Late Oral Mucosa Alterations After Radiotherapy for Head and Neck Cancer Assessed by Exfoliative Cytology

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Abstract. *Aim: Late oral mucosa changes after radiotherapy for head and neck cancer have been poorly studied. This study aimed to determine long-term effects of radiotherapy on oral mucosa using exfoliative oral cytology. Patients and Methods: Fifty patients with cancer were enrolled, five of whom in order to validate microscopic analysis. Smears were collected at programmed visit; a score was used to rank possible cytological alterations. Presence of inflammation was also microscopically described and compared to blood count tests. Results: Epithelial cells revealed a peculiar 'folding' phenotype, not related to chemotherapy, total dose, or to the effective dose delivered to mucosa. Inflammation described was related to the score for 'folding' cells; moreover, score decreased in the presence of a higher lymphocyte count, while it was not altered by neutrophil count. Conclusion: We suggest application of exfoliative cytology to study radiation injury and the variability of individual response of oral mucosa to radiation.*

Normal tissue toxicity is a major concern regarding radiotherapy treatment outcome, with a large spectrum of clinical responses varying among patients, even into the boundaries of rigid dose tolerance limits and schedules. The possibility of predicting the risk of developing severe normal tissue damage would allow individualization of radiation treatment, thus improving radiotherapy delivery. Scientific evidence has revealed that biological factors may determine normal tissue toxicity, with increasing interest in the biomarker research field. Consequently several predictive tests have been proposed to determine the individual sensitivity of patients to radiotherapy schedules (1).

Oral mucositis is a very common tissue side-effect experienced by patients with head and neck cancer during radiotherapy or chemoradiation course, compromising delivery of optimal radiation therapy schedules and quality of life. Many efforts have been made in the past decade to study the pathogenesis of mucositis and awareness has been increasing that oral mucosa toxicity is not only due to direct injury, but is also the consequence of different biological events, many of which involve sub-mucosal tissues (2). Despite the attention given to the acute–subacute radiation injury to oral mucosa, aimed at identifying options for prevention or treatment, late mucosal toxicity has been largely ignored for years, investigations focusing mainly on the consequences of low salivary flow rates (3, 4). The most common late effects of radiation on the mucosal linings of the upper aerodigestive tract are described as paleness and thinning of the epithelium, loss of mucosal pliability, and sub-mucosal induration; due to their similar architecture, these effects have been extrapolated mainly referring to experimental studies on the effects of radiation on skin, since radiation effects on mucosal lining have been historically less investigated (5).

In the past few years, there has been renewed interest in exfoliative cytology in oral pre-cancer and cancer as a diagnostic and predictive method (6). For this reason, we started a study on radiation-induced oral mucosa toxicity based on exfoliative oral cytology, focusing our attention in a first step on the description of morphological features of epithelial cells many years after radiation, exploring the possibility of using insights from cytological analysis to drive further molecular investigations in a second step, even in the on-going radiotherapy course setting.

We present here the first part of the study, aimed at investigating morphological features of long-term effects of radiotherapy on oral mucosa assessed by exfoliative oral cytology.

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Patients and Methods

Patients. A total of 53 patients previously treated for head and neck cancer, consecutively attending a programmed follow-up visit, were asked to participate in the study: three refused to be enrolled, while 50 accepted and completed the evaluation process. Eligibility criteria were: age between 30 and 80 years, a minimum of 24 months' follow-up after completion of 3D conformal radiotherapy for solid head and neck cancer, oral mucosa being included in the fields of exposure of therapy. Exclusion criteria were: diagnosis for second cancer or relapse of head and neck cancer at follow-up, head and neck lymphoma, anaemia, reirradiation of the head and neck region, autoimmune diseases and immunosuppressive therapy, active hepatitis (A, B, C), AIDS and HIV-seropositivity, therapy with immunosuppressive drugs, pregnancy or breast feeding, active smokers (due to the irritative effect of smoke on mucosal lining; smoke is one of the principal risk factor for head and neck cancer by definition so the prevalence of ex-smokers in the enrolled patients was high).

These patients were part of a cohort of 368 patients treated at the Radiation Oncology Department, IRCCS Policlinico San Matteo Foundation, Pavia, Italy, between 2003 and 2010.

Data regarding the primary cancer, types of treatment, radiation fields, total radiation dose and side-effects were collected from the hospital records, together with blood test carried at the follow-up visit. Radiotherapy was performed using a 6 or 18 MeV linear accelerator (X-ray). Doses ranged from 50 to 72 Gy, delivered in daily fractions of 1.8-2.0 Gy, five days per week for 5-7 weeks. The study was approved by the Ethical Committee of our hospital (approval n. 954 General Director 9/16/2011) and all the participants were provided with specific written information about the aims of the study and their written consent was obtained.

Sample collection and staining. Smears were collected at the programmed follow-up visit by scraping left and right cheek mucosa with wooden tongue spatulas, then transferring the scrapings to four dry glass slides (two for each cheek), which were then fixed immediately with Bio-fix® (Bio-Optica, Milano, Italy) and stained with Giemsa (Sigma-Aldrich, St-Louis, MO, USA), diluted 1:5 with double-distilled water, for 7 min, covered with a cover-slip and examined under ×400 power using a light microscope (Leica DM LB2, Wetzlar, Hesse, Germany).

Quantitative/qualitative analysis. Samples were interpreted according to the following criteria: adequacy of the sample amount, mode of smear, nuclear morphology, presence of alterations in epithelial cell phenotype, presence/absence of inflammation (coded as 1 if present or 0 if absent), defined as presence of neutrophils and lymphocytes even in the absence of an infectious background. In order to quantify the possible presence of altered epithelial cell phenotype, these cells were counted as a percentage of all cells per each dry glass slide for each cheek; then the average was taken of the two glass slides obtained for the right and left cheek. Finally a score was used to rank the cytological alterations for each cheek: score 0: absence; score 1: 1-30%; score 2: 31-60%; score 3: 61-100%. This kind of score is derived by the model of scoring routinely used in cytopathology practice, *e.g.* in hematology practice, a score is used to evaluate the immunophenotype of lymphocytes in chronic lymphocytic leukemia, based on the expression or not of a defined antigen group (7).

Table I. *General patients' characteristics (n=5) with glottic squamous cell carcinoma. Inflammation on microscopic samples: r right, l left, 1 presence, 0 absence. Score of "folding" cells: 0 (absence), score 1 (1-30 %), score 2 (31-60%), score 3 (61-100%).*

Patient	Age (years)	Infl r	Infl l	Score r	Score l	Total dose (Gy)	Follow-up (months)	Chemo-therapy
30	70	0	0	0	0	70	29	no
33	70	0	0	0	0	66	72	no
43	64	0	0	0	0	70	45	no
48	57	0	0	0	0	72	48	no
50	78	0	0	0	0	70	93	no

Preliminary analysis. Preliminary observations were made on a subset of patients with the same eligibility criteria to validate further microscopic analysis. These patients were comparable to the others in terms of eligibility criteria, but despite all other patients, the oral mucosa was not included in the fields of exposure of radiotherapy. They had, in fact, early-stage glottic squamous cell carcinoma and none underwent chemotherapy or surgery. They were five patients (four males and one female), their median age was 67.8 (range=57-70) years, median follow-up after radiotherapy was 48 (range=29-93) months, median dose was 70 (range=66-72) Gy with conventional fractionation, median overall treatment time was 58 (range=47-58) days. The characteristics of these patients are presented in Table I. Smears collected from this subset of patients showed a phenotype of epithelial cells and a microbiota very similar to that observed in the normal population never exposed to ionizing radiation in the head and neck region (8) (Figure 1A).

Statistical analysis. Categorical variables were described as counts and percentages; qualitative variables as means and standard deviation or median and range, depending on distribution data. Association between variables was assessed by means of Fisher's exact test or Spearman non parametric correlation test. Rho and *p*-values are reported; all tests were two-sided and a *p*-value of less than 0.05 was considered statistically significant. Stata v. 12 (StataCorp, College Station, TX, USA) was used for computation.

Results

From November 2011 to November 2012, 50 consecutive patients were enrolled in this study at their programmed follow-up visit, five of whom enrolled for further microscopic observation.

In the study group (n=45), patients were 38 males and seven females, their median age was 63.5 (range=46-80) years, median follow-up after radiotherapy was 34 (range=24-112) months, median dose was 66 (range=50-72) Gy with conventional fractionation, median overall treatment time was 58 (range=40-88) days. Primary carcinomas were mainly located in the oropharynx and oral cavity (n=31) and supraglottic or subglottic (n=8), Squamous cell carcinoma (SCC) was the most prevalent

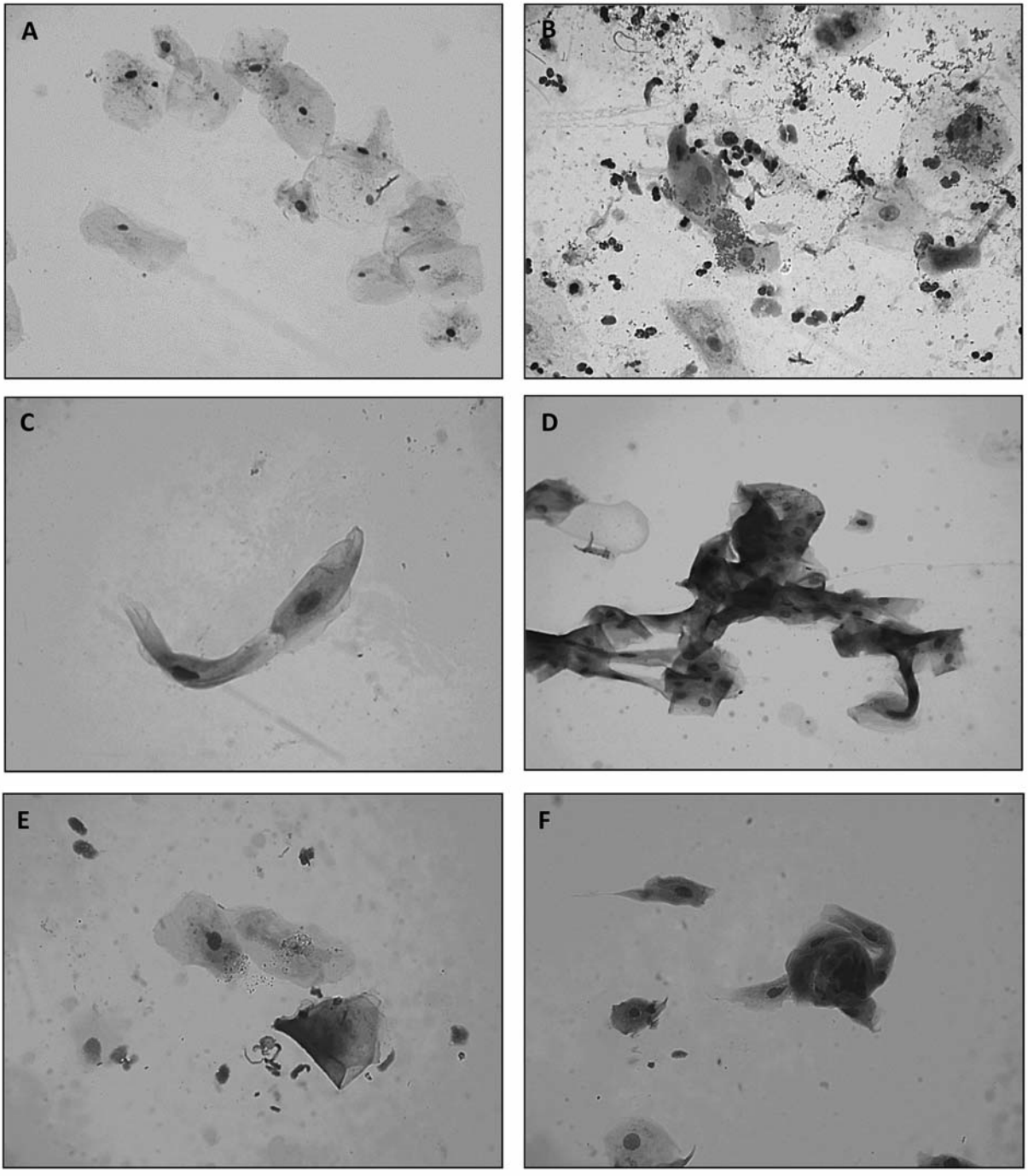

Figure 1. *Smears of exfoliative oral cytology: A, normal superficial epithelial cells of oral mucosa not exposed to radiotherapy; B, background of neutrophils and lymphocytes observed in oral mucosa exposed to radiotherapy; C, D, neutrophils and epithelial cells defined "folding cells"; E, a detail of "folding cells"; F, a cluster of "folding cells". White arrows indicate the "folding cells" and black arrows indicate neutrophils (Giemsa staining; ×400).*

histological subtype (n=42). Twenty-one patients underwent postoperative radiotherapy, seven of whom received concomitant chemotherapy; 24 underwent radical radiotherapy, 18 of whom received concomitant chemotherapy. The characteristics of the study patients are listed in Table II. Acute side-effects recorded during radiotherapy and late side-effects recorded at the time of smear sampling are listed in Table III, according to the Common Terminology Criteria for Adverse Events v3.0 (CTCAE v3.0) (9).

All the smears collected from the group of 45 patients were adequate quantity-wise and showed cytological alterations, not in the sense of atypia, *i.e.* neoplastic transformation, due to a second cancer or relapse of the previous one. Alterations observed in the epithelial cells were a peculiar 'folding' phenotype, with enlarged and hypertrophic nuclei (Figure 1E and F).

The score, *i.e.* the presence of folding cells in our smears, decreased with increasing time from the end of radiotherapy (Spearman's rho=−0.45, p=0.0021 for right cheek; Spearman's rho=–0.35, p=0.0166 for left cheek) but was not related to total dose delivered (Spearman's rho=−0.0389, p=0.7998 for right cheek; Spearman's rho=−0.11, p=0.4859 for left cheek) nor to chemotherapy (Fisher's exact=0.411 for right cheek, 0.184 for left cheek). Moreover, there was no relationship between late toxicity recorded at the time of smearing and score for: mucositis (Fisher's exact=0.187 for right cheek, 1.00 for left cheek), dysphagia (Fisher's exact=0.795 for right cheek, 0.116 for left cheek), xerostomia (Fisher's exact=0.314 for right cheek, 0. 324 for left cheek), salivary gland changes (Fisher's exact=0.439 for right cheek, Fisher's exact=0. 425 for left cheek), dysgeusia (Fisher's exact=0.307 for right cheek, Fisher's exact=0.040 for left cheek). Fisher's exact test confirmed the absence of a relationship with respect to the same side-effects in the acute setting.

Since it was impossible to estimate the effective dose delivered to oral mucosa for all patients because the computerized treatment planning system used for patients treated many years ago was not available, these evaluations were possible on the 25 patients treated more recently (these patients are listed in bold font in Table II). They were 21 males and four females, their mean age was 61.7 (range=46-76) years, mean follow-up after radiotherapy was 28.9 (range=24-39) months, mean dose was 66.6 (range=50-70.2) Gy with conventional fractionation, median overall treatment time was 59 (range=44-88) days. Prevalent tumor localization was in the oropharynx and oral cavity (18 out of 25 patients); prevalent histology was SCC (21 out of 25 patients). Dose to right and left cheek mucosa was calculated on planning computed tomography CT scans using treatment planning system (Oncentra Masterplan v3.3; Nucletron, Veenendal, the Netherlands) dose evaluation tools. This analysis confirmed that the score of folding cells in right and left cheek mucosa was independent from the dose actually delivered according to the radiotherapy treatment planning (Spearman's rho=0.08, p=0.6921 for right cheek, Spearman's rho=0.25, p=0.2362 for left cheek).

In almost all smears, we also observed an inflammatory background with neutrophils and lymphocytes not sustained by an infectious milieu (rarely did we find fungal or bacterial colonies, apart from the resident microbiota), not dependent on the presence of delayed clinically-detectable chronic mucosal alterations, as defined in CTCAE v3.0; none of them had clinical detectable candidiasis (Figure 1B). In our samples, the presence of inflammation influenced the score of folding cells by lowering it

Table II. *General characteristics of the study patients (n=45). Inflammation on microscopic samples: r right, l left, 1 presence, 0 absence. Score of "folding" cells: 0 (absence), score 1 (1-30 %), score 2 (31-60%), score 3 (61-100%). Bold font indicate the 25 patients treated more recently.*

Patient	Age (years)	Infl r	Infl l	Score r	Score l	Total dose (Gy)	Follow-up (months)	Chemo-therapy
1	76	1	0	1	1	64.8	32	no
2	**65**	**1**	**1**	**1**	**1**	**70**	**32**	**yes**
3	**63**	**0**	**0**	**3**	**2**	**70.2**	**24**	**yes**
4	**70**	**1**	**1**	**1**	**1**	**70**	**24**	**no**
5	67	0	0	1	2	66	67	yes
6	52	1	1	1	1	70.2	63	yes
7	71	0	0	2	1	60	42	no
8	70	0	0	1	1	70	65	yes
9	**74**	**0**	**1**	**3**	**2**	**50**	**24**	**yes**
10	60	0	0	1	1	66	54	yes
11	61	0	1	1	1	66	98	no
12	60	0	0	1	1	66	51	no
13	68	0	1	1	1	60	50	no
14	**74**	**1**	**1**	**1**	**2**	**70**	**39**	**yes**
15	58	0	0	1	1	60	39	no
16	**53**	**0**	**1**	**2**	**1**	**66**	**30**	**yes**
17	**68**	**0**	**0**	**1**	**2**	**66**	**31**	**yes**
18	**76**	**1**	**1**	**3**	**1**	**66**	**26**	**yes**
19	**58**	**0**	**0**	**1**	**1**	**60**	**27**	**yes**
20	51	1	1	1	1	50	50	yes
21	57	1	1	1	1	54	59	yes
22	**49**	**1**	**1**	**1**	**1**	**66**	**39**	**no**
23	51	1	1	1	1	60	44	no
24	70	1	0	1	2	50	68	no
25	77	1	1	1	0	72	96	no
26	**50**	**0**	**1**	**3**	**2**	**66**	**24**	**yes**
27	**46**	**1**	**1**	**1**	**1**	**70**	**26**	**yes**
28	**57**	**1**	**1**	**1**	**1**	**70**	**33**	**yes**
29	**58**	**1**	**0**	**1**	**3**	**66**	**27**	**yes**
31	54	1	1	1	1	66	80	yes
32	**54**	**0**	**0**	**1**	**1**	**66**	**24**	**no**
34	**65**	**0**	**0**	**1**	**1**	**70**	**30**	**yes**
35	**72**	**1**	**1**	**1**	**1**	**66**	**26**	**yes**
36	**70**	**0**	**0**	**3**	**2**	**66**	**24**	**yes**
37	80	1	1	1	1	70	91	yes
38	69	0	1	1	1	66	112	no
39	72	0	0	1	0	70	55	yes
40	**51**	**1**	**1**	**1**	**1**	**70**	**38**	**yes**
41	**64**	**0**	**1**	**2**	**1**	**70**	**34**	**yes**
42	**63**	**1**	**1**	**2**	**1**	**59.4**	**27**	**no**
44	**62**	**0**	**0**	**2**	**2**	**70**	**34**	**yes**
45	**47**	**0**	**0**	**2**	**2**	**70**	**29**	**yes**
46	**72**	**1**	**0**	**1**	**1**	**66**	**28**	**yes**
47	**62**	**1**	**0**	**1**	**2**	**66**	**24**	**yes**
49	54	1	1	1	1	70	26	yes

Table III. *Side effects of study patients (n=45) according to CTCAE v3.0 (G1-G5; 0 absence) recorded during radiotherapy (a=acute) and at the time of smear sampling (c=chronic). MUC= mucositis, DYSP= dysphagia. XERO= xerostomia, DYSG= dysgeusia , SGC= salivary gland changes. Bold font indicates the 25 patients treated more recently.*

Patient	MUC a	DYSP a	XERO a	SGC a	DYSG a	MUC c	DYSP c	XERO c	SGC c	DYSG c
1	0	0	G1	G1	G1	0	0	0	0	0
2	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
3	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**0**
4	**G2**	**G1**	**0**	**0**	**0**	**G1**	**0**	**G1**	**0**	**G1**
5	G2	0	G1	0	0	0	0	G1	G1	0
6	G2	0	0	G1	G2	G1	G1	G1	G1	G1
7	G1	0	G1	0	G1	0	0	0	0	0
8	0	0	G1	0	G1	0	0	G1	0	G1
9	**G1**	**0**	**0**	**0**	**0**	**G1**	**0**	**0**	**G1**	**0**
10	G3	0	G1	0	G1	G1	0	G1	0	0
11	0	0	G1	0	0	0	0	0	0	0
12	G2	0	0	0	0	0	0	G1	0	0
13	G1	0	G1	0	G1	0	0	G1	0	G1
14	**G2**	**G2**	**0**	**0**	**0**	**G1**	**0**	**G1**	**G1**	**0**
15	G1	0	0	0	0	0	0	G1	0	0
16	**G1**	**0**	**G1**	**0**	**0**	**0**	**0**	**G1**	**G1**	**0**
17	**G2**	**0**	**G1**	**0**	**0**	**0**	**0**	**G1**	**G1**	**0**
18	**G2**	**0**	**0**	**0**	**G1**	**G1**	**G1**	**G1**	**G1**	**0**
19	**G1**	**0**	**0**	**G1**	**0**	**0**	**0**	**G1**	**0**	**0**
20	G1	G1	0	0	0	G1	0	G1	G1	G1
21	G1	0	0	0	G1	0	0	G1	0	G1
22	**G1**	**G1**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**G1**
23	G1	0	G1	0	0	G1	0	G1	G1	0
24	G1	G1	0	0	0	0	0	G1	G1	0
25	G1	G1	0	0	0	0	0	G1	0	G1
26	**G1**	**G1**	**0**	**0**	**0**	**0**	**0**	**0**	**G1**	**G1**
27	**G3**	**G4**	**0**	**0**	**0**	**G2**	**0**	**G2**	**0**	**G1**
28	**G2**	**G1**	**0**	**0**	**0**	**0**	**G1**	**G1**	**0**	**0**
29	**G2**	**G1**	**0**	**0**	**0**	**0**	**G1**	**G2**	**G1**	**0**
31	G2	G1	0	0	0	G2	G1	G2	G1	G1
32	**G2**	**G1**	**0**	**0**	**0**	**0**	**G1**	**G2**	**0**	**0**
34	**G2**	**G1**	**0**	**0**	**0**	**G1**	**0**	**G2**	**0**	**G1**
35	**G1**	**G1**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**0**
36	**G2**	**G1**	**0**	**0**	**0**	**G1**	**0**	**G1**	**0**	**0**
37	G3	G1	0	0	0	0	0	G1	0	0
38	0	G1	0	0	G1	0	0	G1	0	0
39	G2	G2	G1	0	0	G1	0	G1	0	0
40	**G1**	**G1**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**0**
41	**G2**	**G1**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**G1**
42	**G1**	**0**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**0**
44	**G1**	**G1**	**0**	**G1**	**0**	**0**	**0**	**G1**	**0**	**0**
45	**G4**	**G2**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**0**
46	**G2**	**G1**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**G1**
47	**G2**	**G1**	**0**	**0**	**0**	**0**	**0**	**G1**	**0**	**G1**
49	G3	G2	0	0	0	0	0	G1	0	G1

(Spearman's rho=0–0.37, p=0.0127 for right cheek; Spearman's rho=–0.33, p=0.0254 for left cheek). Then we analyzed blood count tests to see if the relation between the score and presence of local inflammation (neutrophils and lymphocytes) was reflected in the absolute number of neutrophils and lymphocytes in blood. Laboratory tests were considered only if performed within the previous six months; older tests were not taken into account. Laboratory measurements were available for 38 out of 45 patients; they all had normal neutrophil (mean=3.44/μl; SD=1.28/μl) and lymphocyte (mean 1.55/μl; SD=1.03/μl) counts. In these 38 patients, the number of lymphocytes reduced the score of

folding cells (Spearman's rho=–0.36, p=0.0264 for right cheek; Spearman's rho=–0.35, p=0.0297 for left cheek); in contrast, the score was not related to the absolute number of neutrophils (Spearman's rho=–0.001, p=0.9937 for right cheek; Spearman's rho=–0.18, p=0.2911 for left cheek). The absolute number of neutrophils and lymphocytes in blood was not related to the time from the end of radiotherapy (Spearman's rho=0.20, p=0.2235 for lymphocytes; Spearman's rho=0.23, p=0.1591 for neutrophils).

Discussion

Radiotherapy for head and neck region malignancies may induce permanent tissue damage, with continual risk for oral sequelae. The radiation-related changes in the oral mucosa, salivary glands, taste, dentition, periodontium, bone, muscles, and joints are usually divided into early (mucosa, taste, salivary glands), intermediate (taste, salivary glands), and late (salivary glands, dentition, periodontium, bone, muscles, joints) effects (10). Late oral mucosa changes have been poorly-studied, probably because patients are asymptomatic for oral mucosal alterations *per se*, while they are often affected by other symptoms such as dryness of the mouth, caries and periodontal disease, oral functioning and taste alterations. After radiotherapy, the oral mucosal lining lies lifelong in an environment with non-physiological conditions; this well-stabilized setting is variable among patients and in our opinion studying late mucosal alterations would be important to understand the individual variability of response of normal tissue to radiotherapy treatment.

In our study, late mucosal toxicity after radiotherapy for head and neck cancer was assessed by exfoliative oral cytology, a simple and painless procedure, well-accepted by patients, which has been a controversial tool in oral pathology in the past. It has been applied to diagnosis of oral diseases since its validation for diagnosing cancer of the uterine cervix (11, 12), but these two diagnostic fields did not share the same fortune. Exfoliative oral cytology was left aside because it was purported to be inadequate for discriminating dysplasia from malignancy (13). Lately oral cytology is re-emerging as a diagnostic tool in oral precancer and cancer thanks to improved methodology and new molecular techniques (13-15). Despite this renewed interest, few studies have been carried out using exfoliative oral cytology to evaluate oral sequelae due to cancer therapy; for radiotherapy, some studies were carried out from 1950-1980s, mainly focusing on acute/subacute toxicity and response to therapy, or on possible cellular alterations leading to radiation-induced secondary cancer (16-18).

We used exfoliative oral cytology to assess oral mucosa status in a very late setting after radiotherapy in order to describe the features of the mucosal lining in oral cavity with consolidated treatment sequelae. The 5-year relative survival rate for all patients with head and neck cancer is 57%; cancer of the hypopharynx (5-year relative survival rate 30%) and other types of cancer of the oral cavity and pharynx (5-year relative survival rate 30%) have the worst prognoses in terms of relative survival rates (19). The Italian Tumor Registries show a 56% relative 5-year survival rate for patients with head and neck cancer (20). According to these data, a low enrollment rate into the potentially eligible initial cohort of 368 patients was expected, estimating that approximately half of this cohort would have attended follow-up visits during the enrolling time range.

In all 45 patients, we found a strange phenotype of epithelial cells, with hypertrophic nuclei and a strange plicate shape; we defined these as "folding" cells. These cells have not been described in the preliminary observations made in the five patients with oral mucosa not exposed to radiation (score 0 for all the smears) and they were not observed in the general population never exposed to ionizing radiation in the head and neck region (8). Folding cells in our samples decreased with time from the ending of radiotherapy and there was no relation with previous chemotherapy or total dose prescribed, or with the effective dose delivered to cheek mucosa in those patients in which this analysis was possible. Of interest, the number of folding cells was not influenced by the status of the oral cavity according to late or acute side-effects recorded. In contrast, we did not observe cellular atypia, *i.e.* alterations potentially leading to cancer; whether folding cells represent a pattern of very early-stage atypia then giving rise to neoplastic transformation is unclear and beyond the aims of this study. In our opinion, this peculiar cellular phenotype would represent a marker of delayed radiation injury due to chronic-disordered maturation of basal layer epithelial stem cells damaged during radiotherapy, not related to clinical mucosal damage degree during or after radiotherapy.

An inflammatory background (neutrophils and lymphocytes) was found in almost all the smears of the 45 patients, while none of those of the five preliminary patients showed inflammatory cells.

During radiotherapy, the reactive inflammatory process known as mucositis occurs; histomorphologically-infiltrating inflammatory cells, mainly mononuclear leukocytes, are concentrated in the interface between the epithelium and connective tissue (21). In our patients, clinically detectable mucositis was not common and infectious microbiota (candidiasis and massive bacteria colonization) were microscopically described only in three patients, but in absence of clinical manifestations or symptoms. Infiltrating inflammatory cells were described as prevalent in patients with neither mucositis nor

infectious background; their presence is therefore stimulated by the presence of another kind of *noxa*, in our opinion, the radiation-damaged cells (Figure 1C and D). The local inflammation described at microscopy is in fact related to the score of folding cells, acting as a "scavenger" mechanism and reducing the score. When considering this observation in relation to blood neutrophil and lymphocyte counts, in order to identify a correspondence between 'local' and 'systemic' inflammatory indicators, we observed that even within the normal range of laboratory measurements, the score of folding cells was significantly reduced in the presence of a higher lymphocyte blood count, while it was not altered by the neutrophil blood count. Both lymphocytes and neutrophils are furthermore independent from the time passed by the ending of radiotherapy. Even if blood test count was not available for all the patients studied, because we decided to perform this analysis several months after smear collection, we found these observations very interesting for two main reasons. The first is that with a simple, painless and fast method we described microscopically the presence of inflammation of which patients were unaware and which was not evident on clinical examination. The second reason deals with the possible dual role of an inflammatory background in such a context: a role of scavenger of folding cells played by the adaptive immune system in response to the stimuli of damaged cells that might become a continual local promoter of progression to atypia and cancer.

Our data suggest that the immune system plays a key role in the variability of individual response of oral mucosa to radiation damage, even in a very late setting, like the one we studied here. Further investigations are needed in the field of mucosal radiation injury; we suggest the use of exfoliative oral cytology and its related new molecular techniques as an effective approach to studying radiation toxicity, potentially offering new working strategies even in the biological biomarkers research setting.

Declaration of Interest Statement

The Authors report no declarations of interest. The Authors alone are responsible for the content and writing of the paper.

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