ORIGINAL ARTICLE

Expression of salivary biomarkers in patients with oral mucositis: evaluation by SELDI-TOF/MS

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OBJECTIVE: This study aims to evaluate changes in proteomic salivary profile of patients with oral mucositis after adjuvant cancer treatments.

MATERIALS AND METHODS: Samples were collected from patients after adjuvant cancer therapies, and were analyzed by means of SELDI/TOF. Patients were separated in two groups: patients affected by mucositis (MUCOSITIS) and patient without mucositis (NO MUCOSITIS). All patients were divided in function of the anticancer treatment: patients who had radiotherapy (MUCOSITIS RADIO), had not radiotherapy (MUCOSITIS NO RADIO), had chemotherapy (MUCOSITIS CHEMO), and those who had not chemotherapy (MUCOSITIS NO CHEMO). Statistical evaluation PCA (Principal Component Analysis) was conducted with the software BIO-RAD Data Manager™ (Version 3.5).

RESULTS: We found the increased peaks of 3443, 3487, and 4135 m/z in MUCOSITIS group, while 6237 m/z was reduced. These same peaks would the same modifications in MUCOSITIS RADIO, while in MUCOSITIS CHEMIO are increased 3443 and 6237 m/z but 3487, 4135 m/z are reduced. These data were confirmed by the PCA.

CONCLUSION: Anticancer therapy influenced the level expression of many salivary biomarkers in mucositis with a good significance. Therefore, 3443, 3487, 4135, and 6237 m/z are good biomarker candidates of oral mucositis.

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Introduction

Oral mucositis is the most severe complication of anticancer therapies. It occurs in 40–85% of patients during chemotherapy and radiotherapy but also in patients who have undergone hematopoietic stem cell transplantation (Stiff, 2001; Peterson *et al*, 2011).

Many studies have identified four developmental stages of oral mucositis: an initial stage, in which inflammation is triggered by a therapeutic agent; the epithelial stage, in which the action of drug reduces the rate of cell division; the ulceration stage, caused by the loss of the epithelial component; and lastly, the healing stage (Sonis, 2004).

During radio and chemo therapy, smoking and alcohol abuse, poor oral hygiene, burning mouth syndrome, surgical oral procedures, and pre-existing diseases of oral cavity may be significant risk factors; a severe mucositis can lead to partial or complete interruption of radiotherapy before completion of the treatment protocol with a consequent worsened cancer prognosis (Al-Dasooqi *et al*, 2013; Campos *et al*, 2014).

Some patient-related variables, such as age and gender, may influence onset and severity of mucositis. It has been noticed that the risk of developing mucositis is higher in children than in adults (Cheng *et al*, 2001; Sonis and Fey, 2002). In subjects over 50 years old, the risk of developing severe and long-term mucositis is high because reduced renal excretion may alter the elimination of chemotherapeutic drugs (Raber-Durlacher *et al*, 2000).

In women, the risk of mucositis caused by 5-fluorouracile (McGuire, 2002), one of the most important chemotherapeutic agents responsible of mucositis, is higher than in men (Sloan *et al*, 2000, 2002). Moreover, incidence of mucositis is higher in patients treated with continuous infusion 5-fluorouracil compared to those receiving intermittent intravenous administration (Hansen *et al*, 1996). The signs and symptoms of mucositis are oral burning, oral pain, spontaneous bleeding, dysphagia, dysarthria, and odynophagia. Furthermore, the loss of continuous oral barrier is a risk factor for secondary infections (i.e., bacterial, fungal, and viral

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infections) that could disseminate in neutropenic patients (Steinmann *et al*, 2012). In particular, oral burning and pain during swallowing determine worsening of the quality of life in oncologic patients and, in severe cases, may also force the patient to parenteral nutrition (Barber *et al*, 2007; McGuire *et al*, 2013).

Nowadays, in clinical practice there are a number of preventive and therapeutic measures for oral mucositis. The use of local anesthetics and analgesics has been recommended by some authors (Sonis, 2009; Roopashri *et al*, 2011), to reduce pain, which is the most troublesome component of oral mucositis, that negatively affects patient's quality of life. Application of benzydamine hydrochloride, a non-steroidal anti-inflammatory with analgesic, anesthetic, and antimicrobial properties for topical use, has proven to be effective as it reduces the frequency and severity of ulcerative lesions of the mouth and therefore reduces pain (Worthington *et al*, 2007).

Cryotherapy practice is commonly indicated for the management of lesions caused by mucositis. Holding an ice cube in the oral cavity for 5 min before chemotherapy and for 30 min after causes local vasoconstriction and hence reduces the amount of drug reaching the oral mucosa, alleviating the symptoms of mucositis. This method not only provides pain relief, but may also prevent the development of new lesions (Peterson *et al*, 2013).

Helium-neon laser therapy, with low intensity laser, is indicated as a pretreatment to reduce the severity of mucositis in patients undergoing chemotherapy. However, as this type of treatment requires costly equipment and specialized operators, its use is often restricted to a limited number of patients (Migliorati *et al*, 2013; Raber-Durlacher *et al*, 2013).

However, despite these therapies, used alone or in combination with antimicrobials, aimed to prevent or reduce the severity of oral mucositis, there are not evidence-based protocols at the moment (Steinmann *et al*, 2012).

Many researchers are investigating the mechanism that leads to the development of mucositis, and recently have identified proinflammatory cytokines and matrix metalloproteinases as biomarkers of oral mucositis (Logan *et al*, 2007; Al-Dasooqi *et al*, 2010).

In order to prevent a possible outbreak and offer new starting points to find the most effective preventive treatment and therapy, primary aim of this case control study is to identify potential biomarkers of oral mucositis, evaluating changes of the proteomic salivary profile of patients with oral mucositis after adjuvant cancer treatments (chemo and/or radiotherapies).

Furthermore, we sought to understand the extent to which radio- and chemo-therapeutic treatments affect the changes in the expression levels of biomarkers, assessing patients with both mucositis *vs* control.

The saliva is a good diagnostic fluid (Bigler *et al*, 2002; Kaufman and Lamster, 2002; Streckfus and Bigler, 2002; Hu *et al*, 2007a,c), and its collection is easy and non-invasive, contains a wide range of proteins, many of which have been shown to be informative for the detection of oral (Li *et al*, 2004; Hu *et al*, 2007b, 2008; Bigler *et al*, 2009; Park *et al*, 2009)and systemic diseases(Xiao and Wong, 2010; Zhang *et al*, 2010a,b).

The technique that enables a rapid and high-throughput detection of proteins and peptides directly from crude mixtures is SELDI-TOF-MS. This has been used in a number of studies related to diagnostic screening, biomarkers discovery in clinical proteomics researches.

For this reason, the study was developed, thanks to the SELDI-TOF/MS (Surface Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry) technology with ProteinChip array® (Bio-Rad, Hercules, CA, USA) (Streckfus *et al*, 2006).

Salivary samples from patients with cancer who developed oral mucositis following radio- and chemotherapy, and from patients who did not develop the disease, were analyzed to identify different levels of expression of potential biomarkers of oral mucositis.

Materials and methods

Patient population

In this study, 60 saliva samples from patients with several tumor types were recruited, among which 30 patients suffered from oral mucositis (mucositis) and 30 did not (no mucositis). All patients underwent multiple cycles of chemotherapy and/or radiotherapy before saliva collection (the medical records of all patients included in the study are shown in Table 1).

The patients were grouped according to their treatment: subjects with mucositis who underwent radiotherapy (Mucositis R+), patients with mucositis who did not undergo radiotherapy (Mucositis R−), subjects who did not develop mucositis and have underwent radiotherapy (No Mucositis R+), subjects who did not develop mucositis and did not undergo radiotherapy (No Mucositis R−), patients with mucositis who underwent chemotherapy (Mucositis C+), and patients with mucositis who did not undergo chemotherapy (Mucositis C−) subjects who did not develop mucositis and underwent chemotherapy (No Mucositis C+).

The main chemotherapeutic reagents given to patients were the following: 5-fluorouracil, Herceptin (trastuzumab), and cisplatin.

Saliva collection and processing

Saliva samples were collected in the morning after an accurate washing of the mouth with water. All patients abstained from food and beverages, smoking, and oral hygiene for at least 2 h before the sample collection. Saliva was produced spontaneously without any kind of stimulation and was collected by spitting directly inside of a 50-ml sterile test-tube. Before the sample collection, every patient signed a specific informed

Table 1 Medical records of all patients included in the study.

	Mucositis		No Mucositis	
	n	%	n	%
Age (years)				
Mean ± s.d.	63 ± 10.6		56.43 ± 10.95	
Range	34–90		30–83	
Sex				
Male	13	43.3	15	50
Female	17	56.7	15	50
Tumor sites	16	53.3	9	30
Head and neck	5	16.7	12	40
Breast	4	13.3	4	13.3
Lung	2	6.7	5	16.7
Prostate	2	6.7	0	0
Ovary	1	3.3	0	0
Anticancer therapy				
Radiotherapy	12	40	6	20
No radiotherapy	18	60	24	80
Chemotherapy	27	90	30	100
No chemotherapy	3	10	0	0

consent. The study has been approved by the ethical committee of the Azienda Ospedaliera Universitaria della Seconda Università di Napoli – n. prot. 165 – 12 April, 2011.

Saliva collection was stored at −80°C for subsequent analysis.

Before the analysis, samples were gradually freeze thawed, and a cocktail of protease inhibitors (104 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM pepstatin A) in a ratio of 1:1000 was added to each sample.

After a centrifugation of 50 min at 18 130 ×g, the supernatant has been aliquoted, proteins have been assayed according to Bradford's Method, and the supernatant has been stored at −80°C until use.

ProteinChip array® preparation and SELDI-TOF analysis

All samples, appropriately pretreated, were analyzed in duplicate with Q10 ProteinChipArray® SELDI-TOF analysis.

Table 2 Differently expressed mass peaks between MUCOSITIS group and NO MUCOSITIS group.

m/z	P-value	ROC	Trend Mucositis	Fold change
3443	1.48E-04	0.733737564	Increased	3.6
3487	0.033147121	0.660238422	Increased	2.89
4135	0.057320016	0.631334477	Increased	1.9
5133	0.026813933	0.327615780	Reduced	−1.87
6237	0.037	0.311324185	Reduced	−3.07
6318	0.012	0.674603175	Reduced	−2.29
10 091	8.27818E-05	0.700171527	Increased	2.04

Each ProteinChip was subjected to two consecutive washings with 200 μl of binding buffer (100 mM Tris–Hcl pH 8.8) followed by the addition of the sample. Ten micrograms of salivary proteins were added to DB3 denaturing buffer (9 M urea, 2% CHAPS and 100 mM DTT) with ratio vol/vol 2:3, and subsequently diluted in binding buffer to reach a final volume of 150 μl. Each sample was, therefore, spotted in duplicate on the surface of the ProteinChip and incubated for 30 min on the orbital shaker under constant shaking at 250 rpm.

After incubation, washes were performed: first, washes with 150 μl of binding buffer, then a quick wash with 200 μl of Milli-Q water (Millipore, Molsheim, France).

Finally, 1 μl of matrix composed of 50% saturated solution of synaptic acid and 50% ACN/0.1% TFA (50% ACN/0.5% TFA for CHCA) was applied to each spot twice.

All chips were analyzed setting the instrument in the same condition, according to the Protocol Machine (6000 nJ low energy laser, high 6000 nJ; matrix attenuation in 2500 Da, focus mass 10 000 Da; sample rate 800 MHz, covering 25% of the surface area of the spot, acquired mass range from 2500 to 25 000 m/z).

The proteomic profiles of all the samples were analyzed with the software BIO-RAD Data Manager™ (Version 3.5) to identify differentially expressed mass peaks (clusters) in the two groups with a significance of $P < 0.05$.

For this study, we used only the mass peaks in the range between 2500 and 25 000 m/z with a signal-to-noise ratio higher than 5 S/N.

Principal Components Analysis (PCA)

The data sets' proteins were also analyzed by PCA (principal components analysis), a nonparametric statistical analysis method to extract the most relevant information from large data sets. It is used in BIO-RAD Data
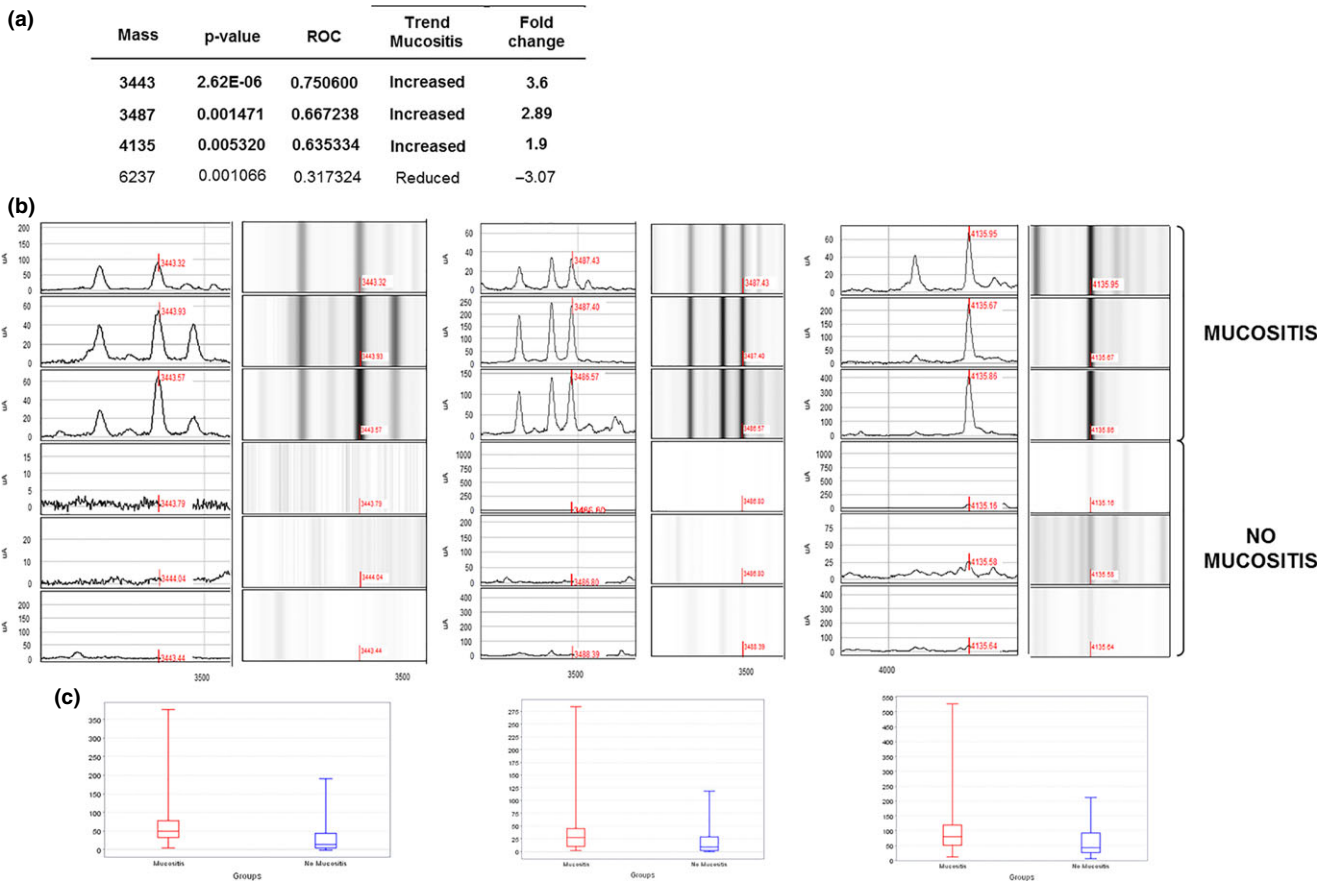

Figure 1 List of the differently excreted mass peaks in MUCOSITIS compared to NO MUCOSITIS patients (**a**). Mass spectra, gel-view of 3443, 3487 and 4135 m/z (**b**). Down, respectively, its group box and whiskers plot indicating 0, 25th, 50th, 75th, and 100th percentiles for sample groups within a cluster (**c**)
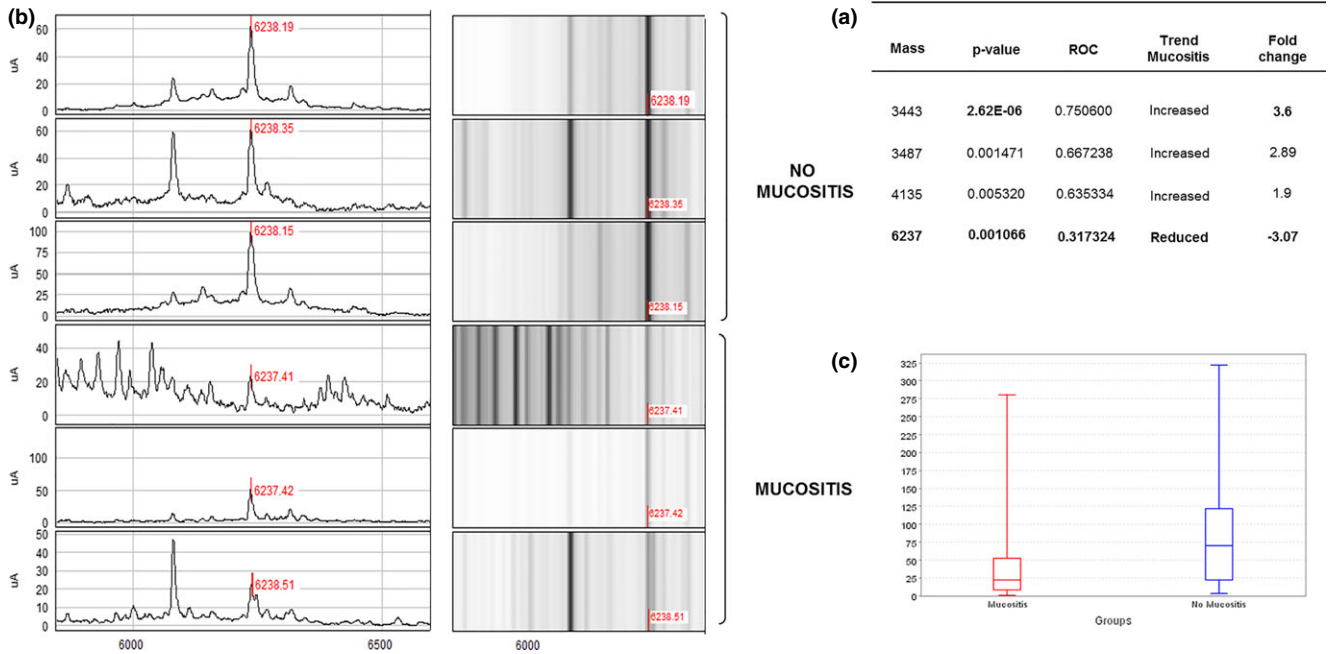
Figure 2 List of the differently excreted mass peaks in MUCOSITIS compared to NO MUCOSITIS patients (**a**). Mass spectra, gel-view of 6237 *m/z* (**b**). Corresponding box and whisker plot (**c**)

Table 3 Differently expressed mass peaks between MUCOSITIS RADIO group and MUCOSITIS NO RADIO group.

m/z	P-value	ROC	Trend Mucosits radio	Fold change
3443	0.043	0.727273	Increased	2.14
3487	0.038	0.597403	Increased	1.25
4135	0.019	0.662338	Increased	2.13
4306	0.037	0.305195	Reduced	−3.1
4472	0.043	0.272727	Reduced	−1.44
4639	0.043	0.272727	Reduced	−1.55
5227	0.049	0.75974	Increased	1.93
5672	0.031	0.532468	Increased	2.25
6237	0.04	0.532468	Increased	1.44
9804	0.049	0.24026	Reduced	−2.41

Table 4 Differently expressed mass peaks between MUCOSITIS CHEMIO group and MUCOSITIS NO CHEMO group.

m/z	P-value	ROC	Trend Mucosits Chemo	Fold change
3443	0.025564	0.424242	Increased	1.34
3487	0.038675	0.424242	Reduced	−1.17
5329	0.086474	0.871212	Increased	2.1
5381	0.03103	0.727273	Increased	2.1
5785	0.021467	0.121212	Reduced	−2.59
6237	0.004772	0.651515	Increased	2.98

Manager™ software (Version 3.5) to visualize spectra in two and three-dimensional graphs.

The principle of this analysis is briefly summarized below.

Each of the mass peaks SELDI presents, both in repeated measurements of the same sample and in different samples, a deviation between the signal and the background noise (variance). Each of the peaks considered may present high variance in repeated measurements of the same sample (low redundancy) or vice versa and maintains a low deviation (high redundancy).

In the PCA, only the peaks that have, in the same sample, a high redundancy are considered, to remove from the analysis the background noise which is a confounding factor. As the analysis SELDI generates a complex data set consisting of hundreds of mass peaks, it is necessary to calculate the covariance or the degree of linear relationship between different variables (mass peaks). The groups of peaks with high correlation are the principal components (PC). PCAs, groups of related mass peaks, are the three main components (PC1, PC2, PC3).

Results

Differential peaks identified in mucositis and in absence of mucositis (MUCOSITIS—NO MUCOSITIS)

From the cluster analysis conducted on the MUCOSITIS group and NO MUCOSITIS group, we found seven peaks of *m/z* that are differentially expressed (Mann–Whitney test $P < 0.05$).

In particular, in Table 2 are reported all values of *m/z*, *P*-value, ROC, trend in mucositis, and fold changes, of mass peaks that are differentially expressed between the two groups: MUCOSITIS and NO MUCOSITIS.

As can be seen in Table 2, there are four peaks mass/charge that are mostly expressed in the group MUCOSITIS's sample (1.9 < fold change > 3.6).

Whereas, Table 2 shows three *m/z* peaks that turned out to be reduced in MUCOSITIS group (−3.07 < fold change > −1.87).

In particular, the expression level of peak 3443 *m/z* seems to be noticeably increased (fold change 3.6) in the samples from subjects affected by mucositis, compared to those who received therapies and were not affected (Figure 1).

Peaks 3487 and 4135 *m/z* also have a higher expression level (fold change, respectively, of 2.89 and 1.9) in MUCOSITIS samples compared to NO MUCOSITIS samples (Figure 1).

Moreover, in Figure 2 the peak 6237 *m/z* is down-regulated in mucositis group (fold change −3.07).

Differential peaks identified in mucositis patients who underwent radiotherapy and those who did not (MUCOSITIS RADIO—MUCOSITIS NO RADIO)

To verify a possible influence of radiotherapy on the onset and development of oral mucositis in patients affected by cancer, we also conducted the cluster's analysis in the group of patients who developed the mucositis and underwent radiotherapy (MUCOSITIS RADIO) and those who developed it, but did not undergo radiotherapy (MUCOSITIS NO RADIO).

In MUCOSITIS RADIO group, we identified six mass change peaks with increased level expression and four mass change peaks with reduced level expression (Table 3).

In fact, the expression level of the peak 3443 *m/z* increased (fold change 2.14) in sample of subjects who underwent radiotherapy and developed mucositis (MUCOSITIS RADIO) compared to those who did not undergo radiotherapy (MUCOSITIS NO RADIO).

In the MUCOSITIS RADIO group, peak 3487 and 4135 *m/z* also have a higher level of expression (fold change 1.25 and 2.13, respectively) compared to MUCOSITIS NO RADIO.

The peak 6237 *m/z* turned out to have a quite high level of expression in the MUCOSITIS RADIO group (fold change 1.44), and was less expressed in the MUCOSITIS group.

Peaks difference in MUCOSITIS CHEMO and MUCOSITIS NO CHEMO

As shown in Table 4, there are 4 peaks of mass-charge that are highly expressed and 2 peaks whose levels are reduced in the MUCOSITIS CHEMO group as well. In particular, the expression level of the peak 3443 *m/z* appears to be increased (fold change 1.34) in samples from to subjects who underwent chemo therapy and developed mucositis (MUCOSITIS CHEMO) compared to those who did not undergo chemo therapy (MUCOSITIS NO CHEMO).

On the contrary, the peaks 3487 and 4135 *m/z* were found to have a reduced level of expression (fold change of −1.13 and −1.3, respectively) compared to MUCOSITIS NO CHEMO.

The peak 6237 *m/z* turned out to be more expressed in the MUCOSITIS CHEMO.

Cluster analysis in MUCOSITIS R+, MUCOSITIS R−, NO MUCOSITIS R+, NO MUCOSITIS R−, MUCOSITIS C−, MUCOSITIS C+, NO MUCOSITIS C+

To confirm the possibility that changes in expression levels of the peaks 3443.98, 3487, 4135, and 6237 *m/z* can be associated with cancer therapies, we analyzed their performance in patients with mucositis who had radiotherapy (MUCOSITIS R+), patients with mucositis and no radiotherapy (MUCOSITIS R−), patients without
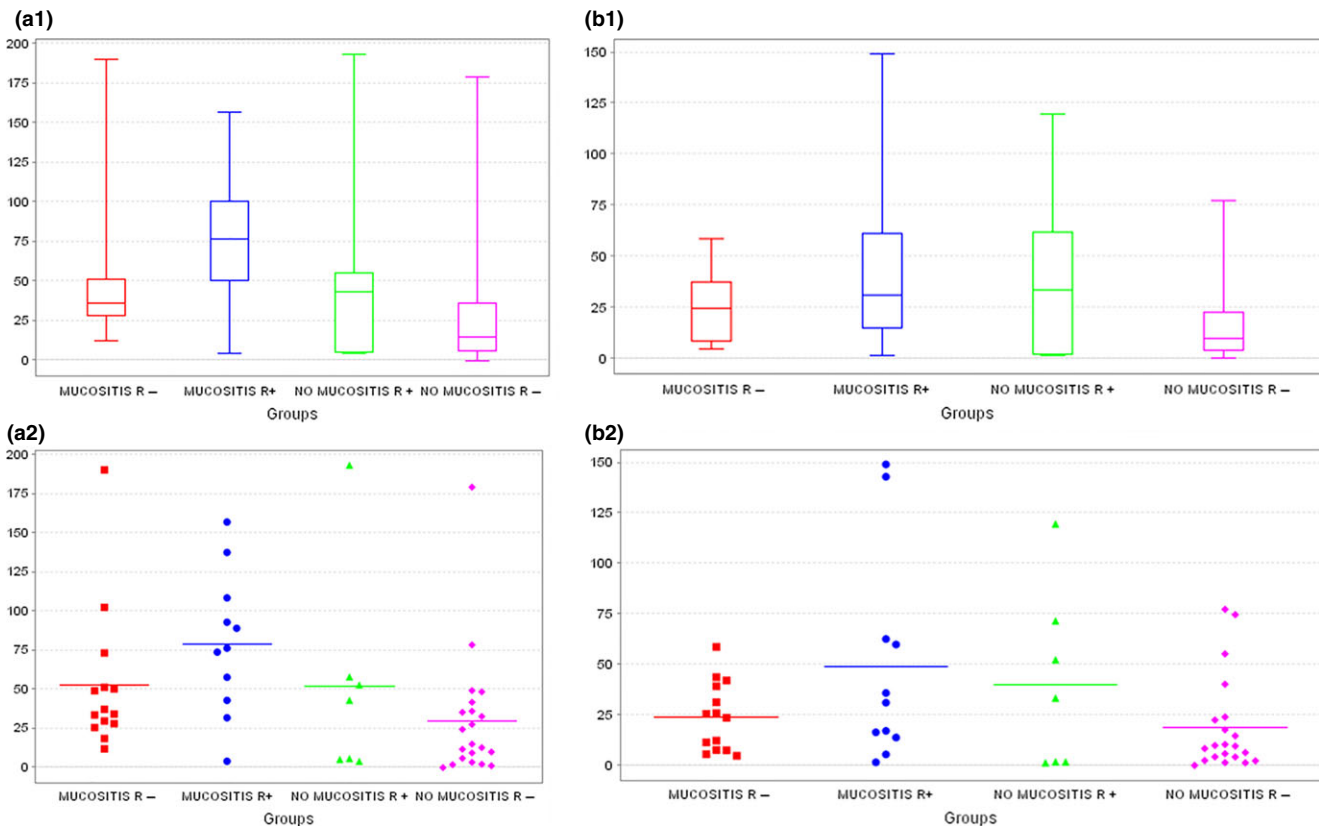

Figure 3 Graphic representations of cluster data in three different groups: MUCOSITIS R−, MUCOSITIS R+, NO MUCOSITIS R+, NO MUCOSITIS R−. Group box and whiskers plot of peaks 3443 (**a1**) and 3487 (**b1**); group scatter plot displaying average group intensities as horizontal lines of peaks 3443 (**a2**) and 3487 (**b2**)

(c1)

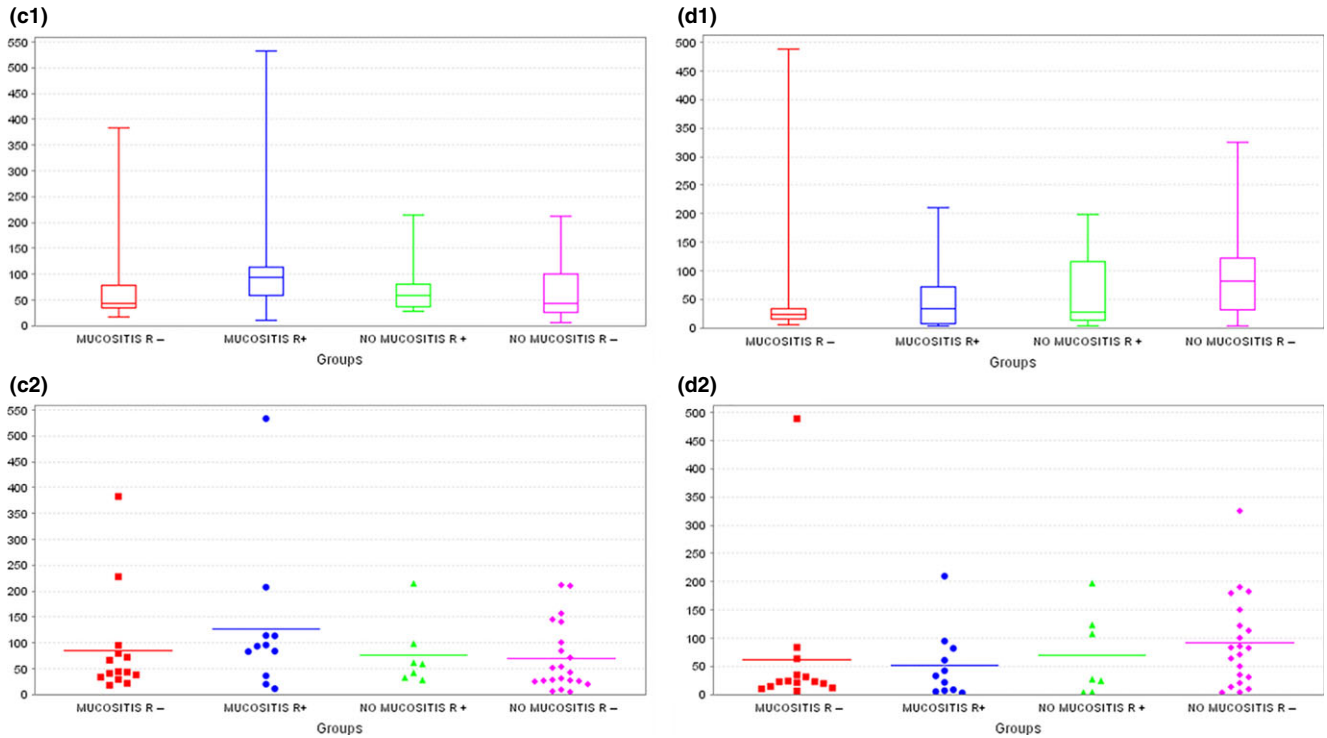**(d1)**

(c2)

(d2)

Figure 4 Graphic representations of cluster data in three different groups: MUCOSITIS R−, MUCOSITIS R+, NO MUCOSITIS R+, NO MUCOSITIS R−. Group box and whiskers plot of peaks 4135 (**c1**) and 6237 *m/z* (**d1**); group scatter plot displaying average group intensities as horizontal lines of peaks 4135 (**c2**) and 6237 *m/z* (**d2**)

mucositis and radiotherapy (NO MUCOSITIS R+), patients without mucositis and radiotherapy (NO MUCOSITIS R−), patients with mucositis who had not chemotherapy (MUCOSITIS C−), patients with mucositis who had undergone chemotherapy (MUCOSITIS C+), and patients who had not mucositis and not chemotherapy (NO MUCOSITIS C+).

From this analysis, we found that the expression of the peaks 3443, 3487, and 4135 *m/z* increased in the MUCOSITIS R+ group more than the others, while the expression of the peak 6237 *m/z* is reduced in MUCOSITIS R+ compared to the other groups (Figures 3 and 4).

In regard to the possible combination with chemotherapy, changes in expression levels of the peaks 3443 and 3487 *m/z* increased in MUCOSITIS C− group, but their level is almost similar to that of the MUCOSITIS C+ group, while the expression level of the peak 4135 *m/z* is higher in MUCOSITIS C+ compared to the other two conditions (NO MUCOSITIS C+ and MUCOSITIS C−) (Figures 5 and 6).

However, the expression of the peak 6237 *m/z* seems to lower in combination with chemotherapy (MUCOSITIS C+) (Figure 6).

Principal Component Analysis (PCA)
We used the principal component analysis (PCA) to confirm the increase in expression of 3443, 3487, and 4135 *m/z* and decrease of 6235 *m/z* in MUCOSITIS compared to NO MUCOSITIS as verificated in cluster statistics analysis (Figure 7).

In PCA, the two groups studied (MUCOSITIS and NO MUCOSITIS) show a distribution potentially correlated with antitumoral treatment because the samples of mucositis group are separate from no mucositis samples.

The net offset between two groups (Figure 8) confirm that the changes in expression of proteins is associated with radiotherapy and chemotherapy treatment.

Discussion

The cytotoxic effects of antineoplastic drugs on tissues with high turnover, such as oral epithelium, and local effects of radiation on oral mucosa are principally responsible of mucositis.

In the major part of case, it compromises seriously the patient's quality of life and may interfere with the management of the primary disease.

It is extremely important to prevent mucositis, or at least treat it to reduce its severity and possible complications.

Currently, several clinical trials to identify possible preventive treatments and protocols of cure and various molecular biological studies to investigate the mechanism of onset and development have been conducting.

However, despite the application of these therapies, alone or combined, there are not yet evidence protocols established.

Therefore, more research is needed especially in the biological field, to contribute to the identification of biomarkers of oral mucositis that can provide a starting point toward the study of the most effective treatments.
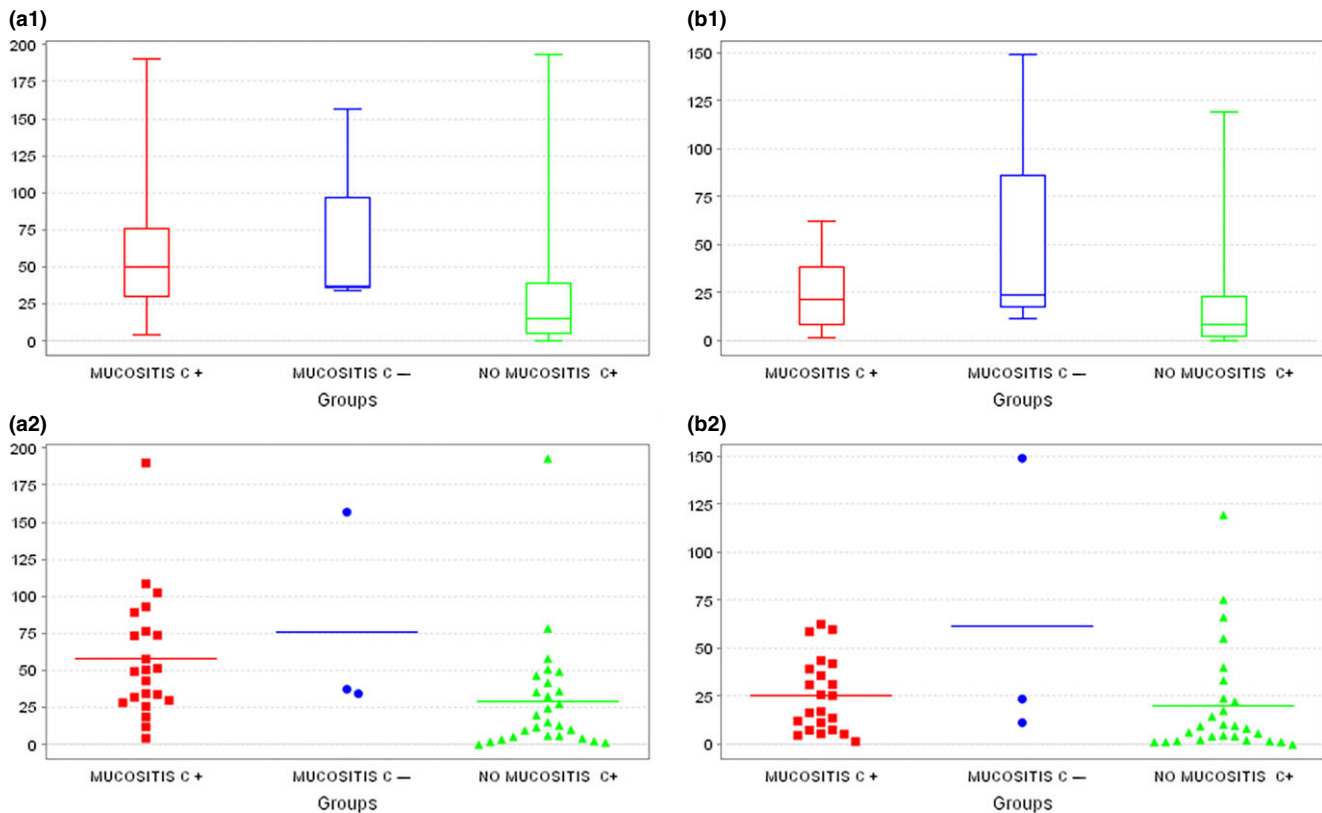
(a1)

(b1)

(a2)

(b2)

Figure 5 Graphic representations of cluster data in three different groups: MUCOSITIS C+, MUCOSITIS C−, NO MUCOSITIS C+. Group box and whiskers plot of peaks 3443 (**a1**) and 3487 (**b1**); group scatter plot displaying average group intensities as horizontal lines of peaks 3443 (**a2**) and 3487 (**b2**)

Aiming at this, we studied 60 saliva samples from cancer patients, 30 of which developed mucositis as a consequence of chemo- and/or radiotherapy (MUCOSITIS group) and 30 did not develop mucositis (NO MUCOSITIS).

All samples were acquired with Q10 ARRAY® ProteinChip SELDI-TOF analysis, and proteomic profiles of all samples were analyzed with the software BIO-RAD Data Manager™ (Version 3.5) to identify mass peaks differentially expressed (clusters) within two groups with a significance of $P < 0.05$.

In mucositis group, the analyzing cluster, we got 3 mass/charge peaks whose expression levels were up-regulated.

In particular, the peaks 3443, 3487, and 4135 m/z were up-regulated in mucositis, while the peak 6237 was down-regulated.

In addition, we further classified the samples included in the group mucositis in patients who got radio (MUCOSITIS RADIO) and patients who did not get radio (MUCOSITIS NO RADIO), in order to evaluate whether the expression of these potential biomarkers was or was not related to cancer treatment.

From this analysis, we identified 6 mass peaks charge whose levels were increased in the MUCOSITIS RADIO group, and 4 mass peaks charge whose levels were reduced in the MUCOSITIS NO RADIO group. Among them, we found peaks 3443, 3487, 4135, and

6237 m/z up-regulated in patients who have developed the disease and obtained radio (MUCOSITIS RADIO) compared with patients who get MUCOSITIS NO RADIO.

The same variation of expression of the markers 3443, 3487, 4135 m/z (up-regulated), and 6237 (down-regulated) in all patients with mucositis and especially in those who got radiotherapy, encourages us to think that this kind of treatment might influence selectively these proteins.

In addition, we conducted cluster analysis dividing the patients with oral mucositis also in function of the chemotherapeutic treatment.

Also in this case, the results were interesting. In fact, in the MUCOSITIS CHEMO group emerged 4 peaks of mass/charge that turn out to be more expressed and 2 peaks whose levels were lower than in the group of patients with mucositis who did not undergo therapy (MUCOSITIS NO CHEMO).

The peaks 3443 and 6237 m/z were increased in the MUCOSITIS CHEMO and MUCOSITIS, the peaks 3487 and 4135 m/z had a reduced level of expression in this group.

This leads us to infer that the peak 3443 may be a good biomarker because it is set up in mucositis and especially in those who develop the disease as a consequent of radio- and/or chemotherapy.

Chemotherapy and radiotherapy have a different influence on the performance of other proteins.
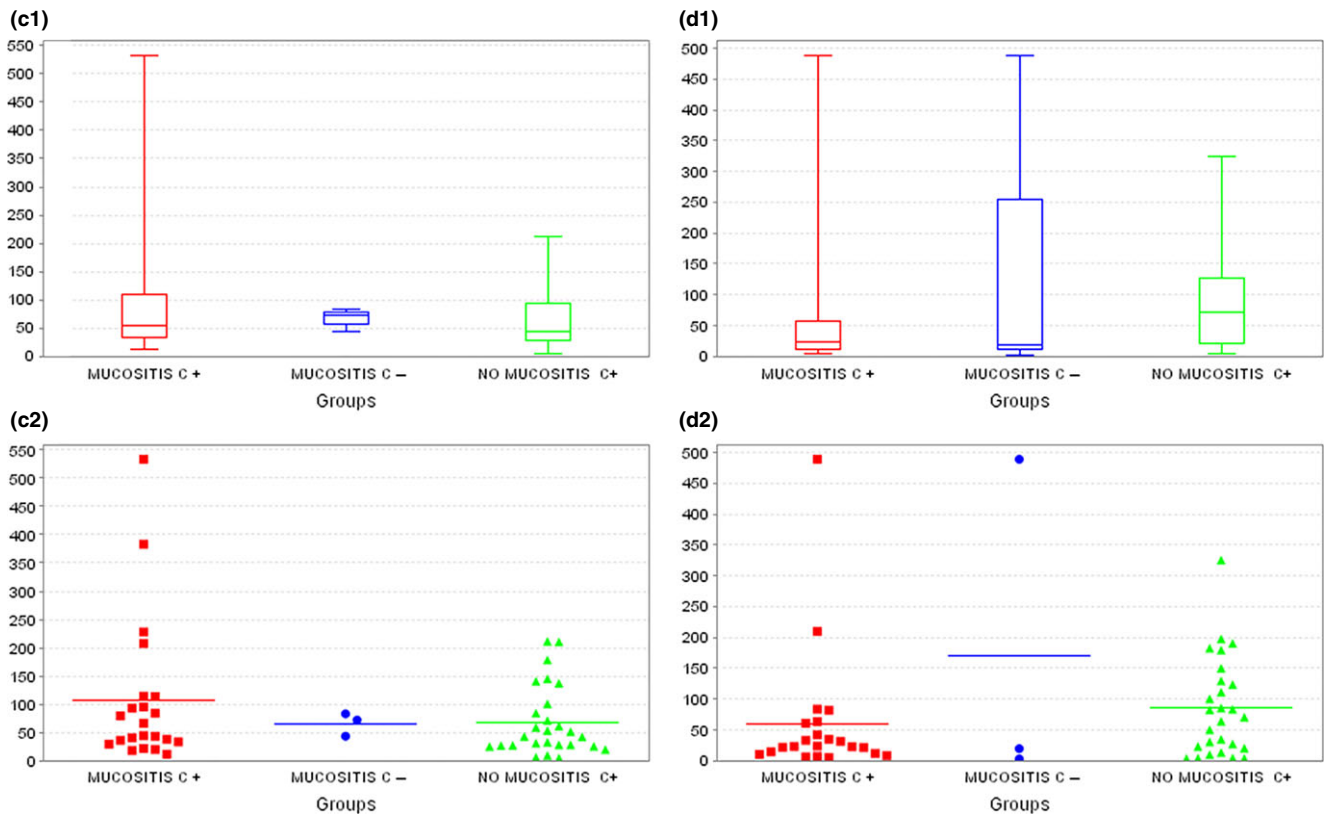
Figure 6 Graphic representations of cluster data in three different groups: MUCOSITIS C+, MUCOSITIS C−, NO MUCOSITIS C+. Group box and whiskers plot of peaks 4135 (**c1**) and 6237 *m/z* (**d1**); group scatter plot displaying average group intensities as horizontal lines of peaks 4135 (**c2**) and 6237 *m/z* (**d2**)

In fact, radiation therapy promotes a higher level of expression of biomarkers 3487 and 4135 *m/z*, while chemotherapy reduces the expression.

The down-regulation of the peak 6237 *m/z* in all patients with mucositis does not seem to be associated with anticancer treatment. In fact, the fold change in the MUCOSITIS RADIO and MUCOSITIS CHEMO are, respectively, 1.44 and 2.98, while it was down-regulated in MUCOSITIS group (fold change −3.07).

In Figure 3, it is possible to assess the trend of the peak 3443 *m/z*. Graphically, we see that the expression of this biomarker is higher in the group MUCOSITIS R+, lower in the MUCOSITIS R−, and even lower in others. A high level of expression in the MUCOSITIS NO RADIO group suggests an increase in the expression of the marker associated with the inflammatory process typical of mucositis, but the expression increased more in people who develop mucositis and obtain radiotherapy.

However, analyzing the Figure 5, we noted that the levels of this same marker are higher in the MUCOSITIS C− group, compared to the MUCOSITIS C+ group and above NO MUCOSITIS C+. Hence, it is surely clear that the increased level of expression can be associated with the inflammatory process characteristic of mucositis, but it is not possible to correlate it with chemotherapy treatments also because in this case, the samples available were few in number.

Instead, the trend of the 3487 peak appears to be significantly related to radiotherapy. In Figure 3, the expression

is greater in the MUCOSITIS R+ and lower in the NO MUCOSITIS R+, but in the latter group, the level is higher than in other groups where patients did not undergo radiotherapy.

The assessment in relation to chemotherapy appears to be not entirely reliable because of the scarcity of samples available.

The expression of the peak 4135 *m/z*, as seen in Figure 4, results to be higher in the MUCOSITIS R+, compared to other groups. Here too, while a high-level group also, MUCOSITIS NO RADIO, suggests a variation of the expression of the marker linked to the inflammatory process typical of mucositis, expression increases when the patient has made radio and mucositis (MUCOSITIS R+).

Moreover, as seen in Figure 6, the peak 4135 *m/z* seems to be associated univocally with patients who have mucositis and had chemotherapy; the peak had an increased expression in MUCOSITIS C+, but the expression seems to be identical in the two other groups.

If this figure will be confirmed on a larger series of patients, we could claim to have found a marker exclusively linked to this condition. This would contribute significantly to the study of targeted therapies and preventing mucositis in patients who have chemotherapy.

In Figure 4 is shown the marker 6237 *m/z*: Its expression in MUCOSITI R+ seems to be low compared to others. This down-regulation of the protein appears to be

Cluster Statistics

Condition: M/Z: 3446.999   Cluster: 3   P-Value: 0.001   ROC area: 0.733   Row 3 of 87

Group	M/Z avg	M/Z std	Intensity avg	Intensity std	# of peaks	# estimated
Mucositis	3443.882	1.225	72.571	80.628	28	0
No	3444.287	3.455	34.628	47.974	27	5

Cluster Statistics

Condition: M/Z: 3486.801   Cluster: 4   P-Value: 0.033   ROC area: 0.660   Row 4 of 87

Group	M/Z avg	M/Z std	Intensity avg	Intensity std	# of peaks	# estimated
Mucositis	3487.775	2.472	37.831	53.091	28	8
No	3487.031	1.548	22.136	29.820	27	16

Cluster Statistics

Condition: M/Z: 4133.556   Cluster: 7   P-Value: 0.057   ROC area: 0.631   Row 7 of 87

Group	M/Z avg	M/Z std	Intensity avg	Intensity std	# of peaks	# estimated
Mucositis	4135.108	1.602	114.023	114.157	28	3
No	4136.111	4.676	72.338	68.200	27	4

Cluster Statistics

Condition: M/Z: 6245.300   Cluster: 23   P-Value: 0.037   ROC area: 0.311   Row 23 of 87

Group	M/Z avg	M/Z std	Intensity avg	Intensity std	# of peaks	# estimated
Mucositis	6241.349	4.956	45.558	62.648	28	5
No	6240.716	3.677	84.532	78.445	27	3

Figure 7 Cluster statistics analysis. The expression levels of peaks 3443, 3487, and 4135 *m/z* is increased in MUCOSITIS group, while 6237 *m/z* is reduced in NO MUCOSITIS group
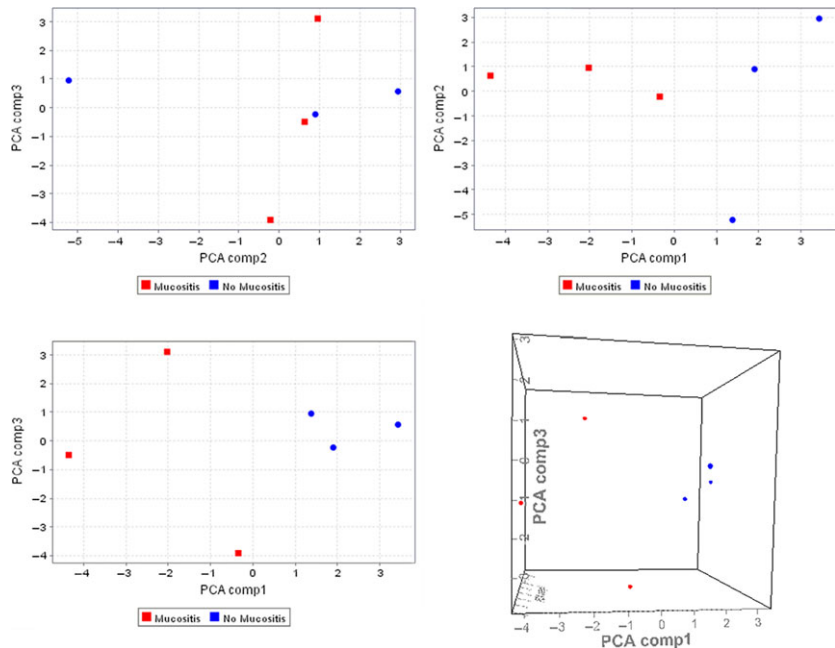

Figure 8 Principal components analysis (PCA) generated using biomarker candidates selected following univariate statistical analysis. These graphics explain the effective differentiation of MUCOSITIS and NO MUCOSITIS samples

associated with the inflammatory process typical of mucositis and radiotherapy. In Figure 6, the peak MUCOSITIS C+ 6237 *m/z* is strongly down-regulated. Therefore, it would be necessary to expand the series and have a comparison with subjects not suffering from mucositis and who did not have chemotherapy.

The data sets' proteins were also analyzed by PCA (principal components analysis), which confirmed that there is no correlation between MUCOSITIS and NO MUCOSITIS groups, for peaks differentially expressed (3443, 3487, 4135, 6237 *m/z*). Therefore, the particular variation of these protein expressions might be associated with one condition rather with another. In conclusion, it would be necessary to deepen this study with a larger number of patients using analytical techniques aimed at identifying these potential biomarkers. Knowing they will serve to plan a protocol for prevention and treatment of oral mucositis.

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Author contributions

F. Ardito and D. Perrone Dr. Ardito and Dr. Perrone did the experiemental sudies, drafted the manuscript and approved the final manuscript as submitted G. Colella, G. Favia and G. Campisi they followed the patient as primary doctor, drafted the initial manuscript, revised, finalized, and approved the final manuscript as submitted. G. Giannatempo Dr. Giannatempo drafted the initial manuscript with Dr. Arito and Dr. Perrone, prepared the tables, and approved the final manuscript as submitted. O. Di Fede and M. Giuliani: Dr. Di Fede and Dr. Giuliani followed the patient as primary doctor, collected data, revised and reviewed the manuscript, and approved the final manuscript as submitted L. Lo Muzio coordinated and supervised data collection, critically reviewed the manuscript, and approved the final manuscript as submitted.

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