 0.36 at D+7, with decreased USSR ($p = 0.04$) (**Figure 1**). BPIFA1 levels were not associated with hyposalivation ($P = 0.65$). There was also no significant difference between the expression of glycosylated ($P = 0.13$), non-glycosylated ($P = 0.41$) or total BPIFA2 ($P = 0.13$) and hyposalivation (**Table 2**).

3.2 Salivary assessment - Allogeneic transplant patients

BPIFA proteins associated with phase of sample collection. Expression of the glycosylated form of BPIFA2 was decreased at D+80 (n = 36) when compared with pre-CR ($P = 0.01$) (**Figure 3a**). No significant difference was seen between pre-CR (n = 50) *x* D+8 (n = 47) and D+8 *x* D+80, ($P = 0.35$) and ($P = 0.09$), respectively.

3.2.1 BPIFA proteins associated with side effects

Mucositis x BPIFA. Mucositis was present in 37 (79%) patients included for the second collection point, with 23 (49%) affected by mild mucositis and 14 (30%) by severe mucositis. There was no significant association between mucositis score and total BPIFA2 expression ($P = 0.30$) **(Table 3)**.

Hyposalivation x BPIFA. Out of 50 patients, 26 (52%) showed hyposalivation at pre-CR as shown by USSR, but only 15 (32%) at D+8 and 19 (53%) at D+80. The median USSR was increased at D+8. Analysis of the quality and quantity of saliva suggested a decrease in total protein even when the salivary flow rate remained near normal at D+8. A statistically significant association between hyposalivation and glycosylated BPIFA2 ($P = 0.02$) and total BPIFA2 ($P = 0.02$) was found at D+8. There was no statistical significance between total BPIFA2 expression and hyposalivation at D+80 ($P = 0.07$) (**Table 3**). A progressive but statistically significant decrease ($P = 0.01$) in USSR was observed from D+8 to D+80 (0.69±0.40, 0.50±0.39 mean and standard deviation respectively) (**Figure 1**).

Acute GVHD onset x BPIFA. Acute GVHD (aGVHD) developed in 24 (67%) patients at D+ 80, but there was no significant association between aGVHD and total BPIFA2 levels (P = 0.09). Patients with mucositis who developed aGVHD showed glycosylated BPIFA2 levels under the median (1.375). However, at the onset of aGVHD, there was no statistically significant association between glycosylated BPIFA2 (P = 0.20), non-glycosylated BPIFA2 (P = 0.62) or total BPIFA2 (P = 0.09) (**Table 3**).

3.3 Chronic GVHD x BPIFA

Eight patients with a diagnosis of cGVHD (four men and four women) had saliva collected. Western blot analysis of BPIFA2 showed that the non-glycosylated form of BPIFA2 was significantly lower in the cGVHD patients when compared to baseline (P = 0.04) (**Figure 3**). Minor salivary glands obtained from cGVHD patients demonstrated acinar atrophy and destruction as well as inflammatory chronic infiltrate in the periductal area **(Figure 4a**). BPIFA1 was expressed in mucous cells (**Figure 4b**), while BPIFA2 in serous cells and serous demilunes (**Figure 4c**). Immunostaining in normal control tissue showed serous acini strongly stained with BPIFA2 in parotid gland parenchymal tissue (**Figure 4d**). Mucous acini in normal minor salivary glands stained less intensively with BPIFA1 in comparison to glands from patients with cGVHD (**Figure 4e**). Demilunes in normal minor salivary glands also stained less intensively with BPIFA2 in comparison to minor salivary glands from cGVHD patients (**Figure 4**). The differences in staining intensity, however, were not statistically significant.

4 DISCUSSION

The proteomic analysis of saliva and salivary gland tissues might be useful for the identification of novel biomarkers and could provide significant information surrounding the oral manifestation of diseases (Presland, 2017; Saxena, Sankhla, Sundaragiri, & Bhargava, 2017). The aims of this clinical and laboratory study were to understand whether changes in innate immune salivary proteins such as BPIFA might be associated with oral mucositis, hyposalivation and GVHD.

Previous studies have shown that BPIFA levels are often decreased in diseased groups when compared with healthy control subjects, for instance in the upper respiratory tract of smokers and workers exposed to reactive epoxy chemicals, in the nasal fluid from persons with allergic rhinitis, in the nasal polyps from patients with chronic rhinosinusitis and in diabetic and rheumatoid arthritis patients with xerostomia (Border et al., 2012; Ghafouri, Irander, Lindbom, Tagesson, & Lindahl, 2006; Ghafouri, Kihlström, Ståhlbom, Tagesson, & Lindahl, 2003; Tsou et al., 2014; Zalewska et al., 2013). On the other hand, BPIFA1 expression was increased in the nasal respiratory epithelium of rats following olfactory bulbectomy, while BPIFA1 mRNA levels were elevated in the lungs of patients with chronic obstructive pulmonary disease (Di et al., 2013; De Smet et al., 2017; Sung et al., 2002). Our study showed increased BPIFA1 expression in patients undergoing auto-HCT at D+7 probably due in part to the increased proportion of mucous saliva present at this time, when saliva was noticeably stickier.

González-Arriagada et al. (2015) showed that higher levels of BPIFA1 were associated with the presence and severity of mucositis in patients submitted to radiotherapy for head and neck cancer. We did not find such association in our groups and differences in relation to BPIFA levels and these cancer therapy side effects could be explained by

the more aggressive and direct effect of head and neck radiotherapy to salivary glands than high dose therapy associated with Total Body Irradiation (TBI). Previous studies have suggested that mucositis-related symptoms were more prominent in the presence of impaired mucosal antioxidant and immunological defence systems (Avivi et al., 2009) justifying our hypothesis that there is an association between BPIFA expression and mucositis.

Allo-HCT and auto-HCT are complex and high-risk procedures requiring significant expertise to manage the potential treatment complications. cGVHD is a life-threatening side-effect of allo-HCT; with 60% of patients present with signs of xerostomia and 57% with lichenoid lesions (Soares, Correa, Cintra, Miranda, & Cintra, 2013). The preservation of salivary gland function is extremely important in maintaining oral health and macrobiotic balance (Dodds, Johnson, & Yeh, 2005). In this study whole saliva was collected as it is a major determinant of the environment of the entire oral mucosa surface. It is well known that saliva plays an important role in physicochemical as well as immune defense of the oral and mucosal surfaces, with both direct antimicrobial actions and agglutination or surface exclusion of microbes.

Interestingly, the most important change in BPIFA expression was found during the neutropenic period (D+7). During the pancytopenia period when the second saliva sample was collected, no patient had HSC engraftment, and host immunity changes were expected (Guinan et al. 2015; Lee, Kang, & Choi, 2017). The success of HCT depends upon infection control during the neutropenic phase, GVHD and relapse (Tanaka et al., 2011). Additionally, the upregulation of some components of the innate immune system, such as the BPI fold-containing family, which are increased in chronically inflamed diseases, may help to explain some of the changes seen during the pancytopenia period (L. Bingle et al., 2012; Yang, Liu, Liu, Cheng, & Li, 2006). It has been established that, during the period of neutropenia, severe mucositis is a cause of septicemia, and thus it is important to fully analyse the patient's saliva. It is possible that, following further analysis, increased BPIFA protein might help to predict the development of infection (systemic or local) in transplant patients.

N-glycosylation of BPIFA2 was demonstrated following PNGase F treatment which removes N-linked sugars. This resulted in the reduction of three bands on a western blot to only one of approximately 25 kDa, the predicted molecular weight based on amino acid sequence. To date no differences in function have been identified for the isoforms, but was hypothesise that the adhesion of the protein to bacteria, teeth or tissues might be facilitated by attached sugars (L. Bingle et al., 2009; Gorr, Abdolhosseini, Shelar, & Sotsky, 2011; Ramachandran et al., 2006). A significant variation for BPIFA expression was observed between three timepoint suggesting a variety of changes in the host immunity of these patients.

BPIFA2 has a very restricted pattern of expression being found only in serous cells of the major salivary glands and in the sero-mucus tubules of the minor salivary glands in the oral mucosa, posterior tongue, and tonsil (C. D. Bingle, Bingle, & Craven, 2011; L. Bingle et al., 2009; Vitorino et al., 2004). Very few studies have investigated BPIFA2 expression in disease subjects, however, reduced levels have been found in the saliva of patients with periodontitis (Wu, Shu, & Liu, 2011) and an increased expression of BPIFA2 was seen in the human immunodeficiency virus patients who were infected with cytomegalovirus or with mycobacteria (da Silva et al., 2011). Some limitations of the current study were that the association between the development of oral infectious during HCT and BPIFA2 expression levels was not considered. This occurred because of saliva sample availability

BPIFA1 analysis was not performed for allo-HCT group. In addition, the inclusion of more patients in auto-HCT group was not possible.

Reduced salivary flow rates due to cGVHD cannot be clinically differentiated in first year after HCT from those due to radiation damage (Dens et al., 1996). Chaushu et al (1994) demonstrated increased USSR at D+8 in patients undergoing allo-HCT as a result of mucositis. Our results also demonstrate increased USSR on day D+8 post-transplantation compared to our baseline assessments. The majority of our patients, however, did not suffer from hyposalivation, but it is important to acknowledge that in the allo-HCT only seven patients were submitted to TBI.

This paper is the first to show localization of either BPIFA1 or BPIFA2 in minor salivary glands of cGVHD patients. Previous studies have shown the expression in the major salivary glands of HIV-infected patients, (da Silva et al., 2011) in the lacrimal gland of patients with Sjogren's Syndrome, (Schicht et al., 2015) and in high-grade mucoepidermoid carcinoma, suggesting a potential diagnostic biomarker of salivary gland disease (González-Arriagada et al., 2012; Vargas et al., 2008). The decrease in BPIFA1 and BPIFA2 in the GVHD group of this study could be explained by the decrease in the number of glands in the pathological tissue due to inflammation. Oral health is directly influenced by adequate salivary gland function (van Leeuwen, Potting, Huysmans, & Blijlevens, 2019). Patients receiving a bone marrow transplant may present with alterations in salivary flow, altered buffering capacity, and changes in the microbiome all resulting in an increased risk of caries (Dens et al., 1996). These findings suggest that for transplant recipients there could be a higher caries risk with oral complications such as mucositis during the early post-transplant period (Coracin et al., 2013; Melkos, Massenkeil, Arnold, & Reichart, 2003). The low incidence of severe oral mucositis in our study might be related to the previous dental care given to all patients.

In summary, we have demonstrated that changes in BPIF levels in patients undergoing auto-HCT, allo-HCT and with oral cGVHD development may be related to changes in saliva composition, suggesting BPIF as a potential biomarker for the oral side effects HCT. This is the first study to analyse expression of these proteins in saliva HCT patients, but further studies aimed at understanding the potential of this protein to predict the development of oral side effects and oral cGVHD are needed.

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CONFLICTS OF INTEREST: none to declare.

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FIGURE LEGENDS

Figure 1. Unstimulated salivary secretion rates in the allo-HCT and auto-HCT groups. (**a)** USSR for auto-HCT showed decreased at D+7 ($P = 0.04$). (**b)** USSR for allo-HCT showed increased at D+8 when compared with the other periods, Pre-CR, D+80 and cGVHD ($P = 0.01$). *$P \leq 0.05$ indicates statistical significance.

Figure 2. BPIFA concentration in saliva from 15 patients in auto-HCT group (**a**) BPIFA 1 Pre-CR *versus* D+7 *versus* D+21 ($P = 0.04$), Pre-CR *versus* D+7 ($P = 0.09$) and D+7 *versus* D+21 ($P = 0.02$). (**b**) BPIFA2 gly (35 kDa) presenting higher expression at D+7, D+7 *versus* D+21 ($P = 0.04$). *$P < 0.05$ indicates statistical significance. The positions of molecular mass markers (kDa) are shown.

Figure 3. Differential expression of BPIFA2, showing four time points comparison pre-CR (n=50), D+8-10 (n=48), D+80-100 (n=37) and cGVHD (n=8) in saliva from allo-HCT group. Comparison at pre-CR *versus* D+80 ($P = 0.01$) for BPIFA2 gly forms (35 kDa) was statistical significant, and at pre-CR *versus* D+8 ($P = 0.003$), pre-CR *versus* D+80 ($P < 0.0001$) and pre-CR *versus* cGVHD ($P = 0.04$) for BPIFA2 non-gly forms (25 kDa). Third gel is correspondent to saliva collected from 8 patients with cGVHD. *$P < 0.05$ indicates statistical significance. The positions of molecular mass markers (kDa) are shown for the stained gel (arrowheads).

Figure 4- BPIFA expression pattern in minor salivary glands of patients with oral cGVHD; **(a)** Minor salivary glands showing inflammatory infiltrate with atrophic and damaged acini, (H&E 100x); (**b)** BPIFA1 expression in mucous acini of minor salivary glands in patients with cGVHD showing stronger staining in mucous acini (IHC, 100x); (**c)** BPIFA2 expression in serous acini and serous part of semilunar acini of minor salivary glands in patients with cGVHD (IHC, 100x). **(d)** Serous acini strongly stained with BPIFA2 in parotid gland tissue; (**e**) **control -** normal minor salivary gland stained with BPIFA1 showing mucous acini stained with less intensity; (**f**) normal minor salivary gland stained with BPIFA2 showing semilunar acini stained with less intensity when compared to minor salivary glands compromised by cGVHD.

TABLES

TABLE 1. Population characteristics of HCT recipients

Variable	Transplant type, N (%)
Male: Female	34 (52): 31 (48)
Age median (range years old)	45.5 (20-68)
Transplant type	
Autologous	15 (23)
Allogeneic	50 (77)
Underlying disease	
NHL	11 (17)
HL	5 (8)
MM	9 (14)
Myelofibrosis	5 (8)
CML	12 (18)
CLL	3 (5)
ALL	3 (5)
AML	11 (17)
Others	6 (9)
Conditioning regimen	
Myeloablative	46 (71)
Reduced intensity	14 (21)
Mini-transplant	5 (8)

Abbreviations: n = number; NHL = non-Hodgkin lymphoma; HL = Hodgkin lymphoma; MM = multiple myeloma; BU = busulfan; FLU = fludarabine; ATG = antithymocyte globulin; BEAM = armustine, etoposide, cytarabine, and melphalan; BEAM-like = lomustine, etoposide, cytarabine and melphalan. Others disease – AA

= aplastic anemia (4), FA = Fanconi anemia (1), PNH = paroxysmal nocturnal hemoglobinuria (1). ¥ - allo-HCT.

TABLE 2. Secondary effects associated to conditioning regimen for auto-HCT (n = 15)

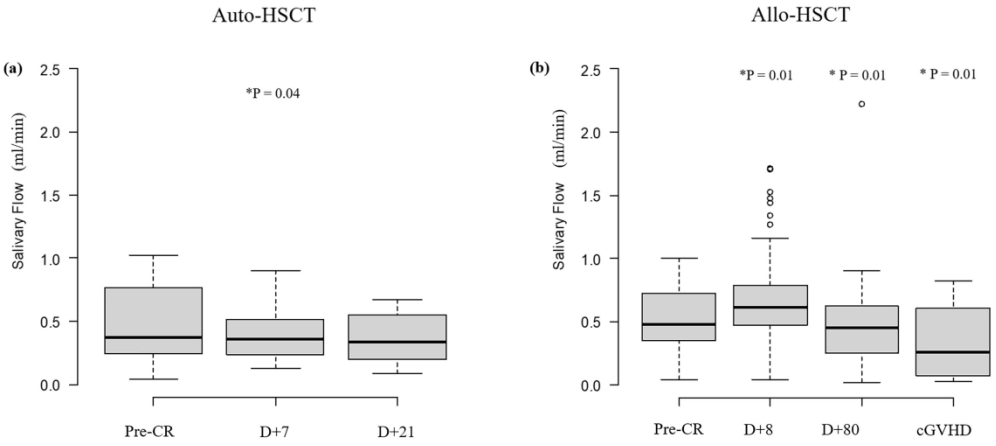
Protein	Hyposalivation			Mucositis		
	Yes (n = 11)	No (n = 4)		Yes (n = 11)	No (n = 4)	
	Median/SD	Median/SD	*P*	Median/SD	Median/SD	*P*
BPIFA1	0.02/1.37	1.12/0.56	0.65	0.96/1.19	0.56/1.19	0.16
BPIFA2 gly	1.48/2.04	3.71/1.14	0.13	2.68/1.93	2.47/2.00	0.39
BPIFA2 non-gly	2.59/4.47	4.91/7.45	0.41	3.47/5.28	2.37/2.60	0.66
BPIFA2 total	4.54/5.94	7.57/8.01	0.13	5.09/7.09	4.68/4.10	0.57

*Western-blotting performed showed BPIFA2 gly-BPIFA2 glycosylated form; BPIFA2 non-gly-BPIFA2 non-glycosylated form. Saliva collected at D+7.

Protein	Hyposalivation*			Mucositis*			aGVHD#		
	Yes (n = 15)	No (n =32)		Yes (n = 37)	No (n = 10)		Yes (n = 12)	No (n=24)	
	Median/SD	Median/SD	*P*	Median/SD	Median/SD	*P*	Median/SD	Median/SD	*P*
BPIFA2 gly	1.34/0.48	1.53/0.75	0.02	1.47/0.70	1.22/0.80	0.27	0.83/1.38	0.78/1.35	0.20
BPIFA2 non-gly	0.49/0.56	0.543/0.59	0.13	0.52/0.61	0.55/0.61	0.90	0.36/0.47	0.36/0.45	0.62
BPIFA2 total	1.628/0.95	2.279/1.05	0.02	2.27/1.03	2.10/1.14	0.30	1.54/0.84	1.27/0.61	0.09

TABLE 3. Secondary effects associated to conditioning regimen for allo-HCT (n = 47)

*Western-blotting was performed in 47 patients in allo-HCT group; BPIFA2 gly - BPIFA2 glycosylated form; BPIFA2 non-gly - BPIFA2 non-glycosylated form. # At D+80 with n = 36. Bold numbers show significant values, $P < 0.05$.



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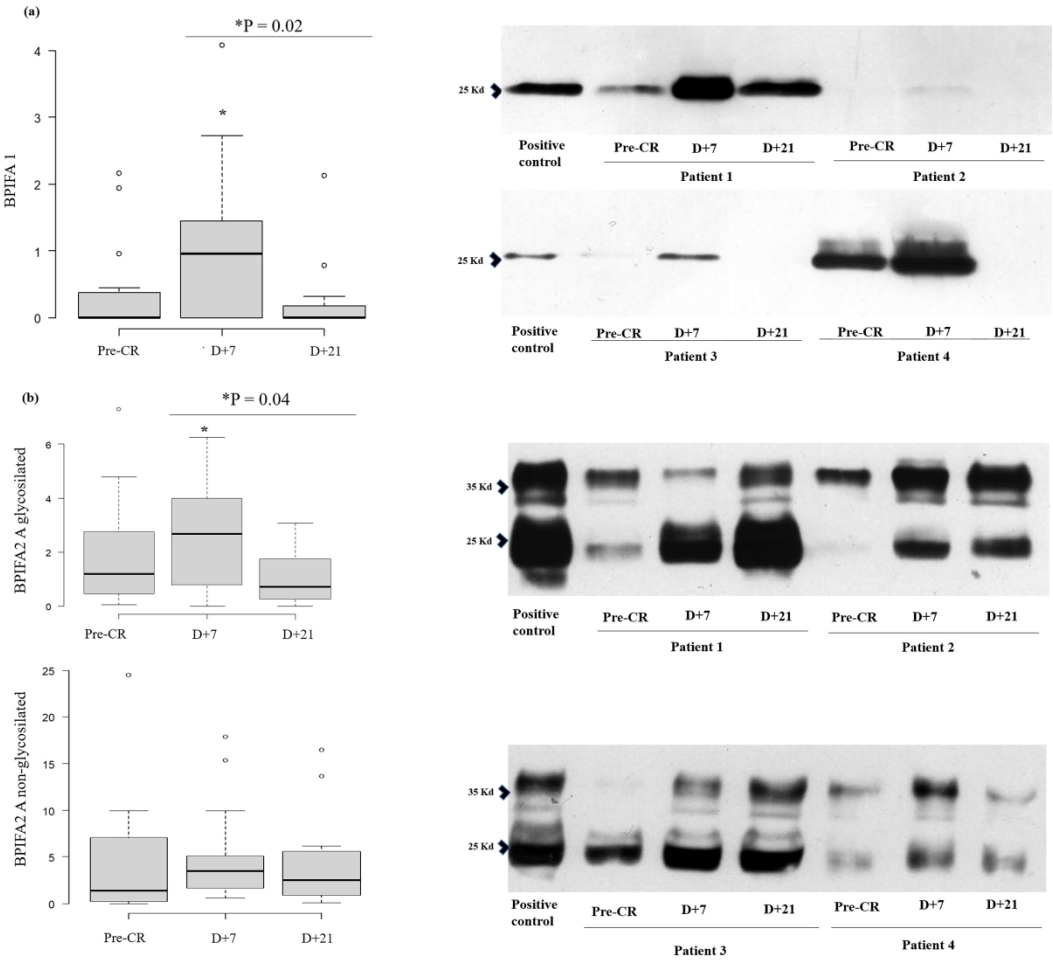

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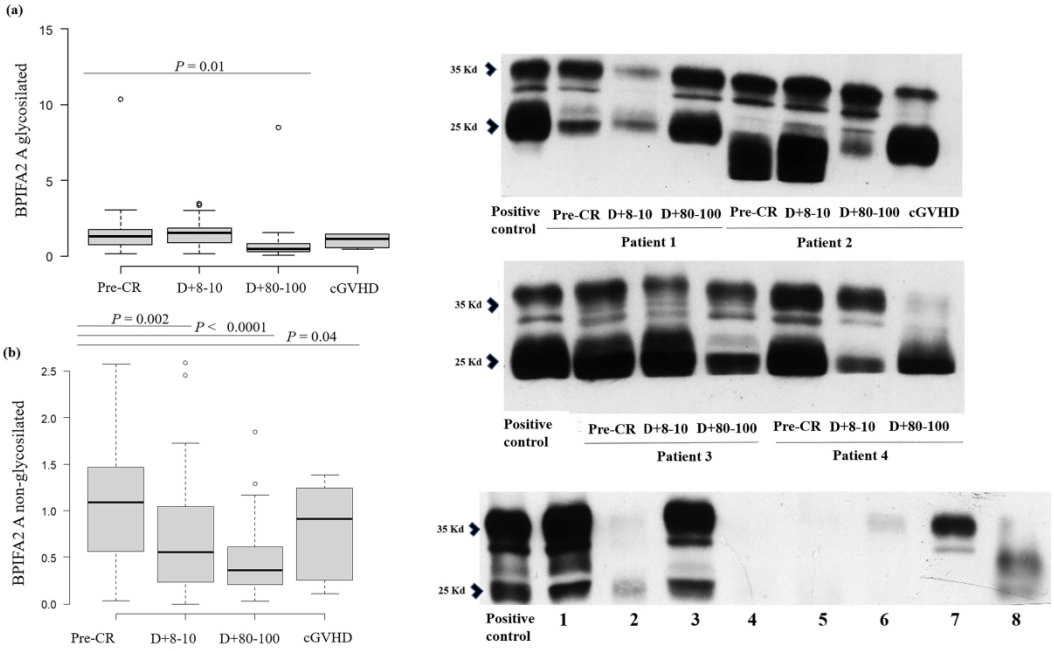

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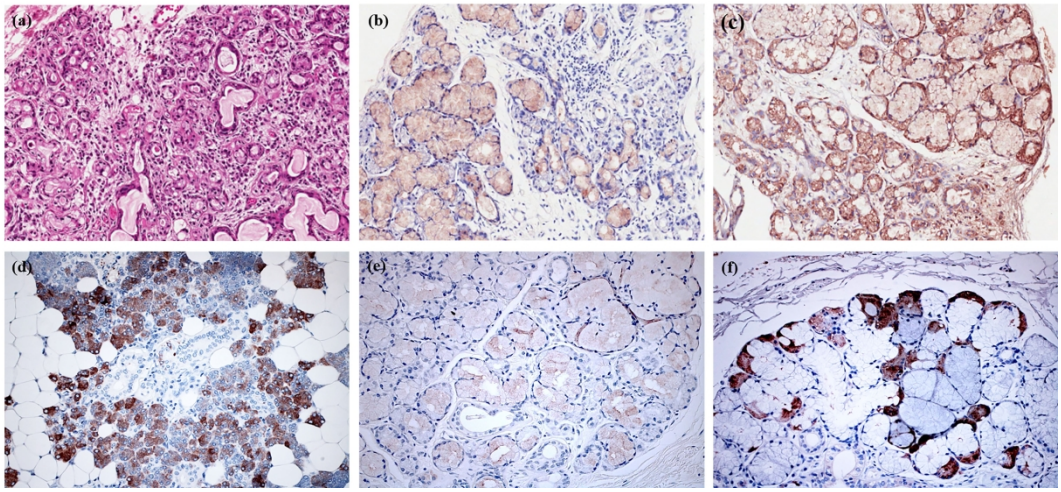

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