“Effect of glucocorticoids on the anti-cancer activity of chemotherapeutic agents in oral squamous cell carcinoma (OSCC) cells”

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Abstract of PhD thesis entitled:

“Effect of glucocorticoids on the anti-cancer activity of chemotherapeutic agents in oral squamous cell carcinoma (OSCC) cells”

submitted by

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Glucocorticoid hormones, such as hydrocortisone, are produced in the adrenal cortex and exert pleiotropic effects in peripheral tissues by regulating the expression of up to 10% of genes that are associated with a broad spectrum of metabolic processes. In addition to the adrenal-derived steroids, it is now recognized that peripheral tissues, such as the epidermis, may also act as steroidogenic organs. Recently has been shown that oral keratinocytes regulate the local concentration of active steroids as well as synthesize hydrocortisone de novo following stimulation with adrenocorticotropic hormone (ACTH). Synthetic corticosteroids are routinely administered during the treatment of several diseases, including pre-malignant and malignant conditions, particularly to alleviate side effects of chemotherapy. However, recent evidence suggests that corticosteroids may have tumour-promoting effects, particularly in epithelial neoplasms.

The aim of this thesis was to assess the influence of the recently characterized tumor-associated glucocorticoid (GC) system on both cell proliferation and migration, and on the efficacy of chemotherapeutic agents in the treatment of oral squamous cell carcinoma (OSCC).

The chemotherapeutic agents used in the present study were 5-fluorouracil (5-FU), an established drug for OSCC treatment and doxorubicin (DOXO), a potential candidate for the treatment of OSCC. Five different human malignant oral keratinocyte cell lines were selected: H314 / H357 / H400 / BICR16 / BICR56. The cell lines were treated with 5µM DOXO, 5 µg/mL 5-FU, 0.5 µg/mL Hydrocortisone (HC), 10 nM Adrenocorticotropic hormone (ACTH), 10 µM 5-pregnen-3-beta-ol-20-one-16-alfa-carbonitrile (PCN) (a Glucocorticoid Receptor antagonist), 25 µM Fasentin (a novel inhibitor of glucose uptake that interacts with GLUT1), and 10 µM WZB-117 (an inhibitor of basal glucose transport; specific GLUT1 inhibitor). The cell lines were tested with both high (4.5 g/L) and low (1g/L) glucose mediums. Moreover in vitro wound healing assays were performed using the H357 human carcinoma cell line to assess cell migration.

The literature review performed showed, in contrast to previous thought, how increased levels of autocrine, paracrine, and exogenous cortisol are important to tumor progression, as well as the expression of enzymes regulating the levels of tumor-derived cortisol. In the experimental part of the project we have clearly demonstrated, for the first time, the importance of cortisol on oral cancer cells ability to survive, migrate, and interestingly combat the effectiveness of chemotherapeutic agents. This effect would appear to be glucose dependent. Finally, Doxorubicin shows promise for the treatment of oral cancer.

In conclusion, glucocorticoids promote oral carcinoma cell proliferation and migration implying an increase in cell invasiveness. This has important implications on the pharmacological use of glucocorticoids, as topical and systemic preparations, for the treatment of a wide variety of oral conditions and in combination with chemotherapy.
Effect of glucocorticoids on the anti-cancer activity of chemotherapeutic agents in oral squamous cell carcinoma (OSCC) cells

Dr. Antonio Celentano

DDS, LDS

January 2017
DECLARATION

I declare that this thesis represents my own work, and that it has not been previously included in a thesis, dissertation, or report submitted to this University or to any other institutions for a degree, diploma or other qualifications.

Signed………………………………………………

ANTONIO CELENTANO
“Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler,
long I stood.................
I shall be telling this with a sigh Somewhere ages and ages hence:
Two roads diverged in a wood, and I took the one less traveled by,
And that has made all the difference”
- Robert Frost

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My love, Tami, for constantly being by my side, holding my hand during this breath-taking journey.
Dedicated to my family
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1. INTRODUCTION

1.1. Introduction and thesis motivation

Glucocorticoid hormones, such as hydrocortisone, are produced in the zona fasciculate of the adrenal cortex and exert pleiotropic effects in peripheral tissues by regulating the expression of up to 10% of genes that are associated with broad spectrum of metabolic processes [1]. In addition to the adrenal-derived steroids, it is now recognised that peripheral tissues, such as the epidermis, may also act as steroidogenic organs [2]. Recently it has been shown that oral keratinocytes regulate the local concentration of active steroids as well as synthesize hydrocortisone de novo following stimulation with adrenocorticotropin hormone (ACTH) [3].

Synthetic corticosteroids are routinely administered during the treatment of several diseases, including pre-malignant and malignant conditions, particularly to alleviate side effects of chemotherapy. However, recent evidence suggests that corticosteroids may have tumour-promoting effects, particularly in epithelial neoplasms [4,5]. Therefore, the role of glucocorticosteroids in oral cancer should be examined.

Oral Squamous Cell Carcinoma (OSCC) is one of the most common forms of epithelial oral cancer in the head and neck area with over 300,000 new cases per year worldwide and its prognosis still remains poor [6-8]. Current treatment strategies of OSCC include surgery, radiation therapy, and adjuvant therapy such as chemotherapy with agents such as cisplatin, carboplatin, 5-fluorouracil, paclitaxel and docetaxel [9]. Among these 5-Fluorouracil and cisplatin are the most used however there are limitations with both of these compounds and as such, other possibilities are being explored. One drug of particular interest could be doxorubicin (DOXO) in the treatment of some forms of OSCC.

Doxorubicin, an anthracycline drug that was discovered in Streptomyces peucetius var. caesius, for decades has been an established chemotherapeutic agent to treat various cancers such as sarcoma,
lung, gastric, ovarian, breast, and thyroid cancers [10]. It has several mechanisms of action including intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair and the generation of free reactive oxygen species (ROS) that cause DNA damage, oxidative stress, lipid peroxidation hence membrane damage, and activate apoptotic pathways [10,11].

Recently, it was shown to work through additional mechanisms, when delivered in combination with are chemicals, in aggressive tumours like Oral Squamous Cell Carcinoma (OSCC) cells, indicating potential for its use to treat OSCC in the near future [12]. Nevertheless, in vitro effects of DOXO on human malignant oral keratinocytes have not previously been explored, and more in general no informations about potential adverse effects of glucocorticosteroids on chemotherapeutics agents are available so far.

Therefore, the aim of this thesis was to assess the influence of the recently characterized tumor-associated glucocorticoid (GC) system on malignant cell survival, migration and also on the efficacy of chemotherapeutic agents in the treatment of oral squamous cell carcinoma (OSCC).

The chemotherapeutic agents used in the present study were 5-fluorouracil (5-FU), an established drug for OSCC treatment and Doxorubicin (DOXO), a potential candidate for the treatment of OSCC.

Our results demonstrated, for the first time, the importance of cortisol on oral cancer cells ability to survive, migrate, and interestingly combat the effectiveness of chemotherapeutic agents. This effect would appear to be glucose dependent.

Finally, Doxorubicin showed promise for the treatment of oral cancer, being effective in all the cell lines tested.
2. LIT REVIEW

2.1 The Non-Conventional Effects of Glucocorticoids in Cancer

Cortisol is an endogenous glucocorticoid (GC) hormone produced by the adrenal cortex in response to adrenocorticotropic hormone (ACTH), and it is released in response to various stress stimuli. ACTH secretion from the pituitary gland is a result of hypothalamus activation. GCs have long been regarded as drugs that promote apoptosis and inhibit cell proliferation and wound healing [13]. However, a body of evidence has mounted over the last 10 years that shows potentially harmful effects of GCs in cancer therapy.

Data from laboratory, preclinical, and clinical studies suggest that glucocorticoids induce treatment resistance in solid tumors, including prostate, ovarian, and breast cancer [5]. Our data also support this view by demonstrating that cortisol significantly reduces the cytotoxic effects of chemotherapeutic agents in several cell lines from oral squamous cell carcinoma (OSCC).

GCs also form the mainstay of the therapeutic armamentarium of a number of diseases with malignant potential, such as oral lichen planus (OLP). Therefore, the potential pro-tumorigenic activity of GCs can have vast clinical implications and warrants urgent attention.

Recent research demonstrates that epithelial cells, including those lining the skin and oral mucosa, can metabolize glucocorticoids de novo from cholesterol or systemic steroid intermediates. It has been shown that there is an endogenous non-adrenal glucocorticoid system existing in human epidermal keratinocytes and they exhibit high levels of corticosteroid metabolizing activity [14]. Keratinocytes express major enzymes involved in the synthesis and metabolism of cortisol, including cytochrome P450 chain, 11βhydroxysteroid dehydrogenases (11b-HSD1/11b-HSD2), adrenocorticotropic hormone receptor (ACTHR/MC2R), and glucocorticoid receptor [3]. (Fig. 1)
Alteration of both the Hypothalamus-Pituitary and Adrenal (HPA) axis and non-adrenal tumor-associated glucocorticoid system has been linked to carcinoma progression as it results in altered cortisol levels [15, 16]. These data may have major implications for cancer pathophysiology and therapy, as GCs are routinely administered during cancer treatment.

This chapter primarily aimed to review the current evidence supporting the traditional (conventional) and non-conventional role of GCs in normal and malignant cells.

GCs are conventionally used to up-regulate anti-inflammatory factors and down regulate pro-inflammatory factors. In oncology, GCs have been widely used in association with other treatment for cancer patients because they have potent proapoptotic properties in lymphoid cells, can reduce nausea and acute toxic effects in healthy tissue [5]. However, a number of secondary effects have been documented that influence a variety of functions such as cell migration, differentiation, apoptosis, and proliferation.
2.1.1 **Role in cell proliferation and apoptosis**

The anti-inflammatory and pro-apoptotic effects of GCs [17,18] are fundamental in their use as therapeutic agents. However, as showed on normal human epidermal keratinocytes, the cortisol can exert both immunostimulatory and immunosuppressive activities depending on its concentration [19].

GCs are able to alter signaling in key survival pathways and this can result in reversible growth arrest or cell death in certain cell types, which may be particularly important in the arrest of tumor cell proliferation [13]. However, although GCs are conventionally used to treat cancer or as adjuvants in cancer treatment, it has been observed that they may in fact induce tumor growth and metastasis formation [20,21]. The underlying mechanism in which GCs induce tumor growth has been explored and it has been proposed that the hyper-secretion of GCs decreases peripheral blood lymphocytes (PBLs); cells which play a central role in killing tumor cells [21].

Alternatively, studies have focused on the fact that GCs are inhibitory to the immune system and thus may inhibit certain molecules which are known to be tumoricidal [22]. While the phenomenon of glucocorticoid-induced apoptosis in hematological cells is well established [23], a growing body of evidence now suggests that glucocorticoids can act as anti-apoptotic agents in epithelial cells to promote cancer progression. It has been demonstrated that elevated levels of GCs during chronic restraint stress mediate an inhibitory effect on the tumor suppressing protein p53 thereby promoting tumorigenesis [24].

Moreover, extraadrenal GCs produced by intestinal epithelium have been implicated in aiding the progression of colon carcinoma cells through an immune evading mechanism [17]. The colon carcinoma cells synthesize and release bioactive immunosuppressive GCs which in turn suppress T-cell activation [17].
Guendisch et al. analyzed the role of glucocorticoids (GCs) in the survival and proliferation of tumor cells. The non-apoptotic actions of glucocorticoids on tumor cell lines, primary tumor cells, and an in vivo model along with molecular signaling studies were examined. The first important finding of this study was that dexamethasone, a commonly used glucocorticoid in cancer patients, enhances tumor cell proliferation in vitro and in vivo. Dexamethasone enhanced tumor cell proliferation in 9/17 cell lines from solid tumors from all three different germ layers [25]. It was found that these dexamethasone-induced proliferative changes were mediated by the glucocorticoid receptor. GCs lead to an activation of intracellular signaling molecules such as N3RC1, AKT, and p38-MAPK which all function in various ways to mediate dexamethasone-induced tumor cell proliferation. In terms of clinical relevance, the findings from this review suggest that the non-use of GCs might actually improve the prognosis of patients with cancer [25].

Moreover, Dexamethasone showed to suppress antitumor immune responses and facilitate tumor progression by enhancing PD-1, a key cell-surface receptor of CD28 superfamily that can attenuate T-cell responses and promote T-cell tolerance, resulting in faster tumor growth and poor prognosis in the clinical setting of anti-cancer therapy [26].

A reported well-known effect of GCs is their ability to inhibit cell proliferation [27-30]. However, it has also been shown that cortisol can increase cell proliferation at certain concentrations. Cortisol concentration modulates the level of Interleukin-6 (IL-6), a cytokine that stimulates the growth of cancer cells via an autocrine mechanism [31]. IL-6 has been associated with angiogenesis and tumor progression [32,33]. In head and neck SCC it can be correlated with recurrence, lymph node recruitment, and a poor prognostic survival [34,35]. At higher concentrations of cortisol (100 and 1000 nM) there is a lower level of IL-6 mRNA expression and secretion in oral SCC. However, at cortisol concentrations that simulate physiological stress levels (10 nM) there is an increase in IL-6 mRNA expression and secretion with a subsequent increase in cell proliferation [31].
There is also support for this increase in cell proliferation, as a result of cortisol treatment, in non-malignant situations. This has been documented in foetal cardiomyocytes and Purkinje fibers [18]. Cell nuclei expressing Ki67, a marker for cell proliferation, were found in greater numbers in foetal heart tissue that were infused with cortisol. The myocyte proliferation due to exogenous GC treatment resulted in cardiac enlargement and hypertrophy leading to increased wall thickness. The proliferative effects of cortisol were blocked by the use of an antagonist to the mineralocorticoid receptor, which is a receptor of particular interest because it can be found in the epidermis of skin and interacts with the 11b-HSD enzyme [36]. In foetal hearts it was found that there was an increase in apoptosis in Purkinje fibers which was blocked through the use of a specific antagonist to the GR [18].

In contrast, GR activation in cardiomyocytes has an anti-apoptotic effect, reflecting another nonconventional effect [37]. Studies on the transcriptional profiles of primary human keratinocytes revealed further evidence for the nonconventional anti-apoptotic effect. The treatment of keratinocytes with dexamethasone promoted anti-apoptotic gene expression and inhibited pro-apoptotic expression. As a result, the treated keratinocytes did not undergo UV-induced apoptosis [38]. To explain the presence of both apoptotic and anti-apoptotic properties of GCs, it has been suggested that they are not mutually exclusive and these effects may occur at different stages, that is, GCs inhibit early stages of differentiation and promote later stages of differentiation [38]. Therefore, GCs can have different effects depending upon cell type, developmental age and cellular environment [18].
2.1.2 Role in wound healing and anchorage independence

Delayed wound healing is a well-known effect of the therapeutic use of GCs. It has been shown that overexpression of the GR in transgenic mice slows down the skin wound healing process. This delay is attributed to the inhibition of keratinocyte proliferation, motility, and migration. Furthermore, delayed wound healing is accompanied by a decrease in granulocyte and macrophage recruitment, as well as a reduction in ERK activity and the expression of TNF-a, IL-b, proangiogenic factor, and vascular endothelial growth factor [38,39].

However, conflicting results have suggested that in fact GCs may have some repair potential. In one particular experiment, mucociliated human bronchial epithelial cell (HBEC) cultures were created to imitate an asthmatic environment and the effects of therapeutic agents such as dexamethasone were studied. As expected, it was found that dexamethasone delayed immediate wound repair of HBEC by lowering the proliferative rate of cells surrounding the injured site. However, this in turn reduced proliferative stress on epithelial stem cells and transit amplifying cells in the basal compartment of the bronchial epithelium during post-wound metaplasia. Hence, the functional lifespan of these replacement cells is enhanced leading to better long term repair potential [40].

GCs have long been used as agents in the treatment and management of particular diseases. The effects of commonly used GC medications to treat pemphigus vulgaris (PV) were studied to demonstrate whether their supposed therapeutic properties in regenerating PV-like lesions could be demonstrated in vitro. Using methylprednisolone (MP) and pyridostigmine bromide (PBr) this study found that MP and PBr significantly improved the rate of keratinocyte wound regeneration associated with PV lesions. However, it was found that these drugs could not accelerate the rate of wound healing in monolayers cultured under no conditions. This suggests that MP and PBr function to specifically offset the effects of PV serum on wounded keratinocytes [41].
Other studies have explored the effects of both endogenous and exogenous corticosteroids on human keratinocyte survival and proliferation rates. The capacity of human keratinocytes to promote the formation of anchorage-independent multicellular aggregates (MCAs) in vitro was studied using endogenously produced and exogenously administered hydrocortisone. Benign/nontumorigenic (I-7/HaCaT) and malignant low metastatic/high metastatic (II-3/RT-3) keratinocytes were treated with ACTH and assessed for determine the levels of cortisol being synthesized. It was found that the malignant keratinocytes secreted higher levels of endogenous hydrocortisone in response to ACTH treatment compared to benign keratinocytes [42]. Overall, this study demonstrates the interaction between glucocorticoids and keratinocytes during cancer progression.

These findings indicate that epidermal glucocorticoid systems are associated with tumor progression which is of clinical relevance as synthetic corticosteroids are so widely used for potentially malignant conditions.
2.2 Alteration of Cortisol and Related Enzymes in Malignancy: Possible Role of the Tumor-Associated Glucocorticoid System

Alteration of key molecules involved in the glucocorticoid pathway in cancer has been reported in previous literature. For instance, elevated cortisol levels have been found in cancer patients where it is believed it affects cancer prognosis by impairing the cellular and humoral immune response and by promoting tumor metastasis [15,16,43].

11b-Hydroxysteroid dehydrogenase (11b-HSD) is the main enzyme that regulates the endogenous activity of GCs and is expressed in two isoforms, type 1 and type 2. These two enzymes are involved in activation and deactivation of GCs (11b-HSD1 and 11b-HSD2, respectively), thus controlling cell proliferation. Expression of 11b-HSD1 generally results in decrease in cell proliferation, whereas expression of 11b-HSD2 is involved in increase in cell proliferation.

Studies have illustrated that expression of these enzymes is altered in tumors and may create a microenvironment favorable for tumor growth. For instance, an earlier study on murine and human epidermal cells observed the involvement of 11b-HSD1 in the natural skin ageing process [44]. It was found that 11b-HSD1 expression increases in ageing skin, resulting in local GC excess and adverse effects such as: altered skin integrity, thinning, and impaired wound healing. This is due to the changes in the extracellular matrix (ECM) including: collagen atrophy, collagen disorganization, shredded appearance of collagen structure, and large inter-fibril spaces. This disordered ECM microenvironment is vital for tumor metastasis. Hence the study suggested that the local increase in 11b-HSD1 in ageing skin may increase the risk of skin cancer.

The GC receptor (GR) also plays a key role in the glucocorticoid pathway. It is a member of the nuclear hormone receptor family normally located within the cytoplasm of cells. Upon binding with cortisol it migrates to the nucleus and functions as a transcription activator or repressor that affects
<table>
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<td>Skin SCC, basal cell carcinoma (BCC) and seborrheic keratosis (SK)</td>
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<td>Reduction in 11b-HSD1 expression with increased keratinocyte proliferation. 11b-HSD2 expression increased in basal cell proliferating conditions such as BCC and SK.</td>
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<td>Glucocorticoid receptor</td>
<td>In early skin papilloma GR expression is reduced but in late papilloma and in SCC, GR levels are similar or higher than normal.</td>
</tr>
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<td>Mouse epidermal papillomas and SCC.</td>
<td>Glucocorticoid receptor</td>
<td>No significant changes in GR gene structure and expression in cancer but resistance to glucocorticoid flucinolone acetonid more likely due to alterations in receptor function.</td>
</tr>
<tr>
<td>Waters et al. (2004) [55]</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Normal expression of GR, however its functionality altered by subtle changes in co-factors such as Nuclear co-repressor which was overexpressed in cancer.</td>
</tr>
<tr>
<td>Ray et al. (1996) [56]</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Mutations in GR gene structure lead to its impaired function.</td>
</tr>
<tr>
<td>Parks et al. (1998) [57]</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Abnormal splicing of the GR transcript causes GR resistance to GC stimulation in cancer cells.</td>
</tr>
<tr>
<td>Kay et al. (2011) [58]</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Increased DNA methylation of promoter c leads to decreased GR expression.</td>
</tr>
<tr>
<td>Li et al. (1996) [59]</td>
<td>Transformed A5 mouse lung cells</td>
<td>Glucocorticoid receptor</td>
<td>Transformed cells contain functional GR but express cjun which antagonizes glucocorticoids.</td>
</tr>
</tbody>
</table>
gene regulation to ultimately cause decreased cell proliferation [13,14]. Mutations in GR structure, aberrant processing of GR pre-mRNA and impaired functionality have been implicated in cancer progression. **Table 1** lists studies that have demonstrated dysregulation of cortisol, 11b-HSD enzymes, and glucocorticoid receptor in different types of cancers.
2.3 Use of Corticosteroids in Malignancy and Premalignant Conditions

GCs have a wide array of uses and effects in premalignant and malignant conditions. As strong anti-inflammatory agents, they have the ability to regulate cell fate by inducing expression of anti-apoptotic genes while suppressing apoptotic factors [60,61]. Additionally, GCs are used as analgesics and antiemetics by patients receiving cancer treatment [38].

There are possible mechanism-based benefits in the use of GCs in pre-malignant conditions. An example of a potentially malignant condition that has potential for transformation is oral lichen planus. Progression to oral SCC can occur in up to 5.8% of cases [62,63]. Lichen planus is an autoimmune condition characterized by chronic inflammation [64]. It is postulated that chronic inflammation is a factor that may lead to the development of malignancy such as oral SCC. The powerful anti-inflammatory effects of GCs may, therefore, be a protective factor in this situation, and they are currently first-line treatment for this condition [65-67].

Nevertheless, the recent finding that GCs may bear cancer-promoting effects warrants further consideration in this area. GCs are used as a monotherapy or in combination with other treatments to manage and treat many different forms of malignancy. GC monotherapy has been shown to have positive treatment outcomes in patients with breast and prostate cancer via proposed mechanisms of adrenocortical inhibition and adrenal androgen suppression, respectively [68].

In a systematic review investigating the combination therapy of chemotherapy ± GCs, evidence supported such added treatment outcomes as decreased leukopenia, decreased thrombocytopenia, and an improved tolerance for increased chemotherapy dose in the group that received GCs as an adjunct to their chemotherapy compared to the group only receiving chemotherapy [68].

Corticosteroids are also prescribed for the treatment of hypercalcaemia associated with malignancy. Renal tubular resistance to endogenous calcitonin during treatment for hypercalcaemia in malignancy can be overcome by the addition of corticosteroids to the treatment [69].
Despite their benefits in cancer treatment, GCs have also paradoxically been implicated in cancer progression [17,70,71]. The postulated benefits of corticosteroids in preventing malignancy should therefore be balanced against the possible carcinogenic effects of immunosuppression warranting further research in this area.

In a study of the primary adult brain tumor, glioblastoma, the use of GCs as part of the standard treatment protocol was examined. The standard treatment protocol for primary adult tumors included maximum surgical resection, radiation therapy, and chemotherapy with about 99% of the patients also receiving perioperative corticosteroids with some patients receiving continued doses throughout the course of care [72]. High dose GCs are generally prescribed for their anti-inflammatory mechanism to help combat radiation therapy associated brain swelling and tumor oedema (a conventional use and effect). However, GCs such as Dexamethasone that are regularly prescribed have been shown to considerably increase blood glucose levels.

The blood glucose is a fuel for glycolysis-dependent tumors and also facilitates production of glutamate, a neurotransmitter linked with causing excitotoxic damage to neurons [72]. Higher levels of blood glucose have been shown to accelerate brain tumor growth and lower treatment prognosis [72]. Glucocorticoid-induced resistance has been identified in cells of solid tumors when used with various anticancer drugs and with radiotherapy.

Such observations were made in established carcinoma cell lines cultured in vitro, in xenografts on nude mice, and in primary cells that had been isolated from fresh surgical samples of solid tumors [73-75]. Tumors analyzed were derived from bladder, brain, breast, cervix, colon or rectum, liver, lung, kidney, ovary, pancreas, and prostate.

The finding that the tumor-associated glucocorticoid system is active in all these tumors (Cirillo et al., manuscript in preparation) adds a further level of complexity and raises the possibility that a deregulated GC metabolism may serve as a tumor promoting mechanism in cancer (Fig. 2).
Overall, it can be ascertained from the wide array of research on this topic that the effects and uses of GCs in malignant conditions can be both beneficial and detrimental in terms of controlling tumor growth and progression.

Conventional uses of GCs have been shown to help treat malignant conditions either alone or in combination with other treatment modalities. Yet the literature also supports the adverse effects of GCs in tumor progression and growth which are linked closely to the non-conventional effects of GCs in malignant conditions.

**Fig. 2:** Schematic representation of cancer-promoting glucocorticoid effects
2.4 Conclusion

Based on preliminary data and evidence obtained from this literature review it can be suggested that in contrast to previous thought, increased levels of autocrine, paracrine, and exogenous cortisol are important to tumor progression. Hence, it is possible that alterations in the expression of enzymes regulating the levels of tumor-derived cortisol take place in cancers.

It will be important, therefore, to assess the levels of expression of steroidogenic molecules, steroids, and receptors among normal and malignant epithelial cells as well as the correlation of the expression levels of steroid-related molecules to the clinical-pathological parameters of cancer.

In addition to shedding light on key patho-physiological mechanisms of SCC, characterization of the tumor-associated GC systems will have salient diagnostic, preventive, and therapeutic clinical implications. Changes in the expression levels of components of the epithelial GC pathway may aid in the selection of novel markers of cancer progression. Identification of the presence of alterations in the steroid pathway in malignant epithelial cells would open new avenues in the management of inflammatory pre-malignant conditions such as lichen planus and provide basis for a mechanism-based approach to cancer treatment.
3. MATERIAL AND METHODS

3.1. Cell lines

Five different human malignant oral keratinocyte cell lines were selected for the study: H314, H357, H400, BICR16, BICR56. The main cell line used in most of the experiments was H357. The other cell lines were used as confirmatory when significant results were obtained with H357.

The H series OSCC adherent cell lines H314/H357/H400 were established at Bristol Dental School, University of Bristol, UK by Prime et al [76], from primary explants of floor of the mouth, tongue and alveolar process squamous cell carcinoma respectively.

The BICR16, BICR56 OSCC adherent cell lines were established at Beatson Laboratories, Glasgow, UK by Parkinson et al [77], from recurrent squamous cell carcinomas of the tongue.

All of the OSCCs were HPV negative. All of the cell lines/strains were derived prior to 2001 and therefore, were not subject to Ethical Committee approval in the UK.

3.2. Culture conditions

At the time of experimentation, all the above mentioned cell lines were cultured in 100-mm Petri plastic dishes (Corning® 430167, Corning, NY, USA) and grown to 60-80% confluence before being split.

Cells were cultured using standard a standard medium composed by:

- Dulbecco’s Modified Eagle’s Medium DMEM (D5796) - F12 (N6658) (1:1) (Sigma-Aldrich, Castle Hill, NSW, Australia) supplemented with
- 10% Fetal Bovine Serum (FBS) (SFBS-F, Bovogen, Keilor East, Vic, Australia),
- 1% pen/strep (P4333, Sigma-Aldrich, Castle Hill, NSW, Australia) and
• 0.5 µg/ml (100nM) hydrocortisone (HC) (H6909, Sigma-Aldrich, Castle Hill, NSW, Australia) in a humidified atmosphere at standard conditions (5% CO2, 37°C).

The epithelial cells at confluency were then detached using a pre-treatment of 10 mM EDTA for 10 minutes, and subsequently 0.25% trypsin - 1 mM EDTA (T4049, Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 minutes.

The viability of the keratinocytes was confirmed by Trypan Blue exclusion (Trypan Blue Dye, 0.4% solution, 1450021, Biorad, UK).

3.3. Glucocorticoid system and chemotherapy

The cell lines were treated with: 5µM DOXO (44583, Sigma-Aldrich, Castle Hill, NSW, Australia), 5 µg/mL 5-FU (F6627, Sigma-Aldrich, Castle Hill, NSW, Australia), 100 nM HC, 10 nM ACTH (A2227, Sigma-Aldrich, Castle Hill, NSW, Australia), 10 µM 5-pregn-3-beta-ol-20-one-16-alfa-carbonitrile (PCN) (a Glucocorticoid Receptor antagonist) (P0543, Sigma-Aldrich, Castle Hill, NSW, Australia), 25 µM Fasentin (a novel inhibitor of glucose uptake that interacts with GLUT1) (F5557, Sigma-Aldrich, Castle Hill, NSW, Australia), and 10 µM WZB-117 (an inhibitor of basal glucose transport; specific GLUT1 inhibitor) (SML0621, Sigma-Aldrich, Castle Hill, NSW, Australia).

The cell lines were tested with both high (4.5 g/L) and low (1g/L) glucose mediums (DMEM D5796 and D6046).

Cell number and viability were assessed at 0/24/48/72 hours (TC10™ Automated Cell Counter & Trypan Blue assay, Bio-Rad, UK).
3.4. Apoptosis assays

Firstly, DNA morphology was evaluated with Hoechst 33342 fluorescence staining, (Sigma-Aldrich) at fluorescence microscopy (EVOS™ FLoid™ Cell Imaging Station, Life technologies). Morphological changes leading to condensed or fragmented nuclei were considered as apoptotic cells.

Annexin V-FITC assay (APOAF, Sigma, Saint Louis, Missouri, USA) was subsequently used to study the apoptotic process in detail. At 0/3/6/12/24/48/72 hours the H357 cells, untreated, treated with DOXO or with DOXO+HC were fixed with 500 µL of 100% ice-cold methanol for 10 min at r/t and stained with annexin V (at the concentration suggested by the supplier). The use of methanol caused the permeabilization of the cellular membranes allowing us to study apoptotic phosphatidylserine exposure and cytoplasmic vesicles trafficking overtime.

The same experiment was also performed at 0/6/12/24 hours, but fixing the cells with 4% formalin and assessing the apoptosis rate with or without the use of FBS in the experimental medium. Confirmatory experiments were performed on BICR16 and BICR56 cell lines in the same experimental conditions.

3.5. Wound healing assay

Cell migration assay to investigate the effect of corticosteroids on cell migration was examined by the in vitro scratch wound healing assay using the H357 cell line.

Keratinocytes were plated on 12 well tissue culture plates (Corning® Costar® 3513, Corning, NY, USA) and allowed to reach 100% confluence. At this point a scratch was introduced into the monolayer using a sterile 1 ml Eppendorf® pipette tip. The cells were then washed in phosphate buffered saline (0.01M PBS, pH = 7.4) to remove the debris before being incubate with either plain
fresh medium (DMEM-F12, 10% FBS, 1%P/S) (control samples), 100nM hydrocortisone, 10 nM ACTH, 5µM DOXO.

Digital microscopy (EVOS™ FLoid™ Cell Imaging Station, Life technologies) was used to observe cell migration across the edges and to capture images of the wound at 0/4/8/24 hours.

10 pictures at each timepoint at x460 magnification were taken to measure wound healing over time.

The captured images were analysed using ImageJ Software (ImageJ v. 1.50i, Wayne Rasband, National institute of Health, USA). All the experiments were performed in triplicate.

3.6. Statistical analysis

The statistical significance of the data was evaluated by using unpaired Student’s t test and ANOVA. Data are reported as mean ± standard deviation and differences were considered to be significant when p was <0.05.
4. PRELIMINARY EXPERIMENTS

At the beginning of this project a series of preliminary experiments was performed with the main aim to assess the best experimental conditions. Each cell line was tested with several types of culture medium, experimental medium, seeding densities (ranging from 50% up to 100%), concentrations of hormones, chemicals and drugs, including different cell splitting procedures and timing.

e.g. for the DOXO the different concentrations tested were 5 µM, 10 µM, 50 µM, while for 5-FU were 0.5 µg/mL, 1 µg/mL.

An example of some of the main protocols and findings from the preliminary experiments is reported below.

4.1. Experiment 1: growth curve, 80% confluent cells

Protocol:
- Cell line: H357
- Cell culture 12-well Plate
- Seed cells (2.5 x 10^5 cells/ml in all the wells) in standard volume of 1 ml
- Wait overnight (12h)
- Change medium (without washing)
- Add the chemotherapeutical agents all together
- At each timepoint:
  - Remove medium and put it in eppendorf
  - Centrifuge it at 200g x 4 min at 4°C
  - Discharge supernatant
  - In each well wash X2 with PBS 500µL
  - Pretreatment with EDTA (10 mM) (5 min)
  - ADD 100 µL of Tripsin-EDTA (wait 10 min)
  - Add 900 µL of medium
  - Add the above to the previous eppendorf in a final standard volume of 1 ml and mix gently.
  - Cell counting (x1)
  - Trypan blue assay (x1)
4.2. Experiment 2: growth curve, 100% confluent cells

Protocol:
- Cell line: H357
- Cell culture 12-well Plate
- 100% confluent cells in a standard volume OF 1 mL
- Change medium (without washing)
- Add the chemotherapeutical agents all together

At each timepoint:
- Remove medium and put it in eppendorf
- Centrifuge it at 800g x 4 min at 4°C
- Discharge supernatant
- In each well wash X2 with PBS
- Pretreatment with µL 150 EDTA (10 mM) (8 min)
- ADD 150 µL of trypsin-EDTA (wait 11 min)
- Add 700 µL of medium
- Add the above to the previous eppendorf in a final standard volume of 1 ml and mix gently.
- Cell counting (x1)
- Trypane blue assay (x1)
4.3. Experiment 5: growth curve, 50% confluent cells

**Protocol:**

- Cell line: H357
- Cell culture 12-well Plate
- 50% CONFLUENT H357 cells in a standard volume of 1 mL
- Change medium (without washing)
- Add the chemotherapeutical agents all together

**At each timepoint:**
- Remove medium and put it in eppendorf
- Centrifuge it at 200g x 4 min at 4°C
- Discharge supernatant
- In each well wash X2 with PBS 500µL
- Pretreatment with µL 150 EDTA (10 mM) (8 min)
- ADD 150 µL of Tripsin-EDTA (wait 11 min)
- Add 700 µL of medium
- Add the above to the previous eppendorf in a final standard volume of 1 ml and mix gently.
- Cell counting (x1)
- Trypane blue assay (x1)
4.4. Experiment 7: DOXO, hydrocortisone, and PCN

Protocol:
- Cell line: H357
- Cell culture 12-well Plate
  - Culture Medium: DMEM 5796 : HAMS F12 (1:1) +
    - 10% FBS
    - 1% Pen/Strept
    - 0.5 µg/ml hydrocortisone
  - Sub-culture passage number = 7
  - 24 hours before the experiments switch the medium (without Hydrocortisone)
  - split the H357 cells and seed them in 12 well plates with a final density of: 2x10^5 cells/mL in a STANDARD VOLUME OF 1 mL
  - wait overnight
  - T=0:
    - wash twice with 500 µL PBS
    - add all together the chemotherapeutic agents already diluted in 1 mL of fresh medium
- At each timepoint in each well:
  - Take picture
  - Remove medium
  - wash X2 with 500 µL of PBS
  - Pretreatment with 150 µL of 10 mM EDTA for 5 min
  - Discharge EDTA
  - Add 150 µL of trypsin-EDTA 0.25% for 5 min
  - Add 700 µL of medium
  - Cell counting with Trypan blue assay (x1) (10 readings each slide)
4.5. Experiment 8: 5-FU, hydrocortisone, and PCN

**Protocol:**
- Cell line: H357
- Cell culture 12-well Plate
- Culture Medium: DMEM 5796 :HAMS F12 (1:1) +
  - 10% FBS
  - 1% Pen/Strept
  - 0.5 µg/ml hydrocortisone
- Sub-culture passage number = 7
- 24 hours before the experiments switch the medium (without Hydrocortisone)
- split the H357 cells and seed them in 12 well plates with a final density of: 2x10^5 cells/mL in a standard volume OF 1 mL
- wait overnight
- Concentrations:
  - **5-FU**: 5 µg/mL
  - **Hydrocortisone**: 0.5 µg/mL
  - **Pregnenolone-16a-carbonitrile (PCN)**: 10 µM
- Replace medium and chemotherapeutic agents every 24 hours
- T=0:
  - wash twice with 500µL PBS
  - add all together the chemotherapeutic agents already diluted in1ml of fresh medium
- At each timepoint in each well:
  - Take picture
  - Remove medium
  - wash X2 with 500 µL of PBS
  - Pretreatment with 150 µL of 10mM EDTA for 5 min
  - Discharge EDTA
  - ADD 150 µL of TRYPsin-EDTA 0.25% for 5 min
  - Add 700 µL of MEDIUM
  - Cell counting with Trypane blue assay (x1) (10 readings each slide)
Controllo 5-FU 5 μg/mL 5-FU + HC 5-FU+HC + PCN 10 μM

Time:
- t=24h
- t=48h
- t=72h
4.6. Experiment 9: 5-FU, hydrocortisone and 3 different experimental mediums

5-FU, HYDROCORTISONE, TRIPLICATE ON 10 READINGS
Medium A- DMEM F12 (1:1) + 1% Foetal Bovine Serum (FBS) +1% Pen/Strept
Medium B- DMEM F12 (1:1) + 10% Foetal Bovine Serum (FBS) +1% Pen/Strept
Medium C- DMEM F12 (3:1) + 10% Foetal Bovine Serum (FBS) +1% Pen/Strept
24 hours before switch the medium (without Hydrocortisone)
48 hours before the experiments switch the medium (1%FBS) (12 well n.1)
density: 1.5x105 cells/mL
4.7. Experiment 10: 5-FU, hydrocortisone and 3 different experimental mediums

DOXO and 5-FU, HYDROCORTISONE, ON 10 READINGS
A- DMEM F12 (1:1) + 1% Foetal Bovine Serum (FBS)
B- ONLY DMEM + 10% Foetal Bovine Serum (FBS)
C- DMEM F12 (3:1) + 10% Foetal Bovine Serum (FBS)
24 hours before switch the medium (DMEM/F12 1:1, 1% FBS, NO HYDROCORTISONE, NO ANTIBIOTIC)
density: 1.5x10^5 cells/mL
5. THE EFFECTS OF GLUCOCORTICOID SYSTEM ON CANCER ENERGY METABOLISM AND RESPONSE TO CHEMOTHERAPY

5.1. DOXO and 5-FU induce apoptosis in malignant oral keratinocytes.

5.1.1. Material methods

All the cell lines were grown in standard medium (see section 3.2). The cells at 60-80% confluency were then detached using a pre-treatment of 10 mM EDTA for 10 minutes, and subsequently 0.25% trypsin - 1 mM EDTA (T4049, Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 minutes. The cells were then plated on 12 well tissue culture plates (Corning® Costar® 3513, Corning, NY, USA) and grown in standard conditions. After an overnight period, at T=0 the cells were washed twice with 500 µL of PBS and were grown in either experimental medium, or experimental medium with DOXO 5 µM or 5-FU 5 µg/mL. At each time point the medium was removed in each well and after two washing with 500 µL of PBS the cells that remain attached were considered as strongly adherent cells hence live. The cells were then splitted and cell number and viability were assessed at each timepoint (TC10™ Automated Cell Counter & Trypan Blue assay, Bio-Rad, UK).

DNA morphology was evaluated with Hoechst 33342 fluorescence staining, (Sigma-Aldrich) at fluorescence microscopy (EVOS™ FLoid™ Cell Imaging Station, Life technologies). Morphological changes leading to condensed or fragmented nuclei were considered as apoptotic cells.

5.1.2. Results

Both DOXO and 5-FU induced cytotoxic effects in all the cell lines tested as early as 24h after treatment, and peaked at 48h (Fig.3a). For this reason 48 hours was selected as the main time point for the further experiments.
Morphological changes, condensed and fragmented dna pattern at fluorescence microscopy confirmed that oscc cells underwent apoptosis (Fig.3b, Fig.4).

**Fig. 3:** a) Effectiveness of chemotherapeutics agents on H357 cell line overtime; b) Hoechst fluorescence staining observed at microscopy at 48 hrs timepoint confirmed the induction of apoptosis in both Doxo and 5-FU groups. Statistical significance is given as follows: *p < 0.05; **p < 0.005.

**Fig. 4:** Fluorescence microscopy showing fragmented and shrinked DNA pattern induced by DOXO at 48 hrs on H357 cell line. (460X magnification)
5.2. Hydrocortisone reduce the apoptosis induced by chemotherapeutic agents

Annexin V-FITC assay (APOAF, Sigma, Saint Louis, Missouri, USA) was subsequently used to study the apoptotic process in detail and to assess the effect of HC administration on the apoptotic process.

Firstly, at 0/3/6/12/24/48/72 hours the H357 cells, untreated, treated with DOXO or with DOXO+HC were fixed with 500 µL of 100% ice-cold methanol for 10 min at r/t and stained with annexin V (at the concentration suggested by the supplier). The use of methanol caused the permeabilization of the cellular membranes allowing us to study apoptotic phosphatidylserine exposure and cytoplasmic vesicles trafficking overtime.

The same experiment was also performed with DOXO and 5-FU at 0/6/12/24 hours, but fixing the cells with 4% formalin and assessing the apoptosis rate with or without the use of FBS in the experimental medium. Confirmatory experiments were performed on BICR16 and BICR56 cell lines in the same experimental conditions.

5.2.1. Material and methods

**Culture Medium:**

- DMEM 5796 : F12 (1:1)
- 10% FBS
- 1% p/s
- Hydrocortisone 100 nM

**Experimental medium:**

- DMEM 5796 : F12 (3:1)
- 10% FBS
- 1% p/s

**Experimental serum free medium (SFM):**

- DMEM 5796 : F12 (3:1)
- 1% p/s

**Concentrations:** DOXO: 5 µM, 5-FU: 5 µg/mL, HC: 100nM
**Protocol:**

Cells were grown in complete culture medium and seeded into 12-well plates at a density of ranging from 1.5 to 2 *10^5 cells/mL in a standard volume of 1 mL (again culture medium).

After overnight, the wells were washed twice with 500 µL of PBS. 1 mL of experimental medium or experimental SFM was placed in each well and the treatments were performed.

At each time point the supernatant was remove, re-suspended in a 1.8 mL eppendorf and the cells counted. Then the cells were fixed with 500 µL of 100% ice-cold methanol or with 4% formalin for 10 min at R/T. Each well was washed 3 times with 500 µL of PBS. 500 µL of PBS were left in the fixed wells to avoid drying.

**Annexin Kit preparation:**

- 0.5 mL of the Binding Buffer was diluted in 4.5 mL of ultrapure distilled water (10977-015, Invitrogen, Life technologies) in a 5 mL falcon tube. 100 µL of Annexin V FITC conjugate was added to 5 ml of the above buffer.

At the end of all the timepoints, 200 µL of annexin diluted were added to each well and incubated for 30 mins at 4°C protected from light. The staining was then removed, the wells were washed 3 times with 500 µL of PBS and observed with fluorescence microscopy. The results were obtained by the analysis of 5 fields from each well. The apoptotic ratio was calculated as a ratio between the average number of positive cells (granular, strongly positive stained cells) divided by the average of the total number of cells found.

1 mM hydroxide peroxide for 1 hr was used as positive control at 0/12/24/48 hours timepoints.
5.2.2. Results

The results were consistent with the previous section. After being treated with doxorubicin or 5-FU up to 24 h, cells were stained with FITC–Annexin V. The strong green fluorescence signal was identified on the treated cells but not on the untreated cells, allowing us to calculate the respective apoptotic ratio.

All the cell lines tested showed to be sensitive to doxorubicin as early as 12 hours after treatment (Fig. 5, Fig. 6, Fig. 7).

In all the experiments the administration of 100nM HC was able to reduce the effectiveness of the chemotherapeutics tested when compared to the groups treated with the chemotherapeutics agents alone. This effect was demonstrated with statistical significance in the biological triplicate (H357, BICR16, BICR56) with DOXO (Fig. 10).

Conversely, an inverted trend has been found in H357 cells when the experimental medium was deprived of the serum (SFM). In this case in fact the effectiveness of DOXO was even enhanced by the addition of HC (Fig. 8).
5-FU also effectively showed to exert citotoxic effects on H357 cells. Also in this case the HC administration was able to reduce the number of apoptotic cells, hence the effectiveness of the drug (Fig. 9).

![Graph](image)

**Fig. 6:** Annexin apoptosis assay on BICR56 cell line with DOXO, and DOXO+HC at 0/6/12/24 hours

![Graph](image)

**Fig. 7:** Annexin apoptosis assay on BICR16 cell line with DOXO, and DOXO+HC at 0/6/12/24 hours
Fig. 8: Annexin apoptosis assay on H357 cell line with DOXO, and DOXO+HC at 0/6/12/24 hours with serum free medium (SFM)

Fig. 9: Annexin apoptosis assay on H357 cell line with 5-FU, and 5-FU+HC at 0/6/12/24 hours
Fig. 10: Annexin apoptosis assay. The results showed were calculated as the average (biological triplicate) of the results obtained with the single cell lines: H357, BICR16, BICR56. Statistical significance is given as follows: *p < 0.05; **p < 0.005

Hydrogen peroxide is a well known inducer of apoptosis in vitro. The positive control was then performed with 1 mM hydrogen peroxide at 0/6/12/24 hours. The results showed that 50% of the cells underwent apoptosis as early as 12 hours after H₂O₂ exposure, and after 24 hours the apoptotic cells were more than 90% of the total (Fig. 11, Fig. 12).
Fig. 11: Positive control of Annexin apoptosis assay with 1 mM hydrogen peroxide at 0/6/12/24 hours. The apoptotic cells showed strong green fluorescence signal increasing overtime at fluorescence microscopy.

Fig. 12: Positive control of Annexin apoptosis assay with 1 mM hydrogen peroxide at 0/6/12/24 hours.

It is well-known that apoptosis is a dynamic process. In fact, as the cell undergoes apoptosis, there are a number of steps in the process that may be investigated using very different imaging modalities [78].
Among them, phosphatidylserine (PS) exposure occurs very early in the apoptotic chain of events, preceding such hallmark events as nuclear condensation and DNA laddering.

Moreover, PS exposure is a near-universal event in apoptosis, and it presents a very abundant target (millions of binding sites per cell) that is readily accessible on the extracellular face of the plasma membrane [79].

Annexin V, an endogenous human protein with molecular weight of 36 kDa, has a high affinity (kd=7 nM) for PS binding [80]. Due to the high affinity for apoptotic cells, no immunogenicity, and lack of in vivo toxicity, Annexin V is the dominant probe to detect and image apoptosis.

In our first apoptosis experiment we decided to use 100% methanol as fixative agent, to permeabilize the cellular membranes and to study apoptotic phosphatidylserine exposure and trafficking overtime.

While at 6 hours the fluorescence was mainly detected with a weak, diffuse cytoplasmic pattern, at 12 hours after the exposure to DOXO a strong green fluorescence signal with a linear pattern was identified on the cell membranes of the treated cells but not on the untreated cells indicating the presence of early apoptosis processes (Fig. 13).
Fig. 13: Phosphatidylserine exposure and trafficking overtime in the annexin apoptosis assay. The early apoptotic events were studied at 0/3/6/12/24/48 hours performing the cells fixation with 100% methanol inducing the permeabilization of the cell membranes.
5.3. Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucose-dependent mechanisms

In this series of experiments the cell lines were grown in standard conditions and treated with the two chemotherapeutic agents DOXO and 5-FU overtime.

We tested the effects of the drugs alone or the effects of the administration of the chemotherapeutic agents in combination with exogenous and endogenous corticosteroids. In particular HC was used as an exogenous corticosteroid, while the study of endogenous corticosteroids was obtained through the stimulation of the cells with ACTH to induce cellular cortisol production [3,52].

Besides the normal controls, 5-pregnen-3-beta-ol-20-one-16-alfa-carbonitrile (PCN), a Glucocorticoid Receptor antagonist, was also used to furtherly assess the coherency of the glucocorticoids effects.

The results suggested us a high correlation between the glucocorticoids effects and the cell culture metabolic conditions. For this reason, the cell lines were also tested with two different metabolic conditions using both high (4.5 g/L) and low (1g/L) glucose grow medium. In this case the results were confirmed with the use of Fasentin and WZB-117, two inhibitors of glucose uptake.

5.3.1. Material and methods

All the cell lines were grown in standard medium (see section 3.2).

24 hours before the experiments the medium was switched with a standard medium deprived of Hydrocortisone. The cells at 60-80% confluency were then detached using a pre-treatment of 10 mM EDTA for 10 minutes, and subsequently 0.25% trypsin - 1 mM EDTA (T4049, Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 minutes. The cells were then plated on 12 well tissue culture plates (Corning® Costar® 3513, Corning, NY, USA) and grown in standard conditions. After an
overnight period, at T=0 the cells were washed twice with 500 µL of PBS and were grown overtime in either experimental medium, or experimental medium supplemented with:

- 5µM DOXO (44583, Sigma-Aldrich, Castle Hill, NSW, Australia),
- 5 µg/mL 5-FU (F6627, Sigma-Aldrich, Castle Hill, NSW, Australia),
- 100 nM HC, (H6909, Sigma-Aldrich, Castle Hill, NSW, Australia),
- 10 nM ACTH (A2227, Sigma-Aldrich, Castle Hill, NSW, Australia),
- 10 µM 5-pregnen-3-beta-ol-20-one-16-alfa-carbonitrile (PCN) (a Glucocorticoid Receptor antagonist) (P0543, Sigma-Aldrich, Castle Hill, NSW, Australia),
- 25 µM Fasentin (a novel inhibitor of glucose uptake that interacts with GLUT1) (F5557, Sigma-Aldrich, Castle Hill, NSW, Australia), and
- 10 µM WZB-117 (an inhibitor of basal glucose transport; specific GLUT1 inhibitor) (SML0621, Sigma-Aldrich, Castle Hill, NSW, Australia).

During all the experiments, the medium and all the above treatments were replaced every 24 hours. The cell lines were tested with both high (4.5 g/L) and low (1g/L) glucose mediums (DMEM D5796 and D6046).

At each time point the medium was removed in each well and after two washing with 500 µL of PBS the cells that remain attached were considered as strongly adherent cells hence live. The cells were then splitted (see section 3.2) and cell number and viability were assessed at each timepoint (TC10™ Automated Cell Counter & Trypan Blue assay, Bio-Rad, UK).

5.3.2. Results

DOXO, a potential candidate for the treatment of OSCC, was effective on all the 5 lines tested (Fig. 14, Fig. 15). 5-FU was slightly more effective than DOXO in three of cell lines tested.

On H357 cell line both endogenous and exogenous corticosteroids significantly reduced the effectiveness of DOXO (Fig. 14a).
Overall, HC administration (100nM) reduced the effectiveness of DOXO and 5-FU in 3 (H314, H357, H400) and 2 (H314, BICR56) of the 5 cell lines tested, respectively.

The increased production of cortisol through ACTH administration instead reduced the effectiveness of DOXO in 2 cell lines (H357, BICR56).

The use of the GR inhibitor PCN drastically deleted these corticosteroids effects, confirming our hypothesis (Fig. 14b).

Fasentin and WZB, novel inhibitors of glucose uptake, were able to partially block the increased resistance to the cytotoxic drugs induced by HC in H357 cell line (Fig. 14b).

Switching the growth medium to a lower glucose dosage (1 g/L), this effect was still present, but only with exogenous HC (Fig. 14b).
Fig. 15: The effect of glucocorticoid system on the effectiveness of DOXO and 5-FU at 48 hours on BICR56 (a), BICR16 (b), H314 (c), and H400 (d) cell lines. Statistical significance is given as follows: *p < 0.05; **p < 0.005

On BICR56 cell line endogenous corticosteroids significantly reduced the effectiveness of doxorubicin while exogenous reduced the effectiveness of 5-FU. Also in this case the use of the GR inhibitor PCN drastically deleted corticosteroids effects (Fig. 15a).

The use of low glucose medium deleted and even inverted the Hydrocortisone effects.

In fact also in BICR56 and H314 cell lines the results showed that while in high glucose metabolic conditions the corticosteroids were able to reduce the effectiveness of both DOXO and 5-FU, the switch to a lower glucose dosage deleted and even inverted this trend, highlighting the key role that glucose transport may play in these effects (Fig. 15b, Fig. 15c).
Paradoxically, in one of the cell line tested, the H400 we found that the use of corticosteroids increased the effectiveness of both the chemotherapeutic tested (Fig. 15d). Interestingly the H400 cell line was primarily isolated from a moderately differentiated, node negative tumour and is the only cell line among those tested to be non-tumourigenic on subcutaneous injection into athymic nude mice.
6. THE EFFECT OF GLUCOCORTICOIDs ON THE INVASIVE BEHAVIOUR OF SQUAMOUS CARCINOMA CELLS

6.1. Exogenous glucocorticoids promote oral carcinoma cell migration implying an increase in cell invasiveness

To assess the effect of glucocorticoids on cell migration in vitro, wound healing assays were performed using the H357 cell line. The scratch assay is a simple and reproducible method to measure cell migration in vitro (Fig. 16).

![Fig. 16: Schematic representation of wound healing assay: the grey areas represent the initial cell fronts (T=0). The blue (T=4hr) and yellow (T=8hr) areas represent the change in the size of the wound, as the cell fronts close. At T=24hr the wound is completely closed because of the cell migration.]

The Cells were grown to 100% confluence, before being scratched and incubated with either plain medium, hydrocortisone, ACTH or 18-β glycyrrhetinic acid.

Digital microscopy was then used to capture images of the wound at 0/4/8/24 hours.

Precisely, 10 pictures at each timepoint at x460 magnification were taken to measure wound healing over time and the captured images were subsequently analysed using ImageJ Software.
6.1.1. Material and methods

Keratinocytes were plated on 12 well tissue culture plates (Corning® Costar® 3513, Corning, NY, USA) and allowed to reach 100% confluence (Fig. 17). At this point a scratch was introduced into the monolayer using a sterile 1 ml Eppendorf® pipette tip. The cells were then washed in phosphate buffered saline (0.01M PBS, pH = 7.4) to remove the debris before being incubate with either plain fresh medium (DMEM-F12, 10% FBS, 1% P/S) (control samples), 100nM hydrocortisone (Exogenous glucocorticoids), 10 nM ACTH (for increasing the levels of endogenous glucocorticoids), 0.02% w/v 18β-Glycyrrhetinic acid (Inhibitor of 11β-HSD2, which inactivates cortisol).

Digital microscopy (EVOS™ FLoid™ Cell Imaging Station, Life technologies) was used to observe cell migration across the edges and to capture images of the wound at 0/4/8/24/48 hours.

10 pictures at each timepoint at x460 magnification were taken to measure wound healing over time. Total of 840 pictures were taken and measured. The captured images were analysed using ImageJ Software (ImageJ v. 1.50i, Wayne Rasband, National institute of Health, USA).

All the images were firstly adjusted with ImageJ software enhancing the contrast to better identify the edges of the wound. Subsequently “Area selection” tool was used to draw the area between the two healing fronts for each image, and the area was then measured in arbitrary units for each image (Fig. 18). All the experiments were performed in triplicate.
Fig. 18: Example of image processing. a) aspect of the original picture (wound at T=0); b) the same image with enhanced contrast; c) the wound edges are detected and the wound area can be correctly measured. x460 magnification

6.1.2. Results

Fig. 19: Aspect of the control and hydrocortisone groups wounds at 0/4/8/24 hours

Fig. 20: Wound closure percentage at 4 and 8 hours in the 4 study groups

Closure of the wound generally occurred between 8 and 24 hours. The wells incubated with hydrocortisone displayed the greatest degree of wound closure (Fig. 19). A statistically significant difference (*, Fig. 20) (one-way ANOVA) was found between different treatment groups ($F(3,8) = 7.35$, $p = 0.011$). Tukey post-hoc tests revealed that wound closure % was statistically significantly higher in the hydrocortisone group ($27.1\% \pm 5.31\%$, $p= 0.15$) compared to that of the control group
(14.2% ± 1.86%). There were no statistically significant differences between the control, ACTH and 18-Beta-glycyrrhetinic-acid groups.

Overall, the results show that Hydrocortisone increase the migration of H357 cells (Fig. 20), while the addition of ACTH and 18-beta glycyrrhetic acid had minimal effect.

ACTH receptor (MC2R), is a G protein-coupled receptor. Ligand-bound G protein-coupled receptor would then undergo conformational change and subsequent downstream transduction cascade that ultimately leads to the increased production of endogenous glucocorticoids. Similarly for 18ß-GA we think that there was lack of an incubation period in our experimental design. Future investigations should take into account an incubation period to allow time for ACTH and 18ß-GA to exert their effects of clinical significance.
7. DISCUSSION AND CONCLUSIONS

At the beginning of this thesis our attention was dedicated to understand through the literature review which was the current evidence supporting the roles of GCs in normal and malignant cells.

The aim was supported by the need to discover which was the current knowledge about less known secondary effects of GCs. In fact, have been documented that GCs can influence a variety of functions such as cell migration, differentiation, apoptosis, and proliferation and may have tumour-promoting effects, particularly in epithelial neoplasms.

Synthetic corticosteroids are widely used nowadays mainly as anti-inflammatory drugs during the treatment of several diseases including potentially malignant disorders. Furthermore they are used in oncology in association with other treatment for cancer patients because of their potent proapoptotic properties in lymphoid cells, or to reduce side effects of chemotherapy.

Therefore, the hypothesis of a possible GCs role in promoting cancer cells survival, proliferation or in reducing the effectiveness of chemotherapy leads to a warring scenario.

The literature review performed showed, in contrast to previous thought, how increased levels of autocrine, paracrine, and exogenous cortisol are important to tumor progression, as well as the expression of enzymes regulating the levels of tumor-derived cortisol. The results also confirmed that a gap exists in the literature of exploration of the role of GCs in the oral cancer field.

Therefore, after the completion of the literature review phase, a 2 years period of in vitro research was carried out, with the attempt to reveal the effects of both endogenous and exogenous glucocorticoids on the behavior of oral squamous carcinoma cells, including the effects of these hormones on the efficacy of two chemotherapeutic agents: 5-fluorouracil, a well established chemotherapeutic agents used for the treatment of head and neck cancer and doxorubicin, a potential candidate for the treatment of OSCC.
Both DOXO and 5-FU induced cytotoxic effects in all the cell lines tested as early as 24h after treatment, and peaked at 48h. Firstly, apoptosis was evaluated with Hoechst stain, and subsequently using the Annexin V-FITC assay at fluorescence microscopy. The experiments performed showed that the cytotoxicity of 5-FU in 5 different malignant cell lines was comparable with the efficacy of DOXO in inducing OSCC cells apoptosis. The DOXO has been recently indicated by Abbasi et al. for its potential use to treat OSCC [12], but nevertheless in vitro effects of DOXO on OSCC cells have not previously been explored. This is then the first piece of work demonstrating in vitro the efficacy of DOXO, and its potential use in oral cancer treatment.

The chapter 5 also clearly established the role of exogenous corticosteroids in reducing the cytotoxicity of the chemotherapeutics tested, confirming our alarming starting hypothesis. These results open new avenues in the future treatment decisions for our patients. On one side, we can already clearly state that the concomitant use of glucocorticoids and chemotherapy for oral cancer treatment should be carefully revised. On the other side there is the stimulus to open an urgent field of research to assess the effects of GCs administration in all those potentially malignant disorders, e.g. oral lichen planus or lupus erythematosus, to elucidate if GCs can actually promote malignant transformation. If that will be the case, we can speculate that it is time to perform a critical revaluation of the pharmacological management of several dangerous conditions not limited to the oral cavity.

In chapter 5 we have also demonstrated the effect of HC administration in reducing the effectiveness of DOXO and 5-FU in 3 (H314, H357, H400) and 2 (H314, BICR56) of the 5 cell lines tested, respectively. An increased endogenous cortisol production, obtained through ACTH administration, also showed to reduced the effectiveness of 5-FU on 2 cell lines tested (H357 and BICR56). Furthermore, through additional experiments in different metabolic conditions, and with the use of fasentin and WZB, novel inhibitors of glucose uptake, we have shown that GCs effects would appear to be glucose dependent.
In chapter 6 we dedicated our attention to the understanding of the effect of glucocorticoids on the invasive behaviour of squamous carcinoma cells. Increased migration, increasing the depth of invasion and influencing the prior chance on nodal metastasis, is one of the features that is enhanced in cancer cells and metastasis, and should be then taken in consideration. Hence in this study the effect of GCs on malignant cells migration was examined by in vitro scratch wound healing assay using the H357 cell line. Statistically significantly faster wound closure rate was found in exogenously administered GC group compared to control. Exogenous glucocorticoids promote oral carcinoma cell proliferation and migration implying an increase in cell invasiveness. This has important implication on the widespread pharmacological usage of glucocorticoids as topical and systemic preparations for the treatment of a wide variety of oral conditions and as a primary combination chemotherapy.

ACTH and 18β-GA groups did not show significance. We believe that ACTH failed to show significance due to the lack of incubation period in the experimental medium, and consequently, 18β-GA failed to show significance due to low baseline endogenous cortisol. Nevertheless, we have demonstrated the ability, at least for exogenous corticosteroids, to increase malignant cell migration.

In conclusion, we have clearly demonstrated, for the first time, the importance of Cortisol on oral cancer cells' ability to survive, migrate, and interestingly combat the effectiveness of chemotherapeutic agents. This effect would appear to be glucose dependent. Finally, Doxorubicin shows promise for the treatment of oral cancer.

Furthermore, in the near future it will be important, therefore, to assess the levels of expression of steroidogenic molecules, steroids, and receptors among normal and malignant epithelial cells as well as the correlation of the expression levels of steroid-related molecules to the clinical-pathological parameters of cancer. The characterization of the tumor-associated GC systems will have salient diagnostic, preventive, and therapeutic clinical implications. Changes in the expression
levels of components of the epithelial GC pathway may aid in the selection of novel markers of cancer progression. Identification of the presence of alterations in the steroid pathway in malignant epithelial cells would open new avenues in the management of inflammatory pre-malignant conditions such as lichen planus and provide basis for a mechanism-based approach to cancer treatment.

Obivously, this study is not free from limitations. What we have demonstrated with one synthetic steroid (hydrocortisone) cannot be assumed as a general rule. For example in other solid tumors (e.g. bladder cancer) has been shown that different steroids can induce different effects on cancer cell viability and invasion. Then, a list of urgent questions is waiting for answers, opening new avenues in the understanding of Head and Neck cancer and creating translational research opportunities on different levels.

In the field of clinical research, retrospective/prospective cohort studies (single or multicenter based) should be performed to assess if there is any possible correlation between malignant transformation of OPMDs, outcome/prognosis of cancer patients and use of glucocorticoids.

In the field of translational research, given the variety of synthetic glucocorticoids commercially available and different chemotherapeutics [81,82], remains to clarify the different effects induced by different steroids on cancer cell proliferation, invasion, migration and to asses which is able to reduce/increase chemotherapy effectiveness.
Bibliography


APPENDIX 1 – JOURNAL PUBLICATIONS


APPENDIX 2 - ORAL PRESENTATIONS


APPENDIX 3 - PUBLISHED ABSTRACTS OF SCIENTIFIC MEETINGS


5) Adamo D, Ruoppo E, **Celentano A**, et al. Socio-demographic characteristics and pain in 75 Burning Mouth Syndrome patients. ORAL DISEASES Volume: 20 Special Issue: SI Supplement: 2 Pages: 11-11, Meeting Abstract: 30 Published: SEP 2014.


APPENDIX 4 - RESEARCH PROCEEDINGS


5) **Celentano A**, Adamo D, Ruoppo E, Leuci S, Mignogna MD. Title: “PTEN hamartoma tumor syndrome (PHTS) in oral medicine”. III Congress of Italian Society of Oral Pathology ad Medicine (3° Simposio Società di Patologia e Medicina Orale) (SIPMO), Torino-Orbassano, Italy. Date: 17-18th/10/2014.


## APPENDIX 5– HONOURS AND AWARDS

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<td>EAOM (European Association of Oral Medicine)</td>
<td>“Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucose dependent mechanisms”</td>
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<td>2016</td>
<td>MTC: Melbourne teaching certificate</td>
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LIST OF ABBREVIATIONS

5-FU: 5-fluorouracil
11b-HSD: 11b-Hydroxysteroid dehydrogenase
11b-HSD1/11b-HSD2: 11b-hydroxysteroid dehydrogenases
18ß-GA: 18ß-Glycyrhetinic acid
ACTH: adrenocorticotropic hormone
ACTHR/MC2R: adrenocorticotropic hormone receptor
DMSO: dimethyl sulfoxide
DOXO: doxorubicin
DMEM: Dulbecco’s Modified Eagle’s Medium
ECM: extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
FBS: Fetal Bovine Serum
GC: glucocorticoid
GR: GC receptor
HBEC: human bronchial epithelial cell
HC: hydrocortisone
HPA: Hypothalamus-Pituitary and Adrenal
IL-6: Interleukin-6
MCAs: multicellular aggregates
MP: methylprednisolone
OLP: oral lichen planus
OSCC: oral squamous cell carcinoma
PBr: pyridostigmine bromide
PBS: phosphate buffer saline
PCN: 5-pregnen-3-beta-ol-20-one-16-alfa-carbonitrile
PV: pemphigus vulgaris
ROS: reactive oxygen species
R/T: room temperature
SCC: squamous cell carcinoma
SFM: serum free medium
Oral lichen planus in childhood: a case series

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<td>09-Nov-2016</td>
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<tr>
<td>Complete List of Authors:</td>
<td>Cascone, Marco; University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences Celentano, Antonio; University federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences Adamo, Daniela; University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences Leuci, Stefania; University federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences Ruoppo, Elvira; University federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences Mignogna, Michele; University federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences</td>
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Title: “Oral lichen planus in childhood: a case series”

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Tables: 5, Figures: 1

Conflict of interest statement: all the authors certify that there is no conflict of interest with any financial organization or personal relationship with any other people that could inappropriately influence their work.

Running head: OLP in childhood
Abstract

**Background:** Although the exact incidence of pediatric oral lichen planus (OLP) is unknown, the oral mucosa seems to be less commonly involved and the clinical presentation is often atypical. The aim of the study is to present a case series of oral lichen planus in childhood.

**Methods:** From our database, we retrospectively selected and analyzed the clinical data of oral lichen planus patients aged under 18 where the diagnosis had been confirmed by histopathological analysis.

**Results:** The case series from our database shows eight patients, four males and four females. The mean (± SD) age at the time of diagnosis of the disease was 13.5 (±2.73) years, ranging in age from 9 to 17. Clinically, a reticular pattern was present in six patients (75%) and the tongue was the most commonly involved oral site (6 cases, 75%). We also report the first case of OLP in a 9 year-old girl affected by autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

**Conclusions:** We report the largest case series of pediatric OLP so far published in literature. Differences in the disease between adults and pediatric patients have been detected but further investigation and a larger case series are needed to establish any detailed differences in clinical outcomes.
Introduction

Lichen planus (LP) is a common chronic inflammatory disorder that affects the skin, mucous membranes, nails, and scalp; the etiology of this condition is still unknown although an immune-mediated pathogenesis has been hypothesized.\(^1\) The age at onset is usually between the third and sixth decade of life and it is predominantly seen in females.\(^2\)

Although the exact incidence of LP is unknown, it seems to vary between 0.1% to 1.2%. Children represent only 1% to 4% of patients with LP and the clinical presentation is often atypical.\(^3\)

Clinically, cutaneous LP is characterized by purple, polygonal, pruritic papules frequently covered by a lacy network of white scales on their surface, known as Wickham striae. The flat-topped papules are often located on the flexor surface of the wrist, the shins, the trunk, and the medial thighs, subdivided into one of the following variants: linear, hypertrophic, annular, follicular, actinic, vesciculobullous and pemphigoid-like. The disease often resolves within 8 to 12 months of treatment and it is not believed to be capable of malignant transformation.\(^4,5\)

Any nail involvement may appear as a thinning of the nail plate, longitudinal fissuring, or distal splitting. Any hair follicle involvement is called lichen planopilaris and if untreated can lead to scarring alopecia. Any involvement of the mucous membranes can affect the oral mucosa, conjunctivae, larynx, esophagus, tonsils, bladder, vaginal vault, vulva, and anus.\(^5\)

In contrast to skin LP, oral lichen planus (OLP) demonstrates a clinical variability\(^6\) and the oral manifestation in adults is more frequently resistant and persistent than the cutaneous type.\(^7\)
The oral lesions are categorized as reticular, papular, plaque-like, atrophic, erosive, or bullous.

The hyperkeratotic variants are commonly asymptomatic while the atrophic/erythematous variant, the erosive/ulcerative variant and the bullous type often have persistent symptoms of pain or stinging aggravated during eating and drinking.

The clinical differential diagnosis depends on the age of the patient, the clinical form of OLP, and the severity and persistence of the lesions and includes: lichenoid drug reaction, leukoplakia, lupus erythematosus, candidiasis, graft-versus-host disease (GVHD), frictional keratosis, autoimmune bullous diseases, erythema multifforme, allergic gingivostomatitis, and gluten sensitivity enteropathy.

In challenging cases more sensitive diagnostic techniques could be useful to achieve diagnosis such as direct and indirect immunofluorescence.

The aim of this study is to provide an update of the oral involvement of the disease in children through the report of a retrospective analysis of pediatric patients referred to our Department during the last four years for whom lichen planus has presented in the oral cavity as the single or as an additional site of involvement. We also conducted a literature review of the topic in order to highlight the similarities and differences between our data and the previously published clinical cases.

**Subjects and Methods**

From our database, we retrospectively selected and analyzed the clinical data of pediatric OLP patients in the outpatient clinic of the Department of Neurosciences, Reproductive and
Odontostomatological Sciences, Federico II University of Naples. The selection was based on
the following inclusion criteria:

- age <18 years old at the time of diagnosis

- a clinical and histological diagnosis of OLP

The exclusion criteria were

- GVHD lichenoid lesions

- the lack of a confirmatory histology

- oral lichenoid drug reaction

- the lack of any results of routine hematological testing including tests for hepatic and
kidney functionality, markers of hepatitis A, B and C viruses, and a red and white blood cell
count and platelet count.

From our database we collected the following data: age at time of diagnosis, sex, preexisting
medical conditions, presence of a positive family history of immunological disorders,
concomitant or previous assumption of drugs, concomitant oral predisposing or iatrogenic
factors, confirmatory histology, clinical pattern, oral sites involved, oral symptoms reported,
extra-oral sites involved, and the treatment and resolution of oral lesions.

Literature review

A PubMed search was carried out of articles published between 1966 and 2015 using the
keywords “lichen” OR “lichenoid” alternatively matched with “oral” OR “lip” AND
“juvenile” OR “child*” OR “familial” OR “pediatric”. 
The selection of the studies was based on the following inclusion criteria:

- the English language
- a case series or case reports
- age <18 years old at the time of diagnosis
- clinical and histological diagnosis of OLP
- an accurate description of the oral sites and clinical features

The exclusion criteria were

- lack of clarity in reporting data about the clinical form(s) of OLP and/or the oral site(s) involved
- GVHD lichenoid lesions
- lack of confirmatory histology
- oral lichenoid drug reaction

The study was approved by the Ethics Committee of the University of Naples “Federico II” in July 2014 and it conforms to the provisions of the Declaration of Helsinki (as revised in Tokyo 2004).

**Results**

The case series from our database shows eight patients, four males and four females. The mean (± SD) age at the time of diagnosis of the disease was 13.5 (±2.73) years, the patients ranging in age from 9 to 17.
A positive familial history of immunological disorders was found in seven cases (87.5%).

Seven patients had been submitted to hepatitis B virus (HBV) vaccination and three (37.5%) patients presented concomitant oral factors. Findings and/or a history of an immune disorder were present in seven patients (87.5%).

For each patient a confirmatory histology was obtained and in no case was dysplasia reported.

A reticular pattern was the one most frequently reported, present in six (75%) patients, followed by the atrophic (50%), plaque-like (37.5%), erosive (12.5%) and bullous (12.5%) patterns; no patient showed a papular pattern. A simultaneous multiple clinical pattern was observed in six cases (75%) and in one patient a mucosal pigmentation was detected.

The tongue was the most commonly involved oral site (6 cases, 75%), followed by the buccal mucosa (4 cases, 50%), gingiva (3 cases, 37.5%), retromolar fossae (1 case, 12.5%), palate (1 case, 12.5%) and lip (1 case, 12.5%). No patient showed any floor of the mouth or extra-oral involvement. Four patients (50%) were symptomatic.

The most commonly used drugs were topical antifungal medications prescribed in order to avoid overlapping fungal overgrowth. Topical steroids were also associated in three cases.

Two patients (25%) showed a complete disappearance of the oral lesions.

The analysis of the literature yielded 344 articles published between 1966 and 2015. After the application of the inclusion criteria, 12 articles were included in our study. We also searched among the references of the aforementioned articles and found a further six articles making a total of 18 articles that fulfilled our inclusion criteria, which described a total of 26 patients.

The flow chart is reported in Table 1.
Data from our cases concerning the epidemiology, predisposing factors, clinical features, diagnosis and treatment are recorded in Table 2.

Data from the PubMed search concerning the epidemiology, predisposing factors, clinical features, diagnosis and treatment are recorded in Table 3.

A comparison between our cases and the review of literature is provided in Table 4.

Discussion

Many previous studies have reported that in LP among pediatric patients the oral mucosa seems to be less commonly involved with a prevalence of approximately 0.03%\textsuperscript{17} compared with 1% to 2% of the general population.\textsuperscript{2}

The present case series confirms the epidemiological data previously collected concerning the pediatric LP population with a balanced M:F ratio\textsuperscript{25} and a greater prevalence for familial LP in children (25%) than in adults. In fact, although LP is usually a sporadic disorder, there is a rare familial form more prevalent in the pediatric population ranging from 1% to 4.3%, with childhood familial LP considered to occur at an earlier age and with a greater severity.\textsuperscript{3} An autosomal dominant mode of inheritance with a variable penetration has been suggested and a linkage of familial LP with HLA-B7 and HLA-BR10 has been observed.\textsuperscript{3}

The exact etiology of LP is unknown but it appears to be complex and multifactorial. Possible cofactors of OLP, such as a hypersensitivity to dental restorative materials (e.g. amalgam and gold), local trauma (the Koebner phenomenon) and several kinds of infections (plaque-causing microorganisms and hepatitis B or C virus infection) have been reported.\textsuperscript{10,25}

Furthermore childhood LP has been documented as a complication of hepatitis B virus (HBV) vaccination, where the recombinant proteins of the HBV vaccine – specifically the viral S epitope – may trigger a cell-mediated autoimmune response targeted at the keratinocytes.\textsuperscript{11}
For these reasons, in Tables 2 and 3 we have recorded the presence of any concomitant systemic and oral factors that could have had a role in the OLP pathogenesis or in its exacerbation; however to date these associations are still unclear.\(^7\)

The medical histories collected from our cases confirm the presence of an increased association between OLP and auto-immune diseases\(^1,3,7,10,11,22\) with seven of our cases showing associated immunological disorders. Among our patients we also report the first case of an oral lichenoid lesion related to autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) also known as polyglandular autoimmune syndrome (PGA) type I, in a 9 year-old girl. Until now there has been only one other case in literature of OLP associated autoimmune polyendocrine syndrome type II\(^26\) in a 42 year-old woman, reinforcing the suggestion of a common immune-mediated pathogenesis between OLP and PGA. (Fig. 1)

From the reported data in our case series the reticular pattern appears to be the most common in childhood followed by the erosive one, in accordance with the literature. Interestingly, we instead recorded a clear difference, when comparing our eight cases to the literature (Table 4), concerning the oral site predilection with 75% of our eight pediatric patients showing lesions on the tongue. Previous studies have reported the buccal mucosa as the most commonly involved oral site in pediatric OLP with the next most common location being the tongue.\(^25\) Finally, confirming the hypothesis of a less common oral involvement in pediatric patients with LP\(^25\) previously reported at a rate of 12.6%\(^27\), none of our eight cases showed any extra-oral involvement.

The histology of OLP has revealed that parakeratosis is the most frequent type of keratosis while the erosive variety has involved the acanthotic epithelium in more than 50% of cases; the rete pegs are predominantly of a wavy pattern while basal cell degeneration and band-like subepithelial lymphocytic infiltration seem to be present in all cases. Our data are consistent
with the previous literature with basal cell degeneration and band-like subepithelial lymphocytic infiltration present in all of our eight cases.25

The treatment of juvenile OLP does not differ significantly from the treatment of adult OLP and is often unnecessary in asymptomatic patients. Oral symptoms are relatively frequent when the erosive and/or atrophic pattern occurs; although patients with a keratotic form can report a roughness, treatment is rarely necessary. In the present paper we have considered prescribing topical antifungal medications to all patients in order to avoid overlapping fungal overgrowth in patients undergoing contemporary topical steroidal therapy. The analysis of previously published studies confirms that topical corticosteroid therapy is the most commonly used treatment in symptomatic OLP, reported in connection with 12 of the 18 symptomatic pediatric patients (66.6%), even if the chronic use of topical steroids can lead to oral candidiasis; an association with retinoid therapy and a plaque control regimen in children have shown favorable responses.11 Systemic steroid therapy and dapsone are typically reserved for refractory and recurrent cases; extreme caution is employed because significant long-term effects are of concern in this young patient population. Of note, tacrolimus ointment, topical tretinoin and topical cyclosporine have also been used with success in some cases6 but the safety of any long term continuous use of some of these drugs in pediatric patients has not been adequately evaluated.25 The effect of the treatment of OLP in children seems to be more favorable than in adults for whom the symptoms usually persist for many years in spite of intensive treatment and a thorough investigation of any associated factors.17 Considering our case series and the previously reported papers, a complete resolution of oral lesions has been observed in 38.2% of cases (Table 4).

In Table 5 we show a summary of the most important similarities and differences in OLP between children and adults so far reported in literature.
In conclusion, our case series mostly mirrors previous epidemiological, clinical and therapeutic knowledge about pediatric OLP but a larger case series is needed to confirm the possibility of a different oral site predilection between adults and children as suggested by our findings. Clinicians must be aware that OLP in childhood may also have a simultaneous or future involvement of the skin and other mucosal sites and, due to a more frequent positive familial history of LP in childhood, close relatives should be examined. Although there have been no OLP-related malignancies described to date in the pediatric population, most previous studies suggest that the schedule of follow-up of pediatric OLP should be of at least one or two examinations per year as long as the OLP persists even if the prognosis seems to be more favorable.

What is new:

- the present paper represents the largest case series so far published
- we report the first case of an oral lichenoid lesion related to APECED in a 9 year-old girl
- our cases suggest a different site predilection in OLP between children and adults
- the accurately tabulated review of the literature relating to pediatric OLP may facilitate further studies on the topic

Limitations of the study:

even if the present paper represents the largest case series so far published, further studies are needed to establish the epidemiological and clinical features in this population of patients. The review of the literature conducted may be conditioned by previous biases in reporting exceptional cases on PUBMED and therefore the results of the present comparison with previous studies should be considered critically.
References


Table 1

344 articles found through searching the PubMed database

- 36 not humans
- 51 not English language
- 22 graft versus host disease

235 articles after research filters application

- 70 title out of topic

156 analyzed abstracts

- 5 unviable
- 7 not accurate clinical design of the study
- 15 no mention of clinical cases
- 4 reported absence of oral lesions
- 13 out of topic

106 full text analysis

- 35 unviable
- 7 lack of clinical data in reporting clinical data
- 14 no pediatric patients
- 3 no oral involvement
- 2 lack of histopathological analysis
- 3 drug induced lichenoid lesions suspicion

12 articles selected

6 additional articles found among references of aforementioned articles

18 articles fulfilling our inclusion criteria
<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Preexisting conditions</th>
<th>Medical history</th>
<th>HIV vaccination status</th>
<th>Drugs</th>
<th>Concomitant oral factors</th>
<th>Clinical pattern</th>
<th>Oral symptoms</th>
<th>Extracutaneous sites</th>
<th>Treatment</th>
<th>Resolution of oral lesions</th>
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<td>1</td>
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<td>F</td>
<td>allergy to NSAIDs (mother)</td>
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<td>vaccinated</td>
<td>none</td>
<td>parafunction</td>
<td>plaque-like + erosive margins of tongue bilaterally</td>
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<td>topical steroids + topical antimycotics</td>
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<tr>
<td>2</td>
<td>17</td>
<td>M</td>
<td>atopy, ↓ Hb (father)</td>
<td>OLP (bilateral)</td>
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<td>none</td>
<td>none</td>
<td>reticular plaque-like + erosive hard palate, retromolar fossae bilaterally</td>
<td>dryness</td>
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<td>14</td>
<td>F</td>
<td>none</td>
<td>none</td>
<td>vaccinated</td>
<td>none</td>
<td>none</td>
<td>reticular plaque-like + atrophic margins of tongue bilaterally</td>
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<td>4</td>
<td>14</td>
<td>M</td>
<td>Hg (NIL6C patch test ++)</td>
<td>autoimmune thyroiditis (mother)</td>
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<td>F</td>
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<td>Sex</td>
<td>Concomitant Oral Factor</td>
<td>Clinical Pattern</td>
<td>Oral Sites</td>
<td>Oral Treatment</td>
<td>Extraoral Site</td>
<td>Extracutaneous Disease</td>
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<td>↓ Hb</td>
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<td>Alam &amp; Hamburger (2004)</td>
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<td>Reticular + papular + pigmentation</td>
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Table 4

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<th>CASE SERIES (n=8)</th>
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<td><strong>AGE</strong></td>
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<td>overall age range (years)</td>
<td>9 - 17</td>
<td>6 - 17</td>
<td>6 - 17</td>
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<tr>
<td>mean (± sd) age</td>
<td>13.5 (± 2.73)</td>
<td>10.41 (± 3.24)</td>
<td>11.18 (± 3.40)</td>
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<tr>
<td><strong>GENDER</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>males</td>
<td>4 (50%)</td>
<td>11 (42.3%)</td>
<td>15 (44.1%)</td>
</tr>
<tr>
<td>females</td>
<td>4 (50%)</td>
<td>15 (57.7%)</td>
<td>19 (55.8%)</td>
</tr>
<tr>
<td><strong>FAMILIAL OLP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (37.5%)</td>
<td>1 (3.8%)</td>
<td>4 (11.7%)</td>
</tr>
<tr>
<td><strong>SYSTEMIC CONDITIONS</strong></td>
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<tr>
<td>preexisting medical conditions</td>
<td>7 (87.5%)</td>
<td>9 (34.6%)</td>
<td>16 (47%)</td>
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<td><strong>IMMUNOLOGICAL DISORDERS</strong></td>
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<tr>
<td></td>
<td>7 (87.5%)</td>
<td>5 (19.2%)</td>
<td>12 (35.2%)</td>
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<tr>
<td><strong>CONFIRMATORY HISTOLOGY</strong></td>
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<td></td>
<td>8 (100%)</td>
<td>26 (100%)</td>
<td>34 (100%)</td>
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<tr>
<td><strong>CONCOMITANT ORAL FACTORS</strong></td>
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<tr>
<td></td>
<td>5 (37.5%)</td>
<td>8 (30.7%)</td>
<td>11 (32.3%)</td>
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<td><strong>OLP CLINICAL PATTERN</strong></td>
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<tr>
<td>reticular</td>
<td>5 (62.5%)</td>
<td>19 (79.1%)</td>
<td>24 (70.5%)</td>
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<tr>
<td>plaque-like</td>
<td>0</td>
<td>1 (3.8%)</td>
<td>1 (3.8%)</td>
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<td>atrophic</td>
<td>3 (37.5%)</td>
<td>3 (11.5%)</td>
<td>6 (17.6%)</td>
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<tr>
<td>erosive</td>
<td>3 (37.5%)</td>
<td>8 (30.7%)</td>
<td>11 (32.3%)</td>
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<tr>
<td>bullous</td>
<td>1 (12.5%)</td>
<td>1 (3.8%)</td>
<td>2 (5.9%)</td>
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<tr>
<td>mixed</td>
<td>3 (62.5%)</td>
<td>10 (38.4%)</td>
<td>13 (38.2%)</td>
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<tr>
<td><strong>OLP SITE INVOLVEMENT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tongue</td>
<td>6 (75%)</td>
<td>15 (57.7%)</td>
<td>19 (55.8%)</td>
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<tr>
<td>buccal mucosa</td>
<td>4 (50%)</td>
<td>19 (73%)</td>
<td>23 (67.6%)</td>
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<tr>
<td>gingiva</td>
<td>3 (37.5%)</td>
<td>3 (11.5%)</td>
<td>6 (17.0%)</td>
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<tr>
<td>labial mucosa</td>
<td>1 (12.5%)</td>
<td>2 (7.7%)</td>
<td>3 (8.8%)</td>
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<tr>
<td>lips</td>
<td>1 (12.5%)</td>
<td>4 (15.4%)</td>
<td>5 (14.7%)</td>
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<tr>
<td>palate</td>
<td>1 (12.5%)</td>
<td>1 (3.8%)</td>
<td>2 (5.9%)</td>
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<tr>
<td>floor of mouth</td>
<td>0</td>
<td>3 (11.5%)</td>
<td>3 (8.8%)</td>
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<tr>
<td><strong>EXTRA-ORAL INVOLVEMENT</strong></td>
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<td></td>
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<td></td>
<td>0</td>
<td>5 (19.2%)</td>
<td>5 (14.7%)</td>
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<td><strong>SYMPTOMS REFERAL</strong></td>
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</tr>
<tr>
<td></td>
<td>4 (50%)</td>
<td>16 (61.5%)</td>
<td>20 (58.8%)</td>
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<tr>
<td><strong>COMPLETE RESOLUTION OF ORAL LESIONS</strong></td>
<td>2 (25%)</td>
<td>11 (42.3%)</td>
<td>13 (38.2%)</td>
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Table 5

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<th>topic</th>
<th>childhood</th>
<th>adulthood</th>
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<tr>
<td>OLP frequency</td>
<td>0.03%&lt;sup&gt;17&lt;/sup&gt;</td>
<td>1.2%&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>familial OLP</td>
<td>1-4%&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.5%&lt;sup&gt;17&lt;/sup&gt;</td>
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<tr>
<td>most common clinical pattern</td>
<td>reticular</td>
<td>reticular (83.5%)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>erosive</td>
<td>erosive (15-39%)&lt;sup&gt;2,6&lt;/sup&gt;</td>
</tr>
<tr>
<td>most common oral sites involved</td>
<td>buccal mucosa and tongue&lt;sup&gt;25&lt;/sup&gt;</td>
<td>buccal mucosa (88%)</td>
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<td></td>
<td></td>
<td>tongue and gingiva (18.7%).&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td>involvement of both skin and oral mucosae</td>
<td>12.6%&lt;sup&gt;17&lt;/sup&gt;</td>
<td>20-34%&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>histology</td>
<td>no dysplasia, basal cell degeneration and band-like subepithelial lymphocytic infiltration&lt;sup&gt;25&lt;/sup&gt;</td>
<td>no dysplasia, basal cell degeneration and band-like subepithelial lymphocytic infiltration&lt;sup&gt;13&lt;/sup&gt;</td>
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<td>treatment</td>
<td>symptomatic&lt;sup&gt;2&lt;/sup&gt;</td>
<td>symptomatic&lt;sup&gt;24&lt;/sup&gt;</td>
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<tr>
<td>resolution</td>
<td>more frequent than in adults&lt;sup&gt;17&lt;/sup&gt;</td>
<td>2-5%&lt;sup&gt;9,10,11&lt;/sup&gt;</td>
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<td>malignant transformation of OLP</td>
<td>never reported&lt;sup&gt;39&lt;/sup&gt;</td>
<td>0.4-5.3%&lt;sup&gt;7&lt;/sup&gt;</td>
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</table>
Table legends

Table 1: Flow chart

Table 2: Data on the 8 patients from our case series: epidemiology, predisposing factors, clinical features, diagnosis and treatment.
†: non-steroidal anti-inflammatory drugs ‡: hemotoxylin eosin §: hemoglobin
¶: Immunoglobulin G versus epstein-barr virus ††: autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy ‡‡: not available

Table 3: Data on the 24 patients from the PUBMED search: epidemiology, predisposing factors, clinical features, diagnosis and treatment.
‡: hemotoxylin eosin §: hemoglobin ‡‡: not available §§: direct immunofluorescence †: non-steroidal anti-inflammatory drugs ¶¶: calcineurin inhibitors †††: antinuclear antibodies
¶¶¶: Immunoglobulin A‡‡‡: basal membrane zone §§§: lichen planus pemphigoides

Table 4: Comparison between our case series and the cases from the PUBMED search

Table 5: Comparison between OLP in childhood and adulthood

Figure legends

Fig 1 Case No.9 a 9-year-old patient affected by APECED. (a) bullous lesions involving the dorsum and margins of the tongue bilaterally and interlaced by reticular keratotic, erythematous and atrophic aspects of the epithelium; (b) bullous lesion involving the left margin of the tongue surrounded by reticular keratotic, erythematous and atrophic aspects of the epithelium; (c) bullous lesion involving the left buccal mucosa surrounded by reticular keratotic, erythematous and atrophic aspects of the epithelium; (d) reticular keratotic lesions of the upper and lower gingiva
Case No. 9 a 9-year-old patient affected by APECED. (a) Bullous lesions involving the dorsum and margins of the tongue bilaterally and interlaced by reticular keratotic, erythematous and atrophic aspects of the epithelium; (b) Bullous lesion involving the left margin of the tongue surrounded by reticular keratotic, erythematous and atrophic aspects of the epithelium; (c) Bullous lesion involving the left buccal mucosa surrounded by reticular keratotic, erythematous and atrophic aspects of the epithelium; (d) Reticular keratotic lesions of the upper and lower gingiva in a 9-year-old patient affected by APECED.
Delayed Diagnosis of a Nasal Type Lymphoma Misdiagnosed as Persistent Sinusitis

Antonio Celentano, DDS,1,2 Massimo Mascolo, MD, PhD,3 Nicola Cirillo, DMD, PhD,2 Gaetano De Rosa, MD,3 and Michele Davide Mignogna, MD, DMD1

Nasal Type T/natural killer (NK)-cell lymphomas are rare clinical entities, highly aggressive with a very poor prognosis. We present a case of a 37-year-old immunocompetent man presenting with deep palatal ulceration and a 3-month history of symptoms, which appear to have been misdiagnosed by physicians. The final diagnosis was achieved by a 15-day diagnostic algorithm, during which the patient’s clinical status worsened severely. In this article, we also provide a succinct update on the clinical and histopathological findings of Peripheral T/NK-cell lymphomas and propose that symptoms that are consistent with these clinical entities should be considered from the early stages to inform a suitable diagnostic pathway.

Keywords: nasal type lymphoma, lymphoma, NHL

Introduction

Lymphoma, or lymphatic cancer, is a broad term encompassing a variety of cancers of the lymphatic system. Lymphomas in humans are generally classified into two main groups: the Hodgkin lymphomas (HL), also known as Hodgkin disease, and the non-Hodgkin lymphomas (NHL). NHL are divided into B- and T-cell neoplasms and natural killer (NK)-cell lymphomas.

NK cells are a third lymphocyte lineage, in addition to B- and T-cells, that mediate cytotoxicity without prior sensitization. NK cells also have phenotypic and genotypic characteristics; they express the NK-related antigen CD56 and T-cell markers such as CD2 and CD3 epsilon, but their T-cell receptor locus is not rearranged.

Included in the NHL group are the Peripheral T-cell and NK-cell lymphomas (PTNKCLs), a wide family of lymphomas with different clinicopathologic features, which represent only 10%–20% of all NHLs in the Western world.

PTNKCLs are defined as angiocentric lymphomas in the revised European American Lymphoma (REAL) classification and include the “nasal” and the “nasal type” varieties, which are very rare clinical entities in the United States and Europe, but more common in Asia and Central America. Nasal T/NK cell lymphoma is a distinct clinicopathologic entity that is highly associated with Epstein-Barr virus (EBV), with a very poor response to treatment and prognosis (5-year overall survival rate of 25%).

Generally, a variety of cells ranging from small or medium-sized cells to large transformed cells can characterize the broad cytologic spectrum of this entity. Tissue damage is a common morphological feature of this form, due to both cytotoxicity of the T/NK lymphoma cells and angiocentricity.

The characteristic immunophenotype of Nasal T/NK cell lymphoma is distinguished by a CD2 and CD56 positivity, but usually a negativity for surface CD3.

The presence of EBV in early diagnosis can be effectively evaluated by *in situ* hybridization.

The differential diagnosis includes lymphomatoid granulomatosis, blast or monomorphic NK cell lymphoma/leukemia, CD56-positive peripheral T-cell lymphoma, and enteropathy-associated T-cell lymphoma.

Extranodal sites such as the skin, the subcutis, and the gastrointestinal tract can be affected by tumors with an identical phenotype and genotype. These extranodal forms should be referred to as nasal-type T/NK cell lymphomas.

Here, we present a rare case of a 37-year-old Caucasian man affected by nasal-type T/NK-cell lymphoma, referred to our unit 3 months after the development of the first symptoms.

The purpose of this study is to update data on the clinical and histopathological findings of this rare disease, pointing out how misdiagnoses are responsible for therapeutic delays and, consequently, of a worse prognosis for these aggressive forms.
Case Report

A 37-year-old Caucasian man was referred to our Oral Medicine Unit, Department of Head and Neck Diseases, Federico II University of Naples, in September 2014 with the main complaint of a wide symptomatic ulcerative lesion of the hard palate.

His family history encompasses three uncles who had died of different forms of cancer in recent years (two of lung cancer, and one of bowel cancer). The patient’s medical history was negative, and he was in apparent good health except for a weak hypercholesterolemia.

His first referred symptoms were a moderate submandibular lymphadenopathy and weak fever starting more than 2 months earlier, for which reason he consulted the family doctor who requested hematological tests with the main suspicion of an EBV/CMV infection. The results of all the tests performed were negative and for 1 month the patient received symptomatic therapy. With no sign of improvement, 4 weeks later the patient started complaining of a sense of nasal obstruction, with breathing difficulty for which he consulted an otorhinolaryngologist who performed an endoscopic examination of the nasal cavities, finding suppurative exudate and inflammation of the nasal floor and lateral/medial walls. It was diagnosed as severe maxillary sinusitis and therapy with corticosteroids through an aerosol, and antibiotics were prescribed. After 1 month, without any improvement of the symptoms, the patient started complaining of the first oral symptom, pain due to swelling, and an ulcerated area in the median area of the hard palate. For this reason, he consulted his dentist who advised him to undergo an oral medicine consultation at our hospital. Our first examination was performed soon after, accordingly 10 weeks after the onset of the first symptoms, and the patient was presented with the clinical condition shown in Figure 1A.

The patient, after providing his written informed consent, was hospitalized and examined by routine hematological tests, which revealed a glucose level of 120 mg/dL (normal range, 60–110), a total cholesterol level of 200 mg/dL (normal value up to 190), a triglyceride level of 218 mg/dL (normal value up to 180), and an iron level of 49 µg/dL (normal range, 55–160).

The physical examination showed a widespread ulcerative and proliferative lesion involving the hard palate, and marginally the soft palate and the left maxillary alveolar process (Fig. 1A). The lesion measured about 4 cm at its maximum diameter and showed a central zone of deep and extensive necrosis, with a surrounding area of weakly brown-colored hyperplastic tissue. Two incisional biopsies of the lesion were performed.

After 2 weeks, while the patient was undergoing a diagnostic pathway, the lesion presented with a considerably worse clinical evolution, as shown in Figure 1B.

At histology, scanning magnification revealed a dense and diffuse proliferation of pleomorphic small and medium-sized lymphocytes within the entire chorion, with overlying extensive squamous epithelium ulceration. An angiocentric pattern of infiltration was evident. The predominant cell type was lymphocyte with round nuclei, one or more nucleoli, and a scant cytoplasm. Numerous mitotic figures were seen, and areas of necrosis were also present (Fig. 2A–C).

The neoplastic cells expressed CD2 and CD3; a weak expression of CD56 and granzyme B was observed (Fig. 2D). CD8 and CD30 were consistently negative. The tumor had a proliferative fraction (Ki-67/MIB1) approaching 70%–75%.

The analysis of the presence of EBV was performed with the FISH (fluorescent in situ hybridization) technique (Dako).
PNA ISH EBERER (PNA Probe) and revealed a widespread positivity for EBV.

A diagnosis of extranodal NK/T-cell lymphoma, nasal type was made.

The patient was immediately referred to the Hematology Department to complete the staging and start treatment of the disease. A head/neck and chest computed tomography (CT) scan and a total body positron emission tomography (PET) scan were negative except for the oral and left nasal cavity involvement. Chemotherapy following the SMILE protocol (Methotrexate 2 g/m² IV, Dexamethasone 40 mg IV + leucovorin 15 mg · 4 doses/day IV + ifosfamide 1500 mg/m² IV + etoposide 100 mg/m² IV, and l-asparaginase 6000 U/m² IV, repeated every 21 days for 3 cycles) was started in early November 2014, 4 months after the onset of symptoms. During chemotherapy treatment, the deep necrosis of the palatal soft tissue evolved into an oronasal fistula, as documented in Figure 1C–F. Four months after chemotherapy, the patient received radiotherapy (at a dose of 56 Gy) for 52 days. From this period until October 2016, the oronasal fistula remained fairly stable (Fig. 1G–I, K), and it was managed with the use of a palatal obturator prosthesis (Fig. 1J, L). Bimonthly follow up occurred in our department. The palatal obturator was changed every 15 days due to a continuous remodeling of the soft tissue and consequent passage of food into the nasal cavity. The management of the fistula included use of chlorhexidine topical gel. Oncology follow up continued every 6 months, and it included a head/neck CT scan and a total body PET. The patient was still alive at the time of writing this article and was about to commence the preparation phase for the bone marrow transplantation.

The study was approved by the Ethics Committee of the University “Federico II” of Naples. Appropriate written informed consent was obtained from the patient included in the study.

Discussion

The head and neck is a common site of extranodal NHL. PTNKCLs are rare and heterogeneous forms of NHL that are usually characterized by a very poor clinical outcome. Furthermore, many subtypes are currently present in the World Health Organization (WHO) classification of PTCL.

Sequential chemotherapy and radiotherapy is the gold standard treatment for extranodal lymphomas at early stages (I–II), proved to be more effective in terms of 5-year overall survival and progression-free survival than chemotherapy or radiotherapy alone. For stage III/IV instead, chemotherapy is the mainstay of treatment. Conventional anthracycline-based regimens are ineffective. Since the advent of l-asparaginase-based regimen, the outcome of extranodal lymphomas was clearly improved. Radiotherapy plays an important role in the treatment of low-grade lymphomas, with curative or palliative intent. In the case of high-grade lymphomas, its combination with chemotherapy is debated. The role of radiotherapy remains, however, undeniable for two specific entities: NK/T-cell lymphoma NK/T nasal type, and primary central nervous system lymphomas.

Nevertheless, the outcome of advanced-stage diseases is still not satisfactory.

As confirmed by this present case, the diagnosis of these forms is difficult for the general practitioner as well as for medical specialists, a difficulty compounded by the frequent underestimation/ misdiagnosis of the first symptoms that are often non-specific and can mimic an inflammatory process. Each failure in a diagnostic step is usually followed by a delay of several weeks for the patient to achieve the correct diagnosis and to undergo the specific therapy. Given that the specific treatment strategy and related prognosis for a patient with NHL strictly depend on disease stage, this diagnostic delay can seriously impact patients’ lives.
Because of the relative rarity of this condition, achieving the correct diagnosis can be a challenge, even for pathologists. In a recent study conducted before the most recent WHO classification, the overall diagnostic accuracy among experts was found to be ~81%, with a value ranging from 67% to 95% depending on the specific subtype.12

In our case, prompt biopsies and histopathological examination led us to a diagnosis in only 2 weeks, but unfortunately the patient had already undergone a 3 month delay since the first medical examination. We speculate that such extended delays could jeopardize a patient’s life, and can surely contribute to the extensive damage to the nasal floor and surrounding structures, with a consequent permanent wound that is hard to manage and impacts the patients’ quality of life.

The head and neck healthcare specialists may be considered the main actors in the diagnostic pathway of these rare diseases. Lack of response to first treatments must induce physicians to increase level of suspicion and to refer their patients for further examination as appropriate. The first observation/waiting period must be not longer than 2 weeks, and it should be followed by a histopathological examination in all uncertain cases.

In this case, the patient had been treated symptomatically for a persistent unspecified peripheral lymphadenopathy for the first 4 weeks without any improvement, and without performing any further investigations. Next, management of the patient by the ENT specialist for a further 4 weeks for an alleged sinusitis was even bordering on negligence. In such a patient, after the first 3–4 weeks of observation, lymph node biopsy was already required. It should be highlighted that imaging, for example, a simple head and neck ultrasound, could have been useful to identify node characteristics more accurately than the physical examination only. It is well established that a change in the long: short axis ratio of a node is a significant sign of metastatic cancer has been reported to be in the range of 76%–100% and 82%–96%, respectively.

The head and neck healthcare specialists may be considered the main actors in the diagnostic pathway of these rare diseases. Lack of response to first treatments must induce physicians to increase level of suspicion and to refer their patients for further examination as appropriate. The first observation/waiting period must be not longer than 2 weeks, and it should be followed by a histopathological examination in all uncertain cases.

Based on this experience, it is arguably undesirable to wait even three days in respect of these forms of lymphomas in which the cytotoxicity and angiocentricity can produce such a fast and deep necrosis. The grievous impairment of the surrounding anatomical structures is the main complication that contributes to the already very poor prognosis.

Furthermore, in these highly aggressive forms, the possibility of starting a therapy before completing the diagnostic pathway should be evaluated. Starting an earlier therapy could make a significant difference in improving such poor survival rates.

Author Disclosure Statement
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References

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“FROM THE BENCH” ARTICLE
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The article by Celentano et al. examines the structural and functional relationship of the desmosome, by providing a comprehensive, yet focused overview of the constituents targets in human disease.
GORDANA BLAGOJEVIĆ ZAGORAC, HANA MAHMUTEFENDIĆ, SENKA MAČEŠIĆ, LJERKA KARLEUŠA, AND PERO LUČIN

Quantitative Analysis of Endocytic Recycling of Membrane Proteins by Monoclonal Antibody-Based Recycling Assays
Accepted manuscript online in Wiley Online Library, 26 July 2016

In this report, an analysis of several recycling protocols based on labeling of membrane proteins with specific monoclonal antibodies (mAbs) is presented. We analyzed recycling of membrane proteins that are internalized by clathrin-dependent endocytosis, represented by the transferrin receptor, and by clathrin-independent endocytosis, represented by the Major Histocompatibility Class I molecules.

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PRIYAMVADA M. PITALE, WAYNE HOWSE, AND MARINA GORBATYUK

Neuronatin Protein in Health and Disease
Accepted manuscript online in Wiley Online Library, 21 July 2016

This literature review provides updates on neuronatin expression in healthy and diseased organs and tissues.

Page 479
Proinflammatory signaling function of thrombin increases secretion of proinflammatory cytokines and chemokines, triggers vascular permeability, promotes leukocyte migration, and induces adhesion molecule expression.

This review summarizes the role of thrombin in the pathogenesis of central nervous system (CNS) inflammatory diseases, including Alzheimer’s disease (AD) and Parkinson’s disease (PD), promoting greater understanding and clinical management of these diseases.

Transforming growth factor beta (TGF-beta) has a clear role in the pathogenesis of diabetic retinopathy, although the underlying mechanisms are poorly established. Here, our current but limited understanding of how signaling by TGF-beta superfamily ligands contributes to the pathogenesis and progression of diabetic retinopathy are discussed.
### Immune receptors CD40 and CD86 in oral keratinocytes: implications for oral lichen planus

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Title: Immune receptors CD40 and CD86 in oral keratinocytes: implications for oral lichen planus

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**Abstract**

Lichen planus (LP) is a chronic T-cell-mediated mucocutaneous inflammatory disease that targets the stratified epithelia, including those lining the oral cavity. The intraoral variant of LP (OLP) is known to be associated with production of IFN-γ by the infiltrating T lymphocytes, however the role of epithelial cells in the etiopathogenesis of the disease is not completely understood. There is however a growing body of evidence about the involvement of a number of epithelial-derived cytokines, immune receptors as well as co-stimulatory molecules in the pathobiological processes that promote and sustain OLP. In the present study, we first used RT-PCR to assess whether CD40, a receptor found mainly on antigen presenting cells, and the co-stimulatory molecule CD86, were expressed in oral keratinocytes (three strains of primary normal oral keratinocytes and the H357 cell line) in the presence or absence of IFN-γ. To further characterize the involvement of CD40 in OLP, expression and distribution of both receptor and ligand (CD40/CD154) were evaluated by immunohistochemistry in tissues from OLP. The results showed, for the first time, that both CD40 and CD86 are constitutively expressed at low levels in oral keratinocytes and that their expression can be enhanced by IFN-γ stimulation. Strong intensity of CD40 staining was found in OLP tissues. Taken together, the results strongly suggest that CD40/CD86 play a role in the pathophysiology of oral inflammatory diseases such as OLP.

**Keywords:** CD40; CD86; oral lichen planus; oral inflammation; Regulatory T Cells; mucosa
Introduction

Lichen planus (LP) is a chronic T-cell-mediated mucocutaneous inflammatory disease whose aetiology and pathogenesis are not completely understood. To further clarify some of the pathological regulatory mechanisms associated with the disease, we investigated the expression of CD40 and CD86 in oral keratinocytes and their role in inflammatory disease of the oral cavity.

CD40 is a 45–50 kDa phosphorylated type I integral membrane glycoprotein that belongs to the tumor necrosis factor (TNF) receptor superfamily which is expressed on a number of different haematopoietic and non-haematopoietic cells (1-11). It has been shown to be involved in several biological functions, such as cell mediated immunity and cell growth regulation. The ligand for CD40 is CD154, also known as CD40 ligand (CD40L), a cell surface molecule mainly expressed by activated T cells. Through interaction with its receptor, CD154 plays a pivotal role in T cell-dependent humoral response as well as cell-mediated immunity and inflammation. Thus, it represents a key element in the pathogenic process of chronic inflammatory diseases, autoimmune disorders and has additional implication in many pathogenic steps of tumorigenesis (1,12,13).

Interferon-gamma (IFN-γ) can cause an increase of CD40 expression in a number of different epithelial cell lines, including buccal oral epithelium (8,11). Due to the increase in CD40 expression after pro-inflammatory cytokine treatment, it is not surprising that CD40 epithelium expression is enhanced in some inflammatory conditions (4).

Focusing on oral epithelium, CD40 has been demonstrated to be involved in many aspects of cell homeostasis, by being constitutively expressed by oral keratinocytes and able to stimulate T lymphocytes by reacting farther with several costimulatory
molecules, including B7.2 (or CD86), one of the most important B7 molecules family member (14). CD86 can bind to the ligands CD28 or to the cytotoxic T-lymphocyte associated molecule-4 (CTLA-4), located on resting and activated T cells, thus determining respective upregulation of T cell stimulation and clonal expansion via multiple cytokines stimulation, or downregulation of this process (15,16).

There is increasing evidence that the expression of CD40 on epithelium plays a role in immune response, stimulating the production of a number of cytokines and chemokines. Previous studies have shown that these chemokines are upregulated in oral lichen planus (OLP) and that this condition is characterised by large numbers of activated T cells present localised near the epithelium or infiltrating the basal epithelium (9,11).

The aim of this study was to investigate the effect of IFN-γ treatment on CD40 mRNA expression in oral epithelial cells. Antigen presentation by keratinocytes is proposed to have role in the pathogenesis of OLP, and the data on CD86 expression remains conflicting (8,17-24). Thus, we investigated CD86 mRNA expression in oral epithelial cells due to its crucial role in this process. The results showed, for the first time, that both CD40 and the co-stimulatory molecule CD86 are constitutively expressed at low levels in oral keratinocytes and that their expression can be enhanced by IFN-γ stimulation.

CD40/CD154 were additionally evaluated by immunohistochemistry in OLP tissue with the intention to further characterize the CD40-CD154 cell-expression and distribution.
Materials & Methods

Patients

All OLP tissue (n = 12) was collected from patients that were attending the Oral Medicine Clinic, Eastman Dental Institute and the Royal dental Hospital of Melbourne. The condition in each case was confirmed as OLP by a pathologist, and only patients without any other systemic diseases were enrolled. All normal oral mucosa was obtained from patients attending the Oral Surgery Clinic, Eastman Dental Institute for routine third molar extraction. The internal Ethical Committee of the UCL Eastman Dental Institute approved the study protocol, which was performed in accordance with the tenets of the Declaration of Helsinki. All patients provided written informed consent.

Cell culture techniques

Normal Human Oral keratinocytes (NHOK) cell culture.

Normal oral mucosal tissue was obtained for this study from healthy patients. Three different NHOK strains (NHOK1, NHOK2, NHOK3) were isolated from the excised normal tissue by separating the connective tissue. The samples were cut into approximately 1mm³ pieces and culturing at 37°C /5% CO₂ in keratinocyte basal medium-2 containing the recommended growth supplements (Biowittaker, Wokingham, UK). The epithelial cells were then detached using 0.25% trypsin-1mM EDTA. The viability of the keratinocytes was confirmed by trypan blue exclusion.
**H357 cell culture**

The oral squamous cell carcinoma cell line, H357, was established by Prime et al. (25), from a primary explant of a tongue squamous cell carcinoma. This cell line was grown in the same medium as described for the NHOK.

**CD40 / CD86 RT-PCR**

mRNA derived from H357 cells and primary oral epithelial cells treated with 1000U/ml IFN-γ for 48 hours was investigated. Primers specific for human CD40 mRNA and CD86 mRNA were generated for this reaction;

CD40 forward 5’ - CTGGGCTAGCGATACAGGAG -3’, reverse 5’- GGAATTTCTG TGGCCAAATCCA -3’ and

CD86 forward 5’ – AGACGCGGCTTTTATCTTCA -3’, reverse 5’- AACTCCAGCTCTGCTCCGTA -3’ (Genosys-Sigma, Poole, UK) and RT-PCR carried out. Magnesium concentration was optimised for each primer as follows; 1µl of cDNA was added to 4µl dNTP (2.5mM), 5µl 10x buffer, 0.225µl AmpliTaq (5.0U/µl) (Perkin Elmer.), 4µl of each specific primer (5µM), 1.5mM, 3.0mM or 4.5mM MgCl in each reaction and dH2O added to give a final volume of 50µl. The thermocycler (Techne Genius; Cambridge, UK) parameters used also vary depending upon the primers utilised, the annealing temperature of the reaction was dependant upon the different guanine-cytosine content of the primers. The general parameters used were: 94°C for 45secs; annealing temperature (57°C-60°C) for 45secs; 72°C for 45secs; Repeated for 35 cycles.

The products were separated on a 2% agarose (GibcoBRL Life Technologies, Paisley, UK) gel and visualised by staining with ethidium bromide (Sigma, Poole, UK), specific bands were visualised by ultra-violet trans-illumination in a MultiImage Light Japan Science and Technology Information Aggregator, Electronic (J-STAGE)
Cabinet (Alphalnnotech Corp., Cannock, UK) and digital images acquired and stored using Alphalnager Software (Alphalnnotech Corp., Cannock, UK). The primers utilised for the study of housekeeping expression encoded a region of 18S ribosomal RNA (5’- tgttggagaagtcctaga-3’, 5’- gcatgccagagtcgcttcg-3’).

**IFN-γ cell treatment assay**

In a modification of the method utilised by Altenburg et al [26], the primary oral epithelial cells and the H357 cell line (at 2nd or 3rd passage) were seeded at 8x10^4 cells/well in a Falcon 6 well plate (Becton Dickinson, Oxford, UK) with 3mls of KBM-2 medium containing no hydrocortisone. The cells were incubated for at least 3-5 days until cell culture was 60-80% confluent. We set up the optimal experimental conditions in preliminary experiments with dose-response curves. Medium containing 1000U/ml IFN-γ was added to 3 wells and control cell culture medium only was added to the remaining 3 wells. The cells were incubated for 48hrs or, in the case of the H357 time course, for the following time-points: 3hrs, 6hrs, 9hrs, 24hrs, 48hrs and 72hrs. The supernatant was extracted, centrifuged and stored at –70°C. The adherent cells were washed with PBS (Gibco Life Technologies, Paisley, UK) before 0.5ml of Trireagent (Sigma, Poole, UK) were added. The suspension was then removed and stored at –70°C.

**Immunohistochemical methods**

**Paraffin section preparation**

Archival paraffin-embedded formalin-fixed (PEFF) oral lichen planus tissue was used. Normal non-diseased skin (leg) sections and oral mucosa (tonsil) sections were also utilised as positive and negative control samples. The paraffin blocks were cut to
a 4 µm thickness using a microtome, and mounted onto Superfrost Plus slides (Thermo Scientific).

**Antigen retrieval methods**

Sections were de-waxed using a xylene wash (BDH, Poole, England) followed by ethanol washes; the sections for CD154 were boiled in citric acid on hot plate for 20 minutes and cooled at room temperature for 20 minutes; for CD40, the sections were boiled in citric acid on hot plate for 10 minutes and cooled at room temperature for 20 minutes. Then the sections were washed in PBS, and the endogenous peroxidase was quenched with 3% H₂O₂ in methanol for 5 minutes.

**Antibodies for immunohistochemistry**

- **Primary antibodies:** Purified anti-human CD154 (C-20) antibody (sc-978, rabbit polyclonal IgG, Santa Cruz Biotechnology Inc., Dallas, Texas; diluted 1:50 in PBS), purified anti-human CD40TNFRSF5 antibody (PA5-32325, rabbit polyclonal IgG, Thermo Fisher, diluted 1:50 in PBS).

- Secondary antibody: Biotinylated Universal (PK-6200, anti-mouse IgG/rabbit IgG) antibody (Vectastain® Universal, Elite, ABC kit, Vector laboratories, Inc, Burlingam, California, USA).

**3', 3'-diaminobenzide (DAB) staining and image acquisition**

The study slides containing OLP sections and positive control were incubated with the primary antibody for 30 minutes at room temperature. The negative control was incubated with no primary antibody (only PBS) for the same time at room temperature. The slides were then washed in PBS 3 times and incubated with the secondary antibody for 30 minutes at room temperature. The slides were then...
incubated with Avidin DH/ Horse biotinylated HRP solution (Vectastain® Universal, Elite, ABC kit, Vector laboratories, Inc, Burlingam, California, USA). The slides were washed 3 times with PBS and DAB (Dako Australia Pty. Ltd, North Sydney, NSW, Australia) was applied for 5 minutes. After this, the slides were washed with PBS, counterstained in Mayer’s Haemotoxylin (Amber scientific, Midvale WA, Australia), and mounted. Consecutive sections of the same samples were also stained with Haemotoxylin and Eosin without Immunohistochemistry. Pictures were taken using Aperio CS2 Digital Pathology Slide Scanner (Leica Biosystems).

**Statistical analysis**

Statistical significance was evaluated using unpaired t-tests.

**Results**

*Time course study of CD40/CD86 expression in H357 cells treated with IFN-γ.*

The production of CD40/CD86 in oral mucosal keratinocytes was first assessed over time in preliminary experiments using the keratinocyte cell line H357 (Figure 1). After three hours of IFN-γ treatment, very low levels of both CD40/CD86 mRNA were detectable in H357 cells. After 6 hours both CD40/CD86 mRNA were slightly higher in the IFN-γ compared to the control cells. Interestingly, both CD40 and CD86 mRNA levels at 9 hours was virtually undetectable in the control cells. The IFN-γ treated cells showed CD86 mRNA levels up to 72 hours, whereas CD86 mRNA transcripts were present up to 48 hours in the control cells.

Taken together, the data show that expression of CD40 and CD86 in H357 cells can be enhanced by IFN-γ stimulation.
Co-stimulatory molecule expression in oral epithelium in vitro

CD40 is expressed constitutively in three different primary oral epithelial cell lines. but can be increased after treatment with IFN-γ treatment in vitro (Figure 2a-2b). The statistical analysis revealed that mRNA expression, was statistically significantly higher (P < 0.001) in the IFN-γ group (0.853 ± 0.025) compared to that of the control group (0.419 ± 0.0134) (Figure 2c). Furthermore, a similar pattern was witnessed for the co-stimulatory molecule CD86 on the same cell lines. Its expression was very low constitutively, but was increased after IFN-γ treatment although this effect was not statistical significant (Figure 2c).

CD40 / CD154 expression in OLP tissue

In sections of OLP lesional tissue, concentrated staining of CD40 is associated with cells within the infiltrate of OLP, although staining of individual cells in proximity to the epithelium are also visible as well as a moderate epithelial staining (Figure 3). Staining was particularly intense within the band-like inflammatory infiltrate (Figure 4). Cells in the basal area appear to express relatively high expression, although positive cells are also present in the supra-basal area. Certain focal areas of the basal epithelium have particularly high expression levels. CD154 is expressed upon infiltrating cells in OLP lesions, but not within the epithelial layers (Figure 5). Positive cells appear to be preferentially located near areas of epithelial cells at the epithelial-connective tissue junction, especially in focal areas of cell damage.

Discussion
There was a strong intensity of CD40 staining in OLP, particularly on cells within the infiltrate and which may represent CD40-positive Langerhans’ cells. CD40 expression was also witnessed in association with oral epithelial cells. CD40 has been reported in other epithelial cell types in vivo, and appears to increase during other inflammatory disorders. Furthermore, it was demonstrated that CD40 expression is increased on primary oral keratinocytes after IFN-gamma incubation, consistent with other studies (8), implicating this cytokine in the stimulation and inflammation of oral epithelium. Furthermore, CD86 mRNA could be enhanced after IFN-gamma treatment of oral keratinocytes and the implications of this expression in oral inflammatory disease could be paramount, as discussed below.

In the light of current knowledge, our findings lead us to conjecture a possible involvement of CD40/CD86 ligation in inflammatory enhancement. T cells in OLP appear to be in an activated state and would thus be expected to express CD154 (27), the ligand for CD40. The proximity of infiltrating T cells and epithelium in OLP, which, in some cases, can extend to interactions between T cells and the epithelium would suggest that ligation of CD40 and CD154 occurs in this disease. This ligation has been shown to induce/enhance a number of effects in epithelial cells, including an increase in the production of pro-inflammatory cytokines (3,28) and chemokines (3,29-31). It is likely that an increased production of these molecules from the epithelial area in OLP would increase the inflammation that occurs. Specifically, the increased expression of the CXC ELR- chemokines witnessed in OLP may be partially due to the ligation of CD40 on oral epithelial cells, as was shown previously in cervical carcinoma cells (26). Furthermore, the increased production of RANTES witnessed from keratinocytes in OLP (32) could also be influenced by CD40 ligation (31, 33).
Interferon-gamma may also play an important contributory role in inflammation of oral epithelium as it can increase the expression of CD40 on epithelial cells, as well as induce the expression of chemokines such as CXCL10 (Marshall et al, manuscript in preparation). These presumably promote migration of activated T cells into the area, which may interact with epithelial cells and bind CD40. In turn, this would synergise to produce more chemokines and cause an increase in the inflammatory status of conditions such as OLP (34-36).

It can be shown that the induction of B7 molecules on keratinocytes can cause an increased immunogenecity to a number of different antigenic stimulants, causing a large influx of stimulated T-cells. This increase in the delayed-type hypersensitivity (DTH) response to reencountered antigens witnessed in B7 transgenic mice displays many similarities to the pathogenic mechanisms visible in OLP, including the large T-cell influx visible in oral lichen planus and the chronic nature of the disease. The fact that CD86 can be upregulated on oral epithelium in vitro by interferon-gamma and that there are many features suggestive of IFN-gamma stimulation on keratinocytes in OLP suggests that this molecule is likely expressed on these cells involved in the increased immunogenecity to antigens, including common oral commensals, such as Candida. Furthermore, the expression of CD86 on oral epithelium may also be relevant to cell migration, as CD28 ligation of CD4+ cells can alter chemokine receptor expression (37).

Although we have shown that CD86 is induced in vitro on oral epithelium, its expression in OLP is unknown. Simon et al (38), investigated the expression of CD28 and B7 in cutaneous lichen planus lesions and discovered that the B7-1 molecule was focally expressed on keratinocytes within the lesion. However, it was found that the
antibody for B7-1 also detected the MHC molecule (39) and it was likely that it was this molecule that was detected in lichen planus.

Furthermore, the role of CTLA-4 and CD28 binding is known to affect Th1/ Th2 differentiation, but this would appear to have a larger effect on naïve cells than memory cell interactions (40) which occur in OLP.

Due to the increase in co-stimulatory molecules in epithelium after inflammatory signals, including oral epithelial cells as shown in this study, it has been proposed that they may act as non-professional antigen presenting cells in OLP (41). However, despite keratinocytes possessing the genes necessary for antigen presentation (42), presumably if oral keratinocytes were capable of antigen presentation, it would have to be immunogenic antigen presentation in OLP to cause the reaction witnessed. However, it has proved difficult to assess the factors that provide even professional antigen presenting cells with either tolerogenic or activating signals to T cells in the periphery (43).

Like bronchial and intestinal epithelium, oral epithelium is also capable of antigen presentation, although by other mechanisms (5,44-46). Particularly, an increase in the expression of co-stimulatory molecules, such as in times of inflammation, is required for oral epithelial antigen presentation.

It is tempting to suggest that those antigens that would be presented at high doses, thus more likely to elicit an effective T cell response (16), would be those at constantly localised high levels in the oral mucosa, such as betel nut antigens in persistent users and amalgam antigens of patients with these fittings. If these antigens were combined with inflammatory signals they may produce active antigen presentation of these antigens, which may produce an immunogenic opposed to tolergenic response.
The proposed activation / tolerance theories in dendritic cells that stimulatory function is either enhanced by toll-like receptors (TLR) on dendritic cells recognising microbial products and upregulating co-stimulatory molecules (47) or that damage in other cells, and the production of molecules, such as heat shock proteins, act as ‘danger signals’ (48) to activate dendritic cells, may also be the case in epithelial cells (49-52).

Furthermore, the induction of co-stimulatory molecules upon the epithelium does not necessarily provide T cell activation, as these molecules may actually bind to CTLA-4, which is thought to be an important molecule in providing tolerance (53). In fact, dysregulation of CTLA-4 in OLP patients may be responsible for a breakdown in tolerance for oral keratinocyte antigens (54). However, in cutaneous lichen planus tissue there is a clear expression of CTLA-4 cells in most samples, whereas there is no expression in a variety of other skin inflammatory disorders (55). The implications of this expression are unclear, however, the findings that many infiltrating T cells in lichen planus express CD28 (38), suggests that these cells could interact with CD86-expressing keratinocytes leading to specific clonal activation of these cells (56).

A subset of CD4+ T cells that express CD25 represent regulatory T cells that are proposed to be involved in the suppression of autoimmunity (57-59). Interestingly, CD25+ cells are upregulated in OLP (58). However, these cells are also thought to involved in a role of the persistence of infection, perhaps in order to permit long-term immunity (59). Therefore, it could be proposed that these cells are involved in causing the chronicity of diseases, with a low-level (and perhaps non-detectable level) of infection occurring. The chronic nature and presence of CD25+ cells in OLP may therefore be related.
Presumably, if oral keratinocytes are capable of antigen presentation it would be ‘non-professional’ antigen presentation i.e. these cells would not be able migrate to the lymph nodes to stimulate naïve T cells. This would suggest that predominately only memory cells could be activated in this manner, in fact, memory T cells are activated by a range of different APC and have less requirement of co-stimulatory function than naïve cells (60). It is interesting to note that memory T cells constitute the majority of infiltrating cells in OLP lesions. Furthermore, the possible increase in chemokine production by T cell ligation of keratinocytes in OLP would presumably promote the migration of further memory cells into this area. This suggests that Langerhans’ cells have an important role in initiating naïve T cell responses, whereas in the secondary response keratinocytes may play a role in re-activating memory T cell responses. However, resident tissue APCs are implicated in presenting self-antigen to Th1 cells during auto-immune conditions (61), and thus keratinocytes may also play a role in initiating the presentation process to Th1 cells. The reaction against oral keratinocytes in OLP suggests that may be a break-down in tolerance to self-antigens of oral epithelial cells. There may be many mechanisms influencing potential breakdown of self-tolerance in the oral mucosa such as the process known as molecular mimicry (62), or the epitope spreading theory (63). Due to the number of agents associated with the onset of OLP and the chronicity of the disease, which may be caused by different shift of epitopes detected in the disease, epitope spreading appears a possible candidate for the pathogenesis of this disease. Furthermore, CD80/86 blockade (64) or CD40-CD154 blockade (65) can inhibit epitope spreading and ease ongoing autoimmunity in animal models. The fact that CTLA-4+ve cells are a positive factor in easing epitope spreading (54), suggests that the CD25+ cells witnessed in OLP may cause a down-regulation in the immune
reaction, however, perhaps further epitope changes promote a wave of inflammatory T cells. As epitope spreading is implicated in chronic diseases like multiple sclerosis, where there is relapses and remission this suggests this pathogenic pattern could occur in OLP.

However, in OLP despite the large number of CD4+ cells present in the lesions, it appears that it is CD8+ cells that are in the proximity to the epithelial area. This suggests that it is actually MHC-class I restricted presentation that is occurring within lesions. However, it may be that prior class II presentation by keratinocytes assist in initiating a CTL reaction. Interestingly, CD40 ligation of antigen presenting cells play an important role in the generation of CTL cells (66,67). Therefore, maybe if keratinocytes are presenting antigen through the MHC-Class II pathway, the ligation of CD40, which is classically associated with antibody-mediated reactions, not only amplifies the inflammation of the area, but may be also involved in the generation of specific CTL.

However, there are a group of CD8+ effector memory cells that are preferentially located in non-lymphoid tissue, that rapidly expand after activation (68), thus may not require further T helper cells. Furthermore, CD8+ memory cells also have a limited requirement for B7 co-stimulatory signals. However, as autoreactive CD4+ and CD8+ T cells often have a weaker affinity to antigen, they may actually require B7-stimulation (40) thus the expression on oral epithelium may still be relevant.

The process of cross-tolerance (69) is thought to be important in the gaining self-tolerance to apoptotic cells. In fact, there is a subset of intestinal dendritic cells that have been shown to transport apoptotic epithelial cells to the lymph nodes (70), in a process thought to provide exposure and induction of tolerance to ‘self’ antigen. Furthermore, Langerhans’ cells have been shown to be capable of phagocytosis of
vaginal apoptotic epithelial cells (71). These findings are of particular interest as it is thought that there are apoptotic keratinocytes in OLP (9), and it may be that the process of transport of antigen from these cells to the lymph nodes may in fact induce an immunogenic response to these antigens instead of the tolerising effect (72). It could be speculated that this process produces auto-reactive T cells to keratinocytes. Additionally, ligation of CD40 on epithelial cells causes growth inhibition and increased apoptosis of cells in ovarian carcinoma cell lines (3), which is of interest due to the increased apoptosis of this cell type in OLP (in conjunction with associated CD40 expression). This may induce the transport of apoptotic cells to the lymph nodes for cross-presentation, a process that was found to be age-related (73).

Also, molecular mimicry and epitope spreading is thought to occur in Class I restricted antigens as well as Class II antigens, so it may an effect of direct MHC-Class I presentation that may cause the cytotoxic effect in OLP. There is increasing evidence from the presence of molecules on epithelial cells that they may in some circumstances be capable of antigen presentation. Whether this antigen presentation produces a tolerogenic or immunogenic response remains to be seen, although the presence of MHC-II, CD40 and the potential of CD86 expression on oral epithelial cells, and CD28 in the T cell infiltrate of OLP suggests that ‘activating’ antigen presentation could occur. The antigen presentation may take the form of ‘sampling’ antigens on the oral mucosa, such as bacterial antigens. The implications for antigen presentation may be very important in conditions such as OLP as there appears to be a breakdown in the tolerance for ‘self’ keratinocyte antigens. This breakdown could occur by a number of mechanisms, including molecular mimicry or epitope spreading. However, CD8+ cells appear to be acting cytotoxically in OLP, therefore, perhaps the Class I pathway is more important in the
disease process. Perhaps the presence of inflammatory signals present during cross-presentation of apoptotic keratinocytes causes normal tolerogenic CD8+ cells to become auto-reactive for these cells. Interestingly, ligation of CD40 on keratinocytes (which is expressed in OLP) is thought to cause apoptosis of this cell type.

**Acknowledgements**

The authors gratefully acknowledge the technical support of Mr Simon Eades, Melbourne Dental School, The University of Melbourne.
References


Figure legends

**Figure 1:** CD40 / CD86 mRNA expression in H357 (timecourse).

**Figure 2:** a) The expression of 18S, CD40 and CD86 mRNA in 3 different normal human oral keratinocytes (NHOK) (indicated as 1, 2 and 3) either after IFN-gamma treatment (IFN-γ 1-3) for 48 hours or non-treated (CON 1-3) or the negative control (-). b) The values were normalized against 18S mRNA expression used as housekeeping. c) Triplicate average with standard deviation and statistical significance (*=Significant .01 < p ≤ .05, **= Highly Significant p ≤ .01).

**Figure 3:** CD40 localisation in OLP tissue using peroxidase staining. Intense staining was associated with subepithelial band-like infiltrate. Epithelial cells within OLP lesions also appeared to demonstrate mild to moderate staining. Magnification x4 and x20. Negative control sections demonstrated no staining. Normal Control and typical H&E staining of OLP are reported in Supplementary Figure 1.

**Figure 4:** CD40 localisation associated with inflammatory infiltrate in OLP tissue. Staining was mostly present within the dense inflammatory infiltrate and also upon single cells in proximity and infiltrating the epithelial layer. Magnification x40.

**Figure 5:** CD154 expression in oral lichen planus tissue. Magnification x10 and x40. Infiltrating cells in proximity to the basal epithelium are associated with anti-CD154 positive staining, however epithelium shows faint or no staining.
Supplementary Figure 1

IHC staining of CD40 (A) and CD154 (B) of non-diseased controls. Haematoxylin-eosin staining of typical OLP tissue (C) and normal control (D).
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Fig. 1

254x190mm (72 x 72 DPI)
Figure 2

595x378mm (72 x 72 DPI)
Fig. 3

226x98mm (72 x 72 DPI)
Fig. 4
254x190mm (72 x 72 DPI)
Fig. 5

177x94mm (72 x 72 DPI)
Suppl Fig. 1

254x190mm (72 x 72 DPI)
CANCER-ASSOCIATED FIBROBLASTS REGULATE KERATINOCYTE CELL–CELL ADHESION VIA TGF-β-DEPENDENT PATHWAYS IN GENOTYPE-SPECIFIC ORAL CANCER

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Abstract

The interrelationship between malignant epithelium and the underlying stroma is of fundamental importance in tumour development and progression. In the present study, we used cancer-associated fibroblasts (CAFs) derived from genetically unstable oral squamous cell carcinomas (GU-OSCC), tumours that are characterized by the loss of genes such as TP53 and p16INK4A and with extensive loss of heterozygosity, together with CAFs from their more genetically stable (GS) counterparts that have wild-type TP53 and p16INK4A and minimal loss of heterozygosity (GS-OSCC). Using a systems biology approach to interpret the genome-wide transcriptional profile of the CAFs, we show that transforming growth factor-β (TGF-β) family members not only had biological relevance in silico but also distinguished GU-OSCC-derived CAFs from GS-OSCC CAFs and fibroblasts from normal oral mucosa. In view of the close association between TGF-β family members, we examined the expression of TGF-β1 and TGF-β2 in the different fibroblast subtypes and showed increased levels of active TGF-β1 and TGF-β2 in CAFs from GU-OSCC. CAFs from GU-OSCC, but not GS-OSCC or normal fibroblasts, induced epithelial–mesenchymal transition and down-regulated a broad spectrum of cell adhesion molecules resulting in epithelial dis-cohesion and invasion of target keratinocytes in vitro in a TGF-β-dependent manner. The results demonstrate that the TGF-β family of cytokines secreted by CAFs derived from genotype-specific oral cancer (GU-OSCC) promote, at least in part, the malignant phenotype by weakening intercellular epithelial adhesion.

Introduction

Intercellular adhesion plays a major structural role in epithelial integrity and is altered in malignancy. Specifically, impairment of cohesion between cells and altered expression of adhesion molecules in epithelial cancers determine an invasive behaviour and are associated with poor prognosis, respectively (1,2). This suggests that the weakening of cell-cell adhesion strength is a feature associated with tumour progression.

Our current understanding of the molecular biology of head and neck cancer, including oral squamous cell carcinoma (OSCC), relates almost exclusively to tumour epithelium. Malignant keratinocytes, however, are heterogeneous. In colorectal cancer, for example, subtypes with microsatellite or chromosomal instability, or neither, have been reported (3). In OSCC, genetically stable (GS-OSCC) and genetically unstable (GU-OSCC) variants have been identified and characterized in cell culture (4). In contrast to the stable variants (GS-OSCC), malignant keratinocytes from GU-OSCC are characterized by TP53 and CDKN2A/p16INK4A dysfunction, telomerase activation, frequent...
inactivation of the NOTCH1 canonical pathway and extensive loss of heterozygosity (4). Furthermore, there is evidence to show that the GS-OSCC and GU-OSCC genotypes have biological relevance in vivo, because the GU-OSCC tumour group have a worst prognosis than GS-OSCC (5). Further, CDKN2A/p16INK4A deletions are detectable in vitro using in situ techniques (6).

With respect to GS-OSCC keratinocytes, it is possible that they are normal contaminants of the tumour tissue, but these cells have an altered transcriptional profile (5) and are resistant to suspension-induced terminal differentiation (5). If the GS-OSCC and GU-OSCC variants have biological relevance, therefore, they may be important with regard to clinical diagnosis, behaviour, treatment and prognosis in OSCC.

It is now recognised that solid tumours are not simply clonally evolved epithelial cells that have accumulated a critical number of mutations but rather act as dysfunctional tissues where the mesenchymal component plays a critical role in tumour pathogenesis (3). The non-malignant cells, together with the extracellular matrix, constitute the tumour stroma and pre-eminent in the stroma are cancer-associated fibroblasts (CAFs). Cancers metastasize by a series of distinct steps that include invasion, intravasation, extravasation and, ultimately, colonization. Epithelial–mesenchymal transition (EMT), a process in which epithelial integrity is lost and mesenchymal attributes are acquired (7), is thought to play a fundamental role in tumour progression. Although the role of EMT has been questioned recently, the fact remains that there is convincing morphological evidence that EMT is present at the invasive front of human tumours. Furthermore, EMT is dynamic, which suggests that the tumour stroma may be remodelled in its regulation, a proposal that requires verification. It is important, therefore, to confirm that CAFs regulate EMT and also to understand the mechanisms by which this occurs.

Recently, we isolated CAFs from GS-OSCC and GU-OSCC and demonstrated that they had distinct transcriptional profiles (8). In GU-OSCC, but not GS-OSCC, malignant keratinocytes induced high levels of senescence in CAFs, which, in turn, induced target keratinocytes to invade collagen gels in vitro (9). This suggests that the epithelial–mesenchymal crosstalk occurring in GU-OSCC involves paracrine signals in the microenvironment. CAFs are known to secrete numerous growth factors, chemokines and cytokines as well as modulating the extracellular matrix through protease activity. Senescent fibroblasts, for example, produce a characteristic secretome termed the senescence-associated secretory phenotype (10), that consists of both soluble and insoluble factors that have the capacity to promote the invasion of transformed cells (11). The key molecules that are secreted by CAFs from GU-OSCC and that are instrumental in inducing epithelial invasion have yet to be identified.

In the present study, we used a systems biology approach to interpret a genome-wide transcriptional profile of CAFs derived from OSCC. We show that members of the TGF-β family of cytokines distinguish CAFs from GU-OSCC and GS-OSCC and from normal oral fibroblasts. We show that CAFs from GU-OSCC overexpress TGF-β1 and TGF-β2 and induce EMT; in addition, they promote a widespread reduction in the expression of cell–cell adhesion molecules and disrupt epithelial cohesion and initiate keratinocyte invasion in vitro in a TGF-β1-dependent manner.

Materials and methods

Cell lines and strains

The cells used for this study were collected and isolated at Bristol Dental School, University of Bristol, UK (4 series) and Beatson Laboratories, Glasgow, UK (BICR series). All of the cell lines/strains were derived prior to 2001 and therefore were not subject to Ethical Committee approval in the UK. All of the OSCC were human papillomavirus negative. Keratinocytes were derived from OSCC (HS57, H129, SCC (HaCaT)), and normal skin (HaCaT). The fibroblasts used in this study were derived from normal oral mucosa (NHOF-1, NHOF-2, NHOF-4, NHOF-5, NHOF-6, NHOF-7), GS-OSCC (BICR-3F, BICR-5F, BICR-6F, BICR-66F, BICR-7F, BICR-73F, BICR-75F, BICR-80F) and GU-OSCC (H314E, H357F, H413F, BICR-3F, BICR-32F, BICR-43F, BICR-62F, BICR-78F, BICR-82F). The mesenchymal origin of the fibroblast strains was confirmed by positive and negative labelling with vimentin and pan-cytokeratin/keratin antibodies, respectively. In contrast, the origin of the keratinocyte lines was confirmed by positive and negative labelling with pan-cytokeratin/keratin 14 antibodies and vimentin, respectively (8,9). We are confident that the fibroblasts are normal because preliminary data from genome-wide copy number analysis have shown that all of the strains have a normal gene copy number (N.Thakker and E.K.Parkinson, unpublished observations). Furthermore, in the many studies where OSCC have been examined in vitro, we have never seen elevated levels of p53 indicative of p53 mutations.

Culture conditions

Details of the keratinocyte cell lines and fibroblast strains have been documented previously (8,9). Fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine and grown in a humidified atmosphere of 5% carbon dioxide/95% air; cells were examined at passages <12. Keratinocytes were grown under the same standard conditions (5% carbon dioxide, 37°C). Previous studies demonstrated induction of fibroblast senescence using this protocol (9), and therefore, the cells were used as positive controls for the non-proliferative, senescent GU-OSCC CAFs.

Collection and conditioned media

Cells were grown in 75 cm² flasks until they were 70–90% confluent, washed with serum-free media (<3) and phosphate-buffered saline (PBS; <3) and then incubated in serum-free media for a further 48 h. The conditioned media (CM) was centrifuged at 800g for 5 min to remove dead cells. The viable attached cells were trypsinised and counted; the CM was normalised for 0.5 × 10⁶ fibroblasts. CM was stored at −20°C.

Neutralization of TGF-β in fibroblast CM

To neutralize TGF-β in fibroblast CM, monoclonal antibodies to TGF-β1, -β2 and -β3 (R&D Systems, cat no. MAB1835), TGF-β1 (R&D systems, cat no. AB-101-NA) or TGF-β2 (R&D Systems, cat no. AB-12-NA) were added to CM at concentrations of 5, 0.5 and 1 μg/ml, respectively, and incubated for 30 min at 37°C. CM was used immediately.

Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CAFs</td>
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<td>CM</td>
<td>conditioned media</td>
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<td>DTG</td>
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<td>GU-OSCC</td>
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<td>H2O2</td>
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By guest on January 13, 2017
Network construction and analysis
The transcriptome of OSCC CAFs (9) was interrogated using a systems biology approach, as described by ourselves and others (12–15). Briefly, in order to prepare a protein interaction map of genes differentially expressed in CAFs, protein–protein interaction (PPI) networks were examined through functional protein association network (STRING) and confirmed by literature searches (PubMed). Only PPI with the highest confidence (0.9 and above) were included. To identify which genes in the databases corresponded to the genes listed in the microarray data, we used either the gene symbol or the SwissProt entry name shown in the protein databases. Proteins and their PPIs were combined to form networks and subnets using Cytoscape 3.4. The resulting network was visualized as a series of graphs in which each node (N) represented a protein and the interactions between proteins were shown by edges (E) in the graph.

We used the differentially transcribed genes (DTG) from our gene expression profile data (9) to build two PPI networks: in the first one, nodes were represented by DTG corresponding to putative secreted molecules with the highest fold change (FC), i.e. logarithmic FC > 3; these nodes were named highly up-regulated secretory molecules (hUSM). The second network, of the same size as the first, was made of DTG corresponding to putative secreted molecules with the lowest significant FC, i.e. logarithmic FC < −3; these nodes were termed mildly up-regulated secretory molecules (mUSM). The average number of connections for each group of DTG was used to describe the degree of connectivity (k). The average connectivity (k) of a whole network was calculated as k = 2E/N, corresponding to the average number of interactive partners for each protein.

Western blotting
Protein extraction and western blotting were undertaken using standard protocols. A list of primary antibodies, concentrations and suppliers is shown in Table 1. Selected proteins were detected using Amersham enhanced chemiluminescence Western Blotting Detection Reagent (Amersham Biosciences, UK) and exposed to radiographic film (Kodak, UK).

In-cell western blotting (In-cell enzyme-linked immunosorbent assay)
In-cell western blotting was performed as described previously (16). First, 2.5 × 10⁵ cells were seeded into each well of 96 well plates, fixed in cold (-20°C) methanol for 20 min, washed with PBS (3×) and permeabilized with 0.1% Triton for 10–15 min. After washing with PBS (3×), a blocking solution consisting of 3% bovine serum albumin (BSA) in PBS was added for 1 h. The samples were incubated with the appropriate primary antibody (1:100 in 2% BSA/PBS for 3 h, washed in 3% BSA/PBS and then exposed to specific secondary antibodies (1:10,000 in 2% BSA/PBS) conjugated to horseradish peroxidase for 1 h. After washing with PBS (3×), the enhanced chemiluminescence mixture was added for 5 min in a dark room and the protein expression was quantified at 415 nm using the ELx808 microplate reader (BioTek Instruments, Winooski, VT) according to the manufacturer’s instructions.

Immunofluorescence microscopy
Immunofluorescence microscopy was performed as reported by us previously (17), with minor modifications. Briefly, the cells were cultured onto four-well plates (NuGene™ Cell-Culture Treated Multidishes, Thermo Fisher Scientific) in standard conditions and at 60% of confluency were treated as reported in the text. Cells were fixed with 100% ice-cold methanol for 10 min at 4°C and then incubated with 0.5 μg/ml of Hoechst staining (H3604, Sigma–Aldrich) for 30 min at 4°C and then blocked with 5% BSA–PBS solution at 4°C for 60 min. The samples were then incubated overnight at +4°C with E-cadherin/CIB1 antibody (FITC conjugate, A157557, Thermo Fisher Scientific) at a 1/100 dilution in 2% BSA–PBS, and with anti-vimentin antibody (Ab154207, Abcam’s RabMAB®) at a 1/1,000 dilution in 2% BSA–PBS. All intermediate washing steps were performed with PBS. Images were taken with a fluorescence microscope (EVOS™ FLoid™ Cell Imaging Station, Life technologies).

Epithelial adhesion
Desmosomal adhesion is crucial for epithelial cells (18) and intercellular adhesion can be specifically measured using the dispase dissociation assay (16). First, 1 × 10⁴ keratinocytes were cultured in six-well plates under standard conditions until ~95% confluent. The culture media were decanted, the cells washed with PBS (3×) and then incubated with 4 ml fibroblast CM for 48 h. The fibroblast CM was decanted from the keratinocyte cultures, and the cells were washed with PBS (3×). Then, the keratinocyte cultures were incubated with 2 ml 0.5% Dispase II (Sigma–Aldrich) at 37°C for 15–20 min such that they were separated from the base of the culture dish as a single sheet. The dispase was carefully decanted, and the cell sheet was gently washed with 3 ml PBS prior to transferring to universal tubes containing 4 ml PBS. The tubes were then sealed and shaken by hand up and down (±20). The content of each tube was returned back to the six-well plates where 10 μl crystal violet was added and the total number of detached fragments was counted. The number of detached fragments (>3 mm), determined by placing the six-well plate over a grid on a white paper, was taken as a measure of intercellular keratinocyte adhesive strength.

Epithelial invasion
Epithelial invasion was examined as described in detail previously (8).

Statistics
Unless otherwise indicated, the experiments were repeated 3×; the values cited were the mean of the repeats ± SD. Data were analysed using the one-way analysis of variance with Tukey`s multiple comparison test being used as a post-test. P < 0.05 was considered statistically significant.

Results
Interactome-transcriptome analysis of the putative secreted molecules
Of the 132 genes differentially expressed in CAFs versus normal fibroblasts, 61 were up-regulated and of these, 16 (~26%) were...
putative ligands/secreted molecules (Supplementary Table 1, available at Carcinogenesis Online), which may interact with OSCC cells. In order to select key secreted molecules for further investigation, we undertook network analysis of interactomes from huSM and muSM. Of the 16 up-regulated molecules, 4 had a logarithmic FC > 3 and therefore were included into the huSM network (INHBA, TGFB2, IGFBP3, IGFBP7). We first built an interactome formed by the 4 huSM plus their first neighbours (Figure 1); the resulting network (Supplementary Table 2, available at Carcinogenesis Online) constituted 87 nodes and 648 interactions, with an average connectivity of $k = 14.9$ edges per node.

An interactome formed by four nodes representing putative secreted molecules with low FC (muSM) was also built, which included STC2, CTGF, SPOCK1 and SERPINE2 (Supplementary Table 2, available at Carcinogenesis Online). As control networks, we used interactomes of comparable size (14), random interactomes from databases (19) and an interactome formed by four random nodes that were up-regulated in CAFs (Supplementary Table 2, available at Carcinogenesis Online). When compared with structured networks such as a cell–cell adhesion interactome (59 nodes, $k = 4.39$), the connectivity of huSM was 4-fold higher. The connectivity of huSM interactome greatly exceeds the $k$ value of publicly available PPI databases: on average, each component has 14.9 direct interactions in the huSM network, compared with 5.54 in BIND, 7.23 in HPRD, 5.14 in PPID, 4.03 in IntAct, 3.53 in MINT and 2.46 in DIP (19).

When compared with an interactome formed by four nodes selected randomly in the CAF up-regulated genes, the huSM interactome displayed a larger size (87 versus 60) but still higher connectivity (14.9 versus 10.07). We then investigated whether the huSM nodes formed a network with different properties than those displayed by the putatively secreted molecules with low FC (muSM), muSM failed to form a single network (Supplementary Figure 1, available at Carcinogenesis Online) as the four nodes either did not interact among each other (STC2, SPOCK1, SERPINE2) or did not have binding partners within the set confidence parameters (CTGF). Overall, this analysis suggests that the most up-regulated secreted molecules in CAFs form a network that is highly interconnected and, therefore, likely to have biological relevance (14).

**CAFs secrete TGF-β1 and TGF-β2**

As huSM nodes were TGF-beta family members, we investigated whether TGF-beta isoforms were secreted by CAFs, as predicted by gene expression data. To do so, we determined TGF-β1 and TGF-β2 protein levels in CM from CAFs derived from GS-OSCC and GU-OSCC, fibroblasts from normal oral mucosa (NHOF) and malignant keratinocyte cell lines; H2O2-treated normal fibroblasts were used as a positive control of GU-OSCC CAFs (8). We show that both TGF-β1 and TGF-β2 were overexpressed by CAFs derived from GU-OSCC relative to GS-OSCC, normal fibroblasts and keratinocyte lines (Figure 2A and B).

**CAFs from GU-OSCC induce EMT**

TGF-β is a well-known inducer of EMT. To examine the capacity of CAFs to induce EMT, we treated a partially transformed keratinocyte cell line (H357) with CM from senescent and non-senescent fibroblasts. We show that CM from GU-OSCC CAFs...
Figure 2. TGF-β1 (A) and TGF-β2 (B) protein expression in CM from fibroblasts derived from GS-OSCC and GU-OSCC, together with fibroblasts from normal oral mucosa and malignant keratinocyte cell lines. Cells were grown to 70% confluence, rinsed in serum-free medium and incubated in serum-free medium for 48 h. The medium was centrifuged and activated prior to the assay TGF-β was quantified using Quantikine TGF-β1 or TGF-β2 enzyme-linked immunosorbent assay (R & D Systems, USA). Each point on the graph represented the mean of >3 repetitions of each fibroblast strain or keratinocyte cell line.

(H357F), but not GS-OSCC CAFs (BICR66F), caused depletion of E-cadherin and up-regulation of vimentin (Figure 3A). The molecular alterations were associated with typical morphological changes such that the keratinocytes assumed a fibroblast-like appearance (Figure 3B). H2O2-treated NHOF1 and untreated NHOF1 served as positive and negative controls, respectively, for the senescent CAFs derived from GU-OSCC. Induction of EMT was then confirmed by immunofluorescence using different CAF strains (Figure 4 and Supplementary Figure 2, available at Carcinogenesis Online). Control cells showed classical intercellular staining of E-cadherin with no or little expression of vimentin (Figure 4A–F). Incubation with CM from BICR31F (GU-OSCC) drastically reduced E-cadherin staining together with a dramatic increase of vimentin (Figure 4G–I). Similar results were obtained with BICR3F (GU-OSCC), however such changes were milder when cells were incubated with CM from BICR66F (GS-OSCC; Supplementary Figure 2, available at Carcinogenesis Online).

Taken together, these data showed that GU-OSCC CAFs induce EMT in target cancer cells.

**CAFs from GU-OSCC inhibit keratinocyte adhesion**

To determine whether CAFs inhibited the expression of adhesion molecules other than E-cadherin, we used an in-cell enzyme-linked immunosorbent assay to show that CM from GU-OSCC CAFs (H357F), but not GS-OSCC CAFs (BICR66F), caused decreased expression of E-cadherin, Desmoglein 1 and 3 (DSG1 and DSG3), Desmoplakin (DSP) and Desmocollin (DSC) in H357 (Figure 5A). Similar findings were noted with H103 and HaCaT-II-3 malignant keratinocytes (Supplementary Figure 3, available at Carcinogenesis Online), but the effect was more pronounced in H357. The results were confirmed by canonical Western blot analysis, which showed a major decrease in the expression of cadherins and DSP (Figure 5B).

The consequences of CAF-induced down-regulation of keratinocyte adhesion molecules were examined in a well-established functional assay of intercellular adhesion strength (16). We show that CM from GU-OSCC CAFs (H357F), but not GS-OSCC CAFs (BICR66F) or normal fibroblasts, reduced the cohesive strength of malignant keratinocytes (H357) as shown by the increase in the number of small fragments (Figure 5C and D). Similar effects were noted using different target keratinocyte lines (H103, HaCaT-II) treated with CM from H357F and BICR66F (Supplementary Figure 3, available at Carcinogenesis Online).

In view of the close association between intercellular epithelial adhesion and the invasive phenotype, we examined the invasion of the partially transformed non-tumorigenic keratinocyte cell line H357 into collagen gels. We confirm our previous observations (9) using alternative fibroblast strains, which show that CM from GU-OSCC CAFs (H357F, BICR18F), but not GS-OSCC CAFs (BICR66F, BICR31F), stimulated the invasion of H357 keratinocytes in vitro (Supplementary Figure 4, available at Carcinogenesis Online).

The data demonstrate that CAFs derived from GU-OSCC induce a pro-invasive phenotype in malignant keratinocytes by the induction of EMT and a reduction in intercellular adhesion.

**TGF-β1 mediates the effect of CAFs on keratinocyte adhesion and invasion**

Since our in silico data indicated that TGF-β1 was an important secretory molecule of CAFs and both TGF-β1 and TGF-β2 were overexpressed by CAFs, we wanted to examine whether TGF-β1
isoforms acted as intermediaries in CAF-induced EMT, inhibition of epithelial dis-cohesion and induction of keratinocyte invasion in vitro. With regard to EMT, we demonstrate that anti-TGF-β antibodies inhibited EMT as demonstrated by the attenuation of E-cadherin and vimentin expression following the treatment of H357 keratinocytes with CM from H357F; the effect was most marked using a pan anti-TGF-β antibody and an anti-TGF-β2 antibody (Figure 6A). With respect to CAF-induced epithelial dis-cohesion (Figure 6B), we show that a pan anti-TGF-β antibody attenuated the dys-cohesive effects of GU-OSCC CAFs (H357F; BICR82F; BICR 18F), where H357 keratinocytes was used as target cells and when small epithelial fragments were counted (Figure 6B); similarly, anti-TGF-β1 and anti-TGF-β2 antibodies attenuated the induction of epithelial dis-cohesion by GU-OSCC CAFs (H357F; Figure 6C). With regard to CAF-induced epithelial invasion in vitro, a pan anti-TGF-β antibody inhibited the invasion of H357 keratinocytes into collagen gels following stimulation by GU-OSCC CAFs (H357F; BICR82F; BICR 18F; Figure 6D), and the effect was also achieved using anti-TGF-β1 and anti-TGF-β2 antibodies (Figure 6E).

Taken together, the data demonstrate that CAFs derived from GU-OSCC induce EMT and, in addition, weaken epithelial cell-cell adhesion and promote invasion of malignant keratinocytes via TGF-β-dependent pathways.

Discussion
In the present study, we identified key molecules secreted by CAFs in oral cancer. Using a network analysis approach we show that the network formed by INHBA, TGFβ2, IGFBP3 and IGFBP7 had high connectivity, thereby implying biological relevance. Previous studies have shown that insulin-like growth factor-binding proteins are expressed at high levels by both activated and senescent fibroblasts (10), but the present study is the first to highlight a potential role for INHBA. INHBA is an important constituent of the senescence-associated secretory phenotype and induces paracrine senescence in normal cells both in vitro and in vivo (21). INHBA is also significantly up-regulated in a broad spectrum of malignant tumours (22–25), including those of the head and neck (26, 27). The present report, however, is the first to show that INHBA is up-regulated in CAFs and has biophysical significance. Our unpublished data also show that INHBA has prognostic significance in terms of patient outcome (N. Cirillo, unpublished observations). The up-regulation of TGF-β2 was also particularly interesting because TGF-β2 has been identified at the tumour-stroma interface (28), stromal regulation of epithelial cell adhesion in the prostate gland occurs in a TGF-β2-dependent manner (29) and the ligand plays a key role in the pathogenesis of a variety of different tumours (30–32).

The role of TGF-β in tumour progression, however, is complex. In the early stages of epithelial tumorigenesis, TGF-β is thought to have a suppressor role by inducing cell cycle arrest and apoptosis whereas in the later stages, when the tumour cells become resistant to growth inhibition by TGF-β, the cytokine acts to promote tumour progression by stimulating invasion, angiogenesis...
and metastases, together with inhibiting immune surveillance (33). In the present study, network analysis identified TGF-β family members as having possible biological relevance to the behaviour of CAFs from OSCC. We extended these observations and showed that both TGF-β1 and TGF-β2 protein expression was up-regulated in CM from GU-OSCC CAFs relative to fibroblasts from GS-OSCC or normal oral fibroblasts. The results demonstrate that overexpression of TGF-β in OSCC is not a ubiquitous characteristic of CAFs as once thought (34) but rather is a specific feature of CAFs in GU-OSCC. The magnitude of TGF-β overexpression could be even bigger in vivo, as suggested by the finding that three-dimensional co-culture of CAFs and cancer cells results in a considerably larger production of TGF-β compared with 2D cultures (35). Previous studies by our group have shown that the majority of human OSCC cell lines remain responsive to TGF-β1 (36) and that overexpression of either TGF-β1 or TGF-β2 in TGF-β-responsive human squamous cell carcinoma lines results in a more aggressive phenotype (37). The results of the present study, therefore, may have clinical implications; it is tempting to speculate that the use of TGF-β inhibitors may be of more therapeutic benefit in GU-OSCC than GS-OSCC.

It is now well established that senescent fibroblasts, generated as a result of replication, oncogenic stress or oxidative DNA damage, induce EMT in breast cancer cell lines (10). In the present study, we show that CM from GU-OSCC CAFs, which are known to be senescent (8), induced EMT in partially transformed keratinocytes. EMT was not induced by the non-senescent GS-OSCC CAFs and was not induced in the immortal HaCaT cell line. The results are in keeping with the previous findings in different cell systems. Interestingly, cells that undergo EMT acquire stem cell-like

![Figure 5](http://carcin.oxfordjournals.org/article-pdf/38/1/82/4053481/82.pdf)
properties such that they gain the capacity to self renew and form secondary tumours at distant sites (38) via TGF-β signalling (39).

Taken together, the results suggest that cancer cell ‘stemness’ is regulated, albeit in part, by the tumour stroma. We believe that this observation raises important clinical issues. The consequences of developing an activated/senescent stroma during healing of a biopsy wound may be the development of EMT and the induction of stem cell-like behaviour in the overlying epithelium. It follows, therefore, that a partially transformed epithelium, as might be seen in a premalignant lesion, would be particularly susceptible to progression after a surgical biopsy. This reasoning argues strongly for the urgent need to develop non-invasive techniques for the assessment of premalignancy.

Desmosome proteins, the main components of keratinocyte intercellular adhesion, are derived from the cadherin, plakin and armadillo gene families; the cadherins are subdivided into desmogleins (DSG1-4) and desmocollins (DSC1-3), the plakins include desmoplakin I/VII and the armadillo proteins consist of plakoglobin (PG) and plakophilins (PKP1-4); armadillo proteins and desmoplakin link desmosomes to the underlying intermediate cytoskeleton. The role of these cell–cell adhesion molecules in cancer is unclear. Low transcript levels of DSG3, DSC2/3, DP, PKP1, for example, have been reported in a large panel of immortal oral dysplasia and carcinoma cell lines (40), but DSG3 appears to be overexpressed in head and neck cancer (41) and is associated with cancer cell migration and invasion (42). Mutations of genes encoding cell adhesion molecules, however, are rare (43-44) suggesting that they may be regulated, at least in part, by the tumour stroma. The results of the present study are consistent with this view because we show that senescent fibroblasts from GU-OSCC down-regulate a broad spectrum of cell adhesion molecules resulting in keratinocyte dis-cohesion.

It is now recognized that there are multiple ways in which stromal fibroblasts influence the behaviour of cancer epithelial cells. CAFs are known to regulate epithelial proliferation (21), disrupt epithelial differentiation (45), alter the metabolism of cancer cells and induce genomic instability (46) and induce EMT (10). In the present study, we show that CAFs from GU-OSCC, but not GS-OSCC, induced EMT in target epithelial cells. Further, we demonstrate down-regulation of a broad spectrum of epithelial cell adhesion molecules in response to CAFs from GU-OSCC leading to epithelial dis-cohesion. There was some overlap in the capacity of TGF-β1 and TGF-β2 to mediate the stromal regulation of epithelial cell adhesion, but this most probably reflects the 70–80% homology of their amino acid sequences (47). The data, combined with our observations that CAFs from GU-OSCC induce keratinocyte invasion in vitro, argue that a primary function of senescent CAFs is to regulate epithelial cell adhesion. This fact that the disruption of epithelial adhesion and promotion of epithelial invasion by CAFs from GU-OSCC occurred in a TGF-β-dependent manner in this study is consistent with previous observations (48,49). More important, the data also agree with the events in vivo, where TGF-β signalling is low in the bulk of primary tumours but is greatest around the tumour margins and blood vessels where it switches cancer cells from a cohesive to single-cell motility (50).

The TGF-β-dependent disruption of epithelial adhesion mediated by CAFs involves the activation of MMPs, including MMP-2 (51). MMP family members are part of the desmosomal

Figure 6. TGF-β acts as an intermediary in fibroblast-epithelial interactions. Western blot analysis demonstrating attenuation of EMT in H357 keratinocytes by GU-OSCC CAFs (H357F) using pan-TGF-β (5 µg/ml), anti-TGF-β1 (0.05 µg/ml) and anti-TGF-β2 (1 µg/ml) antibodies (A). Loss of epithelial cohesion in H357 keratinocytes treated with CM from GU-OSCC CAFs (H357F, BICR82F, BICR18F) using a pan-TGF-β antibody (B), similar effects were demonstrated using anti-TGF-β1 and TGF-β2 antibodies (C). Attenuation of epithelial invasion of H357 keratinocytes treated with CM from GU-OSCC CAFs (H357F, BICR82F, BICR18F) using a pan-TGF-β antibody (D); similar effects were demonstrated using anti-TGF-β1 and TGF-β2 antibodies (E).
References


17. Cirillo, N. et al. (2007) Pemphigus vulgaris immunoglobulin G can recognize a 130 000 MW antigen other than desmoglein 3 on peripheral blood mononuclear cell surface. Immunology, 121, 377–382.


Pathophysiology of the Desmo-Adhesome

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Advances in our understanding of desmosomal diseases have provided a clear demonstration of the key role played by desmosomes in tissue and organ physiology, highlighting the importance of their dynamic and finely regulated structure. In this context, non-desmosomal regulatory molecules have acquired increasing relevance in the study of this organelle resulting in extending the desmosomal interactome, named the “desmo-adhesome.” Spatiotemporal changes in the expression and regulation of the desmo-adhesome underlie a number of genetic, infectious, autoimmune, and malignant conditions. The aim of the present article was to examine the structural and functional relationship of the desmosome, by providing a comprehensive, yet focused overview of the constituents targeted in human disease. The inclusion of the novel regulatory network in the desmo-adhesome pathophysiology opens new avenues to a deeper understanding of desmosomal diseases, potentially unveiling pathogenic mechanisms waiting to be explored.

Desmosomes are intercellular adhesive complexes that have a well-established role in ensuring robust intercellular adhesion in both embryonic and adult tissues subjected to mechanical stress such as the skin and heart. Over 150 years of extensive knowledge has accumulated on intercellular adhesive systems, the desmosome, and its complex structure. The role of the desmosome in human pathophysiology presents the main stimulus for research in this field. Recently more evidence is emerging that desmosomal components are involved in the regulation of cell signaling and the development of cancer (Huber and Petersen, 2015). Therefore, impairment or lack of expression at multiple levels of the desmosomal components can result in diseases of the epithelia, cardiac muscle, and participate in cancer progression. Further, there is an increasing in our understanding of the desmosome as a dense multifunctional component network interacting with regulatory proteins such as kinases (Cirillo and Prime, 2009). This network directly interacts with cellular structural components, and thus broadens the spectrum of targets that may be affected in disease. Impairment or dysfunction of desmosomal structural components directly relates to several conditions, including infectious, autoimmune, genetic, and neoplastic diseases. Table 1 provides a comprehensive summary of these diseases. Exact molecular mechanisms that lead to disease are not completely understood for the majority of these conditions. Further, analysis of molecular networks does not in itself provide enough information about the specific context, such as biological processes and cellular compartments, in which specific pathological processes occur. The wide list of conditions targeting the desmosomal functional unit remains to be fully investigated. This review aims to provide a comprehensive overview of our current understanding of the role of the desmo-adhesome constituents in human disease and associated pathophysiological pathways.

Desmosome Structure and Composition

Desmosomes represent the most prevalent type of adhesive intercellular junction in vertebrate tissues (Marchiando et al., 2010; Kowalczyk and Green, 2013; Khan and Asif, 2015) and are distributed along the cellular membranes. Their integrity, along with their peculiar structural and functional plasticity, contribute to tissue integrity and homeostasis (Fig. 1). More than 6400 papers published to date have defined the major components of the desmosome, although its specific tridimensional architecture remains to be confirmed. At an ultrastructural level, desmosomes appear as multi-layered symmetrical disc-shaped structures of 0.2–0.5 μm in diameter along the cellular membranes of adjacent cells (He et al., 2003).

The classical notion of desmosomes as static adhesive barrier structures has more recently become obsolete. Desmosomes are increasingly being recognized as highly dynamic structures involved in all aspects of epidermal pathophysiology, from tissue homeostasis and morphogenesis, to aging and disease. Desmosomal assembly and disassembly by differential expression and post-synthetic modification of desmosomal components exemplifies both its structural and functional susceptibility. Precision remodeling requires time dependent, bi-directional, signaling mechanisms that requires cross talk with adherens junctions. This is exemplified by the formation of an hyper-adhesive state, that leads to increased difficulty in the dismantling of mature desmosomes compared with those more recently formed. Hyper-adhesion functions to maintain tissue integrity and plays an important role in developmental processes, wound healing, as well as skin diseases (Cirillo, 2016).

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TABLE I. Comprehensive summary of the diseases that target desmosome components

<table>
<thead>
<tr>
<th>Molecular target (molec. weight)</th>
<th>Tissue distribution</th>
<th>Cancer type (regulation)</th>
<th>Autoimmune</th>
<th>Infectious</th>
<th>Genetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmoglein 1 Dsg1 (160 kDa)</td>
<td>Restricted to stratified squamous epithelia</td>
<td>SCC (down); Pancreatic AC (down)</td>
<td>PF, PV</td>
<td>SSSS</td>
<td>SPPK, SAM</td>
</tr>
<tr>
<td>Desmoglein 2 Dsg2 (122 kDa)</td>
<td>All desmosome-bearing epithelia</td>
<td>Familial and gastric (down); Pancreatic AC (down); Melanoma, Prostate, SCC (up)</td>
<td>None known</td>
<td>Respiratory and UT infections (adenoviruses serotypes 3, 7, 11, and 14)</td>
<td>ARVD/C</td>
</tr>
<tr>
<td>Desmoglein 3 Dsg3 (130 kDa)</td>
<td>Restricted to stratified squamous epithelia</td>
<td>SCC (down); Oral SCC (down); Head and neck (up)</td>
<td>PV, PNP, IAP (i-e)</td>
<td>None known</td>
<td>None known</td>
</tr>
<tr>
<td>Desmoglein 4 Dsg4 (14 kDa)</td>
<td>Highly differentiated layers of the epidermis; Hair follicle epithelia, salivary gland, testis, prostate, and skin</td>
<td>Cross-reactivity of anti-DSG1</td>
<td>IgG</td>
<td>None</td>
<td>Localized autosomal recessive hypotrichosis; Recessive monilethrix</td>
</tr>
<tr>
<td>Desmocollin 1 Dsc1 (100 kDa)</td>
<td>Stratified epithelia, hair follicle epithelia</td>
<td>Anal SCC (down); Lung (down)</td>
<td>IAP (i-c) PV</td>
<td>None known</td>
<td>None known</td>
</tr>
<tr>
<td>Desmocollin 2 Dsc2 (120 kDa)</td>
<td>Simple and stratified epithelia, myocardium</td>
<td>Pancreatic ductal AC (down); Oral SCC (down); Lung (down)</td>
<td>PF (Brazilian endemic patients) PV</td>
<td>Giardiasis</td>
<td>ARVD/C;</td>
</tr>
<tr>
<td>Desmocollin 3 Dsc3 (105 kDa)</td>
<td>All desmosome-bearing epithelia</td>
<td>Oral SCC, Prostate (down); SCC (abnormal); Prostate AC (down);</td>
<td>PV, PNP</td>
<td>Bacillus anthracis</td>
<td>Naxos disease Lethal congenital epidermolysis bullosa</td>
</tr>
<tr>
<td>Plakoglobin Pg (86 kDa)</td>
<td>Suprabasal cells of the epidermis</td>
<td>Oral SCC (down); Gastro (down)</td>
<td>PNP</td>
<td>None known</td>
<td>ARVD/C, Brugada syndrome</td>
</tr>
<tr>
<td>Plakoglobin 1 Pplp (75 kDa)</td>
<td>Stratified epithelia</td>
<td>Lung (down)</td>
<td>PF (Brazilian endemic ps)</td>
<td>Giardiasis</td>
<td>None known</td>
</tr>
<tr>
<td>Plakoglobin 2 Pplp 93–97 kDa</td>
<td>Stratified epithelia</td>
<td>Lung (down)</td>
<td>PNP</td>
<td>None known</td>
<td>ARVD/C, Brugada syndrome</td>
</tr>
<tr>
<td>Plakoglobin 3 Pplp (87 kDa)</td>
<td>Stratified epithelia</td>
<td>Lung, Prostate AC, Breast (up)</td>
<td>PNP</td>
<td>None known</td>
<td>None known</td>
</tr>
<tr>
<td>P0071 Plakoglobin 4 (Pkp4) (134 kDa)</td>
<td>Stratified epithelia</td>
<td>Oropharynx SCC</td>
<td>None</td>
<td>None</td>
<td>None known</td>
</tr>
<tr>
<td>Desmoplakin 1 Dsp1 (220 kDa)</td>
<td>All desmosome-bearing epithelia, myocardium</td>
<td>Lung (down),</td>
<td>PNP</td>
<td>&amp; anthracis</td>
<td>Carval syndrome</td>
</tr>
<tr>
<td>Desmoplakin 2 Dsp2 (220 kDa)</td>
<td>Stratified epithelia and myocardium</td>
<td>SCC (abnormal)</td>
<td>EM, SJS, PNP</td>
<td>None known</td>
<td>SAM Naxos-like disease SPPK ARVD/C</td>
</tr>
<tr>
<td>Envolakin EVP (210 kDa)</td>
<td>Stratified epithelia and myocardium</td>
<td>None known</td>
<td>PF, PNP</td>
<td>None known</td>
<td>None known</td>
</tr>
<tr>
<td>Periplakin PPL (195 kDa)</td>
<td>Basal surface of basal keratinocytes (hemidesmosomes); In striated muscle, (as a component of the z-line)</td>
<td>Eosphageal SCC (down)</td>
<td>None known</td>
<td>None known</td>
<td>None known</td>
</tr>
<tr>
<td>Plectin (500 kDa)</td>
<td>None known</td>
<td>None known</td>
<td>PNP,</td>
<td>None known</td>
<td>None known</td>
</tr>
</tbody>
</table>

AC, Adenocarcinoma; ARVD/C, Arrhythmogenic right ventricular dysplasia/cardiomyopathy; EDSFS, Ectodermal dysplasia–Skin fragility syndrome; EM, erythema multiforme; IAP (i-e), IgA pemphigus (intra-epidermal neutrophilic dermatosis); IAP (i-c), IgA pemphigus (subepidermal bullous dermatosis); LA-EB Lethal epidermolysis bullosa; LDAC left dominant arrhythmogenic cardiomyopathy MC, Mucocutaneous type; MD, Mucosal dominant type; PF, Pemphigus foliaceus; PG, Plakoglobin; PNP, paraneoplastic pemphigus; PPK, palmoplantar keratodermia; PV, pemphigus vulgaris; SAP, Skin dermatitis multiple allergies and metabolic wasting syndrome; SCC, squamous cell carcinoma; SJS, Stevens-Johnson syndrome; SPPK, striate palmoplantar keratodermia; SSSS, staphylococcal scalded skin syndrome.

Focusing on its composition, the structure of the desmosomes can be classified according to the location of its main constituents into firstly extracellular and intracellular compartments, as well as a desmosome-associated regulatory network.

The extracellular compartment

The extracellular portion of the desmosome contains the desmosomal cadherins desmoglein (Dsg) and desmocollin (Dsc) that form strong extracellular homo/heterophilic bonds and mediate adhesion (North et al., 1999; Al-Amoudi et al., 2011). Desmosomal cadherins are transmembrane molecules that adhere at their extracellular N-terminal domains. They exist in at least four different isoforms of Dsg (Dsg1–Dsg4) and three isoforms of Dsc’s (Dsc 1–3) (Dusek et al., 2007; Amagai, 2010), and are expressed in a tissue-specific and differentiation-dependent manner (Table I) (Collins et al., 1991; King et al., 1997; Bazzi et al., 2006). At the intracellular level, their extremities interact with plakoglobin (Pg) and plakophilins (Pkp) (Bass-Zubek et al., 2009), which in turn bind to the desmoplakin (Dp) a plakin...
family protein, whose function is to anchor keratins and stabilize desmosomes (Vasioukhin et al., 2001). The seven genes for human desmosomal cadherins are clustered on chromosome 18q12.1 (Nollet et al., 2000; Whittock and Bower, 2003). Dsg’s and Dsc’s show highly homologous structures with a strong similarity to the classical prototype of cadherins, the E-cadherin. The processed extracellular domain of desmosomal cadherins is composed of five extracellular cadherin (EC1–5)-repeat domains with three Ca2⁺-binding sites in between these EC repeats. The fifth and more divergent membrane proximal repeat (EC5) sometimes is also referred to as the extracellular anchor domain.

The transmembrane section of the desmosome simply contains as in the transmembrane protein domain that links the extracellular domain with a cytoplasmic tail. This cytoplasmic tail connects a specific set of desmosomal plaque proteins that provide organization, association with IFs, and have a role in signaling.

The intracellular compartment

The major difference between Dsg’s and Dsc’s resides in their cytoplasmic domains. A membrane proximal to the intracellular anchor domain and an intracellular cadherin segment (ICS) domain are common to both Dsg’s and Dsc’s (except for the short Dscb splice variants). Dsg intracellular domains are extended by an intracellular proline-rich linker region, a repeated unit domain containing different numbers of 29 ± 1 amino acid repeats, and a glycine-rich Dsg-terminal domain (DTD) at the C terminus.

The cytoplasmic portion is well recognizable in electron microscopy as an electron-dense cytoplasmic plaque of multiple proteins lining the membrane known as the “desmosomal plaque.” This plaque is important for the correct assembly and organization of desmosomes, for association with intermediate filaments, and for cell signaling. Besides the intracellular portion of the desmosomal cadherins, the main proteins involved in the formation of the desmosome plaque belong to two families, the armadillo-repeat protein family and the plakin protein family. The armadillo-repeat protein family include plakoglobin (Pg), three plakophilin proteins (Pkp1–3), and a multifunctioning protein known as either p0071 or Pkp4. The plakin protein family consists of desmoplakin (Dsp), plectin, envoplakin (EVP), and periplakin (PPL) (Coates, 2003; Hatzfeld, 2007; Sonnenberg and Liem, 2007).

Plakoglobin, also found to be an important component of the adherens junctions (AJs), is involved in the tridimensional physical link of other plaque proteins and is essential for cross talk between desmosomes and AJs in the regulation of cell-to-cell adhesion (Cowin et al., 1986; Bonné et al., 2003). The Pkp’s, instead, take part in the lateral binding of Dsp and desmosomal cadherins (Kowalczyk et al., 1999). Lastly, the members of the plakin protein family such as Dsp, EVP, and PPL are cytolinker multidomain proteins that complete the assembly between cytoskeletal elements (intermediate filaments) and the junctional complexes (desmosomal cadherine—Pg complex) (Leung et al., 2002).

A schematic representation of desmosomal structure and accessory components is outlined in Figure 2. Correct desmosome assembly with its numerous components, remodeling, and integrity of cross talk network, are essential for tissue integrity.

Fig. 1. Desmosomes are particularly abundant in tissues subject to high mechanical stress such as stratified squamous epithelia (A). Desmosomal molecules localise at the cell-cell border and their presence may be revealed by several techniques, including IF (B). Panel B depicts the classical IF staining pattern of sera from patients with PV, which target desmosomal adhesion molecules. (C) Schematic representation of a desmosome and its correspondent EM image (D) highlighting desmoglea and outer/inner dense plaques.
Desmosome-associated regulatory network

The sophisticated construction of the desmosomal functional unit requires a finely regulated network composed of intrinsic nodes/proteins and accessory proteins such as kinases, phosphatases, and caspases. These accessory proteins must interact directly with the intrinsic nodes/proteins, without any intermediary interactions, so as to be defined as an integral component of the desmosome-associated regulatory network (Paris and Bazzoni, 2008). Regulatory molecules play key roles in the homeostasis of cell adhesion structures, including desmosomes. To fully elucidate the mechanisms that regulate the dynamics of keratinocyte adhesion an understanding of desmosomal structure and function is inadequate without being augmented by a complete understanding of the components of the regulatory network and their responses to perturbations.

The molecular architecture, structure, and signaling of the desmo-adhesome at multiple levels was previously outlined in 2009 by our group through a process of data-mining, database-derived information as well as published studies (Cirillo and Prime, 2009). At that time, we were able to define the functional role and regulation of certain components of the network, distinguishing between directional (either activating or inhibiting binding interactions) and non-directional interactions (Fig. 2) (binding interactions). The desmo-adhesome was found to be formed by a total of 59 proteins (30 intrinsic and 29 accessory components) linked by 128 direct interactions, including membrane and adaptor proteins, enzymes, kinases, phosphatases, and caspases, to form the regulatory subnets (Cirillo, 2014).

Many of these molecules, such as PKC, Src, MAPK, epidermal growth factor receptor (EGFR), and caspase 2/3, are now known to be involved in the assembly and disassembly of desmosomes (North et al., 1999; Green and Simpson, 2007; Kowalczyk and Green, 2013; Kitajima, 2014). It is interesting to note that Pg, one of the main organizers of the desmosome that is crucial for adhesiveness, was highly connected to both protein kinases (n = 5) and phosphatases (n = 4), and this provided an in silico demonstration that the dynamic regulation of the desmosome may be orchestrated by this protein (Cirillo and Prime, 2009). Furthermore, now that the existence of molecular cross talk between adherens junctions and desmosome has been demonstrated, the desmosome-associated regulatory network should be regarded as a constituent part of the desmosome.

Desmosome Diseases

It is rare for single components of the different desmosomal compartments to be involved in one disease process. Conditions typically affect either the extracellular portion (essentially the desmosomal cadherins), the desmosomal
plaque proteins or accessory proteins that arrange the regulatory network. We present description of the diseases categorized by compartmental target.

Diseases targeting the extracellular portion

Autoimmune. Of diseases targeting the extracellular portion, subtypes of the Pemphigus family, particularly the more common Pemphigus Vulgaris, present primary examples. This family of autoimmune conditions is characterized by circulating autoantibodies that target desmosomal components impairing keratinocytes adhesion resulting in acantholysis (Amagai, 2010; Kneisel and Hertl, 2011a). The autoantibodies detected in pemphigus patients may be directed against Dsg1 and/or Dsg3, and can include a wide variety of desmosomal, non-desmosomal, and non-epithelial IgGs (Eyre and Stanley, 1988; Nguyen et al., 2000; Cirillo et al., 2007). These diseases are considered relatively rare with an overall incidence of 1–16 new cases per million people per year (Ruocco et al., 2013; Kitajima, 2014). The age of onset is usually in the fifth or sixth decade of life, although a younger age has been observed in endemic variants of the disease (Rocha-Alvarez et al., 2007, Joly and Litrowski, 2011). Despite the precise pathomechanism of pemphigus remaining elusive, recent studies have indicated that desmosome disassembly involves not only direct inhibition of desmosome formation by IgGs, but also desmosome remodeling. Additionally, several intracellular signaling pathways are possibly triggered by non-desmosomal molecules (Green and Simpson, 2007). The signaling-related events include apoptosis as well as modulation of P6, p38MAPK, heat shock protein 27, cdk2, Src, RhoA, and others (Cirillo, 2016).

The subtypes of pemphigus can be classified in several way, either by the location of acantholysis (i.e., suprabasilar or superficial) or by Ig class and autoantibody targets. Also from the main subtypes, specific clinical, microscopic, and immunopathological features of less common variants including IgG/IgA pemphigus and pemphigus herpetiformis have been described.

The main pemphigus subtypes affecting the extracellular compartment of the desmosome are pemphigus vulgaris (PV), and IgA pemphigus (IAP). PV is the most common subtype attributing for 70% of cases and found mainly in middle-aged and elderly patients, with a female predisposition. Familial cases are rare although a higher incidence has been found in Ashkenazi Jewish and Japanese populations (Joly and Litrowski, 2011). Histologically, PV is characterized by an intra-epidermal cleft between the basal and spinous layers (suprabasal acantholysis) that correspond clinically to painful mucosal lesions and skin blister formation. Autoantibodies primarily target Dsg3 results in a mucosal dominant (MD) variant of PV that is limited to the oral cavity, whereas, when these target both Dsg1 and Dsg3 this leads to the mucocutaneous form (MC), characterized by skin blisters in addition to oral erosions (Sharma et al., 2007; Amagai, 2010). In addition autoimmune in PV target Dsc1, Dsc2 (Dmochowski et al., 1993; Hashimoto et al., 1995), and Dsc3 (Spindler et al., 2009; Mao et al., 2010; Rafei et al., 2011). IgA pemphigus is a further subtype characterized by the presence of circulating IgA autoantibodies that can target desmosomal and non-desmosomal keratinocyte cell surface components. IAP in turn is divided into two subtypes: subcorneal pustular dermatosis (s-c) in which Dsc1 is the target antigen; and intra-epidermal neutrophilic dermatosis (i-e) in which the targets are Dsg1 and Dsg3 (Kneisel and Hertl, 2011b). Recently, a comparative clinico-pathological study described an IgG/IgA pemphigus that is a further overlapping variant between classic IgG pemphigus and IAP, and may be best regarded as a variant of IgG pemphigus as it is distinct from IgA pemphigus (Toosi et al., 2016). Histologically, IAP is characterized by acantholysis and extensive neutrophilic infiltration within the epidermis (Tsuruta et al., 2011). Reports of patients with IAP are sparse, with only about 60 cases described. Diagnosis of this form is considered comparable to classic forms of pemphigus.

Infectious. Several infectious can affect the extracellular compartment of the desmosome:

- **Bullous impetigo** (BI) is a common, highly contagious cutaneous infection that primarily affects children and is caused by *Staphylococcus aureus* through the release of exfoliative toxin (ET). ET is a serine protease that cleaves the extracellular domain of Dsg1 with high specificity, and can be sub-categorized into ETA, ETB, ETC, and ETD, the most common form being ETA (Wiley and Rogolsky, 1977; Sato et al., 1994; Yamaguchi et al., 2002). Dsg1 cleavage by ET results in intra-epidermal cleavage similar to granular layer blistering caused by IgG in Pemphigus Foliacieus (PF) (Amagai et al., 2000).

- **Staphylococcal scalded skin syndrome (SSSS)**, also known as Ritter disease, is a cutaneous infectious disease that commonly affects children under the age of six, particularly neonates, although it may also affect immunocompromised adults (Cribier et al., 1994; Amagai, 2010). SSSS is considered to be a more generalized and severe form of BI (Mockenhaupt et al., 2005). Presentation may range from blisters and severe localized exfoliation at the site of the infection, to involvement of over 90% of body surface area. The latter presentation is due to systemic ETs present in the circulation that cleave desmosomal cadherins, specifically Dsg1, in superficial epidermal sites (Ladhani and Evans, 1998; Handler and Schwartz, 2014). Mucous membranes, including those of the oral cavity and pharynx, are rarely involved. The lips may be involved, especially in the later stages of secondary desquamation, and are characteristically flaky and fissured (Ossoff and Giunta, 1975). Commonly, therapy for both BI and SSSS is based on the use of topically or orally administered antibiotics, with the addition of symptomatic treatment in severe SSSS cases (Bernard, 2008; Hartman-Adams et al., 2014). Mortality rates in neonates and children remain low, but may be over 60% in immunocompromised adults (Patel, 2004).

- Respiratory and urinary tract infections caused by *adenovirusserotypes 3, 7, 11, and 14*, may target Dsg2 (Amagai and Stanley, 2012). Adenovirus 3, for example, can result in disruption of intercellular junctions via interaction with Dsg2 (Wang et al., 2015).

- **Giardiasis**, is an infectious disease characterized by acute or chronic diarrhoea, dehydration, abdominal discomfort, and weight loss. It is caused by *Giardia duodenalis*, a flagellated unicellular eukaryotic protozoan that targets Dsc2 and/or Dsc3. It disrupts the arrangement of tight, adherens, and desmosomal junctions of intestinal cells (Adam, 2001; Maia Brigagio et al., 2012).

Genetic. There are genetic diseases that affect extracellular desmosomal components:

- **Localized autosomal recessive hypotrichosis** is a disease that affects the scalp, chest, arms, and legs and is caused by several mutations in the Dsg4 gene (Moss et al., 2004). Histological features of the scalp skin include thin and atrophic hair follicles and hair shafts that often coil up within the skin (Kljucic et al., 2003a).

- **Recessive monilethrix**, an autosomal dominant human hair disorder, is caused by mutations in three type II hair cortex keratins. When linked to Dsg4 mutations, there is overlap
with localized autosomal recessive hypotrichosis (Schaffer et al., 2006; Schweizer, 2006; Wang et al., 2015b).

- **Woolly hair** is a rare, autosomal recessive abnormality of structure of scalp hair characterized by markedly coiled hair in Caucasians. It has been associated with alterations of Dsc2, but is also a clinical feature shared syndromically with palmoplantar keratoderma and heart anomalies by diseases targeting intracellular components of the desmosome.

### Diseases targeting the desmosomal plaque

#### Autoimmune

Among this class fall two of the main pemphigus subtypes, *Pemphigus vulgaris* (PV) and *Paraneoplastic Pemphigus* (PPN) that can target either extracellular portion of the desmosomal cadherins or the desmosomal plaque constituents.

- PF variant represents about 20–30% of pemphigus cases and is characterized by superficial epidermal blisters without mucosal involvement. The blistering results from autoantibodies directed against Dsg1 and is restricted to the granular layer of the epidermis. Further desmosomal targets, such as Dsc1, Dsc2, Dsc3, Pg, Dsp, and Pkp3, have been described (Korman et al., 1989; Dmochowski et al., 1993; Zhou et al., 1998; Mimouni et al., 2004; Spindler et al., 2009). PF occurs also in an endemic forms (known as “fogo selvagem”) in some regions of Brazil, Columbia, and Tunisia (Joly and Litrowski, 2011).

- PNP is a rare form of pemphigus that typically affects patients diagnosed with malignancies such as non-Hodgkin’s lymphoma and chronic lymphocytic leukaemia (Allen and Camisa, 2000; Yong and Tey, 2013). This variant is the most severe among pemphigus subtypes and shows a distinctive set of clinical features that include a typically polymorphic skin rash, severe mucosal involvement, life-threatening bronchiolitis, and unusual histopathological and immunological findings (Anhalt et al., 1990; Anhalt, 2004; Hata et al., 2013). Patients presenting with immunological profile suggestive of PNP and without evidence of malignancy require close follow-up for the possible subsequent development of cancer (Sprecher, 2015). Cell-mediated immunity plays a key role in PNP, as demonstrated by the presence of keratinocyte necrosis and inflammatory cells within the epidermis (Yong and Tey, 2013). The age of onset is usually 45–70 years, although children and adolescents may be affected. The desmosome proteins targeted by autoantibodies in PNP include Dsg’s (particularly the Dsg3), Dsc’s, Dsp, and plakophilins (Stahley and Kowalczyk, 2015), as well as EVP, PPL, and a number of less well-specified antigens, such as alpha-2-macroglobulin-like-1 (Scheppen et al., 2010). Clinically, PNP is characterized by extremely painful and refractory oral lesions (Amagai, 2010), potentially followed by full body cutaneous involvement. Unlike other pemphigus subtypes, PNP can show the involvement of palms and/or soles (Allen and Camisa, 2000), conjunctiva, and simple squamous epithelia (Amagai, 2010). The prognosis of PNP remains poor, with mortality rates reaching 90% (Yong and Tey, 2013).

#### Infectious

Desmosomal plaque disruption and anomalies have been linked to the presence of many agents, *Bacillus anthracis*. Dsp and Pg of alveolar epithelial cells are targeted by *B. anthracis lethal toxin* (BA-LT), with a consequent impaired desmosome assembly (Langer et al., 2012).

#### Genetic

The desmosomal intracellular compartment has shown to be targeted by a variety of genetic diseases.

- To date more than ten mutations in the Dsg1, Pkp1, and Dsp genes are known to cause Palmpoplantar Keratoderma in its rare form (PFPPK) (SJS). Dsc2, but is also a clinical feature shared syndromically with palmoplantar keratoderma and heart anomalies by diseases targeting intracellular components of the desmosome.

- In SJS, Dsg3 and Dsc3 are targeted by autoantibodies in EM patients (Foedinger et al., 1995; Johnson et al., 1999; Fukuwate et al., 2007; Cozzani et al., 2011; Ellis and Sidhu, 2014). So far, the pathogenetic significance of the circulating Dsp autoantibodies in EM and SJS has not been elucidated. Whether there exists a subset of EM and SJS patients with autoimmune disease or if instead these circulating anti-Dsp antibodies are a manifestation of epitope spreading, remains to be answered.

#### Paraneoplastic

- PNP is a rare form of pemphigus that typically affects patients diagnosed with malignancies such as non-Hodgkin’s lymphoma and chronic lymphocytic leukaemia (Allen and Camisa, 2000; Yong and Tey, 2013). This variant is the most severe among pemphigus subtypes and shows a distinctive set of clinical features that include a typically polymorphic skin rash, severe mucosal involvement, life-threatening bronchiolitis, and unusual histopathological and immunological findings (Anhalt et al., 1990; Anhalt, 2004; Hata et al., 2013). Patients presenting with immunological profile suggestive of PNP and without evidence of malignancy require close follow-up for the possible subsequent development of cancer (Sprecher, 2015). Cell-mediated immunity plays a key role in PNP, as demonstrated by the presence of keratinocyte necrosis and inflammatory cells within the epidermis (Yong and Tey, 2013). The age of onset is usually 45–70 years, although children and adolescents may be affected. The desmosome proteins targeted by autoantibodies in PNP include Dsg’s (particularly the Dsg3), Dsc’s, Dsp, and plakophilins (Stahley and Kowalczyk, 2015), as well as EVP, PPL, and a number of less well-specified antigens, such as alpha-2-macroglobulin-like-1 (Scheppen et al., 2010). Clinically, PNP is characterized by extremely painful and refractory oral lesions (Amagai, 2010), potentially followed by full body cutaneous involvement. Unlike other pemphigus subtypes, PNP can show the involvement of palms and/or soles (Allen and Camisa, 2000), conjunctiva, and simple squamous epithelia (Amagai, 2010). The prognosis of PNP remains poor, with mortality rates reaching 90% (Yong and Tey, 2013).

#### Erythema multiforme

Erythema multiforme (EM) and Stevens-Johnson syndrome (SJS). EM and SJS are two acute immune-mediated disorders that can be triggered by numerous factors. Several reports described circulating autoantibodies to Dsp1 and Dsp2 in EM patients (Opez-Ayala et al., 2014).

#### Arrhythmogenic right ventricular dysplasia/cardio-myopathy

- **Arrhythmogenic right ventricular dysplasia/cardio-myopathy** (ARVD/C) is also considered a disease of the desmosome with several mutations involving Dsg2, Dsc2, Pg, Dsp2, and Pkp2 genes (Awad et al., 2006; Nagaoka et al., 2006; Syrris et al., 2006). ARVD/C is an autosomal dominant primary myocardial disorder characterized by myocardial and fibro-fatty replacement of cardiac myocytes, ventricular arrhythmias, sudden cardiac death, and end-stage heart failure, typically affecting the right ventricle. Dsg2 and Dsc2 are known to be expressed in cardiac myocytes and are the main candidate genes for this disorder (Pilichou et al., 2006; Syrris et al., 2007). ARVD/C was thought to primarily affect the right ventricle, however, in some cases the disease can affect the left ventricle (Left dominant arrhythmogenic cardiomyopathy) and truncating mutations in desmplakin are consistently associated with these aggressive phenotypes (Lopez-Ayala et al., 2014).

- **Woolly hair** is a clinical feature shared syndromically with palmoplantar keratoderma and heart anomalies by diseases such as:...
- **Naxos disease**, if Pg is targeted, which is an autosomal recessive, inherited, cardiocutaneous disorder, characterized by ARVC, woolly hair, and PPK.

- **Carvajal syndrome**, if Dp is targeted, which is characterized by PPK, curly hair, dilated cardiomyopathy, especially on the left ventricle side, and early morbidity.

- **Ectodermal dysplasia–skin fragility syndrome (EDDS)** is an autosomal recessive dermatosis that has been associated with at least eleven different recessively inherited mutations in the Pkpl gene. It is characterized by skin fragility (with trauma-induced erosions and blistering), non-cicatrival alopecia, palmoplantar keratoderma (PPK), onichodystrophy, and in some cases hypohidrosis (McGrath et al., 1999; Hernández-Martin et al., 2013).

- **Lethal acantholytic epidermolysis bullosa** is a rare genetic disease that has also been identified as the result of heterozygosity of two loci containing the C terminus of Dsp and laminin 5, respectively. The absence of a single copy of any of these genes is lethal, thus lacking the entire domain that binds the intermediate filaments. Patients present with cardiomyopathy, severe fragility of the skin and mucous membranes leading to severe mucocutaneous erosions and marked fluid loss, epidermal dislodgment, complete alopecia, neonatal teeth, and nail loss. The prognosis is poor with frequent early demise (Jönkman et al., 2005).

- **Lethal congenital epidermolysis bullosa** is a recently described phenotype of the epidermolysis bullosa disease group. It is caused by a homozygous nonsense Junction Plakoglobin (JUP) mutation that leads to the lack of Pg. Patients suffer from severe congenital skin fragility with generalized epidermolysis and massive transcutaneous fluid loss, but no apparent cardiac dysfunction (Pigors et al., 2011).

- **Brugada syndrome** is an inherited rare disease caused by disturbed function of ion channel subunits or the proteins that regulate them (channelopathies), and is associated with sudden cardiac death at rest, mainly in men in the third–fourth decade of life. This disease has been linked to 18 different gene mutations, with the gene encoding the sodium voltage-gated channel alpha subunit 5, SCN5A gene, represented in the majority. One of the most recently identified genes was PKP2 encoding the protein plakophilin-2. The absence and/or alteration of plakophilin-2 structure in the cardiac desmosomes impair myocyte interactions, inducing myocardium disruption, particularly in response to mechanical stress (Campuzano et al., 2016).

### Diseases targeting the regulatory network

The desmosomal network of accessory proteins is extensive and also populated by numerous protein–protein interactions that play a functional, rather than just a structural role (Cirillo and Prime, 2009). Unlike structural desmosomal proteins, these regulatory proteins are ubiquitous and are therefore associated with diseases of virtually any human tissue. Nevertheless, several examples exist of perturbations of functional nodes that result in phenotypes similar to those seen in typical desmosomal diseases.

The use of a mouse model can be a valuable tool to approach the study of accessory components targeted in disease and as such, phenotypic effects of gene deletions in mice have been reviewed.

Given the central role that desmosomal accessory proteins play, a single deletion of any of specific genes has been associated with marked phenotypic alterations. For example, embryonic/perinatal lethality was found to be associated with gene deletion of AKT1, MAPK1, and CASP2 (Bergeron et al., 1998; Hatano et al., 2003); and postnatal lethality was associated with FYN gene deletion (Yagi et al., 1993).

Gene deletion of Src, EGFR, PRKACA, ERBB2, CASP3 demonstrated both embryonic/perinatal and postnatal lethality. Furthermore, phenotype alterations of the cardiovascular system were associated with deletion of EGFR, PRKCA, ERBB2, CASP3, and MAPK1; digestive/ alimentary phenotype observed with EGFR, ERBB2, CASP3, and MUC1 deletion; and skin, coat, and nails altered in gene deletion of Src, EGFR, PRKACA, and FYN (Soriano et al., 1991; Yagi et al., 1993; Lee et al., 1995; Miettinen et al., 1995; Sibilla and Wagner, 1995; Spicer et al., 1995; Threadgill et al., 1995; Kuida et al., 1996; Erickson et al., 1997; Woo et al., 1998; Dietrich et al., 2000; Chan et al., 2002; Crone et al., 2002; Leitges et al., 2002; Skilhegg et al., 2002; Hatano et al., 2003; Braz et al., 2004; Nolan et al., 2004; Hara et al., 2005; Natarajan et al., 2007).

Further, single perturbations in humans (e.g., deletion) of one accessory protein of the network lead to severe clinical findings. AKT1 mutations have been associated with Proteus syndrome, a very rare and non-inherited condition characterized by progressive, segmental, or patchy overgrowth of diverse tissues of all germ layers, most commonly affecting the skeleton, skin, and adipose and central nervous systems. This syndrome is known to be caused by a single, post-zygotic, somatic mosaic mutation in AKT1 and it is hypothesized that a non-somatic mosaic mutation would be lethal in early development (Lindhurst et al., 2011). Moreover, Cowden syndrome 6 has been shown to be caused by heterozygous mutation in the AKT1 gene (164/70) on chromosome 14q32.3 (Orloff et al., 2013).

Homozygosity from a missense mutation in the EGFR gene has been found in neonatal inflammatory skin and bowel disease-2. The clinical manifestations of this disease was first described by Campbell et al. (2014) and were numerous, from widespread erosions affecting the trunk and limbs, to development of papules and pustules, with frequent S. aureus infections of the skin. Recurrent diarrhea, respiratory difficulties, recurrent bronchiolitis, pulmonary infection, developmental failure, trichomegaly, hypertension, and bilateral renal enlargement were also observed.

Ventricular and atrial septal defects, hypertrophic cardiomyopathy, hypsarhythmia, severe developmental delay, and more clinical features have been associated with specific deletions in the YWHAH gene that encodes for 14-3-3 protein gamma, an accessory protein of the desmosome-associated regulatory network (Kamoiike et al., 2010; Ramocki et al., 2010; Rothlisberger et al., 2010).

Choanal atresia is a rare congenital disorder where the choana (posterior part of the nasal passage) is blocked during development. This has been associated with lymphoedema due to mutation in the protein-tyrosine phosphatase, non-receptor-type, 14 gene (Au et al., 2010).

In summary, although the desmosomal regulatory proteins are pleiotropic and their mutations affect virtually all tissues, alteration of the regulatory network can lead to clinical manifestations resembling desmosomal disease.

### Desmosome and cancer

Perturbation of the complex integrated signaling network of the desmosome has also been strongly linked to carcinogenesis. Late in the last century, first evidence of this link was shown of the role of desmosomes in the suppression of tumour spread (Tselips et al., 1998). The loss of intercellular adhesion, dependent on adhesive intercellular junctional integrity, could be directly related to invasion. Subsequent data evaluating desmosomal components expression in several forms of cancer have been far from easy to interpret. For instance, expression of desmosomal cadherins was
found to decrease in many tumours, such as skin (Chen et al., 2011), head, and neck (Chen et al., 2007), breast (Klus et al., 2001; Oshiro et al., 2005), prostate (Barber et al., 2014), cervix (Alzawi et al., 2003), etc. Paradoxically, the expression of desmocollins cadherin has been reported to increase in the same cancer (Huber and Petersen, 2015).

Nevertheless, dysregulation of desmosomal components frequently correlates with cancer aggression and poor prognosis. Is this the case of renal cell carcinoma (Breault et al., 2005), prostate cancer (Barber et al., 2014; Pan et al., 2014), endometrial carcinoma (Nei et al., 1996), head and neck cancer (Chen et al., 2007; Wong et al., 2008), lung cancer (Savci-Heijink et al., 2009), colorectal cancer (Pan et al., 2014), esophageal adenocarcinoma (Wang et al., 2014), and not least OSSC (Xin et al., 2014).

Interestingly, cell cycle control and triggers for apoptosis by desmosomal cadherin (Cirillo, 2016). To date, several mechanisms by which alteration of desmosomal cadherin expression can occur in cancer have been identified such as transcriptional regulation of desmosomal cadherins, impaired transport, targeting, and assembly into mature desmosomes, as well as, inactivation by proteolytic cleavage. We refer to a several recent comprehensive reviews on this topic (Huber and Petersen, 2015; Shahley and Kowalczyk, 2015), and provide more detail in Table 1.

Concluding Remarks

The desmo-adhesive functional unit offers a valuable model of how basic, translational, and clinical research merge to forward our understanding of human disease. The study of desmosome pathophysiology has salient clinical implications and resulted in the development of novel diagnostic techniques and therapeutic approaches to a number of autoimmune and genetic conditions (Schmidt and Zillikens, 2010; Kalantari-Dahgahi et al., 2013; Otten et al., 2014). This study also has suggested that typical desmosomal disease analysis needs to be extended to the regulatory network compartment that is likely significantly enhance the understanding of desmosomal pathophysiology. The expectation is that an increasingly supportive systems biology and experimental research model could be used to develop novel disease mechanisms that will ultimately be targeted for patients’ benefit.

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INVITED CONCISE REVIEW

Desmosomes in disease: a guide for clinicians

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The large number of diseases occurring when desmosome constituents are impaired provides striking evidence for the key role of desmosomes in maintaining tissue integrity. A detailed understanding of the molecular alterations causing desmosomal dysfunction has, in turn, underpinned the development of novel diagnostic tools. This has salient clinical implications for dentists and oral medicine practitioners because the majority of desmosomal diseases affect the oral cavity. In the present article, we review the autoimmune, infectious, genetic, and neoplastic diseases that target the desmosome, with particular emphasis on clinical manifestations, diagnostic pathways, and relevant laboratory investigations.


Keywords: desmosome; pemphigus; blistering diseases

Giulio Bizzozero, an Italian pathologist, was the first to sketch and describe what would later be called ‘desmosome’ (Bizzozero, 1864). One hundred and fifty years later, vast knowledge has accumulated on the complex biochemical structure and role of the desmosome in health and disease. To date, approximately 30 different conditions including infectious, autoimmune, genetic, and neoplastic diseases are known to involve desmosomal dysfunction. This review presents an update on desmosome pathophysiology, particularly for clinicians involved in the diagnosis of mucocutaneous diseases.

Molecular composition and ultrastructure of the desmosome: the basics

Desmosomes, along with the adherens junctions (AJs), gap junctions, and tight junctions (TJs), are the predominant type of adhesive intercellular junctions in vertebrate tissues, particularly those requiring strength, extensibility, and elasticity such as epithelium. It is now well established that desmosomes are dynamic structures with unique plasticity. Their ability to switch between hyperadhesive and unordered states is essential to maintain tissue integrity and homeostasis.

At the ultrastructural level, desmosomes appear as multilayered symmetrical disk-shaped structures of 0.2–0.5 µm in diameter found between cellular membranes of adjacent cells. These organelles link keratin intermediate filaments (KIFs) to the plasma membrane region and morphologically consist of a central electron-dense midline between two plasma membranes (desmoglea) and intracellular dense plaques, that is, the outer and inner dense plaques. The extracellular portion forming the desmoglea contains desmosomal cadherins desmogleins (Dsg1-4) and desmocollins (Dsc1-3). These calcium-dependent proteins are expressed in a tissue-specific and differentiation-dependent manner and mediate adhesion by forming strong extracellular homo-/heterophilic bonds. At the intracellular level, their extremities interact with plakoglobin (Pg) and plakophilins (Pkps) (Bass-Zubek et al, 2009), thus forming the outer dense plaque. These molecules bind to components of the inner dense plaque, namely desmoplakin (Dp), plectin, envoplakin (EVP), and periplakin (PPL), whose function is to anchor KIFs and stabilize desmosomes (Vasioukhin et al, 2001). In addition to this structural role, components of desmosomal plaques are also important for cell signaling and interaction with regulatory molecules such as kinases and proteases. This finely regulated network of intrinsic and accessory molecules constitutes the desmosomal functional unit, or desmo-adhesome (Cirillo and Prime, 2009).

To date, almost 30 different diseases (Table 1) in which desmosomes are impaired have been identified. These can be categorized into infectious, autoimmune, and inherited. Additionally, recent evidence has shown that desmosomal molecules are often dysregulated in cancer.

Autoimmune disease of the desmosome

Pemphigus disease

Among the acquired desmosomal diseases, a predominant role is surely represented by Pemphigus, a family of autoimmune diseases characterized by circulating autoantibodies that target desmosomal components as
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<tr>
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<tbody>
<tr>
<td>Autoimmune</td>
<td>PV</td>
<td>Dsg1, Dsg3, Dsc1, Dsc2, Dsc3</td>
<td>Painful refractory erosions and ulcers of the oral mucosa</td>
<td>Flaccid bullae and erosions on skin. Lesions of lips, pharynx, larynx, esophagus, eyelid conjunctiva and vagina.</td>
<td>Suprabasal acantholysis; 'row of tombstones' pattern of basal keratinocytes; sparse inflammatory infiltrate in the dermis with eosinophils</td>
<td>DIF: Intra-epidermal intercellular deposition of IgG and/or C3, in a 'cobbledstone' or 'fishnet' pattern</td>
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<tr>
<td></td>
<td>PF</td>
<td>Dsg1, Dsc1, Dsc2, EVP, PPL</td>
<td>Mucosal involvement absent</td>
<td>Fragile blisters, shallow erosions, erythematous patches, crusts most frequently affecting head, face, chest and back</td>
<td>Subcorneal or granular layer acantholysis; mixed inflammatory infiltrate in the superficial dermis</td>
<td>DIF: Intercellular deposition of IgG</td>
</tr>
<tr>
<td></td>
<td>PNP</td>
<td>Dsg1, Dsg3, Dsc2, Dsc3, Pg, Pkp1, Pkp2, Pkp3, Dsp1, Dsp2, EVP, PPL, Plectin, epiplakin, BP230, A2ML1</td>
<td>Severe and extensive mucosal erosions and ulcerations crusting of vermillion border</td>
<td>Variable cutaneous findings, for example blisters, erosions, lichenoid lesions, broncholiolitis obliterans; severe mucous membrane involvement</td>
<td>Variable findings; suprabasal acantholysis, keratinocyte necrosis, and lichenoid interface dermatitis are most common</td>
<td>DIF: Intercellular and/or basement membrane zone deposition of IgG and/or C3</td>
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<tr>
<td>IAP (i-e)</td>
<td></td>
<td>Dsg1, Dsg3, Dsc1</td>
<td>Mucosal involvement usually absent</td>
<td>Vesicles, pustules, crusts on skin; annular, circinate, or herpetiform morphology</td>
<td>Intra-epidermal pustules; minimal acantholysis; mixed infiltrate in dermis</td>
<td>DIF: Intercellular deposition of IgA</td>
</tr>
<tr>
<td>IAP (s-e)</td>
<td></td>
<td>Pkp1</td>
<td>Subcorneal clefts and pustules; minimal acantholysis; mixed infiltrate in dermis</td>
<td>Variable mucocutaneous lesions: minimal acantholysis; mixed infiltrate in dermis</td>
<td>Subcorneal clefs and pustules; minimal acantholysis; mixed infiltrate in dermis</td>
<td>DIF: Intercellular deposition of IgA</td>
</tr>
<tr>
<td>EM/SJS</td>
<td>Dsp</td>
<td>Variable erosive/bullous lesions. The buccal mucosa, vermilion border, and labial mucosa are the most commonly affected sites</td>
<td>Variable mucocutaneous involvement; isolated symmetric targetoid lesions commonly distributed on the extensor surfaces of the Extremities, hands, elbows and knees with extensive involvement of the arms, legs, and trunk. can affect &gt;10% of BSA</td>
<td>Variable: usually non-specific inflammatory infiltration with rare eosinophils with/without subepidermal blistering; confluent keratinocyte apoptosis and focal basal vacuolar degeneration, necrosis, intra-epithelial exocytosis, Civatte bodies</td>
<td>DIF: Strongly positive linear staining for C3 and weakly positive immunoglobulin G</td>
<td>IIF: Can be positive</td>
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<tr>
<td><strong>Infectious</strong></td>
<td>BI</td>
<td>Dsg1</td>
<td>Flaking and fissuring of the lips</td>
<td>ELISA: No apparent IgM reactivity against Dsg1 or Dsg3</td>
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<td></td>
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<td></td>
<td>Small vesicles that evolve in large flaccid bullae usually affecting the trunk, perineum, periumbilical area and the extremities. Lesions rupture and leave a thin brown crust.</td>
<td>Gram stain and culture: of pus or exudate is recommended</td>
</tr>
<tr>
<td></td>
<td>SSSS</td>
<td>Dsg1</td>
<td>Lack of mucous membrane involvement; Perioral manifestation: Flaking and fissuring of the lips</td>
<td>ELISA: low titers of anti-Dsg1 IgG autoantibodies</td>
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<tr>
<td></td>
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<td></td>
<td>Diffuse erythema, fever, positive Nikolsky’s sign. Rash that evolves in lesions ranging from localized blisters to severe exfoliation of &gt;90% of BSA</td>
<td>Gram stain and culture: swab of the skin, orificial areas, and mucous membranes</td>
</tr>
<tr>
<td><strong>Respiratory and</strong></td>
<td>Respiratory and urinary tract infections (ad 3, 7, 11 &amp; 14),</td>
<td>Dsg2</td>
<td>Exudative tonsillitis</td>
<td>ELISA: adenovirus-specific ELISA kit is commercially available. Viral culture: of the nasopharynx or throat PCR and real-time PCR adenovirus detection</td>
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<td></td>
<td>Pharyngitis and coryza, acute respiratory disease, bronchitis, pneumonia, pharyngoconjunctival fever, conjunctivitis, laryngotracheitis, fever, malaise, headache, myalgia, abdominal pain, periumbilical area and the trunk, perineum, periumbilical area and the extremities. Lesions rupture and leave a thin brown crust.</td>
<td>Not usually indicated</td>
</tr>
<tr>
<td><strong>GD</strong></td>
<td>GD</td>
<td>Dsc2, Dsc3</td>
<td>50% of patients clear the infection in absence of clinical symptoms</td>
<td>ELISA: Monoclonal ELISA detect G. intestinalis cyst antigen in the stool (sensitivity 88–99%, specificity 100%). Various nucleic acid amplification techniques: (NAAs), MICROSPHERES (Luminex), or LAMP (loop-mediated isothermal amplification)</td>
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<td>Acute giardiasis: diarrhea (90%), malaise (86%), foul-smelling and fatty stools (steatorrhea) (75%), abdominal cramps and bloating (71%), flatulence (75%), nausea (69%), weight loss (66%), vomiting (23%), fever (15%) constipation (13%), urticaria (10%)</td>
<td>Not usually indicated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chronic giardiasis: loose stools but usually not diarrhea, steatorrhea, profound weight loss (10–20% of body weight), malabsorption, malaise, fatigue, depression, abdominal cramping, borborygmi, flatulence, burping</td>
<td>Not usually indicated</td>
</tr>
<tr>
<td><strong>BA-LT</strong></td>
<td>BA-LT (Anthrax),</td>
<td>Pg, Dsp</td>
<td>Onpharyngial: Pseudomembranous ulcer and necrosis reported to be localized at base and dorsal surface of the tongue, hard palate, soft palate, uvula, and tonsils</td>
<td>ELISA: detection of antiprotective antigen (PA) immunoglobulin (IgG) Standard culture and Susceptibility testing PCR: LRN, Standard or RT PCR performed on a variety of specimen types</td>
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<td></td>
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<td>Fever, malaise, lymphadenopathy and headache. Cutaneous: small, painless, often pruritic papule with central vesicle or bulla, followed by erosion, painless necrotic ulcer with a black, depressed eschar. Gastrointestinal: Ulcers can occur in the stomach, esophagus, and duodenum Respiratory: hemorrhagic necrosis of the thoracic lymph nodes draining the lungs</td>
<td>Bacilli presence easily identifiable. Focal hemorrhage, necrosis, edema, and congestion. Infiltration by neutrophils and prominent immunoblasts, various degrees of mononuclear inflammation, cyst formation</td>
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**Table 1 (continued)**
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<tr>
<td>Genetic</td>
<td>PPK</td>
<td>Dsc2, Pkpl</td>
<td>Perioral hyperkeratotic plaques with severe paritia, hypodontia and dental defects, periodontitis, leukokeratoses, and leukoplakia can be present</td>
<td>Thick, mild, or severe diffuse hyperkeratosis, hyperhidrosis</td>
<td>Not indicated</td>
<td>Genetic test: responsible gene: Dsg1, KRT1, KRT6c, KRT9, KRT16, SERPINB7, AQP5, SLURP1, TRPV3, AAGAB, COOL14A1</td>
</tr>
<tr>
<td></td>
<td>SPPK</td>
<td>Dsg1, Dsp</td>
<td>Keratinization of sites exposed to trauma (knees ankles, knuckles etc.), mild nail dystrophy. Skin thickening is prominent in a linear pattern along the flexor aspects of the fingers and over pressure points on the soles</td>
<td>Keratinization of sites exposed to trauma (knees ankles, knuckles etc.), mild nail dystrophy. Skin thickening is prominent in a linear pattern along the flexor aspects of the fingers and over pressure points on the soles</td>
<td>Keratinization of sites exposed to trauma (knees ankles, knuckles etc.), mild nail dystrophy. Skin thickening is prominent in a linear pattern along the flexor aspects of the fingers and over pressure points on the soles</td>
<td>Not indicated</td>
</tr>
<tr>
<td></td>
<td>SAM</td>
<td>Dsg1</td>
<td>Limited oral involvement</td>
<td>Recurrent infections, severe metabolic wasting, severe PPK, severe erythroderma, severe dermatitis, skin erosion and scaling, multiple allergies, Elevated IgE, hard and curly hair, microcephaly, cardiac defects, and hypotrichosis</td>
<td>Reduced in the number of desmosomes, accompanied by widening of intercellular spaces between suprabasal keratinocytes, and retracted KIFs</td>
<td>Not indicated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dsp</td>
<td>Marked hypodontia, poor periodontal health</td>
<td>Recurrent infections, erythroderma, ichthyosis, PPK, nail dystrophy, diffuse hypotrichosis macrocephaly, a widespread superficial pustulosis, development delay and nystagmus, multiple food allergies, atopy, persistent eosinophilia, increased IgE levels</td>
<td>Irregular psoriasiform hyperplasia, hyperkeratosis and parakeratosis, and a mild superficial dermal inflammatory infiltrate, folliculitis pustular dermatosis</td>
<td>Not indicated</td>
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<tr>
<td>ARVD/C</td>
<td>Dsg2, Dsc2, Pkp2, Pkp4, Dsp</td>
<td>Oral involvement usually absent</td>
<td>Myocardial atrophy and fibrofatty replacement of cardiac myocytes, ventricular arrhythmias, sudden cardiac death, syncope, and end-stage heart failure, typically affecting the right ventricle</td>
<td>Endomyocardial biopsy (EMB) is not recommended</td>
<td>Not indicated</td>
<td>Genetic test: truncating mutations in Dsp ECG Cardiac magnetic resonance</td>
</tr>
<tr>
<td>Left dominant arrhythmogenic cardiomyopathy</td>
<td>Dsp</td>
<td>Oral involvement usually absent</td>
<td>Palpitations, less frequent syncope, chest pain, dyspnea, and, rarely, sudden cardiac death</td>
<td>Endomyocardial biopsy (EMB) is not recommended</td>
<td>Not indicated</td>
<td>Genetic test: truncating mutations in Dsp ECG Cardiac magnetic resonance</td>
</tr>
<tr>
<td>LAH</td>
<td>Dsg4</td>
<td>Oral involvement usually absent</td>
<td>Hypotrichosis of the scalp, chest, arms and legs</td>
<td>Histology of scalp skin revealed thin and atrophic Hair Follicles and hair shafts that often coil up within the skin</td>
<td>Not indicated</td>
<td>Genetic test: several mutations in the Dsg4 gene (18q12.1; 3q27.3; 13q14.11;13q21.32)</td>
</tr>
<tr>
<td>Recessive monilethrix</td>
<td>Dsg4</td>
<td>Oral involvement usually absent</td>
<td>Moniliform hairs, pili torti, trichoschisis, trichorrhexis nodosa-like defects, tapered hairs</td>
<td>Carded ingrown hair shafts within the hair follicle</td>
<td>Not indicated</td>
<td>Genetic test: mutations in three type II hair cortex keratins (KRT8&lt;sub&gt;1&lt;/sub&gt;,KRT8&lt;sub&gt;3&lt;/sub&gt;, KRT8&lt;sub&gt;6&lt;/sub&gt;), and Dsg4</td>
</tr>
<tr>
<td>Wooly hair</td>
<td>Dsc2</td>
<td>Oral involvement usually absent</td>
<td>Hair with elliptical cross section, variations in caliber, axis rotation, kinked formation, and non-homogeneous keratinization</td>
<td>Scalp biopsy is usually not necessary. An ophthalmologic examination is recommended for all patients with wooly hair. If a syndrome is suspected, an accurate cardiological exam is mandatory</td>
<td>Not indicated</td>
<td>Genetic test: can be carried out when the gene locus is known: Hair examination: is carried out by light and electron microscopy Trichogram</td>
</tr>
<tr>
<td>Naxos disease</td>
<td>Pg, Dsp</td>
<td>Leukokeratoses, and hypotrichodontoia can be present</td>
<td>Familial wooly hair, diffuse non-epidermolytic PPK, ARVC with heart-rhythm disruptions</td>
<td>Not usually indicated</td>
<td>Not indicated</td>
<td>Genetic test: can be carried out when the gene locus is known: Pg genetic defect located on chromosome 17q21; Dsp genetic defect located on chromosome 6p23-24</td>
</tr>
<tr>
<td>Carvajal syndrome</td>
<td>Dsp</td>
<td>Hypotrichodontoia can be present</td>
<td>Dilated left ventricular Cardiomyopathy, wooly hair, keratoderma</td>
<td>Not usually indicated</td>
<td>Not indicated</td>
<td>Genetic test: utosomal-dominant mutations in the DSP gene</td>
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<td></td>
<td>EDFSF</td>
<td>Pkp1</td>
<td>Perioral fissuring</td>
<td>Orthokeratosis, hypergranulosis and wide intercellular spaces between keratinocytes,</td>
<td>Not indicated</td>
<td>Genetic test: At least eleven different recessively inherited mutations in the Pkp1 gene (chromosome 1q32) Electron Microscopy</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Widespread skin fragility, non-cicatricial alopecia, PPK, nail dystrophy, hypohidrosis</td>
<td></td>
<td></td>
<td>Genetic test: homozygous nonsense JUP mutation, c.1615C&gt;T, p.Q539X, that leads to the lack of Pk. Electron Microscopy</td>
</tr>
<tr>
<td></td>
<td>Lethal congenital epidermolysis bullosa</td>
<td>Pg</td>
<td>Tongue mobility reduction</td>
<td>Pronounced acantholysis, and cleavage within the epidermis, with the loss of upper spinous, granular and horny layers.</td>
<td>DIF: abnormal expression and distribution of desmosomal and adherens junction protein. Abnormalities of the dermal-epidermal basement membrane. (keratin 5 and 14, positive). Loss of immunoreactivity to Dsp and Dsg3.</td>
<td>Genetic test: homozygous nonsense JUP mutation, c.1615C&gt;T, p.Q539X, that leads to the lack of Pg. Electron Microscopy</td>
</tr>
<tr>
<td></td>
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<td>Severe skin fragility, generalized epidermolysis, massive transcutaneous fluid loss, esophageal stenosis, Pyloric atresia</td>
<td></td>
<td></td>
<td>Genetic test: homozygous nonsense JUP mutation, c.1615C&gt;T, p.Q539X, that leads to the lack of Pg. Electron Microscopy</td>
</tr>
<tr>
<td></td>
<td>Lethal acantholytic epidermolysis bullosa</td>
<td>Dsp</td>
<td>Possible neonatal teeth</td>
<td>Acantholytic cell-poor intra-epidermal blister with a single row of preserved but partially necrotic basal cells on the blister floor</td>
<td>DIF: on fresh frozen skin samples with anti-DP C-terminal antibodies provide a sensitive and quick screening method for DP C-terminal truncating mutations</td>
<td>Genetic test: genomic DNA (gDNA) can be extracted from peripheral blood leukocytes, or from formalin-fixed paraffin-embedded (FFPE) skin tissue Electron Microscopy</td>
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<td></td>
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<td></td>
<td>Complete alopecia, nail loss, cardiomypathy, ears malformation, extensive neonatal epithelial shedding (&gt;50% BSA), syndactyly, neonatal death</td>
<td></td>
<td></td>
<td>Genetic test: sequencing SCN5A gene commercially available</td>
</tr>
<tr>
<td></td>
<td>Brugada syndrome</td>
<td>Pkp2</td>
<td>Absence of oral involvement</td>
<td>From autopsy: Absence of active myocardial inflammation, moderate myocardial hypertrophy, moderate fibrosis and fatty replacement of the myocardium (predominantly in the septum)</td>
<td>Not indicated</td>
<td>Genetic test: sequencing SCN5A gene commercially available</td>
</tr>
<tr>
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<td></td>
<td>Life-threatening ventricular arrhythmias, sudden cardiac arrest, atrial fibrillation, syncope, Nocturnal agonal respiration</td>
<td></td>
<td></td>
<td>Case by case evaluation of: drug challenge (e.g., sodium channel blockers), signal-average ECG invasive electrophysiology testing</td>
</tr>
</tbody>
</table>

ARVDC, Arrhythmogenic right ventricular dysplasia–cardiomyopathy; BA-LT lethal toxin of Bacillus anthracis; BSA, Body surface area; ECG, electrocardiography; EDSFS, Ectodermal dysplasia–Skin fragility syndrome; EM, erythema multiforme; Gd, Giardiasis; IAP (i-e), IgA pemphigus (intra-epidermal neutrophilic dermatosis); IAP (s-c), IgA pemphigus (subcorneal pustular dermatosis); LAH, Localized autosomal recessive hypotrichosis; MC, Mucocutaneous type; MD, Mucosal dominant type; PCR, polymerase chain reaction; PF, Pemphigus foliaceus; PG, Plakoglobin; PNP, paraneoplastic pemphigus; PPK, palmoplantar keratoderma; PV, pemphigus vulgaris; SAM, Skin dermatitis multiple allergies and metabolic wasting syndrome; SJS, Stevens-Johnson syndrome; SPPK, striate palmoplantar keratoderma; SSSS, staphylococcal scalded skin syndrome.

aIndirect immunofluorescence is negative in around 50 percent of patients with IgA pemphigus.

bTest availability restricted to specialized laboratories.
well as other epithelial antigens and impair keratinocytes adhesion with subsequent acantholysis. The antidesmosomal antibodies detected in pemphigus patients are directed against Dsg1 and/or Dsg3 (Cirillo et al., 2007). These diseases are considered relatively rare with an overall incidence of 1–16 new cases per million people/year (Ruocco et al., 2013), and the age of onset is usually the fifth or sixth decade of life. Although the precise pathomechanisms of pemphigus have not yet been fully elucidated, recent studies have shown that desmosome disassembling involves not only the direct inhibition of desmosome formation caused by IgGs, but also desmosome remodeling. Additionally, several intracellular signaling pathways possibly triggered by non-desmosomal molecules. The signaling-related events include apoptosis as well as modulation of Pg, p38MAPK, heat-shock protein 27, cdk2, Src, RhoA, and others (Kitajima, 2014; Cirillo, 2016).

The main subtypes of Pemphigus are pemphigus vulgaris (PV), pemphigus foliaceus (PF), paraneoplastic pemphigus (PNP), and IgA pemphigus (IAP).

- **PV** is the most common subtype of pemphigus (~70% of cases), found mainly in middle-aged and elderly patients, with a female predisposition. Familial cases are rare although a higher incidence was found in Ashkenazi Jewish and Japanese populations (Joly and Litrowski, 2011). Histologically, PV is characterized by an intra-epidermal cleft between the basal and spongy layers (suprabasal acantholysis), which manifests clinically as painful mucosal lesions and skin blister formation. Oral mucosal lesions appear early and associate with the presence of anti-Dsg3, but not anti-Dsg1, antibodies. Early diagnosis and treatment are associated with a better prognosis (Mignogna et al., 2010) and may prevent the spreading of the disease to skin tissues. The mucocutaneous type of PV is characterized by skin blisters in addition to oral erosions and associates with an autoantibody switch to both anti-Dsg1 and anti-Dsg3 IgG (Amagai, 2010). In addition, autoantibodies in PV can target Dsc1, Dsc2, and Dsc3 (Stahley and Kowalczyk, 2015).

- **PF variants** represent about 20–30% of pemphigus cases and are characterized by superficial epidermal blisters without mucosal involvement. The blistering results from autoantibodies directed against Dsg1 and is restricted to the granular layer of the epidermis. Further desmosomal targets such as Dsc1, Dsc2, Dsc3, Pg, Dsp, and Pkp3 have been described in the literature.

- **PNP** is a rare form of pemphigus that typically affects patients diagnosed with malignancies such as non-Hodgkin’s lymphoma and chronic lymphocytic leukemia (Yong and Tey, 2013). This variant is the most severe among pemphigus subtypes and shows a distinctive set of clinical features including a typically polymorphous skin rash, severe mucosal involvement, life-threatening bronchiolitis, and unusual histopathological and immunological findings (Hata et al., 2013). The age of onset is usually 45–70 years, and intraorally, PNP is characterized by extremely painful and refractory lesions potentially followed by full body cutaneous involvement. Unlike other pemphigus subtypes, PNP can show the involvement of palms and/or soles, conjunctiva, and simple squamous epithelia.

The desmosome proteins targeted by autoantibodies in PNP are several and include Dsgs (particularly the Dsg3), Dscs, Dsp, and plakophilins (Stahley and Kowalczyk, 2015), as well as EVP, PPL, and a number of less well-specified antigens such as alpha-2-macroglobulin-like-1 (Schepps et al., 2010). Patients presenting with immunological profiles suggestive of PNP and without evidence of malignancy deserve strict follow-up for the possible subsequent development of cancer. The prognosis of PNP remains poor, with mortality rates reaching 90% (Yong and Tey, 2013).

- **IAP** is a subtype characterized by the presence of circulating IgA autoantibodies that can target desmosomal and non-desmosomal keratinocyte cell surface constituents. IAP in turn is divided into two subtypes: the subcorneal pustular dermatosis (s-c), in which Dsc1 is the target antigen, and the intra-epidermal neutrophilic dermatosis (i-e) in which the targets are Dsg1 and Dsg3. Recently, a comparative clinicopathologic study has established that IgG/IgA pemphigus, a further overlapping variant between classic IgG pemphigus and IAP, may best be regarded as a variant of IgG pemphigus and distinct from IgA pemphigus (Toosi et al., 2016). Histologically, IAP is characterized by anacantholyis and extensive neutrophilic infiltration within the upper layers or all layers of the epidermis. Reports of patients with IAP are sparse, with only about 60 cases described, although the prognosis of this form was rated as comparable with the classic forms of pemphigus.

### Erythema multiforme (EM) and Stevens–Johnson syndrome (SJS)

Besides the pemphigus group, desmosomes have shown to be targeted in erythema multiforme (EM) and Stevens–Johnson syndrome (SJS) cases. EM and SJS are two acute immune-mediated disorders that can affect the skin and mucous membranes through a type 4 cytotoxic reaction, mediated by T lymphocytes and triggered by numerous factors. Several reports described circulating autoantibodies to Dsp1 and Dsp2 in EM patients (Johnson et al., 1999; Ellis and Sidhu, 2014). So far, the pathogenic significance of the circulating Dsp autoantibodies in EM and SJS has not yet been elucidated. The unsolved question is whether there is a subset of EM patients with autoimmune disease or they are a manifestation of the phenomenon of epitope spreading.

### Infectious diseases of the desmosome

Disruption of desmosome integrity can be caused by infective agents. *Bullous impetigo* (BI) and *Staphylococcal scalded skin syndrome* (SSSS) are caused by *Staphylococcus aureus* through the release of exfoliative toxin, a serine protease that specifically cleaves the extracellular domain of Dsg1 (Amagai et al., 2000).
Respiratory and urinary tract infections caused by adenovirus serotypes (Ad) 3, 7, 11, and 14 can target Dsg2 (Amagai and Stanley, 2012).

*Giardiasis (Gd)* targets Dsc2/3 and disrupts tight, adherens and desmosomal junctions of intestinal cells (Adam, 2001). Lastly, Dsp and Pg of alveolar epithelial cells can be targeted by *Bacillus anthracis* lethal toxin (BA-LT), with a consequent impaired desmosome assembly (Langer et al., 2012). These infectious diseases have limited oral involvement and their discussion goes beyond the scope of this review. More details can be found in Table 1. Desmosome disruption and anomalies have also been linked to coxsackievirus B3 (CVB3) and infections of the oral mucosa, although the specific targets remain unknown (Stahley and Kowalczyk, 2015).

### Genetic diseases of the desmosome

Diseases resulting from genetic alterations in desmosomal components involve skin and heart tissues but have limited oral involvement. Details are reported in Table 1.

*Striated* palmoplantar keratoderma (PPK) and *Palmoplantar keratoderma* (PPK) feature mutations in the Dsg1, Pkp1, and Dsp genes (Cirillo, 2016). *Severe skin dermatitis, multiple allergies, and metabolic wasting syndrome (SAM)* are linked to mutations in Dsg1 (Samuelov et al., 2013) and Dsp genes (McAuley et al., 2015). *Localized autosomal recessive hypotrichosis (LAH)* is caused by several mutations in the Dsg4 gene (Moss et al., 2004). *Recessive monilethrix* may also be linked to Dsg4 mutations. *Ectodermal dysplasia–skin fragility syndrome* (EDDFS) is caused by mutations in the Pkp1 gene, whereas *Lethal congenital epidermolysis bullosa* features homozygous nonsense mutation, which leads to the lack of Pg.

Desmosome-like structures are also present in cardiac muscle cells, and mutations of desmosomal components result in heart disease. *Arrhythmogenic right ventricular dysplasia/cardiomopathy (ARVD/C)* and left dominant arrhythmogenic cardiomopathy involve several mutations in the Dsg2, Dsc2, Pg, Dsp2, and Pkp2 genes (Awad et al., 2006; Nagaoka et al., 2006; Syrris et al., 2006). Another variant of the disease named *lethal acantholytic epidermolysis bullosa* has also been identified as the result of heterozygosity of two loci containing the C terminus of Dsp and leading to the formation of a truncated protein, thus lacking the entire IF-binding domain. *Brugada syndrome* has been linked to 18 different gene mutations, one being *PKP2* (ENSG00000057294), which encodes for the protein plakophilin-2 (PKP2).

In certain cases, both epidermis and heart can be affected. *Woolly hair*, an autosomal recessive structural abnormality of scalp hair, is a clinical feature shared syndromically with palmoplantar hyperkeratosis and heart anomalies by diseases targeting Dsc2, Pg (e.g., *Naxos disease*), and Dp (e.g., *Carvajal syndrome*).

### Desmosome and cancer

David Garrod’s group first proposed a mechanistic role of desmosomes in cancer progression (Tselepis et al., 1998). To date, at least three mechanisms through which alteration in desmosomal cadherins occurs in cancer have been identified, namely: (i) transcriptional regulation of desmosomal cadherins; (ii) impaired transport, targeting, and assembly into mature desmosomes; and lastly, (iii) inactivation by proteolytic cleavage. Furthermore, the control of cell cycle as well as apoptosis can be indirectly affected by desmosomal cadherin signaling function.

The idea that the loss of intercellular adhesion could be directly related to cancer invasiveness is fairly predictable. Surprisingly, analysis of the expression levels of desmosomal components in cancer has yielded conflicting results. Specifically, expression of desmosomal cadherins was found to be decreased in many tumors such as skin, head and neck, lung, breast, prostate, cervix but reported as increased by others (Huber and Petersen, 2015).

There is, however, compelling clinical evidence that correlates desmosomal deregulation with clinicopathologic features of the tumor including staging and grading, and consequently prognosis. This is the case of renal cell carcinoma, prostate cancer, endometrial carcinoma, head and neck cancer, lung cancer, gastric and colon cancer, pancreatic ductal adenocarcinoma, SCC of the sinonasal cavity, cutaneous SCC, esophageal adenocarcinoma, and not least OSSC.

Thus, current evidence strongly suggests that desmosomal proteins will soon assume a main role as cancer markers/prognostic factors, but also as potential molecular targets for development of novel therapies.

We refer the reader to a couple of recent review articles for a comprehensive discussion of this topic (Huber and Petersen, 2015; Stahley and Kowalczyk, 2015).

### Diagnostic pathway

The aim of this section is to provide guidance to dentists and oral medicine specialists for the diagnosis of desmosome-related conditions, especially blistering diseases, and to outline the rationale for the diagnostic investigations required.

The diagnosis of blistering disease is reached from the combination of clinical, histological, immunopathological, and serological findings, the latter being a prerequisite for the diagnosis of immunobullous disorders. Genetic disease can be confirmed by gene mutation analysis. Additional tests may include immunoblotting, immunoprecipitation, and electron microscopy.

#### Clinical evaluation

Diseases of the pemphigus group dominate the field of desmosome-related conditions with oral manifestations and hence are more relevant to dental practitioners. Clinical evaluation includes examination of the skin, mucous membranes, and nails, and patients should be also questioned for symptoms suggestive of extraoral mucosal involvement. The patient’s medical history and medications should be thoroughly reviewed, as clinical and laboratory studies may not discriminate between idiopathic and drug-induced pemphigus. Despite some clinical findings being suggestive of specific immunobullous diseases, for example, flaccid blisters, and a positive Nikolsky sign
for PV, laboratory-based detection of tissue-binding and circulating autoantibodies is always required. A standard laboratory work-up for these patients would include the following:

- A lesional skin/mucosal biopsy for routine hematoxylin and eosin (H&E) staining
- A perilesional skin/mucosal biopsy for direct immunofluorescence (DIF), and/or
- Serum collection for indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA).

**Sample collection**

The biopsy for routine histopathology should ideally be performed on an intact lesion, and a punch biopsy (4 mm) is usually sufficient. Clinicians may prefer to employ a stab-and-roll technique using size 15 scalpel blade to maintain the epithelial roof in the sample. The sample should be placed immediately in 10% buffered formalin.

A tissue sample for DIF microscopy should be taken from the perilesional area to contain both epithelium and stroma. The sample should be placed this time in optimal cutting temperature (OCT) compound and frozen immediately at −20°C and stored at −80°C until processing. If OCT compound is not available, the tissue may alternatively be placed in normal saline-soaked gauze and kept at 4°C, or in Michel’s medium that can preserve the sample at room temperature for approximately 6 months (Vodegel et al., 2004).

Biopsy for transmission electron microscopy (TEM) and immunomapping are identical to that for routine histopathology. The sample should be placed immediately in an electron microscopy-specific medium, and in OCT, respectively.

ELISA and IIF assays are used for serodiagnosis and require the collection of a 5–10 ml blood sample without anticoagulants. This is then centrifuged to separate plasma (which contains the gamma globulins) from blood cells.

**Histology**

The characteristic histopathological findings in PV include intra-epithelial cleavage with loss of keratinocytes adhesion, known as acantholysis, localized primarily to the suprabasal region. Retention of basal keratinocytes along the basement membrane zone results in an appearance that resembles a ‘row of tombstones’. There is additionally sparse inflammatory infiltrate in the dermis with eosinophils. In the variant known as pemphigus vegetans, the suprabasal cleavage is accompanied by hyperkeratosis, papillomatosis, and prominent acanthosis with downward proliferation of the rete ridges.

The characteristic histopathological findings of PF include intra-epithelial acantholysis beneath the stratum corneum or within the granular layer, occasional presence of neutrophils within the blister cavity and mixed inflammatory infiltrate in the superficial dermis with neutrophils and eosinophils.

Typical histopathological findings of IAP include intra-epidermal clefts and pustules located in a subcorneal location (s-c) or in the entire or mid-epidermis (i-e), slight or absent acantholysis and a mixed inflammatory infiltrate in the dermis.

The most common histopathological findings in PNP are suprabasal acantholysis, keratinocyte necrosis, and a lichenoid interface dermatitis. However, in PNP the histopathological findings are variable.

The istopathological features of all other desmosomal diseases with limited involvement of the oral cavity are reported in Table 1.

**Direct immunofluorescence microscopy (DIF)**

DIF is a technique that aims to detect any in situ deposition of immunoreactants (typically immunoglobulins and/or complement components) in patients’ epidermis or mucous membranes. Detection of the immune deposits in the sample allows confirmation of presumed immunological pathomechanism, and classification of the disease according to the exact location of the immune deposits. The most common antibodies used for DIF comprise fluorescein-conjugated antibodies against IgG, IgM, IgA, C3, and fibrinogen. IF microscopy is still considered the diagnostic gold standard for pemphigus given that the tissue-fixed intercellular antibodies are present in about 90% of the patients. In both PV and PF, an intercellular binding of IgG and/or C3 is found in a typical ‘cobblestone’ or ‘fishnet pattern’ in the epidermis/epithelium, while in PNP, in addition to the above findings, an abnormal band-like deposit of immunoglobulin/complement is detected in the dermal—epidermal junction (Chiorean et al., 2014). In IAP, the main finding is the deposition of IgA with an intercellular pattern. Importantly, cryosections of perilesional biopsies are required for direct IF microscopy.

**Indirect immunofluorescence microscopy (IIF)**

IIF aims to detect circulating autoantibodies in patients’ sera that target skin constituents. The procedure applies patient serum to normal epithelial substrate in a two incubation steps. One of the most used epithelial substrates is the monkey esophagus. In general, it is well established that the type of substrate used influences the sensitivity of the test. More in detail, for PV and PF the best substrates are monkey and guinea pig esophagus, respectively. For PNP, rat or monkey bladder is used due to their high expression of plakins. Interestingly, more than 80% of PV and PF patients have detectable circulating autoantibodies (Payne and Stanley, 2012), which makes IIF a reliable technique for the diagnosis of these autoimmune blistering diseases.

In IAP, the preferred substrate is monkey esophagus, and binding of autoreactive IgA from patient’s sera to epithelial cells with an intercellular pattern may be found. Only recently a novel IF assay using desmocollin-transfected COS-7 cells is available to characterize autoreactive IgA molecular specificity (Otten et al., 2014).

The staining patterns are similar to those described for DIF.

**ELISA**

ELISA is a technique that measures specific autoantibodies in serum. Test serum is incubated in microtiter plate
wells that are coated with the antigen(s) of interest. Matched autoantibodies will bind to the antigen and the incubation with an enzyme conjugated secondary anti-IgG antibody, resulting in proportional color change. In PV and PF, serum levels of anti-Dsg1 and anti-Dsg3 antibodies may be related to disease activity (Cirillo et al., 2012). This test can also be useful to assess response to treatment (Zone, 2009). In general, anti-Dsg1 levels better correlate to disease course in PV and PF patients than anti-Dsg3 levels, which may remain high even in phases of remission (Abasq et al., 2009). ELISA kits are commercially available for detection of serum IgG antibodies to Dsg1/ Dsg3. ELISA assay, with a sensitivity that exceeds 90 percent, has been demonstrated to be more sensitive and specific than IIF for the diagnosis of PV and PF (Payne and Stanley, 2012).

In general, the following outcomes may be expected:

- In PV with exclusive mucosal involvement, anti-Dsg3 will be detected, whereas in mucocutaneous variant both Dsg1 and Dsg3 autoantibodies can be found.
- PF is characterized by exclusive involvement of Dsg1 autoantibodies.
- In most cases of PNP, IgG autoantibodies against Dsg1 and Dsg3 are found but also against Dsc1/Dsc3/EVP/ PPL, followed by Dsp1/Dsp2/plectin, and BP230.
- IgA pemphigus is characterized by the detection of IgA autoantibodies against Dsg1 and Dsg3 in the i, e-type and against Dsc1 in the s-type. (Kneisel and Herl, 2011).

The ELISA system for detection of anti-envoplakin antibodies is now commercially available. Conversely, ELISA for detection of other molecules such as desmocollins, periplakin, while already available, has not yet been released for routine clinical diagnostic use.

Additional test: immunoblotting and immunoprecipitation, protein arrays, PCR, gene sequencing

Together with ELISA, immunoblotting (also referred to as Western Blotting) and immunoprecipitation are highly sensitive and specific detection techniques of autoantibodies that use recombinant autoantigens or keratinocyte extracts from healthy human skin. Recently, protein microarrays have proven to be a powerful technique to detect large number of autoantibodies in blistering disease (Kalantari-Dehaghi et al., 2013).

Immunoprecipitation is more sensitive than immunoblotting and in particular is used for immunosero logical follow-up and as definitive serological confirmatory test. Remains, however, to consider that Western blotting analysis is not necessarily useful in pemphigus diagnosis given that most of the immunogenic epitopes on Dsg1 and 3, are conformational. The increasing complexity of autoantibody profiles in blistering diseases will probably be addressed by protein arrays in future years.

Together with clinical and histological findings, the diversity and heterogeneity of some genetic desmosomal diseases make genetic testing indispensable for diagnosis, assuming that the causative genes have been identified. In general, single base pair mutations are identified by direct sequencing, DNA hybridization, and/or restriction enzyme digestion methods.

Concluding remarks

Alteration in desmosomal components leads to diseases that feature skin, cardiac and oral involvement. The desmosome offers a precious example of how basic, translational, and clinical research integrate and support each other to gain insight into the understanding of human diseases and, ultimately, to improve patient management.

Author contributions

NC designed the manuscript and revised the text, AC drafted the manuscript.

References


Synthetic corticosteroids are widely used for the treatment of a variety of diseases, including pre-malignant and malignant conditions. In striking contrast, recent evidence suggests that corticosteroids can bear tumor-promoting effects in solid tumors of epithelial origin. Epithelial cells, including the mucosa of the oral cavity and the skin, are able to modulate the local concentration of active corticosteroids and to produce steroids de novo. This has important clinical and physiopathological implications, because tissue-specific regulation of glucocorticoids plays a key role in the overall effect of these molecules. In the present review of the current English literature, performed using MEDLINE/PubMed/Ovid databases, we collected published evidence to demonstrate that corticosteroids influence pathophysiological processes that are strictly related to malignancy, providing the rationale for further investigation.


Cortisol is an endogenous glucocorticoid hormone produced by the adrenal cortex in response to adrenocorticotrophic hormone (ACTH), and it is released in response to various stress stimuli. ACTH secretion from the pituitary gland is a result of hypothalamus activation. GCs have long been regarded as drugs that promote apoptosis and inhibit cell proliferation and wound healing (Schlossmacher et al., 2011). However, a body of evidence has mounted over the last 10 years that shows potentially harmful effects of GCs in cancer therapy. Data from laboratory, preclinical, and clinical studies suggest that glucocorticoids induce treatment resistance in solid tumors, including prostate, ovarian, and breast cancer (Herr and Pitzzenmaier, 2006). Our data also support this view by demonstrating that cortisol significantly reduces the cytotoxic effects of chemotherapeutic agents in several cell lines from oral squamous cell carcinoma (OSCC) (Celentano et al., manuscript in preparation). GCs also form the mainstay of the therapeutic armamentarium of a number of diseases with malignant potential, such as oral lichen planus (OLP). Therefore, the potential pro-tumorigenic activity of GCs can have vast clinical implications and warrants urgent attention.

Recent research demonstrates that epithelial cells, including those lining the skin and oral mucosa, can metabolize glucocorticoids de novo from cholesterol or systemic steroid intermediates. It has been shown that there is an endogenous non-adrenal glucocorticoid system existing in human epidermal keratinocytes and they exhibit high levels of corticosteroid metabolizing activity (Slominski et al., 2014). Keratinocytes express major enzymes involved in the synthesis and metabolism of cortisol, including cytochrome P450 chain, 11β-hydroxysteroid dehydrogenases (11β-HSD1/11β-HSD2), adrenocorticotropic hormone receptor (ACTHR/MC2R), and glucocorticoid receptor (Cirillo and Prime, 2011). Alteration of both the Hypothalamus-Pituitary and Adrenal (HPA) axis and non-adrenal tumor-associated glucocorticoid system has been linked to carcinoma progression as it results in altered cortisol levels (Rasmuson et al., 2001; Bernabé et al., 2012). These data may have major implications for cancer pathophysiology and therapy, as GCs are routinely administered during cancer treatment.

This paper primarily aimed to review the current evidence supporting the traditional (conventional) and non-conventional role of GCs in normal and malignant cells.

Conventional and Non-Conventional Effects of GCs on Epithelial Cells

GCs are conventionally used to up-regulate anti-inflammatory factors and down regulate pro-inflammatory factors. In oncology, GCs have been widely used in association with other treatment for cancer patients because they have potent proapoptotic properties in lymphoid cells, can reduce nausea and acute toxic effects in healthy tissue (Herr and Pitzzenmaier, 2006). However, a number of secondary effects have been documented that influence a variety of functions such as cell migration, differentiation, apoptosis, and proliferation.

Role in cell proliferation and apoptosis

The anti-inflammatory and pro-apoptotic effects of GCs (Sidler et al., 2011; Feng et al., 2013) are fundamental in their use as therapeutic agents. However, as showed on normal human epidermal keratinocytes, the cortisol can exerts both immunostimulatory and immunosuppressive activities depending on its concentration (Itoi et al., 2013).

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GCs are able to alter signaling in key survival pathways and this can result in reversible growth arrest or cell death in certain cell types, which may be particularly important in the arrest of tumor cell proliferation (Schlossmacher et al., 2011). However, although GCs are conventionally used to treat cancer or as adjuvants in cancer treatment, it has been observed that they may in fact induce tumor growth and metastasis formation (Romero et al., 1992; Deguchi et al., 1998). The underlying mechanism in which GCs induce tumor growth has been explored and it has been proposed that the hyper-secretion of GCs decreases peripheral blood lymphocytes (PBLs); cells which play a central role in killing tumor cells (Deguchi et al., 1998). Alternatively, studies have focused on the fact that GCs are inhibitory to the immune system and thus may inhibit certain molecules which are known to be tumoricidal (Hogan and Vogel, 1988).

While the phenomenon of glucocorticoid-induced apoptosis in hematological cells is well established (Olnes et al., 2016), a growing body of evidence now suggests that glucocorticoids can act as anti-apoptotic agents in epithelial cells to promote cancer progression. It has been demonstrated that elevated levels of GCs during chronic restraint stress mediate an inhibitory effect on the tumor suppressing protein p53 thereby promoting tumor progression (Feng et al., 2013). Moreover, extra-adrenal GCs produced by intestinal epithelium have been implicated in aiding the progression of colon carcinoma cells through an immune evading mechanism (Sidler et al., 2011). The colon carcinoma cells synthesize and release bioactive immunosuppressive GCs which in turn suppress T-cell activation (Sidler et al., 2011).

Guendisch et al. analyzed the role of glucocorticoids (GCs) in the survival and proliferation of tumor cells. The non-apoptotic actions of glucocorticoids on tumor cell lines, primary tumor cells, and an in vivo model along with molecular signaling studies were examined. The first important finding of this study was that dexamethasone, a commonly used glucocorticoid in cancer patients, enhances tumor cell proliferation in vitro and in vivo. Dexamethasone enhanced tumor cell proliferation in 9/17 cell lines from solid tumors from all three different germ layers (Guendisch et al., 2012). It was found that these dexamethasone-induced proliferative changes were mediated by the glucocorticoid receptor. GCs lead to an activation of intracellular signaling molecules such as N3RC1, AKT, and p38-MAPK which all function in various ways to mediate dexamethasone-induced tumor cell proliferation. In terms of clinical relevance, the findings from this paper suggest that the non-use of GCs might actually improve the prognosis of patients with cancer (Guendisch et al., 2012). Moreover, Dexamethasone showed to suppress antitumor immune responses and facilitate tumor progression by enhancing PD-1, a key cell-surface receptor of CD28 superfamily that can attenuate T-cell responses and promote T-cell tolerance, resulting in faster tumor growth and poor prognosis in the clinical setting of anti-cancer therapy (Xing et al., 2015).

A reported well-known effect of GCs is their ability to inhibit cell proliferation (Spiegelman et al., 1997; Newton, 2000; Rabbitt et al., 2003; Yuan et al., 2016). However, it has also been shown that cortisol can increase cell proliferation at certain concentrations. Cortisol concentration modulates the level of Interleukin-6 (IL-6), a cytokine that stimulates the growth of cancer cells via an autocrine mechanism (Bernabé et al., 2011). IL-6 has been associated with angiogenesis and tumor progression (Heikilä et al., 2008, Jobe et al., 2016). In head and neck SCC it can be correlated with recurrence, lymph node recruitment, and a poor prognostic survival (Duffy et al., 2008; Nagata et al., 2003). At higher concentrations of cortisol (100 and 1000 nM) there is a lower level of IL-6 mRNA expression and secretion in oral SCC. However, at cortisol concentrations that simulate physiological stress levels (10 nM) there is an increase in IL-6 mRNA expression and secretion with a subsequent increase in cell proliferation (Bernabé et al., 2011).

There is also support for this increase in cell proliferation, as a result of cortisol treatment, in non-malignant situations. This has been demonstrated in foetal cardiomyocytes and Purkinje fibers (Feng et al., 2013). Cell nuclei expressing Ki67, a marker for cell proliferation, were found in greater numbers in foetal heart tissue that were infused with cortisol. The myocyte proliferation due to exogenous GC treatment resulted in cardiac enlargement and hypertrophy leading to increased wall thickness. The proliferative effects of cortisol were blocked by the use of an antagonist to the mineralocorticoid receptor, which is a receptor of particular interest because it can be found in the epidermis of skin and interacts with the 11β-HSD enzyme (Kenouch et al., 1994). In foetal hearts it was found that there was an increase in apoptosis in Purkinje fibers which was blocked through the use of a specific antagonist to the GR (Feng et al., 2013). In contrast, GR activation in cardiomyocytes has an anti-apoptotic effect, reflecting another non-conventional effect (Ren et al., 2012).

Studies on the transcriptional profiles of primary human keratinocytes revealed further evidence for the non-conventional anti-apoptotic effect. The treatment of keratinocytes with dexamethasone promoted anti-apoptotic gene expression and inhibited pro-apoptotic expression. As a result, the treated keratinocytes did not undergo UV-induced apoptosis (Stojadinovic et al., 2007). To explain the presence of both apoptotic and anti-apoptotic properties of GCs, it has been suggested that they are not mutually exclusive and these effects may occur at different stages, that is, GCs inhibit early stages of differentiation and promote later stages of differentiation. (Stojadinovic et al., 2007). Therefore, GCs can have different effects depending upon cell type, developmental age and cellular environment (Feng et al., 2013).

Role in wound healing and anchorage independence

Delayed wound healing is a well-known effect of the therapeutic use of GCs. It has been shown that overexpression of the GR in transgenic mice slows down the skin wound healing process. This delay is attributed to the inhibition of keratinocyte proliferation, motility, and migration. Furthermore, delayed wound healing is accompanied by a decrease in granulocyte and macrophage recruitment, as well as a reduction in ERK activity and the expression of TNF-α, IL-β, proangiogenic factor, and vascular endothelial growth factor (Stojadinovic et al., 2007; Sanchis et al., 2012). However, conflicting results have suggested that in fact GCs may have some repair potential. In one particular experiment, mucosal human bronchial epithelial cell (HBEC) cultures were created to imitate an asthmatic environment and the effects of therapeutic agents such as dexamethasone were studied. As expected, it was found that dexamethasone delayed immediate wound repair of HBEC by lowering the proliferative rate of cells surrounding the injured site. However, this in turn reduced proliferative stress on epithelial stem cells and transit amplifying cells in the basal compartment of the bronchial epithelium during post-wound metaplasia. Hence, the functional lifespan of these replacement cells is enhanced leading to better long term repair potential (Wadsworth et al., 2006).

GCs have long been used as agents in the treatment and management of particular diseases. The effects of commonly used GC medications to treat pemphigus vulgaris (PV) were studied to demonstrate whether their supposed therapeutic properties in regenerating PV-like lesions could be demonstrated in vitro. Using methylprednisolone (MP) and

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promoting tumor metastasis (Rasmuson et al., 2001; impairing the cellular and humoral immune response and by instance, elevated cortisol levels have been found in cancer pathway in cancer has been reported in previous literature. For Alteration of key molecules involved in the glucocorticoid pathway in cancer has been reported in previous literature. For instance, elevated cortisol levels have been found in cancer patients where it is believed it affects cancer prognosis by impairing the cellular and humoral immune response and by promoting tumor metastasis (Rasmuson et al., 2001; Mazzoccoli et al., 2003; Bernabé et al., 2012). 11β-Hydroxysteroid dehydrogenase (11β-HSD) is the main enzyme that regulates the endogenous activity of GCs and is expressed in two isoforms, type 1 and type 2. These two enzymes are involved in activation and deactivation of GCs (11β-HSD1 and 11β-HSD2, respectively), thus controlling cell proliferation. Expression of 11β-HSD1 generally results in decrease in cell proliferation, whereas expression of 11β-HSD2 is involved in increase in cell proliferation. Studies have illustrated that expression of these enzymes is altered in tumors and may create a microenvironment favorable for tumor growth. For instance, an earlier study on murine and human epidermal cells observed the involvement of 11β-HSD1 in the natural skin ageing process (Tiganescu et al., 2013). It was found that 11β-HSD1 expression increases in ageing skin, resulting in local GC excess and adverse effects such as: altered skin integrity, thinning, and impaired wound healing. This is due to the changes in the extracellular matrix (ECM) including: collagen atrophy, collagen disorganization, shredded appearance of collagen structure, and large inter-fibril spaces. This disordered ECM microenvironment is vital for tumor metastasis. Hence the study suggested that the local increase in 11β-HSD1 in ageing skin may increase the risk of skin cancer.

The GC receptor (GR) also plays a key role in the glucocorticoid pathway. It is a member of the nuclear hormone receptor family normally located within the cytoplasm of cells. Upon binding with cortisol it migrates to the nucleus and functions as a transcription activator or repressor that affects gene regulation to ultimately cause decreased cell proliferation (Schloossmacher et al., 2011; Slominski et al., 2014). Mutations in GR structure, aberrant processing of GR pre-mRNA and impaired functionality have been implicated in cancer progression.

Table 1 lists studies that have demonstrated dysregulation of cortisol, 11β-HSD enzymes, and glucocorticoid receptor in different types of cancers.

**Use of Corticosteroids in Malignancy and Premalignant Conditions**

GCs have a wide array of uses and effects in premalignant and malignant conditions. As strong anti-inflammatory agents, they have the ability to regulate cell fate by inducing expression of anti-apoptotic genes while suppressing apoptotic factors (Sung et al., 2009; Oakley and Cidliowski, 2011). Additionally, GCs are used as analgesics and antiemetics by patients receiving cancer treatment (Stojadinovic et al., 2007).

There are possible mechanism-based benefits in the use of GCs in pre-malignant conditions. An example of a potentially malignant condition that has potential for transformation is oral lichen planus. Progression to oral SCC can occur in up to 5.8% of cases (Kesic et al., 2009; Kaplan et al., 2012). Lichen planus is an autoimmune condition characterized by chronic inflammation (Ismail et al., 2007). It is postulated that chronic inflammation is a factor that may lead to the development of malignancy such as oral SCC. The powerful anti-inflammatory effects of GCs may, therefore, be a protective factor in this situation, and they are currently first-line treatment for this condition (Scully et al., 2000; Usatine and Tintiğan, 2011; Lodi et al., 2012). Nevertheless, the recent finding that GCs may bear cancer-promoting effects warrants further consideration in this area.

GCs are used as a monotherapy or in combination with other treatments to manage and treat many different forms of malignancy. GC monotherapy has been shown to have positive treatment outcomes in patients with breast and prostate cancer via proposed mechanisms of adrenocortical inhibition and adrenal androgen suppression, respectively (Keith, 2008). In a systematic review investigating the combination therapy of chemotherapy ± GCs, evidence supported such added treatment outcomes as decreased leukopenia, decreased thrombocytopenia, and an improved tolerance for increased chemotherapy dose in the group that received GCs as an adjunct to their chemotherapy compared to the group only receiving chemotherapy (Keith, 2008). Corticosteroids are also prescribed for the treatment of hypercalcaemia associated with malignancy. Renal tubular resistance to endogenous calcitonin during treatment for hypercalcaemia in malignancy can be overcome by the addition of corticosteroids to the treatment (Hosking et al., 1990). Despite their benefits in cancer treatment, GCs have also paradoxically been implicated in cancer progression (Nakane et al., 1990; Zhang et al., 2006a; Sidler et al., 2011). The postulated benefits of corticosteroids in preventing malignancy should therefore be balanced against the possible carcinogenic effects of immunosuppression warranting further research in this area.

In a study of the primary adult brain tumor, glioblastoma, the use of GCs as part of the standard treatment protocol was examined. The standard treatment protocol for primary adult tumors included maximum surgical resection, radiation therapy, and chemotherapy with about 99% of the patients also receiving perioperative corticosteroids with some patients receiving continued doses throughout the course of care (Seyfried et al., 2010). High dose GCs are generally prescribed for their anti-inflammatory mechanism to help combat radiation therapy associated brain swelling and tumor oedema (a conventional use and effect). However, GCs such as Dexamethasone that are regularly prescribed have been shown to considerably increase blood glucose levels. The blood glucose is a fuel for glycosis-dependent tumors and also
facilitates production of glutamate, a neurotransmitter linked with causing excitotoxic damage to neurons (Seyfried et al., 2010). Higher levels of blood glucose have been shown to accelerate brain tumor growth and lower treatment prognosis (Seyfried et al., 2010).

Glucocorticoid-induced resistance has been identified in cells of solid tumors when used with various anticancer drugs and with radiotherapy. Such observations were made in established carcinoma cell lines cultured in vitro, in xenografts on nude mice, and in primary cells that had been isolated from fresh surgical samples of solid tumors (Herr et al., 2003; Sui et al., 2006; Zhang et al., 2006b). Tumors analyzed were derived from bladder, brain, breast, cervix, colon or rectum, liver, lung, kidney, ovary, pancreas, and prostate. The finding that the tumor-associated glucocorticoid system is active in all these tumors (Cirillo et al., manuscript in preparation) adds a further level of complexity and raises the possibility that a de-regulated GC metabolism may serve as a tumor promoting mechanism in cancer (Fig. 1).

Overall, it can be ascertained from the wide array of research on this topic that the effects and uses of GCs in malignant conditions can be both beneficial and detrimental in terms of controlling tumor growth and progression.

Conventional uses of GCs have been shown to help treat malignant conditions either alone or in combination with other treatment modalities. Yet, the literature also supports the adverse effects of GCs in tumor progression and growth which are linked closely to the non-conventional effects of GCs in malignant conditions.

Conclusion

Based on preliminary data and evidence obtained from this literature review it can be suggested that in contrast to previous thought, increased levels of autocrine, paracrine, and exogenous cortisol are important to tumor progression. Hence, it is possible that alterations in the expression of enzymes regulating the levels of tumor-derived cortisol take place in cancers. It will be important, therefore, to assess the levels of expression of steroidogenic molecules, steroids, and receptors among normal and malignant epithelial cells as well as the correlation of the expression levels of steroid-related molecules to the clinical-pathological parameters of cancer.

In addition to shedding light on key patho-physiological mechanisms of SCC, characterization of the tumor-associated GC systems will have salient diagnostic, preventive, and

**TABLE I. Dysregulation of serum cortisol, 11β-HSDs, and glucocorticoid receptor in different types of cancers**

<table>
<thead>
<tr>
<th>Study</th>
<th>Type of cancer</th>
<th>Dysregulated molecule</th>
<th>Specific findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bernabe et al. (2012)</td>
<td>Oral squamous cell carcinoma (SCC)</td>
<td>Cortisol</td>
<td>Elevated plasma and salivary cortisol levels in SCC and also higher levels in advanced stage compared to initial stage.</td>
</tr>
<tr>
<td>Rasmussen et al. (2001)</td>
<td>Renal cell carcinoma (RCC)</td>
<td>Cortisol</td>
<td>Serum cortisol levels higher in RCC and positively correlated with tumor diameter and grade. Elevated cortisol levels also had worse prognosis.</td>
</tr>
<tr>
<td>Sephton et al. (2013)</td>
<td>Lung cancer</td>
<td>Cortisol</td>
<td>Reduction in 11β-HSD1 expression with increased keratinocyte proliferation. 11β-HSD2 expression increased in basal cell proliferating conditions such as SCC and SK.</td>
</tr>
<tr>
<td>Terao et al. (2013)</td>
<td>Skin SCC, basal cell carcinoma (BCC) and seborrheic keratosis (SK)</td>
<td>11β-HSD1 and 11β-HSD2 enzyme</td>
<td>11β-HSD1 normally not found in healthy lung tissue, was expressed in cancerous lung tissue.</td>
</tr>
<tr>
<td>Parks et al. (1998b)</td>
<td>DMS-79 cells (cell line derived from an ACTH-producing small cell lung)</td>
<td>11β-HSD2 enzyme</td>
<td>11β-HSD2 enzyme not found in normal post-menopausal ovarian tissue was increased in cancer cells.</td>
</tr>
<tr>
<td>Temkin et al. (2006)</td>
<td>Ovarian epithelial cancer</td>
<td>11β-HSD2 enzyme</td>
<td>Overexpression of 11β-HSD2 enzyme in cancer, compared with predominant expression of 11β-HSD1 enzyme in normal human osteoblast cells. Decrease in the abundance of 11β-HSD2 mRNA and enzyme activity in cancer tissue. Also demonstrated increase in 11β-HSD1 in some samples.</td>
</tr>
<tr>
<td>Bland et al. (1999)</td>
<td>Osteosarcoma</td>
<td>11β-HSD2 enzyme</td>
<td>In early skin papilloma GR expression is reduced but in late papilloma and in SCC, GR levels are similar or higher than normal.</td>
</tr>
<tr>
<td>Zbinkova et al. (2004)</td>
<td>Colorectal cancer</td>
<td>11β-HSD2 and 11β-HSD1 enzyme</td>
<td>No significant changes in GR gene structure and expression in cancer but resistance to glucocorticoid fluorocinolone acetonide more likely due to alterations in receptor function.</td>
</tr>
<tr>
<td>Lu et al. (2011)</td>
<td>Breast cancer</td>
<td>11β-HSD1 enzyme</td>
<td>In early skin papilloma GR expression is reduced but in late papilloma and in SCC, GR levels are similar or higher than normal.</td>
</tr>
<tr>
<td>Budunova et al. (1997)</td>
<td>Mouse epidermal papillomas and SCC.</td>
<td>Glucocorticoid receptor</td>
<td>Normal expression of GR, however its functionality altered by subtle changes in co-factors such as Nuclear co-repressor which was overexpressed in cancer.</td>
</tr>
<tr>
<td>Spiegelman et al. (1997)</td>
<td>Mouse epidermal papillomas and SCC.</td>
<td>Glucocorticoid receptor</td>
<td>No significant changes in GR gene structure and expression in cancer but resistance to glucocorticoid fluorocinolone acetonide more likely due to alterations in receptor function.</td>
</tr>
<tr>
<td>Waters et al. (2004)</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Mutations in GR gene structure lead to its impaired function.</td>
</tr>
<tr>
<td>Ray et al. (1996)</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Abnormal splicing of the GR transcript causes GR resistance to GC stimulation in cancer cells.</td>
</tr>
<tr>
<td>Parks et al. (1998b)</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Increased DNA methylation of promoter c leads to decreased GR expression.</td>
</tr>
<tr>
<td>Kay et al. (2011)</td>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Glucocorticoid receptor</td>
<td>Transformed cells contain functional GR but express cjun which antagonizes glucocorticoids.</td>
</tr>
<tr>
<td>Li et al. (1996)</td>
<td>Transformed A5 mouse lung cells</td>
<td>Glucocorticoid receptor</td>
<td>Normal expression of GR, however its functionality altered by subtle changes in co-factors such as Nuclear co-repressor which was overexpressed in cancer.</td>
</tr>
</tbody>
</table>
therapeutic clinical implications. Changes in the expression levels of components of the epithelial GC pathway may aid in the selection of novel markers of cancer progression. Identification of the presence of alterations in the steroid pathway in malignant epithelial cells would open new avenues in the management of inflammatory pre-malignant conditions such as lichen planus and provide basis for a mechanism-based approach to cancer treatment.

Fig. 1. Schematic representation of cancer-promoting glucocorticoid effects.

Keith BD. 2008. Systematic review of the clinical effect of glucocorticoids on nonhematologic malignancy. BMC Cancer 8:1


Antimicrobial activity and regulation of CXCL9 and CXCL10 in oral keratinocytes


Chemokine (C-X-C motif) ligand (CXCL)9 and CXCL10 are dysregulated in oral inflammatory conditions, and it is not known if these chemokines target microorganisms that form oral biofilm. The aim of this study was to investigate the antimicrobial activity of CXCL9 and CXCL10 on oral microflora and their expression profiles in oral keratinocytes following exposure to inflammatory and infectious stimuli. Streptococcus sanguinis was used as a model and Escherichia coli as a positive control. The antimicrobial effect of CXCL9/CXCL10 was tested using a radial diffusion assay. mRNA transcripts were isolated from lipopolysaccharide (LPS)-treated and untreated (control) oral keratinocyte cell lines at 2-, 4-, 6-, and 8-h time-points of culture. The CXCL9/10 expression profile in the presence or absence of interferon-γ (IFN-γ) was assessed using semiquantitative PCR. Although both chemokines demonstrated antimicrobial activity, CXCL9 was the most effective chemokine against both S. sanguinis and E. coli. mRNA for CXCL10 was expressed in control cells and its production was enhanced at all time-points following stimulation with LPS. Conversely, CXCL9 mRNA was not expressed in control or LPS-stimulated cells. Finally, stimulation with IFN-γ enhanced basal expression of both CXCL9 and CXCL10 in oral keratinocytes. Chemokines derived from oral epithelium, particularly CXCL9, demonstrate antimicrobial properties. Bacterial and inflammatory-stimulated up-regulation of CXCL9/10 could represent a key element in oral bacterial colonization homeostasis and host-defense mechanisms.

Bacteria colonize all surfaces of humans, but colonization is particularly dense in the lower gastrointestinal tract and in the oral cavity where streptococci represent a large proportion of the resident microflora.

It has previously been shown that bacteria can adhere to and invade host oral epithelial cells (1) and also that there are bacterial receptors present in saliva that can be absorbed onto oral mucosal surfaces (2). Among them, the Toll-like receptors (TLRs), expressed on host cells, are involved in the recognition of conserved bacterial patterns such as lipopolysaccharide (LPS), the cell-wall component of Gram-negative bacteria (3).

Following bacterially mediated tissue damage, keratinocytes produce a wide range of molecules, differentially regulated to across epidermal layers, including antimicrobial peptides (AMPs) and proinflammatory cytokines (4).

The chemokines monokine, chemokine (C-X-C motif) ligand (CXCL)9 and CXCL10, which are induced by interferon-γ (IFN-γ) and IFN-γ-induced protein-10, respectively, are two chemokines belonging to the CXC family, and both bind the same receptor, namely chemokine (C-X-C motif) receptor (CXCR)3 (5). Chemokines play an important role in directing the migration of specific immune-cell populations and, for some, direct antibacterial and/or antifungal activities have been demonstrated (6–8). As oral epithelial cells are known to produce chemokines (9–11), these may play a direct role in microbial defense.

It is known that CXC ELR-negative chemokines can be induced by LPS in some cell types (12) and that these chemokines are expressed during bacterial infections (13). It is also known that LPS is capable of inducing a range of cytokines and chemokines from epithelial cells predominately by signaling through TLR-4 (14).

The induction of these chemokines may be triggered in response to an alteration in the microbial flora, which could, in turn, cause an ensuing immune-cell infiltration. We recently found that CXCL9 and CXCL10 are dysregulated in oral inflammatory disease (A. Marshall, A. Celentano, N. Cirillo, M. McCullough, S. Porter, personal communication), but nothing is known about their antibacterial activity. Furthermore, while the induction of several different types of cytokine has
been demonstrated in oral epithelial cells after stimulation with LPS (11, 14–17), the roles of CXCL9 and CXCL10 in the infection and immunity of the oral cavity have never been investigated.

Therefore, the aim of this paper was to investigate the potential of CXC ELR-negative chemokines to mediate microbicidal activity on the Gram-positive species, *Streptococcus sanguinis*, one of the most prevalent residents of the oral microflora, and on the expression of chemokines in oral keratinocytes after exposure to infectious and inflammatory stimuli.

The chemokine (C-C motif) receptor (CCR)10 ligands – chemokine (C-C motif) ligand (CCL)27 (CTACK) and CCL28 (MEC) – are two C-C chemokines that bind the CCR10 receptor found to exert potent antimicrobial activity against *Candida albicans*, Gram-negative bacteria, and Gram-positive bacteria (7). We used these two chemokines as positive controls.

**Material and methods**

**Cell-culture techniques**

*Cell culture of normal human oral keratinocytes: All normal oral mucosa was obtained from healthy patients attending the Oral Surgery Clinic, Eastman Dental Institute (London, UK) for routine third-molar extraction. Three different normal human oral keratinocyte (NHOK) strains (NHOK1, NHOK2, and NHOK3) were isolated from the excised normal tissue. The samples were cut into pieces of approximately 1 mm² and cultured at 37°C/5% CO₂ in keratinocyte basal medium-2 containing the recommended growth supplements (Biowhiitaker, Wokingham, UK). The epithelial cells were then detached using 0.25% trypsin/1 mM EDTA. The viability of the keratinocytes was confirmed by Trypan Blue exclusion. All cell lines/stains were derived before 2001 and therefore were subject to ethics committee approval in the UK (18). The study was approved by the internal research committee at the Eastman Dental Institute, University College London (London, UK).*

*H357 cell culture: The oral squamous cell carcinoma cell line, H357, was established by Prine et al. (19). This cell line was grown in the same medium as described for NHOKs.*

**Bacterial cell culture and antimicrobial assessment**

All bacterial stocks were maintained frozen at −70°C in trypticase soy broth (TSB) (Becton Dickinson, Oxford Science Park, Oxford, UK) supplemented with 0.6% yeast extract (Oxoid, Basingstoke, UK) and 10% glycerol (BDH Chemical, Dorset, UK). Cultures were checked weekly, both visually and by Gram staining, for contamination with other bacteria. Stocks of *Escherichia coli* NCTC JM22 and *S. sanguinis* NCTC 10904 (provided by Dr Rod McNab at the Eastman Dental Institute, University College London) were plated on agar plates containing 5% TSB. They were grown for 48 h at 37°C/5% CO₂ and maintained by twice-weekly subculture onto TSB agar plates.

Each *E. coli* colony was collected and resuspended in 50 ml of 3% TSB. For the oral streptococcal species, three streaks of each species on a culture plate were resuspended in 10 ml of 3% TSB. The cultures were shaken, on an orbital shaker, at 250 rpm for 15–18 h at 37°C. Then, 50 ml of the *E. coli* culture or 2 ml of the oral streptococcal species culture was transferred into 50 ml or 10 ml of 3% TSB, respectively. This was shaken, on an orbital shaker, at 250 rpm for 3.5 h at 37°C. At this point, the culture was adjusted to an optical density (OD) of 1 at 620 nm.

To prepare the underlay, 50 ml of 100 mM sodium phosphate buffer, 5 g of agarose [low electroendosmosis (EEO); Sigma, Poole, UK], and 5 ml of 3% TSB were added to 1 l of distilled water. The pH was then adjusted to 7.4 and the agarose was dissolved by heating the solution in the microwave. The solution was dispensed into 50-ml aliquots and autoclaved. The overlay aliquots were then stored at room temperature until required for use in the radial diffusion assays, at which point they were heated in a microwave until fluid and then stored in a 60°C water bath.

Eight milliliters of *E. coli* or 16 ml of the streptococcal species was added to 5 ml of molten underlay and dispensed into a Petri dish, using a leveling tray. When the underlay was set, 3-mm holes were punched in the gel, using 10-ml pipettes (Sarstedt, Leicester, UK). Then, 5 μl of test solution diluted in 0.01% acetic acid was added to the wells.

For overlays, 10 g of agarose, low EEO (Sigma), was added to 6% TSB, dispensed into 50-ml aliquots, and autoclaved. The overlay aliquots were then stored at room temperature before use in radial diffusion assays. This plate was incubated for 3 h at 37°C, then 5 ml of overlay was added to the plate and incubated at 37°C overnight.

Radial diffusion assays were then performed, adding 5 μl of either recombinant human CXCL9/CXCL10/ CCL27/CCL28 (all Peprotech EC, London, UK) or 0.01% acetic acid to the wells before incubating the plates. The positive control for the assay was 100 μM tetracycline.

Images of the plates were taken using AlphaImager software (AlphaInnotech, Cannock, UK) and the zones around the cultures were measured from three different points from the end of the well.

**IFN-γ cell-treatment assay**

In a modification of the method utilized by Altenburg et al. (20), the H357 cells were seeded at 5 × 10⁵ cells per well in a Falcon 6-well plate (Becton Dickinson) in 3 ml of KBM-2 medium containing no hydrocortisone. The cells were incubated for at least 3–5 d until the cell culture was 60–80% confluent. Medium containing 1,000 U ml⁻¹ of IFN-γ was added to three wells and control cell-culture medium only was added to the remaining three wells. The cells were incubated for 48 h. The supernatant was extracted, centrifuged, and stored at −70°C. The adherent cells were washed with PBS (Gibco Life Technologies, Paisley, UK) before addition of 0.5 ml of TRI Reagent (Sigma). The suspension was then removed and stored at −70°C. The RNA was isolated as described below.

**CXCL9 and CXCL10 mRNA transcripts in oral epithelial cells in response to the presence of LPS and IFN-γ: mRNA isolation and semiquantitative RT-PCR**

mRNA transcripts for *18S, CXCL9*, or *CXCL10* were isolated from H357 cells, exposed or not exposed (control) to
LPS for 2, 4, 6, or 8 h. The RNA was extracted using TRI Reagent (Sigma) and 2 ml of Pellet Paint Co-precipitant (Novagen, Nottingham, UK) to visualize the RNA pellet.

Single-strand cDNA synthesis was performed. Two milliliters of RNA was added to 4 ml of deoxynucleotides (dNTPs) (2.5 mM) (Sigma), 2 ml of random hexamers (50 mm) (Ambion, Austin, TX, USA), and 9.5 ml of distilled H₂O (dH₂O). Then, 1 ml of RNAaseIN (Ambion), 2 ml of 10× MultiVRRT buffer, and 0.5 ml of M-MuLVRT (200 U/ml⁻¹) (Boehringer-Mannheim, Lewes, UK) was added and the reaction mix was incubated at 42°C for 1 h.

RT-PCR for 18S, CXCL9, and CXCL10

The magnesium concentration was optimized for each primer as follows: 1 ml of cDNA was added to 4 ml of dNTPs (2.5 mM), 5 ml of 10× buffer, 0.225 ml of AmpliTaq (5.0 U/ml⁻¹) (PerkinElmer, Waltham, MA, USA), 4 ml of each specific primer (5 mM), and 1.5, 3.0, or 4.5 mM MgCl₂ in each reaction, and dH₂O was added to give a final volume of 50 ml. The products were separated on a 2% agarose (GibcoBRL Life Technologies, Paisley, UK) gel and visualized by ethidium bromide (Sigma). Specific bands were visualized by ultraviolet transillumination in a MultiImage Light Cabinet (AlphaInnotech, Cannock, UK), and digital images were acquired and stored using Alphamager software (AlphaInnotech).

The CXCL9 (forward: 5'-ccaacccccacaggtge-3'; reverse: 5'-gcatgccagagtctcgttcg-3'), CXCL10 (forward: 5'-gcaatatttgcgacttgcttg-3'), and 18S ribosomal RNA (forward: 5'-ttcgagaatcttgacctg-3'; reverse: 5'-gcctgagcttcgctg-3') primers were generated for use in this study (Genosys-Sigma, Poole, UK). The thermocycler (Techne Genius, Cambridge, UK) parameters utilized were 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s.

For each primer the linear range was determined by repeating the above reaction with optimized magnesium concentration for each primer and stopping the reaction at 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, and 35 cycles. The mid-point of each linear range was determined by using intensity analysis of the bands with Alphamager software (AlphaInnotech), and this cycle length was utilized for each primer in subsequent reactions. 18S primer and 18S competitor primers (Ambion) were combined at ratios of 1:9, 2:8, and 3:7, respectively. For each of the primers CXCL9 and CXCL10, 4 ml of the 18S primer/competitor mix was added to the RT-PCR reaction. The band intensity of the 18S and specific primers was quantified for each primer in each sample using Phoretix 1D software (Phoretix, Newcastle, UK).

Unless specified otherwise, all experiments were performed at least in triplicate.

Results

Production of CXCL9 and CXCL10 mRNA transcripts in an oral epithelial cell line in response to IFN-γ

The production of CXCL9 and CXCL10 in oral mucosal keratinocytes was first assessed over time in preliminary experiments using the keratinocyte cell line H357 (Fig. S1). The expression of CXCL10 mRNA was detected in H357 cells as early as 3 h after treatment with IFN-γ and appeared to peak at 24 h. In contrast, the control cells showed virtually undetectable levels of mRNA over the same time period. The patterns shown by CXCL9 and CXCL10 mRNA transcripts were similar, being biphasic patterns that showed an initial rapid induction of mRNA in the stimulated cells followed by a second peak at 24/48 h. Thus, the expression of CXCL9/CXCL10 in H357 cells can be significantly enhanced by IFN-γ in a time-dependent manner, with a peak after 48 h (Fig. 1). Similar results were obtained in primary NHOKs (Fig. 2). These data were confirmed at the protein level by ELISA (data not shown). These results indicate that the expression of CXCL9 and CXCL10 in oral epithelial cells is significantly enhanced by IFN-γ in a time-dependent manner.

Antimicrobial effect of CXCL9, CXCL10, CCL27, or CCL28 on S. sanguinis and E. coli

CCL27 and CCL28 were used as positive controls. The clear zones of bacterial growth depletion as a result of the antimicrobial activity of the chemokines are indicated in Fig. 4 and Fig. 5. All chemokines investigated demonstrated a level of antimicrobial activity at the tested concentration (Table 1). It was found that CXCL9 was the most effective chemokine against both S. sanguinis and E. coli; CCL27 and CXCL10 had a

![Image](image-url)

Fig. 1. Expression of 18S, chemokine CXCL9, and chemokine (C-X-C motif) ligand 10 (CXCL10) mRNAs in the H357 cell line, either treated with interferon-γ for 48 h (IFN) or untreated for 48 h (CON). The left column ‘m.w.’ indicates the molecular weight. The molecular weight of CXCL9 expression is located at 351 bp, CXCL10 expression is at 601 bp.
less effective antimicrobial action against *S. sanguinis* than against *E. coli*; and CCL28 had more effective antimicrobial action against *S. sanguinis* than against *E. coli*. These results clearly demonstrate that the epithelial-derived chemokines CXCL9 and CXCL10 exert antimicrobial activity.

**Discussion**

Chemokines are known to have antimicrobial effects but little is known of the action of chemokines derived from the oral mucosal epithelium. The present study is the first to examine the expression of CXC ELR-positive chemokines by oral epithelial cells when stimulated with bacteria-derived products such as LPS. The present study has established that a cell line of oral origin is capable of expressing CXCL9 and CXCL10 mRNAs when stimulated with LPS. The production of these chemokines was also enhanced by inflammatory stimuli such as IFN-γ. Expression of CXCL10 mRNA was increased in H357 cells after stimulation with LPS, whereas expression of CXCL9 mRNA was not induced over the same time-period. Previously, we have shown that CXCL10 can act as a potent chemoattractant of lymphocytes; hence, this local production of CXCL10 by oral epithelial cells in response to LPS could have important effects on oral inflammation, perhaps crucially in the initial stages of inflammation.

Previous studies have also shown that LPS treatment alone can induce or enhance expression of CXCL10 mRNA in several different cell types (12, 21–23). However, in contrast to the present findings, LPS stimulation did not induce production of CXCL10 from cultured skin keratinocytes (24), suggesting perhaps that oral keratinocytes are more responsive than cutaneous keratinocytes to LPS stimulation. This difference in expression may reflect the high bacterial load in the oral cavity.

The rapid expression of CXCL10 mRNA observed in the present study is in accordance with that of murine macrophages (17, 25) although LPS stimulation may be more transient than IFN-γ relative to stimulation of...
CXCL10 (10). This short-term effect may be essential to avoid over-stimulation of CXCL10 in response to resident bacteria in proximity to the epithelium. Interleukin-10 is known to be able to down-regulate production of LPS-induced CXCL10 in macrophages (22). This cytokine is present within oral lichen planus lesions and is increased in serum and saliva from patients with oral lichen planus (26), and thus may act to down-regulate expression of CXCL10 in oral inflammation.

There are few reports of LPS-induced production of CXCL9. The present study revealed that CXCL9 mRNA was not expressed by H357 cells when stimulated with LPS. In contrast to the present study, CXCL9 mRNA was found to be expressed in LPS-stimulated murine dendritic cells (12); however, in another study, this chemokine was not induced in the same murine cell line by LPS, despite induction of CXCL9 by IFN-γ (25).

It is possible that CXCL9 displays a delayed response, in comparison with CXCL10, as induction of CXCL9 mRNA in lung tissue of mice treated intravenously with LPS shows a later induction than CXCL10 mRNA, and is never expressed at the same levels as CXCL10 (25).

Many studies report that LPS and IFN-γ act synergistically to induce the production of high levels of CXCL10 mRNA, for example, in breast carcinoma cells (21, 22). It is then possible that CXCL10 levels could be enhanced in oral inflammation where there is both LPS and IFN-γ, perhaps through the enhancement of specific TLRs (27).

Gram-positive bacteria, such as S. sanguinis, contain components other than LPS that are known to stimulate chemokine release from various cell types and it would be interesting to determine whether these are also capable of stimulating production of CXC ELR-negative chemokines in oral epithelium. Many Gram-positive bacterial components act on a different Toll-like receptor, TLR-2, which is functionally expressed on keratinocytes (28). However, TLR-2 agonists do not induce production of CXCL10 in macrophages (29) or dendritic cells (30) in vitro. This suggests that TLR-2 agonistic bacterial products would also not induce CXCL10 in epithelial cells. Therefore, only products bound by TLR-4 would be influential in up-regulating CXCL10 production in epithelial cells. In addition, as LPS-mediated CXCL10 production is TLR-4 dependent, this strongly suggests that oral keratinocytes bear functional TLR-4, and therefore stimulation of epidermal cells with LPS is not caused by TLR-2 agonist contaminants in LPS preparations, as previously suggested (28).

Chemokine modulation in oral cells by bacterial products is thus complex and many different factors, including T-cell contact, may play a factor in chemokine induction during an immune response.

The present studies suggest that in certain circumstances, bacterial products could stimulate oral

Table 1

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>CXCL9</th>
<th>CXCL10</th>
<th>CCL27</th>
<th>CCL28</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>34.6 ± 6.80</td>
<td>14.9 ± 5.65</td>
<td>20.6 ± 0.90</td>
<td>15.4 ± 6.04</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>44.4 ± 14.05</td>
<td>8.4 ± 3.52</td>
<td>10.0 ± 5.45</td>
<td>21.0 ± 5.70</td>
</tr>
</tbody>
</table>

Values are mean ± SD of three experiments and represent the diameter of bacterial clearance (minus the well diameter) multiplied by 10 to convert to units. The negative control had no effect on the microbial growth in any of the experiments tested.
epithelial cells to produce an inflammatory response through TLR-4 agonists (perhaps after continuous stimulation with TLR-2) and this may induce epithelially derived CXCL10-mediated inflammation. This inflammation would presumably be characterized by activated memory T-cell infiltration localized under the basal epithelium, reminiscent of the pathology of oral lichen planus.

The chemokine CXCL9 was shown to be a potent antimicrobial agent against both S. sanguinis and E. coli, as was CXCL10, but to a lesser degree than CXCL9, confirming the results of the study by Cole et al. (6). Although only one oral bacterial species was tested in the present study, the results hint that the antimicrobial properties of these chemokines may assist in countering bacterial growth in the oral cavity.

The choice of using S. sanguinis as a model has been driven by the delicate role played by this bacterium in maintaining the balance of the oral flora. Streptococcus sanguinis is commonly found in healthy tissues as a pioneer colonizer, and it is implicated in modulating the virulence of bacterial biofilms (31). Furthermore, significant inhibitory effects of the intracellular proteins produced by S. sanguinis on the growth and the morphology of many other components of the oral flora, such as Prevotella intermedia, Porphyromonas gingivalis, C. albicans, and Candida tropicalis, and their biofilms, have been demonstrated (32, 33). This allows us to conjecture and expect that a series of chain effects of the antimicrobial activity of CXCL9 and CXCL10 would occur in vivo. As previously shown for the two chemokines, CCL27 and CCL28, for which a wide spectrum of antimicrobial activity is well established, the action of CXCL9 and CXCL10 expected against other microorganisms of the oral cavity should follow a similar pattern (7).

The low production of CXCL9 by oral epithelial cells following stimulation with IFN-γ or LPS could potentially be a means of avoiding an overactive antimicrobial response as a result of the potent antimicrobial activity of CXCL9.

In our study, both CCL27 and CCL28 exerted antimicrobial properties against E. coli and S. sanguinis. The chemokine CCL28 has been shown to have microbicidal activity against a wide range of bacteria (both Gram-negative and Gram-positive) and yeasts (7, 34), and our findings confirmed that this chemokine was also effective against the oral commensal, S. sanguinis. This is only the second report of the antimicrobial effect of CCL28 upon enterobacterial E. coli after the study reported by Berra et al. in 2014 (34). The production of CXCL10 by oral epithelial cells, in inflammatory conditions, such as oral lichen planus, may be induced by resident bacteria that contain TLR-4 agonists. If this inflammation resulted from constant TLR-2 stimulation, this may cause a CXCL10-based inflammation and a resultant influx of memory T-helper 1 CD4+ cells. Furthermore, CXCL10 is antimicrobial at high concentrations. It may be up-regulated by the presence of bacteria, thus playing a role in the defense of oral epithelial cells. The antimicrobial activity of the chemokines tested is likely to be an important mechanism in the homeostasis of oral bacterial colonization. Any IFN-γ in the oral epithelial area (which could presumably be produced by the infiltrating T-helper 1 cells) may synergize with TLR-4 agonists to cause an increased inflammatory state. Further studies are still warranted to confirm these novel findings.

References


15. RYU OH, CHOI SJ, LINARES AM, SONG IS, KIM YJ, JANG KT, HART T. Gingival epithelial cell expression of macrophage


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Figure S1. 18S, CXCL9 and CXCL10 mRNA expression in the H357 cell line.
Erythema multiforme (EM) is a group of acute immune-mediated disorders that can affect the skin and mucous membranes. It has previously been classified into 4 major variants: erythema multiforme minor (EMm), erythema multiforme major (EMM), Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN, also known as Lyell disease). 1-3

Many authors still consider EMm, EMM, SJS, and TEN to be a single disease continuum, varying on a spectrum of clinical severity. Others consider EM a separate entity to SJS and TEN, particularly due to its strong association with infections, such as herpes simplex virus (HSV). 3 This is in contrast to the majority of cases of SJS and TEN, which are commonly medication induced. Additionally, cutaneous findings may be distinct, although clinical overlap does exist. 3,9

The development of EM has been linked to a type 4 cytotoxic reaction, mediated by T lymphocytes and triggered by numerous factors. These include infections (particularly HSV-1 and HSV-2), medication use, malignancy, autoimmune diseases, radiation therapy, and immunizations. 10

Many pathogens have been associated with EM, including Mycoplasma pneumoniae, Chlamydophila pneumoniae, hepatitis viruses, Epstein-Barr virus, Orf virus, human immunodeficiency virus, cytomegalovirus, Mycobacterium leprae, and variella zoster virus, as well as several vaccination agents (small pox, rabies, and human papillomavirus). 11-31 Additionally, endocrine triggers have been implicated in the occurrence of EM. 32,33

The differential diagnosis of EM encompasses a wide range of diseases, including urticaria, fixed drug

Statement of Clinical Relevance

Prompt recognition of erythema multiforme by all oral health clinicians is important to prevent diagnostic delay as oral mucosal involvement may precede extension of the disease. Identification of trigger factors and diagnostic features is pertinent as illustrated by this case series.
eruption, bullous pemphigoid, paraneoplastic pemphigus, Sweet syndrome, Rowell syndrome, polymorphous light eruption, and cutaneous small vessel vasculitides. To improve the diagnostic accuracy, histopathologic analyses and others laboratory tests can be used.\(^\text{1,14}\) Cutaneous involvement is variable, ranging from isolated symmetric targetoid lesions, which are commonly distributed on the extensor surfaces of the extremities; on the hands; around the elbows and knees with extensive involvement of the arms, legs, and trunk; and with or without oral or other mucous membrane involvement.\(^\text{1,14}\) Both SJS and TEN can be fatal, with a reported mortality rate of 1% to 5% and 25% to 35%, respectively.\(^\text{3,4}\)

EM is usually a self-limiting disease, resolving within weeks without significant sequelae. However, in a minority of cases, the disease may recur frequently, establishing a well-defined variant known as “recurrent EM.”\(^\text{4,5,30}\)

Most patients with EM can be managed with symptomatic therapy along with identification and modification of all the suspected initiating factors. However, patients with severe EM may require hospitalization for hydration, analgesia, antiviral therapy, and systemic therapy with corticosteroids, immunosuppressants, and/or antiviral suppressive therapy.\(^\text{1,14,35}\) Daily antiviral therapy has been used successfully to control the disease in patients with recurrent EM.\(^\text{5,7}\)

Knowledge about EM is full of conflict. Diagnostic criteria are not universally accepted, and the diagnosis continues to be one of exclusion, based on clinical history. Epidemiologic data and case series in the literature are dated. Additionally, there is a lack of extensive EM case series demonstrating the range of oral presentations. Here, we present the analysis of a series of 60 patients affected by EM with oral involvement. The range of clinical presentation is illustrated.

**MATERIAL AND METHODS**

Data were collected from clinical records of 60 EM patients, diagnosed and treated between 1982 and 2014 in 2 centers: the Oral Medicine Unit, Federico II University of Naples in Naples, Italy (Center 1), and the Oral Medicine-Oral Pathology Department, University of Medicine and Pharmacy in Bucharest, Romania (Center 2).

The study was approved by the Ethics Committee of the University “Federico II” of Naples in June 2014. Data were collected by 2 blinded researchers and confirmed by 1 supervisor, the heads of the respective Oral Medicine departments. A digital template, developed at Center 1, was utilized at both centers. Data collected included age, gender, habits, number and duration of outbreaks, previous or concomitant infections, antecedent drugs or other precipitants, presence of mucosal and cutaneous lesions, oral sites involved, histopathologic findings, and details of hospitalization.

Inclusion criteria of the first phase of the study required a definitive diagnosis of EM and/or SJS in the clinical discharge summary. Acknowledging the absence of unique validated diagnostic methods for EM,\(^\text{3,7}\) this study included only those cases in which other diseases were clearly excluded from the differential diagnosis. Cases were only included in the study if the following data were clearly recorded: patient medical history, medication history, outbreaks details, course of the illness (self-limiting and well-responder to symptomatic therapy), evidence of mucocutaneous lesions (fixed targetoid lesions, raised atypical papules, mucosal involvement, or a combination of these) with clinical and/or photographic morphology descriptions and/or information on the extent of mucocutaneous involvement, signs, and symptoms. The clinical-based approach described by Al-Johani et al (2007) was used to classify the clinical forms of our cases.\(^\text{3}\) The analysis of site distribution was performed with the Pearson \(\chi^2\)-squared test. The dependence analysis between lesion site and age and significant difference between medians was determined using a Mann-Whitney U test.

**RESULTS**

Initially, 67 EM medical records were included; however, 7 cases were inadequate in description or did not satisfy the diagnostic criteria and were excluded from the study, thus leaving 60 patients. Fifteen EM patients were obtained from Center 1 and 45 patients from Center 2.

Thirty-one patients (51.7%) were male, and 29 (48.3%) were female, with a mean (±SD) age of 37.9 years (±18.1) at the time of diagnosis. Seventeen patients were smokers, and 1 case of alcohol abuse was reported.

Fifty-one (85.0%) patients were classified as EMm, and 9 (15%) were classified as EMM.

The mean duration of the EM outbreaks was 7 ± 6 days (ranging from 2 to 42 days). The number of previous outbreaks ranged from 0 to 10 (mean ± SD = 1.4 ± 2.0). Twenty-nine patients (48%) had not experienced a previous outbreak, 9 patients (15%) had a single previous outbreak, and 22 patients (37%) had 2 or more outbreaks. Sixteen cases had accompanying histopathology with predominant findings of necrosis, intraepithelial exocytosis, necrotic keratinocytes, Civatte bodies, edematous corium hyperemic vessels, lymphohistiocytic inflammatory infiltrate with rare eosinophils, and subepidermal clefts. No deeper extension of the infiltrate or prominent melanin incontinence was observed, which allowed us to exclude cases of fixed drug eruptions. Drugs were suspected as
Table I. Data of 60 patients: epidemiology, predisposing factors and clinical features

<table>
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<tr>
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<th>Other infections</th>
<th>Drug antecedents/other precipitants</th>
<th>Other affected mucosae</th>
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<th>Classification</th>
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<td>K sigma 1 year before</td>
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Involved oral sites: B, buccal mucosa; L, labial mucosa; V = vermilion border; T, tongue; A, alveolar mucosa; F, floor of the mouth; P, palate; TP, tonsillar pillar.
EMm, erythema multiforme minor; EMM, erythema multiforme major; SJS, Stevens-Johnson syndrome; HBV, hepatitis B virus; HCV, hepatitis C virus; HSV, herpes simplex virus.
precipitants in 28 patients (46.7%), the most implicated being antipyretics, food allergens, and antibiotics.

HSV infection was suggested to be a triggering factor in 18 patients (30.0%), 10 of whom had supportive serologic HSV testing (8 enzyme-linked immunosorbent assay test and 2 polymerase chain reaction [PCR]). In 13 patients (21.6%), a medical history of previous infections was reported, 11 of which were concomitant to the EM outbreak. These infections included pneumonia (3 cases), urinary infection (2 cases), and *M. pneumoniae* (1 case). Two patients were found to be positive for hepatitis C. A concomitant history of candidiasis was reported in 3 cases (2 oral and 1 genital). Hospitalization of the patient was required in 7 cases (11.6%). Table I presents the summary of clinical data from case series. All infections reported in the clinical record within 30 days from the diagnosis of EM were recorded under consideration as “concomitant” infections. The infections described in Table I thus occurred within 30 days before the EM presentations, unless otherwise specified.

Exclusive oral involvement was observed in 29 patients (46.6%). All but 1 patient had involvement of...
multiple oral sites. The buccal mucosa was the most commonly involved oral site (75%) followed by the vermilion border (71.7%) and labial mucosa (61.7%). Twenty-four of 60 patients (40.0%) had concomitant involvement of all of these sites, and 44 (73.3%) patients had involvement of at least 2 of 3 sites. Details of sites involved for each case are described in Table I.

The floor of mouth was significantly more commonly involved in females (37.9%) than in males (6.5%) ($P = .003$) (Table II). Involvement of the tongue was significantly related to age (median interquartile range: Yes = 32-33 years; no = 25-21 years; $P = .013$). Clinical forms were significantly associated with gender (female: EMM = 24.1%; EMm = 75.9%; male: EMM = 6.5%; EMm = 93.5%; $P = .045$). Other significant results of the dependence analysis are shown in Tables III and IV.

**DISCUSSION**

Current literature regarding the epidemiology of EM remains scarce and controversial. This is reflective of the lack of universally accepted classification criteria. Additionally, there may be a component of underreporting, particularly of cases of short duration when hospitalization is not required. This study presents the largest oral EM case series in last 2 decades,\(^{38-39}\) describing the multiple clinical features that characterize this group of diseases, and the dependence analysis of associated variables.

The consensus is that EM and related disorders occur predominantly in young adults, with majority of cases occurring between the second and fifth decades of life.\(^{1,39}\) There is no a clear predilection for gender or race. However, variation in age at presentation should not be underestimated, as several cases of pediatric patients have been reported, including neonates.\(^{20,40-45}\) In our case series, the mean age was 37.9 years (range 7-78 years), with no significant difference between males and females. Interestingly, there was a significant gender predilection for the clinical forms with EMM being more frequent in the females and EMm being much more frequent in males. Clinical forms were additionally significantly related to age (see Table IV).

The most commonly reported triggers are infections and medications, with HSV-1 and HSV-2 being the most commonly reported precipitators of EMm and EMM. Medications and HSV, taken together, were implicated in approximately 67% of cases in our series.

Typically, the onset of EMm and EMM lesions begins 10 to 14 days after the clinical manifestation of an HSV infection.\(^3\) Unfortunately, the number of studies using confirmatory PCR to assess for the presence of HSV-DNA is low, with conclusion of infection based predominantly on clinical history. The reported association of HSV with the recurrent variant of EM is between 61% and 100%.\(^{14}\) The percentage of patients affected solely by HSV without a history of medication use in our series was 21.6% (13 cases), which is similar to the 23% reported by Wetter and Davis in 2010, but dissimilar from the 70% to 100% values reported by Schofield in 1993 and Huff in 1983.\(^{2,35,46}\)

Five more cases were also potentially associated with HSV, but the patients were using medications simultaneously. It is not uncommon for patients affected by EM, in association with a concomitant or previous infection, to have started drug therapies (e.g., antibiotics, antivirals, or anti-inflammatory drugs). Of the total of 18 patients (30.0%), 10 patients (16.7%) were screened for HSV exposure by using serologic tests. Some authors have suggested the use of a Tzanck smear test, which is an easy and inexpensive test to identify viral balloon cells.\(^3\) However, PCR assays are much more sensitive and target a wide range of infectious agents. Thus, PCR should form part of the mandatory criteria to clearly identify the trigger and to support the use of antivirals in particular patients.

Currently, the literature consistently supports medications as precipitants in more than 50% of EM episodes.\(^1,3,48\) Some authors have reported figures as low as 10% of cases, although this has not been our experience.\(^49,50\) Moreover, the list of the medications associated with the induction of EM continues to expand and include new categories of drugs, such as tyrosine-kinase inhibitors; biologic agents, such as tumor necrosis factor-\(\alpha\) inhibitors, phosphoinositide 3-kinase inhibitor, retinoids.\(^51-59\) In our experience, medication use has a marked role as a triggering factor in 28 patients (46.7%). The most commonly implicated medications were nonsteroidal anti-inflammatory drugs, antibiotics, antifungals, and antivirals. In 1 case, the oral contraceptive pill was also considered to be associated. Alcohol consumption has been reported as a risk factor in drug-induced EM, particularly if it is associated with antiepileptic therapies.\(^60,61\) Our case series included 1 case of alcohol abuse with unknown significance.

Food-borne antigens, triggers first suggested by Lozada in 1978, were supported by evidence as playing an important role in 7 cases of our series.\(^62\) Another suggested trigger is radiotherapy, although concomitant medication use is an expectant common confounder.\(^63-65\) There was no history of radiotherapy present in our case series.

In a previous case series, *M. pneumoniae* infection was reported to be responsible for almost two-thirds of SJS cases, particularly in childhood cases, although it was never associated with a typical EM eruption.\(^66\) In contrast, the role of this infection in EM has recently been challenged and re-evaluated, suggesting that the *M. pneumoniae*-induced rash and mucositis may
represent a distinct syndrome from EM and SJS. An established association with *M. pneumoniae* was clearly reported in only 1 of our 3 pneumonia cases, a 73-year-old female. Positive culture tests for *Candida* were only found in only 3 (5%) of our cases (2 oral and 1 genital), a lower rate than the 20% reported by Lozada-Nur in 1989. Cases of “persistent EM,” a rare variant of the disease characterized by uninterrupted lesion eruptions often linked with Epstein-Barr virus infection, were not present in our series. The character of presenting clinical features of EM potentially can change during the course of the disease, leading to an overlap of the variants. Oral involvement in EM is reported in 60% to 70% of cases. In our series, exclusive oral involvement was observed in 29 patients (46.6%). Lesions were predominantly erosive or bullous with the buccal mucosa, vermilion border, and labial mucosa being the most commonly affected sites. Figure 1 presents typical crusting of the vermilion border seen in exclusively oral EM. This site may
revised to include its particular epithelial and connective tissue structures and the immunologic composition. In these cases of exclusive oral involvement, clinicians should consider the differential diagnosis with focus on the timing of the outbreak, history of drug consumption, the course of the illness, atypical sites involved, and symmetric distribution of the lesions. Eight cases (13.3%) of our series presented with at least 1 additional site of mucosal involvement, genital in 4 cases (6.7%), ocular in 2 cases (3.3%), nasal in 1 case (1.7%) and genital and ocular in 1 case (1.7%). Cutaneous involvement was found in 25 cases (41.7%), particularly perioral lesions in 10 cases (16.7%), followed by acral in 5 cases (8.4%) and arm lesions.

According to the current classification criteria, different clinical forms are broadly categorized on the basis of the presence, morphology, and extension of the mucocutaneous disease. Some authors accept a diagnosis of EM in patients with less than 10% of body surface area (BSA) involvement, defining the disease beyond this as SJS/TEN. All the cases described in our case series had no more than 10% of BSA involvement. EM is further subdivided into EMm and EMM and may be defined by either the presence or absence of mucous membrane involvement or the extent or number of mucosal sites involved. In our study, we utilized the classification system described by Al-Johani et al. (2007), which distinguishes EMm and EMM. Fifty-one (85.0%) of our patients were classified as EMm, and 9 (15%) were classified as EMM.

The most accepted criteria to differentiate the diagnoses of EMM and SJS, regardless of BSA involvement, are centered on the basis of the presence of systemic symptoms and a positive Nikolsky sign. Utilizing such criteria, 3 of our EMM cases that required hospitalization could be reclassified as mild forms of SJS.

More than half of the patients had experienced at least 1 previous outbreak, which supports the commonality of recurrence. Seven of our cases (11.6%) could be considered the recurrent variant of EM, which is lower than the rate reported by Cretu et al (20%).

The limitations of this retrospective study include acceptance of the variations in the use of clinical tests and the limited use of HSV PCR and dietary records across the 30 years during which these patients were observed. Furthermore, the difficulty in collecting recent data on the same patients through recall affected our ability to better define the clinical behavior of the disease and patient responses to previous exposures to triggers, and therefore the outcome of our cohort.

CONCLUSIONS
EM may present initially with oral mucosal involvement before an increase in disease severity. Prompt recognition, particularly of bilateral bullous and ulcerative involvement of the buccal mucosa, the labial mucosa, or the vermilion border, is important to avoid any delay in diagnosis. Although approximately half of those presenting with EM report no previous episodes, it remains unclear which of these patients will progress to recurrence, so all patients should be informed and encouraged to have an awareness of their exposures to drug and food antigens. With the lack of universally accepted classification criteria and the absence of specific diagnostic tests, EM, especially in its mild forms, continues to be an underestimated disease.

REFERENCES


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LETTERS TO THE EDITOR

Oral erythema multiforme: trends and clinical findings of a large retrospective: European case series

To the Editor:

Celentano et al. are to be congratulated on their recent publication. This excellent addition to the literature reports a retrospective case series of 60 oral erythema multiforme (EM) patients. However, presently, diagnostic criteria that distinguish between EM major (EMM), EM minor (EMm), Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN) are available. Celentano et al. have combined all of these diagnostic entities within the category of EM. This grouping is certainly appropriate, since the study patient population was initiated in 1982, when all such diagnoses would have been described within the category of EM. The authors commented that diagnostic criteria between EMM, EMm, SJS, and TEN presently are not universally accepted. However, a case can be made that such diagnostic criteria are presently relatively well established, although not universally accepted.

In 2005, Williams and Conklin summarized the dermatologic diagnostic standards for EM, SJS, and TEN, as reported by Bastuji-Garin et al., Assier et al., and Auquier-Dunant et al., and Cote et al. These standards describe EM, SJS, and TEN as separate diagnostic entities with relatively well-defined diagnostic characteristics. Furthermore, Ayangco and Rogers defined the similarities and differences between EMm and EMM.

Hopefully in the near future, the oral medicine and oral and maxillofacial pathology communities will undertake confirmation of universally accepted diagnostic standards for EMM, EMm, SJS, and TEN, possibly at the next World Workshop of Oral Medicine.

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REFERENCES

In reply: Oral erythema multiforme: trends and clinical findings of a large retrospective: European case series

In reply:

The authors are very appreciative of comments presented by Professor Brown regarding our recent publication, which highlighted the applicability of diagnostic criteria proposed by several authors in the last two decades.

We agree that future studies should consider the use of these presently well-established diagnostic criteria differentiating between erythema multiforme minor (EMm), erythema multiforme major (EMM), Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN). This may allow the clinical community to achieve more accurate epidemiologic data that can contribute to a better understanding and management of the disease.

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Simultaneous removal of third molars and completion of a sagittal split osteotomy: effects of age and presence of third molars—a commentary

To the Editor:

This was a new research hypothesis, and the authors tried to address the same with a retrospective cohort study design.1 We congratulate the authors on their approach and effort to address the same. We could not draw any conclusions from the article and request the authors to provide clarifications in the some areas as described below.

The authors have stated in the results section: “When correlating the occurrence of adverse fractures by the number of patients, slight evidence of a difference was noted for presence or absence of third molar or degree of impaction and no statistical differences were noted for sex (P = .073; P = .069; and P = .336, respectively) (Table 4)” and later in the results section again stated: “As shown above, there was no relation between presence of third molar and adverse fracture when the occurrence per patient was considered.” These two statements contradict each other. Can statistical difference be considered in this comparison?

Another instance was in the results section: “However, when evaluating the occurrence of adverse fracture correlated to the presence of third molar with the number of osteotomies performed, there was a significance association between the bad splits with presence of third molar in the site of the osteotomy (P = .05)” but later below Table 5, the authors stated, “The association between rows (groups) and columns (outcomes) is considered to be not quite statistically significant.” Can statistical difference be considered in this comparison? Can we compute the odds ratio and confidence intervals to quantify the association?

In Table 4, the authors have compared the mean age between “yes” and “no” of a bad split variable using the analysis of variance F test. Can this comparison be done with the help of independent sample t test or Student t test? The mean ages in the tables and the text did not match. We request the authors to address the above queries so that readers can have a definitive conclusion from this manuscript.

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REFERENCES


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Lichen planus of the lips: an intermediate disease between the skin and mucosa? Retrospective clinical study and review of the literature

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Conflicts of interest: None.

Abstract
Background Lichen planus of the lips (LPL) is not frequently described in the literature. The objective of this study is to investigate the clinical outline, behavior, and prognosis of LPL.

Methods Clinical data of patients with true oral lichen planus (LP) involving the lips, diagnosed and treated at our Oral Medicine Unit (University Federico II of Naples, Italy), have been collected and analyzed. Concurrently, a PubMed search was carried out from 1950 to March 2014 to assess epidemiological and clinical data about LPL.

Results Our case series revealed 13 patients (female/male ratio 0.4) with a mean (±SD) age of 71.85 years (± 6.72). The lower/upper lip involvement ratio was 9, mainly with mixed clinical patterns (76.9%), generally including erosion and mild keratosis. In most cases, the lips were involved with other oral sites but displayed a better evolution of the lesions. The literature review showed 21 reports of LPL (35 patients, female/male ratio 0.4) with a mean (±SD) age of 45.35 years (± 16.19).

Conclusions In the literature, erosive (28.57%) lower lip lesions showed a clear predominance (lower/upper lip ratio 6.5). One case of malignant transformation was also reported. The prevalence of isolated LPL was clearly reported only in two studies, ranging from 0.51% to 8.9%. In our patients, lesions were mostly found at the inner border of the lower vermillion and presented a tendency for self-limitation, or to regression after treatment, like cutaneous lesions. The lip lesions were small and easy to overlook, and therefore the prevalence of these lesions may have been underestimated.

Introduction

Lichen planus (LP) is a chronic T-cell-mediated mucocutaneous inflammatory disease with an etiology and pathogenesis that is not completely understood. It affects 1–2% of the general adult population, with the highest frequency in women over 40. Histological characters pathognomonic for LP are the liquefactive degeneration of the basal cell layer, a juxtaepithelial band-like zone of cellular infiltration, predominantly lymphocytic, and the absence of epithelial dysplasia. An interruption of the basement membrane, appearance of eosinophilic Civatte bodies, parakeratosis, acanthosis, and histological cleft formation may also be present.

The clinical presentation is complex, with white, red, or mixed lesions. Six variants for oral LP (OLP) have been described: reticular, papular, plaque-like, erosive, atrophic, and bullous. The reticular form, with white striations (Wickham’s striae) is the most typical. These variants can also coexist and change during the course of the disease.

Oral involvement is quite common and is often the only site of manifestation of the disease. OLP typically affects the buccal mucosa, tongue, and gingiva, with symmetrical and bilateral lesions, and less frequently the lips and the palate.

Coincident cutaneous lesions appear in approximately 15% of the patients, presenting as purple, polygonal, pruritic papules on the wrists, ankles, and genitalia. Other dermatological features are nail pitting, pterygium formation, nail loss, and scarring alopecia.

The diagnosis results from integration of the histological and clinical data, as well as the medical history, which...
is necessary to exclude lichenoid reactions to drugs, dental materials, or graft-versus-host disease.3

Lip involvement, particularly if isolated, is not common, and few case reports are described. Lip lesions are probably subject to a variety of injuries, such as biting, application of makeup, or sun exposure, which can change the clinical features and mimic lesions of a different nature. Therefore, LP of the lips (LPL) is difficult to detect, and it is often misdiagnosed.

On the other hand, it has been suggested that injuries acting on lip lesions in OLP could increase the risk of malignant transformation,4 so that the diagnosis and management of such lesions are mandatory.

In this paper, we present a retrospective study of patients affected by LPL, who were diagnosed and treated at the Oral Medicine Unit of the University Federico II of Naples, Italy.

Contextually, a review of the literature about lip involvement in the course of OLP has been performed to integrate the clinical data discussed. The purpose of this paper is to identify distinct features of LPL relating to its clinical presentation, evolution, and response to treatment, and concurrent oral or skin lesions.

Materials and methods

All the clinical records of OLP treated at the Oral Medicine Unit of the University Federico II were scanned, and all the cases in which lips (upper, lower, or both) were involved were selected for a retrospective analysis. Cases of allergic mucositis, associations with dental fillings/amalgams, lichenoid lesions, and graft-versus-host disease were excluded.

All the patients were diagnosed and treated by teams experienced in oral medicine and dermatology from the University of Naples Federico II for oral, and skin and genital exams, respectively.

The diagnostic pattern for LP at our unit includes medical history, thorough skin and oral exam, and the realization of a biopsy for histomorphologic confirmation; no direct immunofluorescence is usually performed. If the case is consistent with a diagnosis of LP and oral lesions are present, a diagnosis of OLP is realized. Exclusion of allergic or lichenoid lesions is possible through confrontation of medical history and absence of local irritating factors (i.e., drugs, dental fillings); differential diagnosis with discoid lupus is done through the integration of clinical and histological data. Within the group of cases of OLP, if lesions on the lips were present, the case was considered as LPL.

For each file selected, these variables have been considered: age and sex, presence of any concurrent oral lesions, clinical form of LP, the symptoms, any skin involvement, systemic pathologies, hepatitis C virus (HCV) infection, drug therapy, realization of a biopsy specimen of the lip lesion, treatment, and outcome.

Concurrently, a review of the literature has been realized using the MEDLINE database via PubMed for articles about LPL published from inception (1950) to March 2014. The key words we have used are association, OLP and cutaneous LP, lip involvement, and clinical feature, in various combinations.

The inclusion criteria were the English language and the relevance of the title or the abstract to the field of research, including lip lesions in OLP/LP, both as sole manifestations of the disease and with concurrent lesions in other sites, in patients of either sex, and of any age and nationality.

The exclusion criteria were papers describing oral lichenoid lesions, graft-versus-host-disease, or other forms of LP different from OLP.

For each article reporting a case of LPL, these variables have been considered: year and country of publication, number of cases described, sex and age of the patient, presence of any concurrent oral lesions, clinical form of LP, symptoms, any skin involvement, systemic pathologies and HCV infection, a confirmatory biopsy, treatment, and outcome.

Epidemiological data about isolated lip lesions or concurrent lip lesions in OLP have also been investigated, and the related articles have been classified according to the year and country of publication, and number of patients involved in the study. Articles producing only narrative data were excluded.

Two reviewers selected the studies then extracted and classified the data. Another independent reviewer checked the selection and the data classification.

Results

Of the 388 OLP files recorded from 2002, 63 were excluded for incomplete information about the clinical data considered in this study. Thirteen clinical records of patients affected by true OLP involving the lips were found and reviewed, representing 4% of the remaining 325 OLP files, which had been considered elective for the selection, with a mean follow-up of 5.15 years (Figs. 1 and 2; Table 1). The patient’s mean age at the last follow-up was 71.85 years, and the female/male ratio was 0.4. In all but two cases, the lips were not the only site of oral involvement, i.e., tongue, buccal mucosa, gingiva, and mucobuccal fold; the other localizations of the lesions, in order of frequency (69.23% of cases for both tongue and buccal mucosa; 53.85%, the gingiva; 23.08%, mucobuccal fold). The lower lip was more frequently affected than the upper lip, with a ratio of 9:1. With the exception of two erosive forms and one keratotic form, all the other patients showed mixed clinical patterns, generally including erosion and mild keratosis. Nine patients complained of pain and burning (one also complained of xerostomia), while four of them were reported to be asymptomatic.
Only two patients showed concurrent skin lesions or lesions in other mucous epithelia. Two other patients reported a previous skin involvement, which had spontaneously disappeared.

HCV infection was detected in six patients. Moreover, all but three patients showed some systemic pathologies and had followed some chronic, often multidrug therapy. Three patients were former smokers, but none reported smoking at the time of examination.

No history of lichenoid lesions in near family members was recorded except for one doubtful case.

Biopsies were made on the lip lesions to exclude actinic cheilitis in some cases with medical history of prolonged ultraviolet exposition.

Most of the patients were treated with nystatin and cortisone ointments. One patient with OLP and cutaneous LP also reported a previous therapy with cyclosporine. One asymptomatic patient was given no treatment and only scheduled for a regular follow-up. One patient with a solitary lesion on the upper lip was treated with surgical excision due to the suspicion of malignancy, which proved positive.

Most of the lesions remained constant during the course of time, while four of them showed signs of regression or complete remission. Interestingly, the lip lesions in a few cases showed a different and more favorable course than other lesions of the mouth in the same patient, appearing later on in the development of the disease, or regressing earlier.

In the review of the literature, the data of 17 case reports and four case series of LPL that met the inclusion criteria were analyzed, for a total of 35 patients.
Table 2). All the cases were presented as true LP, but in three cases, the lesions were diagnosed as concurrent LP and fungal infection,\textsuperscript{7} morphea,\textsuperscript{8} and systemic lupus erythematosus.\textsuperscript{9} A histological specimen was provided in 17 articles.\textsuperscript{7–23}

The age of the patients ranged from 7 to 75 years (mean ± SD 45.35 ± 16.19), and the female/male ratio was 0.4. As for the geographical distribution, nine reports were from Europe,\textsuperscript{8,15,19,21–23,25–27} seven from Asia,\textsuperscript{7,9–11,16,24} and five from America.\textsuperscript{13,14,17,18,24}

Of the 21 case reports and series, 17 described an isolated lip involvement.\textsuperscript{7–12,14,17–22,24–27} The lower/upper lip involvement ratio was 6.5, while in two cases both the lower and upper lip presented lesions.\textsuperscript{11,25}
Discussion

In the literature, the most frequently reported clinical form of LPL is the erosive (10 cases),\textsuperscript{7,10,14,16,20,25,28,29,30,31} followed by the reticular/annular (three),\textsuperscript{10,15,23} nodular (one),\textsuperscript{21} and bullous (one).\textsuperscript{15} Accordingly, symptoms such as pain, burning, bleeding, and crusting were reported in 13 cases,\textsuperscript{7,10,14,20,22,23,27} while only two cases were completely asymptomatic.\textsuperscript{20,26} Only four cases of concurrent skin lesions have been described.\textsuperscript{15,16,25,27}

It is worth noting that for two reports it was impossible to determine if a confirmatory biopsy had been made for the diagnosis,\textsuperscript{25,26} while two other papers describe cases in which the diagnosis was only clinical.\textsuperscript{24,27}

<table>
<thead>
<tr>
<th>HCV</th>
<th>Drugs</th>
<th>Biopsy</th>
<th>Diagnosis</th>
<th>Familial</th>
<th>Treatment</th>
<th>Outcome</th>
<th>Notes</th>
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<tbody>
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<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>K lip, lichenoid infiltrate</td>
<td>No</td>
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<td>Remission</td>
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<td>ASA, bisphosphonates</td>
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<tr>
<td>No</td>
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<td>Yes</td>
<td>LP</td>
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<td>LP</td>
<td>No</td>
<td>Nystatin</td>
<td>Regression</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>LP</td>
<td>No</td>
<td>Nystatin</td>
<td>Stable</td>
<td>Spontaneous remission of skin lesions. Koebner + (buccal lesions) Keratosis on the lip only. Skin lesions appeared after oral lesions</td>
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<td>No</td>
<td>Yes</td>
<td>LP</td>
<td>No</td>
<td>Cortisone, nystatin, cyclosporine</td>
<td>Worsening in the mouth, Stable on the lip</td>
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<td>No</td>
<td>Yes</td>
<td>LP</td>
<td>No</td>
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<td>No</td>
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<td>Allopurinol, bisoprolol, warfarin</td>
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<td>LP</td>
<td>No</td>
<td>Nystatin</td>
<td>Stable in the mouth, regression on the lips</td>
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<tr>
<td>No</td>
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<td>No</td>
<td>LP</td>
<td>No</td>
<td>Cortisone + nystatin</td>
<td>Stable</td>
<td>Lip lesions appeared after oral lesions</td>
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<tr>
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<td>No</td>
<td>LP</td>
<td>No</td>
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<tr>
<td>Yes</td>
<td>Immunosuppressors</td>
<td>No</td>
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<td>No</td>
<td>Nystatin</td>
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<tr>
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<td>Amiloride + hydrochlorothiazide</td>
<td>No</td>
<td>LP</td>
<td>No</td>
<td>None</td>
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</tr>
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</table>

\textsuperscript{12} Muzzolo et al. Lichen planus of the lips: a case series Report e477

\textsuperscript{10,11}
<table>
<thead>
<tr>
<th>Year</th>
<th>First author/reference</th>
<th>Country</th>
<th>No. of patients</th>
<th>Age/sex</th>
<th>Isolated (oral mucosa)</th>
<th>Symptoms</th>
<th>Clinical features</th>
<th>Skin involvement</th>
<th>Systemic pathologies</th>
<th>HCV</th>
<th>Biopsy</th>
<th>Diagnosis</th>
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<td>Domingues[10]</td>
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<td>1</td>
<td>44/M</td>
<td>Yes (lower)</td>
<td>Painful, bleeding</td>
<td>Enaive</td>
<td>Yes</td>
<td>Treatment with imiquimod</td>
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<td>No</td>
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<td>India</td>
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<td>40/M</td>
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<td>No</td>
<td>Annular</td>
<td>No</td>
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<td>No</td>
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<td>LPL</td>
<td>Tacrolimus</td>
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<td>1</td>
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<td>No</td>
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<td>4</td>
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<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>LPL</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2011</td>
<td>De Moraes[14]</td>
<td>Brazil</td>
<td>1</td>
<td>7/F</td>
<td>No (upper)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>OLP</td>
<td>Corticosteroid</td>
<td>Remission</td>
</tr>
<tr>
<td>2008</td>
<td>Johnson[15]</td>
<td>USA</td>
<td>1</td>
<td>42/F</td>
<td>Yes (lower)</td>
<td>Dryness, peeling</td>
<td>Erosive</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>LPL</td>
<td>Tacrolimus</td>
<td>NA</td>
</tr>
<tr>
<td>2007</td>
<td>Petrutz[16]</td>
<td>USA</td>
<td>1</td>
<td>75/F</td>
<td>No (lower)</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>5/10</td>
<td>NA</td>
<td>No</td>
<td>OLP</td>
<td>Remission</td>
<td>Stable</td>
</tr>
<tr>
<td>2007</td>
<td>van Twy[17]</td>
<td>Netherlands</td>
<td>1</td>
<td>75/F</td>
<td>No (lower)</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>OLP</td>
<td>Remission</td>
<td>Stable</td>
</tr>
<tr>
<td>2006</td>
<td>Shichinohe[18]</td>
<td>Japan</td>
<td>2</td>
<td>64, 68/M, 60/M</td>
<td>No (lower), no (lower)</td>
<td>Painful</td>
<td>Erosive</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>LPL</td>
<td>Tacrolimus</td>
<td>NA</td>
</tr>
<tr>
<td>2005</td>
<td>Donovan[19]</td>
<td>USA</td>
<td>1</td>
<td>51/M</td>
<td>Yes</td>
<td>Painful</td>
<td>Erosive</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
<td>LPL</td>
<td>Tacrolimus</td>
<td>Remission</td>
</tr>
<tr>
<td>2003</td>
<td>Yu[20]</td>
<td>USA</td>
<td>1</td>
<td>44/M</td>
<td>Yes (lower)</td>
<td>Burning</td>
<td>Erosive</td>
<td>No</td>
<td>Hypertension</td>
<td>No</td>
<td>Yes</td>
<td>LPL</td>
<td>Tacrolimus</td>
<td>Remission</td>
</tr>
<tr>
<td>2002</td>
<td>Chi[21]</td>
<td>Taiwan</td>
<td>1</td>
<td>36/F</td>
<td>Yes (lower)</td>
<td>Painful</td>
<td>Erosive</td>
<td>No</td>
<td>No</td>
<td>NA</td>
<td>Yes</td>
<td>LPL</td>
<td>Tacrolimus</td>
<td>Remission</td>
</tr>
<tr>
<td>2002</td>
<td>Ciocchi[22]</td>
<td>Italy</td>
<td>1</td>
<td>45/M</td>
<td>Yes (lower)</td>
<td>Buming, swelling</td>
<td>Erosive</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>LPL</td>
<td>Betamethasone</td>
<td>NA</td>
</tr>
<tr>
<td>2000</td>
<td>Malat[23]</td>
<td>Italy</td>
<td>1</td>
<td>NA</td>
<td>Yes (upper)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Vitiligo</td>
<td>NA</td>
<td>Yes</td>
<td>LPL</td>
<td>Betamethasone</td>
<td>NA</td>
</tr>
<tr>
<td>1997</td>
<td>De Angli[24]</td>
<td>Spain</td>
<td>1</td>
<td>51/F</td>
<td>Yes (lower)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>LPL</td>
<td>Chloroquine phosphate</td>
<td>Regression</td>
</tr>
<tr>
<td>1997</td>
<td>Ito[25]</td>
<td>Japan</td>
<td>1</td>
<td>54/M</td>
<td>Yes</td>
<td>Painful</td>
<td>Erosive</td>
<td>Yes</td>
<td>NA</td>
<td>NA</td>
<td>No</td>
<td>LPL</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1996</td>
<td>Allan[26]</td>
<td>UK</td>
<td>1</td>
<td>51/M</td>
<td>Yes (lower)</td>
<td>Irritation, naalness</td>
<td>Erosive</td>
<td>Yes</td>
<td>NA</td>
<td>NA</td>
<td>No</td>
<td>LPL</td>
<td>Betamethasone</td>
<td>Remission</td>
</tr>
<tr>
<td>1995</td>
<td>Bay[27]</td>
<td>Switzerland</td>
<td>1</td>
<td>44/NA</td>
<td>Yes (lower)</td>
<td>Burning</td>
<td>Erosive</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>LPL</td>
<td>Remission</td>
<td>Regression, recurrence, K</td>
</tr>
<tr>
<td>1992</td>
<td>Harland[28]</td>
<td>UK</td>
<td>1</td>
<td>23/M</td>
<td>No (lower)</td>
<td>NA</td>
<td>Nodular</td>
<td>Yes</td>
<td>Former smoker</td>
<td>NA</td>
<td>Yes</td>
<td>OLP</td>
<td>Remission</td>
<td>Stable</td>
</tr>
<tr>
<td>1978</td>
<td>Ramphongsan[9]</td>
<td>Thailand</td>
<td>2</td>
<td>NA</td>
<td>Yes (lower)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>Yes</td>
<td>LPL - LES</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1937</td>
<td>Nuzzolo[10]</td>
<td>USA</td>
<td>1</td>
<td>51/M</td>
<td>Yes (lower)</td>
<td>Irritation, plaque</td>
<td>Anal mucosa</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>LPL</td>
<td>Mercury, arsenic, x-ray</td>
<td>Stable</td>
</tr>
</tbody>
</table>

LES; LPL, lichen planus of the lip; NA, not applicable; OLP, oral lichen planus.
HCV, hepatitis C virus; LP, lichen planus; NA, not applicable.

Most of the patients are reported to have no systemic pathology, and only in six cases, a serological positivity for HCV infection was reported.\(^{17,15}\) As for the treatment, tacrolimus was used in five cases,\(^{7,13,14,16,17}\) and the reported outcome was regression in two cases,\(^{14,16}\) remission in one,\(^{17}\) and the persistence of the lesion in one\(^{14}\) (in one where there was no reported outcome); corticosteroids, alone or in association with other drugs, were reported to have been used in 10 articles,\(^{7,13,15,16,17,21,25,26}\) causing remission of the lesion in most cases.\(^{7,13,14,15,16,17,24,25}\) However, in one article, recurrence and malignant transformation was described.\(^{53}\) Imiquimod and chloroquine phosphate were also reported to have been used.\(^{12,26}\) In the first reported uncertain case of LPL, Whittle described the use of mercury, arsenic, and x-rays in its treatment.\(^{7,17}\)

Few data about the prevalence of lip involvement in LP can be found in the literature (Table 3). The prevalence of isolated LPL was assessed only in two studies, with very different results: Aminzadeh et al. in 2009\(^{28}\) reported a prevalence of 0.51% in a total of 186 Iranian patients, while Xue et al. in 2005\(^{29}\) reported a prevalence of 8.9% in a total of 674 Chinese patients. Lip involvement, concurrently with other oral sites, is reported to have a prevalence between 3.2.3% and 6.3%,\(^{28,29}\) being the third most common site of involvement according to Xue et al.’s epidemiological study, or the fourth according to Eisen and Carrozzo’s reviews.\(^{53,54}\) LPL is unanimously considered to affect the lower lip far more frequently than the upper lip.

In accordance to these data, in the case series described, the lesions were almost always erosive, or erosive and keratotic, and consequently, symptomatic.

The particular predilection for the male gender and lower lip, as well as the clinical features of the lesions, seem to suggest some environmental and behavioral influence on the development of these lesions, such as solar radiation, wind exposure, air pollution, and the habit of smoking. For this reason, attention has been focused on detecting the cancerization of LPL, reported, in fact, in a 23-year-old former smoker by Harland et al.\(^{53}\) as well as in one of our patients.

LPL seems to be rare but still somewhat underestimated. In our case series, the lesions appeared as small areas of mild keratosis and/or moderate erosion, often associated with atrophy, erythema, and exfoliation, and were mainly located at the limit between the vermillion and the labial mucosa. This is a very rough area due to the exposure of the inner part of the vermillion to oral irritants, such as saliva, food, and tooth margins. Clinical features could be a combination of dystrophia and inflammatory conditions overcoming the lichenoid aspects.

Additionally, the lip involvement in more than one case resembled the course of cutaneous LP, disappearing or regressing spontaneously after some years, or appearing after the other oral lesions, even though skin involvement affected only a small percentage of patients. In accordance with this finding, the isolated lip lesions described in the literature underwent remission or regression.

The transitional mucosa of the lip, with its distinct antigenic structure, might be responsible both for the mildness of the lesions and for their cutaneous-like progression.

Another possibility, supported by more evidence, is that the difference in the clinical behavior of cutaneous LP and OLP, between which LPL might stand, is to be found...
in the immunological composition and molecular expression of the two epithelia, T-helper 22 cell-produced interleukin-22 and -23 have been proven to be more expressed in oral lesions, probably because of the massive presence of T-helper 22 cells in the oral mucosa. In the same way, the cytotoxic molecules interleukin-17 and Foxp3, perforin, and granzyme B, and finally caspase 3, Bax, and Bcl-2 associated with apoptosis have been found to be highly expressed only in oral lesions. In addition, a concentration of CD4-positive cells in the oral mucosa has been related to the entity of these lesions. Possibly, the turning point between these different molecular patterns, which can be related to the different clinical behavior of the skin and oral lesions, might be at the interface of the skin and oral mucosa, namely the vermilion.

On the other hand, the milder presence of microbiota and environmental factors might act on lip lesions, with a beneficial effect: ultraviolet B radiation is known to reduce the lesions in LP, and phototherapy is also used to treat skin lesions. It is possible that such factors act in multiple ways, on the one hand controlling the immunological response of the epithelia but also, on the other hand, acting as a chronic irritating stimulus on the lesions.

Given all these considerations, lip lesions in LP, showing transitional characteristics between the oral and cutaneous forms, might need independent categorization and could be the starting point for a better understanding of the immunopathogenesis, prognosis, and treatment of this disease. They should be detected very carefully by the clinician because they are insidious and easily overlooked and might undergo malignant transformation.

References


The GOCCLES® medical device is effective in detecting oral cancer and dysplasia in dental clinical setting. Results from a multicentre clinical trial

Il dispositivo medico GOCCLES® è in grado di individuare displasie e cancro orale se impiegato nel setting odontoiatrico. Risultati da uno studio multicentrico

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1 Unit of Maxillofacial Surgery, Nuovo Ospedale San Giovanni Battista, Foligno, Italy; 2 Unit of Maxillofacial Surgery, Policlinico Gemelli, C.I. Columbus, Università Cattolica del Sacro Cuore, Rome, Italy.

SUMMARY

The purpose of this study is to demonstrate that the GOCCLES® medical device allows proper autofluorescence examination of the oral mucosa in a dental care setting. This is a non-randomised multicentre clinical trial on consecutive patients at risk for oral cancer. Patients underwent a classical naked eye inspection of the oral cavity followed by autofluorescence examination with each source of light used during the study. These results suggest that the role of the autofluorescence visualisation is that of a complementary inspection following naked eye examination when dealing with patients at risk for oral cancer. The device allows detection of otherwise invisible lesions and otherwise impossible complete resections.

KEY WORDS: Oral cancer • Early Detection of Cancer • Dentistry • Fluorescence • Curing Lights • Dental

RIASSUNTO

Scopo di questo studio è dimostrare che il dispositivo medico GOCCLES® permette di condurre l’esame dell’autofluorescenza del cavo orale nel setting odontoiatrico. Si tratta di uno studio multicentrico non randomizzato su pazienti consecutivi a rischio di cancro orale. I pazienti sono stati sottoposti ad ispezione del cavo orale ad occhio nudo seguita dall’esame dell’autofluorescenza condotto indossando gli occhiali GOCCLES® mentre una lampada fotopolimerizzante illuminava la mucosa orale. Le lesioni sono state definite come qualunque lesione precancerosa del cavo orale visibile ad occhio nudo o area di perdita di fluorescenza visibile con GOCCLES®. Tutte le lesioni presenti sono state sottoposte a biopsia escissionale o incisionale. Sono stati reclutati 61 pazienti e analizzati i dati da 64 lesioni. Delle 62 lesioni identificate dal dispositivo, 31 erano veramente positive. Il dispositivo ha identificato 31 delle 32 lesioni veramente positive. Una lesione (un carcinoma invasivo) non era visibile ad occhio nudo. Tutte le lesioni classificate come displasia trasformazione moderata e severa e ogni carcinoma sono stati correttamente identificati dal dispositivo. Nel 56,7% delle lesioni identificate dal dispositivo mostravano margini più ampi rispetto a quelli visibili ad occhio nudo. Il dispositivo medico GOCCLES® permette di osservare il fenomeno della perdita di fluorescenza in pazienti affetti da displasia o cancro del cavo orale. Ha permesso di effettuare l’esame dell’autofluorescenza con ciascuna lampada fotopolimerizzante testata. I risultati suggeriscono di impiegare GOCCLES® come esame complementare rispetto all’ispezione ad occhio nudo del cavo orale su pazienti a rischio per cancro orale. Il dispositivo permette di identificare lesioni altrimenti visibili o i cui margini sono sottostimati dall’ispezione ad occhio nudo.

PAROLE CHIAVE: Cancro orale • Diagnosi precoce del cancro • Odontoiatria • Fluorescenza • Lampade fotopolimerizzanti

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Introduction

No oral cancer screening test on large populations is currently recommended for oral cancer. However, studies on low-cost oral cancer diagnostic techniques are currently ongoing. The autofluorescence examination is among these techniques.

Autofluorescence of the oral mucosa consists of a dim light coming from oxidised flavin adenine dinucleotide (FAD) and other fluorophores when excited by blue-violet and ultra-violet (UV) light. Healthy tissues produce a 515 nm (green) light. Conversely, tissues with disorders of cell metabolism (such as dysplastic mucosa) appear as dark spots on a green background. The disruption of the extracellular matrix, hyperemia and neo-angiogenesis also contribute to reduce fluorescence emission.

Persistent areas of decreased fluorescence can therefore be a sign of dysplasia or cancer and should be treated accordingly. Today, it is commonly accepted that potentially malignant lesions of the oral cavity must be treated if they persist for more than 2 weeks, and many researchers are trying to find a role for autofluorescence not only in the early detection, but also in the complete surgical resection of cancerous or pre-cancerous lesions.

The GOCCLES® (Glasses for Oral Cancer – Curing Light Exposed – Screening) medical device was created in order to provide comfortable, easy and low cost direct visualisation of abnormalities of the oral cavity tissue fluorescence.

This device has already been tested in a clinical trial, with promising results. However, that study suffered from several limitations, and was a pilot study on just 32 patients. Two-thirds of enrolled patients were in follow-up after the surgical resection of an oral cancer, and therefore at high risk of showing dysplastic tissues at histological examination regardless of the results of autofluorescence examination because of disease relapse or incomplete resection. Moreover, during this trial only a prototype of the device was studied, and it was tested with only one dental curing light (an Elipar 2500 3M ESPE). Furthermore, the study was conducted only by the research team responsible for the creation of the device, with obvious conflicts of interest.

In order to demonstrate the capability of the GOCCLES® medical device to allow proper autofluorescence examination of the oral mucosa, which is the aim of this multicentre trial, it is therefore necessary to involve more patients, multiple research groups and curing lights, and to test the final model of the device.

Materials and methods

The GOCCLES® device (Pierrel S.p.A, Italy) consists of a pair of glasses equipped with filters (Fig. 1) that highlight autofluorescence when the oral mucosa is illuminated by the light emitted by any dental curing light.

The device was studied in a non-randomised multicentre clinical trial in which eligible consecutive patients underwent oral examination with GOCCLES®.

Subjects above the age of 17 years were eligible for the study if showing potentially malignant lesions of the oral mucosa or if they were in follow-up after surgical resection for oral cancer. Patients who underwent radiotherapy for oral or head and neck cancer in the previous three months were excluded from the study.

All eligible subjects were informed in detail on the study protocol and participants joined the study voluntarily after signing an informed consent form. All eligible subjects showing lesions at naked eye oral inspection or on autofluorescence analysis were asked to participate in the study. For ethical reasons, subjects with totally negative naked eye and autofluorescence examinations did not undergo biopsy of the oral mucosa and were excluded.

Enrollment took place at the following six Centers: Unit of Maxillofacial Surgery, C.I. Columbus, Università Cattolica del Sacro Cuore (Rome); Department of Reconstructive and Diagnostic Surgical Sciences, Unit of Oral Pathology and Medicine and Complex Unit of Maxillofacial Surgery, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, University of Milan (Milan); Department of Integrated Activities Head-Neck, Unit of Maxillofacial Surgery and Unit of Oral Medicine, Università di Napoli Federico II (Naples); Unit of Maxillofacial Surgery, Nuovo Ospedale San Giovanni Battista (Foligno). Enrollment lasted one year and started in September 2013.

Patients underwent a naked eye classical inspection of the oral cavity followed by the autofluorescence examination wearing the GOCCLES® medical device while the light from a dental curing light irradiated the oral mucosa. All examinations were performed by skilled physicians and surgeons with patients lying in a dental chair in a setting similar to that of the dental practice. All operators were asked to hold the light at 20-40 cm
detection of oral cancer in a dental clinical setting.

**Results**

Sixty-one patients were enrolled and all underwent both naked eye inspection and autofluorescence examination of the oral cavity mucosa. Data. From all 61 patients entered the analysis. Main characteristics of the patients are summarised in Table I.

Autofluorescence of the oral mucosa was analysed using a Led.B curing light on 29 patients (47.6% of the sample), an Elipar S10 on 21 patients (34.4%) and an Optilux 501 on 11 patients (18.0%).

Naked eye inspection of the oral cavity detected 60 suspect lesions, while autofluorescence examination with the GOCCELS® device detected 62 suspect lesions. A total of 65 lesions were detected: in 59 cases (90.8% of observed suspected lesions) they were visible to both naked eye and autofluorescence, while 2 suspected lesions (3.1%) were only visible during the naked eye inspection and 4 (6.2%) were only visible on the autofluorescence examination. No significant differences in terms of detected suspect lesions were observed between the naked eye inspection and the autofluorescence examination.

**Statistical analysis**

Differences in terms of diagnostic performance between the autofluorescence analysis and naked eye inspection of the oral mucosa were assessed using the two-tailed McNemar test. Data analysis was performed with the IBM SPSS 22 Statistics Software for Windows. Statistical significance was set at \( p = 0.05 \).

Sample size calculation was based on the results of the previous study. Assuming that the examination of autofluorescence showed larger margins compared to the naked eye in 25% of the lesions and that the opposite occurs in about 5% of cases, a sample of 100 patients was set in order to observe a significant difference between the two examinations with a probability of 95% (accepting a probability of type I error of 5%). An interim analysis was planned at one year. After analysis of the first results the study was discontinued given the low probability of achieving more conclusive results with the preset sample of 100 patients.
and autofluorescence examination of oral mucosa (McNemar test \( p = 0.687 \)).

Sixty-five biopsies of the oral mucosa were thus taken. Fifty-eight patients underwent one oral mucosa biopsy, while two underwent two biopsies and another patient underwent three biopsies because of multiple lesions. An invalid sample was excluded from statistical analyses and data from 64 of 65 biopsies (98.5% of all biopsies) were analysed. Thirty-two of 64 samples (50.0% of the valid samples) were classified as false positives. Of the 32 truly positive samples, 11 (17.2% of the valid samples) were classified as mild dysplasia, 4 (6.3%) as moderate dysplasia, 3 (4.7%) as severe dysplasia, 2 (3.1%) as carcinoma in situ and 12 (18.8%) as invasive cancer.

Of the 60 suspected lesions detected by naked eye inspection of the oral cavity, 31 were true positive lesions (51.6%), while of the 62 lesions identified on autofluorescence examination, 31 (50.0%) were true positives. In particular, autofluorescence examination identified all the lesions classified as moderate to severe dysplasia and all lesions classified as cancer. Main results of the study are summarised in Table II. Both techniques identified 31 of 32 (96.9%) actual lesions. One lesion (a carcinoma) was not visible to the naked eye, while one lesion (a mild dysplasia) was not visible on the autofluorescence examination. No significant differences in terms of diagnostic performance were observed between the two techniques (McNemar test \( p = 1.000 \)).

Thirty true positive lesions were visible by both classical inspection and autofluorescence analysis. In 17 cases (56.7% of the 30 true positive lesions), extension of the lesion detected on the autofluorescence examination was greater than that observed at naked eye inspection, while in two cases (6.7%) the margins overlapped and in nine cases (30.0%) autofluorescence examination showed a smaller lesion. In two cases (6.7%), the operator was unsure whether the margins were overlapping or not.

The resection margins identified during the naked eye inspection were infiltrated in 21 cases (67.7% of the 31 lesions identified). Of the 31 true positive lesions identified by autofluorescence examination, 24 (77.4%) showed infiltrated margins. No significant differences were observed between the two techniques in terms of free resection margins (McNemar test \( p = 0.754 \), see Tab. III). No statistically significant differences were observed in the performance of the autofluorescence test using different curing lights (Tab. IV).

### Discussion

The GOCHEL® medical device allowed visualisation of fluorescence loss in patients suffering from mild to severe dysplasia and oral cancer (Fig. 2). The device worked

<table>
<thead>
<tr>
<th>Table II. Autofluorescence examination.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion description</td>
</tr>
<tr>
<td>False positive [N (%)]</td>
</tr>
<tr>
<td>True positive [N (%)]</td>
</tr>
<tr>
<td>Of which:</td>
</tr>
<tr>
<td>Mild dysplasia [N (%)]</td>
</tr>
<tr>
<td>Moderate dysplasia [N (%)]</td>
</tr>
<tr>
<td>Severe dysplasia [N (%)]</td>
</tr>
<tr>
<td>Carcinoma in situ [N (%)]</td>
</tr>
<tr>
<td>Invasive cancer [N (%)]</td>
</tr>
<tr>
<td>TOTAL [N (%)]</td>
</tr>
</tbody>
</table>

* The percentages under the "total" column relate to the whole sample. † Includes other non-precancerous and non-cancerous disorders of the oral mucosa.

Definitive diagnoses are based on histological findings. No patients were negative for both naked eye and autofluorescence analysis because no subject with totally negative physical examination underwent biopsy of the oral mucosa.

### Table III. Proportions of free and infiltrated margins of the lesions identified during naked eye inspection of the oral cavity and on autofluorescence examination.

<table>
<thead>
<tr>
<th></th>
<th>Not visible to AF</th>
<th>AF margins not infiltrated</th>
<th>AF margins infiltrated</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not visible to the NE</td>
<td>-</td>
<td>0 (0%)</td>
<td>1 (3.1%)</td>
<td>1 (3.1%)</td>
</tr>
<tr>
<td>NE margins not infiltrated</td>
<td>1 (3.1%)</td>
<td>3 (9.4%)</td>
<td>6 (18.8%)</td>
<td>10 (31.3%)</td>
</tr>
<tr>
<td>NE margins infiltrated</td>
<td>0 (0%)</td>
<td>4 (12.5%)</td>
<td>17 (53.1%)</td>
<td>21 (65.6%)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>1 (3.1%)</td>
<td>7 (21.9%)</td>
<td>24 (75.0%)</td>
<td>32 (100%)</td>
</tr>
</tbody>
</table>

NE: naked eye inspection; AF: auto fluorescence examination.
No patients showed lesions invisible to both techniques because no subject with totally negative physical examination underwent the biopsy of the oral mucosa.
GOCCLLES® is effective in detecting oral cancer in a dental clinical setting. Properly with each source of light used in this study, which have different technical characteristics reproducing the variety of available dental curing lights. The Led.B is a LED lamp with a wavelength of 440-490 nm and an intensity of 1,000-1,200 mW/cm²; the Elipar S10 LED lamp has a 430-480 nm wavelength and an intensity of 1,000 mW/cm²; the Optilux 501 is a halogen lamp with a wavelength of 400-505 nm and an intensity of 850-1,000 mW/cm². The GOCCLLES® device was also previously successfully tested with another halogen lamp with different characteristics (400-500 nm wavelength and 600 mW/cm² intensity).

The study was performed with patients lying in dental chairs in order to reproduce the settings of common dental practice. The intended purpose of the device was to allow any dentist equipped with a curing light to perform low-cost autofluorescence examination. According to the scientific literature, autofluorescence examination shows otherwise invisible characteristics of the oral mucosa that are associated with oral cancer. However, the current evidence suggests that the role of the autofluorescence examination is that of a complementary inspection following the naked eye examination, which should not be replaced by any screening test. Moreover, given the high risk of false positive findings (50% in this study), it is recommended that every dentist equipped with the device is properly trained before using it and that only patients at risk for oral cancer or showing potentially malignant lesions undergo examination with GOCCLLES®. Remarkably, however, in one case GOCCLLES® allowed the detection of an otherwise invisible lesion, and in four cases it allowed otherwise impossible complete resections of lesions with infiltrated margins.

We hope that the availability of additional low cost screening techniques encourages more careful and more frequent inspections of the oral cavity among dentists, and further promotes a much needed culture of oral cancer prevention: naked eye inspection of the oral cavity alone could, in fact, save about 37,000 lives worldwide each year.

Furthermore, data on its diagnostic performance are lacking because of the small sample size and study de-
sign (it was impossible to assess the proportion of false negatives in this study as no follow-up of negative patients was planned). Further studies on much larger samples (possibly randomised clinical trials comparing the device with other techniques) are needed to define its diagnostic performance. The previous pilot study (in which patients underwent a follow-up of one year) showed 100% sensitivity, 95% specificity, 93% positive predictive value and 100% negative predictive value. However, the device was studied on patients at very high risk, and most had a history of oral cancer. It is likely that the diagnostic performance is heavily affected by the population tested, being poorer in the general population and improving greatly when dealing (as appropriate) with subjects at risk.

Conflict of interests
This study was funded by Pierrel S.p.A., owner of the rights on the GOCCLES medical device. Three researchers involved in this study (SP, AM, FDN) have royalty percentages on the sales of the device.

References

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Original Research Article
The Relationship Between Sociodemographic Characteristics and Clinical Features in Burning Mouth Syndrome

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All persons and only persons who meet authorship criteria and participated in the preparation of the manuscript are listed as authors.

Abstract

Objective. To compare sociodemographic and clinical characteristics in patients with burning mouth syndrome (BMS) and their relationship with pain.

Design. Cross-sectional clinical study.

Setting. University-Hospital.

Subjects. 75 BMS patients were enrolled.

Methods. The study was conducted between September 2011 and March 2012 at the “Federico II” University of Naples. Demographic characteristics and clinical information including age, sex, educational level, marital status, job status, age at disease onset, oral symptoms, and triggers were collected via questionnaire interviews. To assess pain intensity the visual analogue scale (VAS) was administered. Descriptive statistics were collected, and Pearson Chi-square tests, Kruskal–Wallis nonparametric tests and the Spearman bivariate correlation were performed.

Results. The mean age was 61.17 (±11.75, female/male ratio = 3:1). The mean age at disease onset was 56.75 (±12.01). A low educational level (8.57 ± 4.95) and 80% of unemployment were found. Job status and age at disease onset correlated with the VAS scale (P = 0.019 and P = 0.015, respectively). Tongue morphology changes, taste disturbances, and intraoral foreign body sensation have a significant dependence on gender (P = 0.049, 0.001, and 0.045, respectively); intraoral foreign body sensation has a significant dependence on marital status (P = 0.033); taste disturbances have a significant dependence on job status (P = 0.049); xerostomia has a significant dependence on age (P = 0.039); and tongue color changes and a bitter taste have a significant dependence on educational level (P = 0.040 and 0.022, respectively). Marital status and educational level have a significant dependence on the triggers (P = 0.036 and 0.049, respectively).

Conclusions. The prevalence of BMS is higher in women, and in married, unemployed, and less highly educated patients. Burning is the most frequent symptom while stressful life events are the most frequent trigger reported.

Key Words. Burning Mouth Syndrome; Sociodemographic Factors; Oral Burning; Educational Level; Gender; Oral Pain

Introduction

Burning Mouth Syndrome (BMS) is a complex chronic orofacial pain disorder characterized by an intraoral
burning sensation for which no medical or dental cause can be found, unremitting for at least 4 to 6 months [1,2]. It is identified by three diagnostic criteria: pain in the mouth present daily and persisting constantly or for most of the day, oral mucosa of a normal appearance, and the absence of local and systemic diseases [3,4].

It occurs more commonly in middle-aged and elderly women, and rarely affects individuals under the age of 30 years. The prevalence may vary but ranges from 0.7% to 3.6% in men and 0.6 to 12.2% in women [5,6]. Other studies have reported a higher prevalence ranging from 0.6% to 15%[7–12].

The main symptom is a burning sensation in the oral mucosa and perioral regions that usually has a bilateral and symmetric distribution. Sometimes the pain is described as scalding, tingling, or numbing [13]. Other oral symptoms, such as dysesthesia, a bitter/metallic taste, subjective xerosis, and foreign body sensation have been reported [14]. Pungent or hot food or beverages, stress, and tiredness have been reported to worsen pain. The corresponding pain alleviating factors are eating, sucking pastilles, drinking cold beverages, and relaxation [15].

The onset is generally spontaneous, and without any recognizable precipitating factors. However, some BMS patients report antecedent dental procedures, the initiation of medications, or stressful life events [16]. Spontaneous remission is rare [17].

The pain has precise physical, anatomical, and pathological dimensions [18], but is also characterized by cultural or universal components in its expression and manifestation, with different interpretations from the social or cultural perspective [19]. This is due to the psychological, social, and cultural dimensions of nociception. In this context, the pain is conditioned by personal and particular elements affecting the individual suffering from the condition, and also by the social elements that identify the individual, such as his or her sociodemographic characteristics [20].

Although BMS has been extensively studied, little is known about the relationships between sociodemographic characteristics, symptoms, and triggers in the onset of disease. Therefore, this study aims to explore 1) the sociodemographic profiles in a sample of BMS patients; 2) the patients’ perceived pain intensity, the oral symptoms reported, the triggers in the onset of the disease and the relationships with sociodemographic variables such as gender, age, educational level, marital status, and employment status and; 3) the diagnostic delay comparing the age of the patients with their age at the onset of the disease.

To the best of our knowledge, this is the first study that analyzes the relationships between clinical outcomes and sociodemographic variables.

Materials and Methods

This was a cross-sectional single-assessment clinical study performed at the oral medicine unit of the “Federico II University of Naples.”

One hundred and ten BMS cases were screened for possible participation between September 2011 and March 2012. Seventy-five cases were included in the trial in accordance with the inclusion/exclusion criteria. All patients received written information and provided their written informed consent for the management of personal data before their participation.

The study was approved by the Ethics Committee of the Federico II University (approval number 177/08).

The inclusion criteria for BMS were 1) either sex, aged 18 or older; 2) the presence of chronic pain in the oral mucosa, subjective xerosis, or foreign body sensation; 3) pain lasting more than 4 months, continuous throughout the day, with no paroxysm and not following any unilateral nerve trajectory; and 4) the absence of any abnormalities from the following laboratory investigations: salivary flow rates, laboratory tests, and tests for the detection of candidiasis. The exclusion criteria encompassed patients presenting with organic conditions that could be considered a causative factor, such as diabetes, anemia, thyroid disease, hypophysial-related systemic disorders (e.g., Sjogren’s syndrome), contact allergies, psychotic illness, organic brain syndrome, or neurological disease; subjects with signs of parafunctional habits; or patients regularly treated with anxiolytic, antidepressant, anticonvulsant, or psychotropic drugs. Even in the absence of mucosal lesions, a local effect of dental materials related to contact hypersensitivity was excluded by means of patch tests when the symptoms had started after any dental rehabilitation. A final diagnosis of BMS was established only after all other possible causes of the oral complaints had been ruled out.

At admission, each subject underwent a medical anamnesis (including history, clinical features, and age at disease onset), a general medical examination, an intraoral examination and extraoral examination, and laboratory tests (e.g., a full blood cell count, and analyses of serum levels of iron, ferritin, folate, vitamin B12, and glucose).

The data were obtained using face-to-face questionnaire interviews addressing sociodemographic variables, age at disease onset, oral symptoms, and triggers at disease onset. To assess the pain intensity the visual analogue scale (VAS) was administered. The VAS is usually presented as a 10-cm horizontal line, with each point clearly marked. The patient marks on a line the point that they feel represents their perception of their pain at the current state. It is a test which is widely used in psychiatry.

By using social measurements to assess subjective phenomena, it is easy to administer, fast to complete, and with a high response rate [21].
The demographics of the patients such as gender, age, educational level, marital status, and employment status were compared with data relating to South Italy from the recent ISTAT (Italian National Institute of Statistics) census (2011) [22].

Regarding the symptoms, we requested the patients to report the presence of: any change of tongue morphology, change of tongue color, bitter taste, scaling, burning, pain, taste disturbances, xerostomia, sialorrhea, or intraoral foreign body sensation. The patients could indicate one or more symptoms. Furthermore, regarding the triggers, patients were asked to report any precipitating factors such as the starting of any new medications, dental procedures or stressful life events [23] occurring in the time frame of 1–12 weeks before the onset of BMS. In such a case, we suggested that the patients should indicate preferentially one trigger.

Finally, we analyzed the diagnostic delay comparing the age of the patients at their first medical appointment with their age at the onset of the disease.

An oral medicine specialist was responsible for determining the diagnosis of BMS, and for collecting all the demographic and medical data from the patients.

**Statistical Analysis**

Descriptive statistics, including means, standard deviations, medians, and interquartile range, were used to summarize all the variables. The Pearson Chi-square test was used to verify the significance of any dependence between qualitative variables. Because numerical variables do not have a normal distribution we chose to use nonparametric tests. To verify the significance difference between medians, we performed the Kruskal–Wallis nonparametric test while to measure the degree of correlation we used the Spearman bivariate correlation index.

**Results**

Table 1 summarizes the demographic and clinical parameters. The demographics of the patient group were compared with data relating to South Italy from the ISTAT 2011 census Website [22].

The number of study years of the patients was 8.57 ± 4.95 (8.12 ± 4.83 for the females and 9.89 ± 5.20 for the males). These data were similar to those of South Italy.

Some differences were detected in marital status and in job status. We found that 6.7% of our patient group were single compared with 29.6% in South Italy, 5.3% were divorced compared with 1.3%, and 12.1% were widowed compared with 7.1%.

There was a higher level of unemployment (60 patients, 80%) compared with the data relating to South Italy (45%; P < 0.001). The percentage of male unemployed patients was 84% compared with 28% in South Italy. The percentage of unemployed women was 80% compared with 60%.

Comparing the age of the patient group with the age at disease onset we found a diagnostic delay of 4.42 ± 0.26 years.

**Dependence Analysis of Age at Disease Onset**

As shown in Table 2, job status and educational level correlated (P < 0.001 and P = 0.006, respectively) with age at disease onset.

**Dependence Analysis of VAS Scale**

Table 3 shows the dependence analysis of the VAS scale in relation to gender, job status, and marital status.
status. Only job status correlated with the VAS scale ($P = 0.019$). Furthermore, the table shows the dependence analysis of the VAS scale in relation to age, educational level, and age at disease onset. Only age at disease onset correlated with the VAS scale ($P = 0.015$).

### Analysis of Symptoms and Dependence

Table 4 shows the dependence analysis of the symptoms in relation to gender, marital status, job status, age, educational level, and age at disease onset. Changes of the tongue morphology, taste disturbances, and intraoral foreign body sensation have a significant dependence on gender ($P = 0.049$, $P = 0.001$, and $P = 0.045$, respectively); intraoral foreign body sensation has a significant dependence on marital status ($P = 0.033$); taste disturbances have a significant dependence on job status ($P = 0.049$); xerostomia has a significant dependence on age ($P = 0.039$); and changes of tongue color and a bitter taste have a significant dependence on educational level ($P = 0.040$ and $P = 0.022$, respectively).

No symptom correlated positively with age at disease onset.

### Analysis of Triggers and Dependence

Table 5 shows the dependence analysis of the triggers in relation to gender, marital status, job status, age, educational level, and age at disease onset. Marital status and educational level have a significant dependence on the triggers ($P = 0.036$ and $P = 0.049$, respectively).

Initiation of drugs and stressful life events were the most common triggers reported by single BMS patients (40%), stressful life events was the most common trigger reported by married patients (46%) and by widowed BMS patients (44%), and antecedent dental procedures and stressful life events were the most common triggers in divorced BMS patients with the same percentage (50%).

In terms of educational level, the trigger most commonly reported by patients with the lowest level of education (0–4 years) was antecedent dental procedures (50%). The patients with study periods from 5 to 13 frequently reported as a trigger stressful life events while the patients with the highest educational level (14–18) did not identify any specific cause of their disease (50%).

### Discussion

Several pain conditions show a remarkable gender-related difference in their prevalence [24]. Men and women do not suffer from the same illnesses or do so with a different intensity and risk [25]. Epidemiological studies have shown that the female gender is itself a risk factor for chronic pain, although these findings appear to be pain site dependent. Gender differences are more consistently found in relation to abdominal pain and headache while instead for orofacial pain

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Median–IQR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VAS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6.0–3.0</td>
<td>0.597</td>
</tr>
<tr>
<td>Female</td>
<td>6.5–6.0</td>
<td></td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>5.8–4.0</td>
<td>0.495</td>
</tr>
<tr>
<td>Married</td>
<td>6.0–4.0</td>
<td></td>
</tr>
<tr>
<td>Divorced</td>
<td>7.0–6.0</td>
<td></td>
</tr>
<tr>
<td>Widowed</td>
<td>4.0–3.0</td>
<td></td>
</tr>
<tr>
<td>Job Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>5.0–3.0</td>
<td>0.019*</td>
</tr>
<tr>
<td>Unemployed</td>
<td>7.0–4.0</td>
<td></td>
</tr>
<tr>
<td>Educational level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(in years) vs VAS</td>
<td>–0.124</td>
<td>0.288</td>
</tr>
<tr>
<td>Age (in years) vs VAS</td>
<td>0.223</td>
<td>0.055</td>
</tr>
<tr>
<td>Age at disease onset</td>
<td>0.279</td>
<td>0.015*</td>
</tr>
<tr>
<td>(in years) vs VAS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IQR = interquartile range. The significance difference between the medians was measured by the Kruskal–Wallis nonparametric Anova. The correlation was measured by the Spearman correlation coefficient.

* Significant $0.01 < P < 0.05$.

** Significant $P < 0.01$. 

---

**Table 2** Dependence analysis of age at disease onset in relation to gender, job status, marital status, and educational level

<table>
<thead>
<tr>
<th>Age at Disease Onset</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>60.74 ± 14.06</td>
</tr>
<tr>
<td>Female</td>
<td>55.39 ± 11.05</td>
</tr>
<tr>
<td>Job status</td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>46.33 ± 10.66</td>
</tr>
<tr>
<td>Unemployed</td>
<td>59.35 ± 10.96</td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>49.80 ± 23.08</td>
</tr>
<tr>
<td>Married</td>
<td>57.82 ± 10.45</td>
</tr>
<tr>
<td>Divorced</td>
<td>47.25 ± 10.05</td>
</tr>
<tr>
<td>Widowed</td>
<td>58.00 ± 13.45</td>
</tr>
<tr>
<td>Educational level</td>
<td>Spearman $\rho = 0.314$</td>
</tr>
</tbody>
</table>

The significance difference between the means was measured by the Anova test procedure.

* Significant $0.01 < P < 0.05$.

** Significant $P < 0.01$.

---

**Table 3** Dependence analysis of VAS in relation to gender, job status, marital status, age, educational level, and age at disease onset

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Median–IQR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6.0–3.0</td>
<td>0.597</td>
</tr>
<tr>
<td>Female</td>
<td>6.5–6.0</td>
<td></td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
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<tr>
<td>Single</td>
<td>5.8–4.0</td>
<td>0.495</td>
</tr>
<tr>
<td>Married</td>
<td>6.0–4.0</td>
<td></td>
</tr>
<tr>
<td>Divorced</td>
<td>7.0–6.0</td>
<td></td>
</tr>
<tr>
<td>Widowed</td>
<td>4.0–3.0</td>
<td></td>
</tr>
<tr>
<td>Job Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>5.0–3.0</td>
<td>0.019*</td>
</tr>
<tr>
<td>Unemployed</td>
<td>7.0–4.0</td>
<td></td>
</tr>
<tr>
<td>Educational level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(in years) vs VAS</td>
<td>–0.124</td>
<td>0.288</td>
</tr>
<tr>
<td>Age (in years) vs VAS</td>
<td>0.223</td>
<td>0.055</td>
</tr>
<tr>
<td>Age at disease onset</td>
<td>0.279</td>
<td>0.015*</td>
</tr>
<tr>
<td>(in years) vs VAS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IQR = interquartile range. The significance difference between the medians was measured by the Kruskal–Wallis nonparametric Anova. The correlation was measured by the Spearman correlation coefficient.

* Significant $0.01 < P < 0.05$.

** Significant $P < 0.01$. 

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<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Change of Tongue Morphology</th>
<th>Change of Tongue Color</th>
<th>Bitter Taste</th>
<th>Scalding</th>
<th>Burning</th>
<th>Taste Disturbances</th>
<th>Xerostomia</th>
<th>Sciarora</th>
<th>Introral Foreign Body Sensation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td>Male</td>
<td>52.6%</td>
<td>31.6%</td>
<td>42.1%</td>
<td>21.1%</td>
<td>57.9%</td>
<td>42.1%</td>
<td>15.8%</td>
<td>63.2%</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>35.7%</td>
<td>39.3%</td>
<td>41.1%</td>
<td>26.8%</td>
<td>66.1%</td>
<td>42.9%</td>
<td>33.9%</td>
<td>58.9%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.049*</td>
<td>0.377</td>
<td>0.572</td>
<td>0.434</td>
<td>0.521</td>
<td>0.566</td>
<td>0.001**</td>
<td>0.482</td>
<td>0.153</td>
</tr>
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<td><strong>Marital status</strong></td>
<td>Single</td>
<td>20.0%</td>
<td>40.0%</td>
<td>20.0%</td>
<td>20.0%</td>
<td>40.0%</td>
<td>80.0%</td>
<td>60.0%</td>
<td>60.0%</td>
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<td></td>
<td>Married</td>
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<td>35.1%</td>
<td>42.1%</td>
<td>28.1%</td>
<td>70.2%</td>
<td>40.4%</td>
<td>28.1%</td>
<td>57.9%</td>
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<tr>
<td></td>
<td>Divorced</td>
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<td>25.0%</td>
<td>0.0%</td>
<td>50.0%</td>
<td>50.0%</td>
<td>25.0%</td>
<td>25.0%</td>
</tr>
<tr>
<td></td>
<td>Widowed</td>
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<td>55.6%</td>
<td>55.6%</td>
<td>22.2%</td>
<td>44.4%</td>
<td>33.3%</td>
<td>22.2%</td>
<td>88.9%</td>
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<tr>
<td><strong>P value</strong></td>
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<td>0.350</td>
<td>0.443</td>
<td>0.259</td>
<td>0.308</td>
<td>0.310</td>
<td>0.082</td>
<td>0.907</td>
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<tr>
<td><strong>Job status</strong></td>
<td>Unemployed</td>
<td>43.3%</td>
<td>38.3%</td>
<td>43.3%</td>
<td>25.0%</td>
<td>68.3%</td>
<td>41.7%</td>
<td>33.3%</td>
<td>60.0%</td>
</tr>
<tr>
<td></td>
<td>Employed</td>
<td>26.7%</td>
<td>33.3%</td>
<td>33.3%</td>
<td>26.7%</td>
<td>46.7%</td>
<td>46.7%</td>
<td>13.3%</td>
<td>60.0%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.229</td>
<td>0.719</td>
<td>0.482</td>
<td>0.895</td>
<td>0.118</td>
<td>0.727</td>
<td>0.049*</td>
<td>1.000</td>
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<tr>
<td><strong>Age (in years)</strong></td>
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<td>0.0%</td>
<td>100.0%</td>
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<tr>
<td></td>
<td>30–39</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>50.0%</td>
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<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>40–49</td>
<td>44.4%</td>
<td>44.4%</td>
<td>22.2%</td>
<td>33.3%</td>
<td>66.7%</td>
<td>33.3%</td>
<td>22.2%</td>
<td>33.3%</td>
</tr>
<tr>
<td></td>
<td>50–59</td>
<td>31.8%</td>
<td>27.3%</td>
<td>40.9%</td>
<td>31.8%</td>
<td>63.6%</td>
<td>40.9%</td>
<td>31.8%</td>
<td>50.0%</td>
</tr>
<tr>
<td></td>
<td>60–69</td>
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<td>&gt; 79</td>
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<td>69.2%</td>
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<td>60–69</td>
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<td>36.4%</td>
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<td>72.7%</td>
<td>36.4%</td>
<td>45.5%</td>
<td>81.8%</td>
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<tr>
<td><strong>P value</strong></td>
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<td>0.238</td>
<td>0.276</td>
<td>0.246</td>
<td>0.203</td>
<td>0.397</td>
<td>0.516</td>
<td>0.076</td>
<td>0.805</td>
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</tbody>
</table>

The significance difference between the conditional distributions was measured by the Pearson Chi-square test.

* Significant 0.01 < P ≤ 0.05.

** significant <0.001.
consistent gender differences in prevalence have not been established [26].

The true prevalence of BMS is difficult to establish due to the lack of rigorous diagnostic criteria in many studies which do not make any distinction between the secondary symptoms of oral burning and the syndrome itself. For this reason, estimates of the prevalence of BMS range from 0.7% to 7% of the general population and increase to 12–18% for postmenopausal women [27–29]. BMS occurs at a 90% higher rate in women than in men [30,31] and usually presents from 3 years before to 12 years after menopause [5]. The reported gender ratio of the affected patients (females to males) has ranged from 3:1 to 16:1. [32,33]. In our study, the male/female ratio was 1:3.

Multiple biopsychosocial mechanisms contribute to gender differences in BMS conditions, including sex hormones, endogenous opioid function, genetic factors, pain coping, and catastrophizing, and gender roles [28].

BMS occurs in middle aged and elderly subjects with an age range from 27 to 87 but rarely affects individuals under the age of 30 years, never having been described in children or adolescents. It usually occurs in the fifth to seventh decade of life [6].

The mean age of our patients was 61 years with a peak between 50 and 59 (22 patients); we had only one patient of 29 years. Furthermore, BMS occurs later in men than in women (65 compared with 60 years).

In this study, we found the same educational level in BMS patients as in the general South Italy population, revealing a higher percentage of BMS patients with a low educational level (less than 8 years, 35 patients, 46%).

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**Table 5** Gender, age, educational level, job status, marital status, and age at disease onset differences in relation to triggers of BMS

<table>
<thead>
<tr>
<th>Trigger</th>
<th>Gender</th>
<th>Male</th>
<th>Female</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 47.4%</td>
<td>5.3%</td>
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<td>10.5%</td>
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<tr>
<td>Female 23.2%</td>
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<tr>
<td>Married 31.6%</td>
<td>1.8%</td>
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<td>9.3%</td>
</tr>
<tr>
<td>Divorced 0.0%</td>
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<td>50.0%</td>
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<tr>
<td>Widowed 33.3%</td>
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<td>22.2%</td>
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<tr>
<td>Unemployed 30.0%</td>
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<td>25.0%</td>
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<td>Employed 26.7%</td>
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<tr>
<td>Male 47.4%</td>
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<td>Married 31.6%</td>
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<td>Female 23.2%</td>
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<td>Married 31.6%</td>
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<td>Employed 26.7%</td>
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<td>0.0%</td>
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</tbody>
</table>

The significance difference between the conditional distributions was measured by the Pearson Chi-square test.

* Significant 0.01 < P ≤ 0.05.
** Significant P ≤ 0.01.
On the contrary, in terms of marital status and job status, the data of the sample were different from those reported for South Italy. We found a higher percentage of married patients and widowed patients, and a lower percentage of single patients. Moreover, we found a higher percentage of unemployment (80%). Unemployment seems to be the most important stressful life event in our patients.

Through a comparison of the age at BMS onset and the age of the patients, we evaluated the professional diagnostic delay. Mignogna et al. in 2005, found that it usually takes 34 months to arrive at a definitive diagnosis (range, 1–348 months; median, 13 months). The average number of medical and dental practitioners consulted by each patient over this period and who initially misdiagnosed BMS was 3.1 (range, 0–12; median, 3) [34].

In this study, the diagnostic delay was 4.42 ± 0.26 years (median 53 months). Therefore, we can argue that the health care providers’ awareness of BMS, in 9 years, has not improved.

Gender and marital status did not influence age at disease onset. Conversely, educational level and job status were correlated with this factor (P < 0.001 and P = 0.006, respectively). In fact, BMS is recognized earlier in patients who have a higher educational level and are employed. A higher level of education seems to lead to a more informed patient with a better understanding of the relationship between stressful events and somatic disorders.

Furthermore, stress in the workplace could predispose some people to somatic symptoms [35].

In literature, oral mucosal burning pain represents the principal symptom of BMS. Pain levels may vary from mild to severe [36,37]. The mean severity of BMS pain has been assessed at about 5–8 cm (or 50–80 mm) on a 10-cm (100 mm) VAS scale [38,39].

Our data were in line with the current literature, because oral burning was present in 59 patients (70%); the Median-IQR of VAS in men was 6.0–3.0 and in women 6.5–6.0.

Consistently, the literature suggests that females may rate their pain as being more intense than males [40]. However, we did not observe any statistically significant difference in pain intensity in relation to gender. The pain intensity is equally high in men and women, being independent of gender (P = 0.597).

Hungria et al., in relation to temporomandibular disorders (TMD) found that patients with a lower educational level suffer more from pain than people with a higher educational level. However, in our patients pain was not related to educational level (P = 0.288) [41].

In other chronic pain conditions, Goulet et al. found that age influences the intensity of pain. In that study, the authors found that individuals in the elderly group (55+) were three times more likely to rate jaw pain as severe than the younger age group (18–34) [42]. In contrast, in our study, the age of the patients was not related to the pain intensity (P = 0.223, P = 0.055). Job status and age at disease onset were correlated with the VAS scale in this study (P = 0.019 and 0.015, respectively). Generally, pain intensity in orofacial pain syndromes (such as temporomandibular disorders) will increase with the duration of the illness [43]. This tendency was observed in our sample of BMS patients; it is conceivable that the prolonged time for diagnosis (median 53 months), and the absence of any treatment could contribute to increasing the psychological stress of the patients and consequently the pain intensity.

Unemployed patients showed higher values on the VAS scale compared with employed patients. Unemployment could contribute to the psychological stress of patients and, consequently, could influence directly the pain intensity.

Clinical presentations of BMS may vary as some patients can be monosymptomatic (burning or pain only) or oligosymptomatic [29]. Generally, in BMS, oral burning alone is rare, it being more frequently associated with one or more other oral symptoms, such as xerostomia, paresthesia, and altered taste [3].

Other authors have reported xerostomia in approximately 46–67% of BMS patients [2,33]; in our study, xerostomia was the most frequent symptom after oral burning (45 patients, 60%). In contrast with the current literature that has reported dysgeusia in almost 70% of BMS patients [1,38,44], in our study taste disturbances was ranked in eighth place (22 patients, 30%). A bitter taste, a very specific oral sensation, was in third place (32 patients, 43%). We found that taste disturbances were frequent in women (34% compared with 16%) and in the unemployed (33% compared with 13%).

Another factor to consider is that our patients show an excessive concern about their health and are focused on inspecting their mouth many times during the day, noting any changes of the tongue morphology and tongue color. They evaluate mainly the tongue probably because this is the site most affected by symptoms. A changing of the tongue morphology was noted principally by men (52% compared with 36%) A changing of the tongue color was observed frequently in patients with a lower educational level (5–7; years; 55%).

The age at disease onset did not modify the quality of the symptoms reported by our patients.

The onset of BMS is spontaneous although some patients report antecedent dental procedures, the initiation of medications, or other illnesses [16,29,44].

Sociodemographic Characteristics and Burning Mouth Syndrome
In this study, the most frequent trigger reported was stressful life events (34 patients, 45%); in contrast with the current literature, antecedent dental procedures were in third place (15 patients, 20%).

Through the analysis of dependence, we can see that gender, job status, and age at disease onset did not influence the triggers in BMS. On the contrary, marital status and educational level were associated.

The surprising dependence on marital status needs to be addressed by further studies: at the moment no hypothesis seems to be appropriate.

Antecedent dental procedures was the most common trigger in patients with the lowest educational level (50%) while patients with the highest level of education fail to find a cause for their BMS (50%).

In conclusion, BMS remains an important medical condition which often places a significant burden on both the patient and the health care system.

This article reports that BMS occurs more frequently in women than men. Nevertheless, gender does not influence pain intensity perception, which is equally high in women and in men. Pain intensity is higher in the unemployed and is related to diagnostic delay. A longer time in reaching a diagnosis is connected with higher pain intensity.

Burning is the most frequent symptom while stressful life events is the most frequent trigger reported in patients with BMS.

Within the limitations of the study, it can be concluded that the sociodemographic characteristics of patients could play a role in determining the clinical features of BMS. However, further investigations and multicentric studies are needed to support our hypothesis.

References
Sociodemographic Characteristics and Burning Mouth Syndrome

Nodular fasciitis of the tongue

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ABSTRACT: Background. Nodular fasciitis is a non-neoplastic proliferation within the subcutaneous tissue and the deep fascia of the fibroblasts, probably of a reactive nature characterized by apparent infiltration of the connective tissues by a mitotically active spindle cell lesion. Nodular fasciitis in the head/neck region is rarely found and only 2 previous cases affecting the tongue have been reported.

Methods and Results. The purpose of this study was for us to report a very rare case of a 67-year-old man with a history of a 3-month subepithelial asymptomatic nodule of the tongue tip with an ulcerated surface. An excisional biopsy of the mass was performed with 0.5-mm surgical margins.

Conclusion. The clinical and histological features of nodular fasciitis may mimic a head and neck malignancy, but it is often misdiagnosed as a malignant mesenchymal neoplasm. Even if it is a rare entity, nodular fasciitis should be considered in cases of rapidly growing masses of the head and neck region. 

KEY WORDS: nodular fasciitis, tongue, lingual fasciitis, mesenchymal neoplasm, pseudosarcomatous fibromatosis

INTRODUCTION

Nodular fasciitis is a non-neoplastic proliferation within the subcutaneous tissue and the deep fascia of the fibroblasts. It is probably of a reactive nature and occurs typically in the third to fifth decades of life with no sex predilection. 1,2 The clinical and macroscopic appearance is nonspecific and microscopic examination shows an apparent infiltration of the connective tissues by a mitotically active spindle cell lesion. It can develop in almost any site, except the viscera, and is most commonly encountered in the upper extremities. 1,3 Nodular fasciitis in the head and neck region is rarely found, but is more common in children. In the oral mucosa, it has also rarely been reported, with the most common involvement being in the buccal mucosa. Only 2 previous cases of nodular fasciitis of the tongue have been described in literature by Takagi and Ishikawa 4 in 1982 and by Martinez–Blanco et al 5 in 2002.

CASE REPORT

In July 2014, a 67-year-old white man was referred to our Oral Medicine Unit, Department of Head and Neck Diseases, Federico II University of Naples, presenting with an asymptomatic lesion of the tongue tip that had been growing rapidly for 3 months and with no history of trauma.

Clinically, the lesion appeared as a firm subepithelial myxoid nodule of approximately 2 cm, with an ulcerated surface and hard consistency upon palpation (Figure 1A).

His medical history included hypercholesterolemia. Routine hematologic tests revealed a phosphorus level of 2.9 mg/dL (normal range, 3–4.5), a total cholesterol level of 206 mg/dL (normal value, up to 190), and an immunoglobulin G4 subclass deficiency with a level of 0.004 g/L (normal range, 0.08–1.40).

Because of the anatomic position of the lesion and of its volume, it occupied the entire thickness of the muscle.

An excisional biopsy of the mass was performed with 0.5-mm surgical margins of excision. Histology revealed a proliferation of plump spindle cells arranged in irregular fascicles with a focal storiform pattern and no characteristic vascular pattern. The cells had vesicular and hyperchromatic nuclei and small to medium sized nucleoli. Brisk mitotic activity was observed with no sign of necrosis (Figure 1B–1D). Immunohistochemistry showed positivity for vimentin and smooth muscle actin (Figure 1E).

Based on its morphological features and immunohistochemical profile, it was diagnosed as a fasciitis-like myofibroblastic proliferation.

This study was approved by the Ethics Committee of University “Federico II” of Naples on October 2014 with the protocol number 173/14. Appropriate written informed consent and permission were obtained from the patient described in this article.
DISCUSSION

The pathogenesis of nodular fasciitis remains unknown, but it seems that it is a self-limiting lesion, reactive or inflammatory in nature, rather than a true neoplasm, often triggered by trauma. The clinical and histological features of nodular fasciitis may mimic a malignancy because of its acute onset, rapid growth, rich cellularity, and mitotic activity, thus leading to it often being misdiagnosed as a malignant mesenchymal neoplasm.

As a result, the diagnosis is often a challenge for many clinicians as well as for the pathologists.

Differential diagnosis encompassed reactive proliferations, such as pyogenic granuloma, inflammatory myofibroblastic tumors (historically known as “pseudotumors”), several soft tissue sarcoma-like leiomyosarcoma, rhabdomyosarcoma, fibrosarcoma, myxoid neurofibroma, myxoid hematologic malignancies like myeloid sarcomas, non-Hodgkin lymphomas and multiple myeloma, and sarcomatoid variant of squamous cell carcinoma.5,6

The usefulness of the fine-needle aspiration cytology in nodular fasciitis diagnosis is supported by many authors, but is not shared by several other authors because it can easily lead to misdiagnoses of malignant neoplasms of spindle cells or salivary glands or cutaneous tumors, so this aspect remains to this day discordant.7

Conservative surgical excision remains the standard treatment of these lesions. Self-resolution has widely been reported in literature, spontaneous or after partial resection.8

A rational clinical algorithm to manage these kind of patients could be the following: after a mandatory incisional biopsy, a 4 to 6-week period of follow-up should be considered because of the possibility of spontaneous regression of the tumor. If resolution does not occur after this period, complete conservative surgical therapy should be performed.

After the first surgery, our patient has until today a 5-month negative follow-up.

Even if it is a rare entity, nodular fasciitis should be considered in cases of rapidly growing masses of the head and...
neck region. The comprehensive knowledge of this entity is indispensable to correctly plan the appropriate therapy and to avoid a misdiagnosis of malignant mesenchymal neoplasms for which more aggressive treatment options are needed.

REFERENCES

Short communication

Primary oral leishmaniasis mimicking oral cancer: a case report

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Abstract

Primary mucosal leishmaniasis is a rare infectious disease, particularly in immunocompetent patients. We present a 50-year-old patient with a 6-week history of a painful lesion of the left buccal mucosa that mimicked cancer. The exophytic lesion looked invasive, and we took an incisional biopsy specimen to exclude cancer. The diagnosis of leishmaniasis was unexpected, and the patient was successfully treated with amphotericin B for five weeks. After five months the patient had a visceral recurrence. Chronic exophytic and ulcerated mucosal lesions that do not heal within 3–4 weeks should be regarded as the first signs of oral cancer, but primary oral leishmaniasis can easily mimic it.

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Keywords: Exophytic lesion; Infectious diseases; Mucosal leishmaniasis; Oral cancer; Oral leishmaniasis

Leishmaniases are neglected tropical parasitic diseases that are classified clinically into three forms, visceral, cutaneous, and mucosal leishmaniasis.1,2 They can all affect the head and neck region with primary mucosal lesions that can imitate a wide range of oral infectious diseases and neoplasms.1–3

We present the case of a 50-year-old, immunocompetent, white man who was referred to us in January 2014 with a six-week history of a symptomatic lesion of the left buccal mucosa, which looked malignant (Fig. 1). He was a heavy smoker and had no contributory history. He was from an area of Naples, Southern Italy, where leishmaniasis is endemic, and had no history of recent travels abroad. He complained of pain, spasm of the jaw, and weight loss of 6 kg. His dental history included poor oral hygiene and the presence of a crown fracture of 28.

Routine haematological tests showed no abnormality except for increased activity of lactate dehydrogenase (650 U/L).

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0266-4356/© 2015 The British Association of Oral and Maxillofacial Surgeons. Published by Elsevier Ltd. All rights reserved.
Leishmaniases are a worldwide threat with about 58,000 cases of visceral and 220,000 cases of cutaneous disease reported annually, high rates of mortality and morbidity.\(^3\)\(^\text{5}\) and (according to the WHO) an estimated 350 million people at risk of contracting the infection.\(^4\)

The female sand fly (genus *Phlebotomus* or *Lutzomyia*) transmits the parasite.\(^2\) People that have to stay in endemic areas (such as inhabitants, migrant workers, soldiers, and refugees) are considered at risk.\(^6\)

Systemic spread of the disease is common, and may involve the liver, spleen, abdominal lymphatic system, lymph nodes, and bone marrow. Oral involvement with mucosal disease, and any mucosal leishmaniasis of the head and neck, is rare in immunocompetent patients, at least as the only manifestation. Differential diagnoses encompass a range of other diseases including oral squamous cell carcinoma (SCC).\(^7\)

The standard treatment of mucosal forms of the disease is based on pentavalent antimonials.\(^8\)

Oral SCC is among the 10 most common cancers worldwide, and affects about 700,000 people. Alcohol, smoking, and HPV are risk factors.\(^9\) Any indurated, heterogeneous lesion of the oral mucosa, particularly one that presents with white patches mixed with erythematous, erosive, or ulcerated areas, should lead to a suspicion of malignancy and in particular oral SCC.

Examination of a biopsy specimen is an essential first step. However, this will not always be sufficient to diagnose an infectious disease, and in all uncertain cases procedures such as an ELISA or polymerase chain reaction will be necessary.

**Conflict of interest**

We have no conflict of interest.

**Ethics statement/confirmation of patient’s permission**

The study was approved by the Ethics Committee of the University. The patient gave written informed consent to publication.

**References**

Oral manifestations of phosphatase and tensin homolog hamartoma tumor syndrome

A report of three cases

Antonio Celentano, DDS; Daniela Adamo, DDS; Stefania Leuci, DDS, PhD; Michele Davide Mignogna, MD, DMD

Germline mutations in the phosphatase and tensin homolog (PTEN) gene are linked to a spectrum of disorders known as “PTEN hamartoma tumor syndrome” (PHTS). These disorders are Cowden disease (CD), Bannayan-Riley-Ruvalcaba syndrome (BRRS), adult Lhermitte-Duclos disease (LDD) and autism spectrum disorders associated with macrocephaly. The bulk of the clinical data regarding these disorders comes from studies of patients with CD and, less commonly, with BRRS.

Here, we present a case series of three patients: a 26-year-old man affected by CD and a 60-year-old man and his 33-year-old daughter, both affected by BRRS and having the complaint of multiple oral papillomatous lesions and cutaneous trichilemmomas. The purpose of this study is to add new data to the current literature on the clinical findings of these rare diseases, considering that oral manifestations are identified as major diagnostic criteria of PHTS.

CASE DESCRIPTIONS

All three cases described here involve patients treated in the Oral Medicine Unit, Department of Head and Neck Diseases, University of Naples Federico II, Italy. The internal institutional review board at the University at Naples Federico II approved this study, and we obtained written informed consent from all participants before completing study assessments.

Case 1. In October 2013, a 26-year-old white man was referred to the oral medicine unit with the main complaint of asymptomatic keratotic lesions of the maxillary and mandibular gingiva (Figure 1). His medical history included a mild form of celiac disease, as well as multiple papillomatous lesions of the penis, hands and plantar skin. His dental history was negative for oral disease. He had never undergone endoscopy or colonoscopy.

ABSTRACT

Background. Phosphatase and tensin homolog (PTEN) hamartoma tumor syndrome (PHTS) encompasses several rare disorders linked to mutations of the PTEN gene, including Cowden disease (CD) and Bannayan-Riley-Ruvalcaba syndrome (BRRS). The authors present a case series involving patients with characteristic periodontal features.

Case Descriptions. The authors assessed three patients, two of whom already had been diagnosed with BRRS: a 60-year-old man and his 33-year-old daughter, both of whom had pathognomonic oral and cutaneous manifestations, and a 26-year-old man affected by multiple micropapillomatous and keratotic periodontal lesions, through which the diagnosis of CD was made. All three patients were referred to the oral medicine unit of the authors’ institution because of asymptomatic lesions of the oral mucosa, and two of them underwent incisional biopsy.

Conclusions. This series of cases emphasizes that oral health care workers always should perform a more careful visual inspection of the oral cavity without neglecting a macroscopic analysis of the gingival pattern. The knowledge of these diseases and their clinical features, associated with a multidisciplinary approach, allows clinicians to achieve remarkable diagnostic success.

Practical Implications. Gingival manifestations may represent one of the primary clinically detectable manifestations of these rare systemic diseases, in respect of which an early diagnosis could decrease the associated mortality and morbidity.

Key Words. Oral medicine; genetics; neoplasms; phosphatase and tensin homolog; PTEN hamartoma tumor syndrome.

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The patient, after providing his written informed consent, was hospitalized and examined by three of the authors (A.C., D.A., M.D.M.) by means of routine hematologic tests, including glucose, sodium, potassium, chloride, calcium, creatinine, albumin, hemoglobin, alanine aminotransferase, alkaline phosphates, aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase, hematocrit, red blood cell (RBC) count, mean corpuscular hemoglobin, mean corpuscular volume, platelet count, mean platelet volume, RBC distribution width, white blood cell count and differential, human immunodeficiency virus, and hepatitis B and C viruses. All laboratory test values were normal.

The physical examination showed a widespread whitish papillomatosis of the maxillary and mandibular gingiva with a micropapillary, at times cobblestonelike, pattern and a mild keratosis. The same three clinicians performed an incisional biopsy of the maxillary gingiva, and the histopathological examination revealed a proliferation of multiple benign fibromas with overlying hyperkeratosis. This histopathological result, together with the widespread aspect of lesions in the mouth, the presence of multiple dermatologic lesions and the absence of risk factors or inflammatory processes, led us to conjecture a different diagnosis than keratotic lesions. Because we suspected CD, we conducted further investigations. A gastrointestinal endoscopy showed two gastric polypoid lesions, and, four weeks later, a gene study by means of direct sequencing established the diagnosis of CD caused by a germline mutation in exon 8. A close surveillance was established and, at press time, the patient continues to be monitored closely for manifestations of CD.

**Case 2.** In November 2013, a 33-year-old white woman was referred to the oral medicine unit with the main complaint of a one-year history of bilateral hamartomatous lesions of the buccal mucosa. Her medical history included moderate obesity (body weight, 103 kilograms; height, 1.75 meters; body mass index, 33.6), secondary amenorrhea (treated by means of ethinyl estradiol and drospirenone), diffuse gastric polyposis, osteoporosis and Hashimoto thyroiditis (treated by means of levothyroxin 125 micrograms per day). The patient has BRRS, diagnosed through genetic tests at the age of 25 years after the discovery of this disease in her father. After providing her written informed consent, the patient was hospitalized and examined by means of routine hematologic tests. All laboratory test values were normal.

Physical examination of the patient revealed bilateral exophytic lesions of the buccal mucosa (Figure 2) (maximum diameter, about 1 centimeter) resulting from the confluence of multiple papillomas, widespread papillomatous lesions of the maxillary and mandibular gingiva (Figure 3) and micropapillomatosis of the tongue with a cobblestonelike pattern.

The skin inspection revealed a trichilemmoma localized on the left nasolabial fold, multiple cutaneous papillomas of the left hemithorax and the homolateral abdominal skin, skin-colored scars on the site of an inframammary incision on the right side (results of a surgical procedure performed four months previously) and a hemangioma on the elbow. A yearly follow-up protocol has been established.

**Case 3.** In November 2013, a 60-year-old white man visited the oral medicine unit, initially not as a patient but rather to accompany the woman described in case 2, who was his daughter, on her visit. His medical history included hypertension, colon cancer (in 2005) and a series of surgical removals of multiple cutaneous trichilemmomas. Physical examination revealed widespread papillomatous lesions of the

maxillary and mandibular gingiva with the same clinical periodontal pattern as in case 2, as well as multiple papillomas of the dorsum of the tongue, all of which had a cobblestonelike pattern (Figure 4). Skin inspection revealed multiple papillomas of the abdomen and some macular pigmentations of the glans penis.

**Follow-up for all three patients.** Three of the authors (A.C., D.A., M.D.M.) properly informed the patients about the need to undergo a regular follow-up for gastric and colon polyps, thyroid nodules, and breast and cutaneous diseases because these conditions usually are symptomatic of PHTS and also are associated with higher cancer risk.

**DISCUSSION**

CD is a rare, multisystem disease that causes an increased risk of malignancies (breast, thyroid and endometrial) as well as a benign hamartomatous overgrowth of tissues (such as skin, colon and thyroid). CD was first described in one family in 1963, a description then extended by Weary and colleagues in 1972, who added an additional set of five patients and expanded the spectrum of component features. Germline PTEN mutations first were reported in people with CD in 1997.

BRRS is a rare congenital disorder whose primary clinical features include macrocephaly, hamartomatous intestinal polyps, lipomas and pigmented macules on the penis. Other features include developmental delay, vascular anomalies, large birth weight and joint hyperextensibility. Diagnoses are based on the presence of several of the primary clinical features. BRRS has been shown to be allelic to CD, with approximately 60 percent of patients with a clinical diagnosis of BRRS having PTEN mutations. There are relatively few data regarding the clinical features of BRRS patients with documented PTEN mutations, however; only 30 published cases have been identified (in a 2003 review).

The National Comprehensive Cancer Network, Fort Washington, Pa., has established testing criteria denoting when PTEN testing is indicated; these criteria are based on the clinical features present in a patient. It also has established management and screening recommendations for people who have been found to have a PTEN mutation. However, in clinical practice, it often is necessary to provide treatment for patients on the basis of their clinical diagnosis alone, either because testing is not possible or because it has been done but no mutation has been found. Thus, accurate clinical diagnostic criteria are a necessary adjunct to genetic testing.

Box 1 presents a summary of the diagnostic criteria for PHTS proposed by Pilarski and colleagues.

Patients affected by PHTS require lifelong follow-up, and family members should be screened for the disease. For example, mammography at regular intervals and monthly breast self-examination are of great importance for the early diagnosis of breast cancer. Some authors even suggest bilateral prophylactic mastectomy for patients with PHTS.

Thyroid function tests, thyroid ultrasonography, complete blood count, complete urine analysis, Papanicolaou (Pap) test and abdominopelvic ultrasonography should be performed with all patients at the first stage and repeated at regular intervals. If thyroid nodules are present, fine-needle biopsy or surgical biopsy should be performed.

Box 2 presents a brief summary of the management of CD.

As is well known from the literature and confirmed by our experience, alterations in the oral mucosa and skin usually are present in patients with PHTS. Starink and colleagues reported that 100 percent of patients with PHTS developed oral papillomatosis by the second decade of life, and, for this reason, in the majority of cases such features may precede malignancy in other sites.

A differential diagnosis of oral papillomatous lesions includes multiple traumatic fibromas, oral fibromas in tuberous sclerosis, Darier-White disease, Heck disease, lymphangioma, pyogenic granuloma, fibroepithelial polyps, lipid proteinosis, oral florid papillomatosis, oral papillomas in Goltz syndrome, mucosal neuromas of
**BOX 1**

**Revised clinical diagnostic criteria for PTEN* hamartoma tumor syndrome.†**

### MAJOR CRITERIA

- Breast cancer
- Endometrial cancer (epithelial)
- Thyroid cancer (follicular)
- Gastrointestinal hamartomas (including ganglioneuromas, but excluding hyperplastic polyps; three or more gastrointestinal hamartomas total)
- Lhermitte-Duclos disease (adult)
- Macular pigmentation of the glans penis
- Multiple mucocutaneous lesions (any of the following), proven by means of a biopsy or diagnosed by a dermatologist:
  - Multiple trichilemmomas (three or more, at least one of them proven by means of a biopsy)
  - Acral keratoses (three or more palmpoplantar keratotic pits, acral hyperkeratotic papules or a combination of the two)
  - Mucocutaneous neuromas (three or more)
  - Oral papillomas (particularly on tongue and gingiva), multiple (three or more)

### MINOR CRITERIA

- Autism spectrum disorder
- Colon cancer
- Esophageal glycogenic anacanthosis (three or more lesions)
- Lipomas (three or more)
- Intellectual disability (that is, an intelligence quotient test score of 75 or lower)
- Renal cell carcinoma
- Testicular lipomatosis
- Thyroid cancer (papillary or follicular variant of papillary)
- Thyroid structural lesions (for example, adenoma, multinodular goiter)
- Vascular anomalies (including multiple intracranial developmental venous anomalies)

### OPERATIONAL DIAGNOSIS IN AN INDIVIDUAL: EITHER OF THE FOLLOWING

- Three or more major criteria, but one must be macrocephaly, Lhermitte-Duclos disease or gastrointestinal hamartomas
- OR
- Two major and three minor criteria

### OPERATIONAL DIAGNOSIS IN A FAMILY IN WHICH ONE PERSON MEETS REVISED CLINICAL DIAGNOSTIC CRITERIA FOR PTEN HAMARTOMA TUMOR SYNDROME OR HAS A PTEN MUTATION

- Any two major criteria with or without minor criteria
- OR
- One major criterion and two minor criteria
- OR
- Three minor criteria

*PTEN: Phosphatase and tensin homolog.
† According to the diagnostic criteria for PTEN hamartoma tumor syndrome proposed by Pilarski and colleagues.1

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**BOX 2**

**Management of Cowden disease.***

### WOMEN

- Breast self-examination starting at age 18 years
- Clinical breast examination starting at age 25 or five to 10 years before the earliest breast cancer in the family occurred, whichever comes first
- Patient education about endometrial cancer symptoms and clinical screening
- Discussion of prophylactic mastectomy and hysterectomy

### MEN AND WOMEN

- Annual physical examination starting at age 18 years or five years before the youngest age at which cancer occurred in family history, whichever comes first
- Thyroid sonogram at age 18, then once per year
- Colonoscopy, starting at the age of 35 years, then every five to 10 years
- Annual dermatologic examination

### RISK TO RELATIVES

- Explanation of possible inherited cancer risks to relatives, as well as options for risk assessment and management (the parents of an affected proband must be examined clinically and genetically; if a parent is affected, his or her family members are at risk)
- Genetic counseling

* Source: National Comprehensive Cancer Network.10

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**CLINICAL IMPLICATIONS**

The purpose of this article is to stimulate general dental practitioners to perform an accurate oral examination focusing mainly on the periodontal mucosa and to investigate the differential diagnosis of widespread oral papillomatous lesions carefully. These can represent one of the primary clinical features of this diagnostic challenge, even in young people.

Early diagnosis is the most important element in the management of PHTS: affected people have a lifetime risk of up to 50 percent for breast cancer, 10 percent for thyroid cancer and 5 to 10 percent for endometrial cancer. More than 90 percent of affected people will show some clinical manifestations by their 30th or later years.11-19

Dental practitioners who suspect the presence of a PTEN-related syndrome, or more generally of a multiple hamartoma syndrome, should request from specialists a better screening of their patients to meet major and minor diagnostic criteria before resorting to genetic investigation, which is expensive. The important diagnostic steps after the oral examination are an accurate anamnestic evaluation, an accurate total-body dermatologic examination, breast and thyroid sonographic imaging, a genitourinary screening, gastroscopy and colonoscopy.

Establishing a clear diagnosis means giving these patients the opportunity to undertake an earlier self-surveillance, thereby decreasing the mortality and morbidity associated with these conditions. Once again, dentists may play an important role in the diagnosis and management of rare systemic diseases.

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multiple endocrine adenomatosis, acanthosis nigricans, pseudoepitheliomatous hyperplasia and squamous cell carcinoma.14

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CONCLUSIONS
Our experience highlights that oral health care workers could represent the first line of defense in early diagnosis of rare disorders. In the patients in our three cases, gingival clinical features and a widespread oral papillomatosis led us to suspect PHTS or, in the already diagnosed patients, PHTS-related lesions.

Three cases of PHTS, two of them in patients of relatively young ages, are not a small number to be seen in a single center, and this aspect stresses the importance of intercepting disease early.

Nowadays, the visual inspection of the oral cavity requires higher standards, commensurately greater time, and a wider knowledge of these diseases and their main clinical features. A multidisciplinary approach is key in these diagnostic challenges and allows clinicians to achieve a remarkable diagnostic success. If physicians are able to provide an early diagnosis, people with rare disorders can achieve an adequate quality of life, reducing the systemic complications of their conditions.

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REVIEW ARTICLE

Mucosal leishmaniasis with primary oral involvement: a case series and a review of the literature

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OBJECTIVE: To analyze retrospectively a case series of primary oral leishmaniasis and to review the literature on head–neck primary mucosal leishmaniasis (ML) in immunocompetent patients.

SUBJECTS AND METHODS: A PUBMED search was carried out from 1950 to 2013. Clinical records of patients with primary head–neck mucosal manifestations of leishmaniasis were analyzed. In addition, clinical records between 2001 and 2012 of patients with primary oral manifestations were collected from two independent hospitals.

RESULTS: Our multicenter case series revealed seven patients with oral leishmaniasis. The most commonly affected site was the tongue (four patients, 57%), and the most common clinical presentation was an exophytic lesion (six patients, 85%). The literature review showed 11 reports published between 2005 and 2013, describing 13 patients (100% male) affected by head–neck primary ML (54% laryngeal, 31% oral, 23% pharyngeal, and 15% endonasal). The most common clinical presentation was an exophytic lesion (69%).

CONCLUSIONS: The literature analysis revealed that in immunocompetent patients, the oral mucosa is the second most frequently affected site of the head and neck region. In the oral cavity, the tongue is the most affected site. Diagnosis of oral leishmaniasis represents a challenge but must be considered in any differential diagnosis of exophytic lesions of oral mucosa.

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Keywords: primary oral leishmaniasis; mucosal; immunocompetent; head–neck leishmaniasis

Introduction

Leishmaniasis is a parasitic disease caused by several protozoan species of the genus Leishmania, belonging to the family Trypanosomatidae. After malaria and African trypanosomiasis (‘sleeping sickness’), the leishmaniases are the third most important group of vectorborne diseases and are ranked ninth in terms of the global burden of disease of all infectious and parasitic diseases (Prabhū et al., 1992; Stockdale and Newton, 2013).

The leishmaniases are widely dispersed, with transmission to humans on five continents, and are endemic in 98 countries. However, the human disease burden is concentrated mainly in a few major foci. The epidemiological data on leishmaniasis are very complex, with intra- and interspecific variations in transmission cycles, reservoir hosts, sand fly vectors, and clinical forms (World Health Organization, 2010).

Travelers and soldiers who have to stay in endemic areas, foreign citizens, migrant workers, asylum seekers, refugees from endemic areas, migrants, and immunosuppressed patients are considered as risk groups (Grimaldi and Schottelius, 2001).

In humans, the clinical forms of the leishmaniases are broadly categorized into three groups: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucosal leishmaniasis (ML). However, a more complete classification encompasses 11 different clinical forms (World Health Organization, 2010). Among these, mucocutaneous leishmaniasis (MCL) or ML pure forms can occur with a latency of months, even years, after exposure in endemic areas and because of this huge latency, the diagnosis is often seriously delayed. All the clinical forms of leishmaniasis can start with primary mucosal lesions in the head–neck region, sometimes affecting the oral cavity, or certain significant symptoms detectable by the dental practitioner. Such symptoms include swallowing difficulties, dysphonia, and dyspnea. Therefore, dentists play an important role in the early diagnosis of oral leishmaniasis, to avoid the systemic spread of the disease (Pellicioli et al., 2012).

In any such systemic spread, the sites most commonly involved are the liver, spleen, abdominal lymphatic system, lymph nodes, and bone marrow.
Management of leishmaniasis still represents a big challenge. Presently, the main target of any drugs used is to fight the parasite in its different physiological and biochemical aspects and to promote the immune response of the host. The therapeutic strategies currently available include pentavalent antimony derivatives, systemically or intralesionally administered, pentamidine, metronidazole, amphotericin B, azoles, and miltefosine (Masmoudi et al., 2013).

The primary objective of this study is to present a retrospective multicenter case series describing seven cases of primary oral leishmaniasis. The secondary objective is to review the literature on head and neck primary ML in immunocompetent/otherwise healthy patients, as no similar data are currently available in the literature, and to update the current literature with our seven new cases.

Patients and methods
Multicenter case series assessment
We retrospectively selected and analyzed the clinical data of leishmaniasis patients from the archives of two independent hospitals, the outpatient clinic of the Oral Medicine Unit, Department of Head and Neck Diseases, Federico II University of Naples and the Department of Dental Sciences and Surgery, University of Bari, reporting seven new cases with a history of ML with exclusive and primary oral involvement, diagnosed and treated between 2001 and 2012.

The study was approved by the Ethics Committee of University of Naples ‘Federico II’.

All patients, after providing their written informed consent, were hospitalized and examined by routine hematological testing, HIV, HBV, HCV, and oral biopsies with histopathological evaluation. Abdominal CT and bone marrow biopsies were made in some cases to exclude any suspected visceral forms. The study was performed in accordance with the Declaration of Helsinki.

Literature review

The inclusion criteria of this review encompass (i) articles published in the English language reporting primary ML for all patients described; (ii) healthy or immunocompetent patients who contracted an infection of leishmania- sis between 1950 and 2013; (iii) mucosal lesions that presented in the regions of the nasal mucosa, oral mucosa, nasopharynx, oropharynx, hypopharynx, esophageal, and laryngeal mucosa; (iv) the absence of any visceral or cutaneous manifestations; and (5) documentation on the leishmania infection.

A flow chart is shown in Figure 1.

In relation to the reviewed articles, we collected, tabulated, and depicted the following information: country and year of publication, age, sex, underlying or past diseases, predisposing risk factors, local conditions, involved sites, symptoms, lesion description, diagnostic procedures, treatment, follow-up period, and outcomes.

Results
The case series from our database shows seven patients, three of whom were treated at our institution.

All the patients underwent incisional or excisional biopsy (cases 2, 4, and 7), and in all cases, the biopsy showed inflammatory lesions, with the presence of parasites belonging to Leishmania spp., allowing the diagnosis of leishmaniasis. Subsequently, all patients underwent routine laboratory examinations, CT scanning, and bone marrow biopsy, revealing no significant findings or evidence of leishmaniasis elsewhere except for splenomegaly in case 5 (Figures 2–6).

Case reports
Case 1 – A 55-year-old Caucasian man with a history of a 4-week painful swelling affecting the left side of the tongue. The patient had lived in Senegal for 20 years and was a smoker (25 cigarettes daily for 30 years).

Physical examination showed a nodular, whitish, and ulcerated lesion on the left edge of the tongue, 1.7 cm of maximum diameter, with some mild perilesional inflammatory lesions, with the presence of para-

The patient was treated with 28 intramuscular injections of meglumine antimoniate at a daily dose of 5 mg kg⁻¹ body weight.

After 1 month, the patient had a complete remission of the tongue lesion, but after 8 months, the disease recurred with the onset of a cutaneous form of leishmaniasis. The patient was referred to the Infectious Diseases Department for treatment.

Case 2 – A 48-year-old Caucasian man with a history of a 7-week painful lesion of the tongue. The patient was...
a former smoker (40 cigarettes daily for 25 years), and 2 years before, he had been treated with polychemotherapy for B-cell lymphoblastic NHL.

Physical examination revealed a nodular and erythematous lesion, with a hard consistency and a mild bleeding upon palpation, affecting the left lateral border of the tongue, near the tongue tip.

Surgical treatment with an excisional biopsy was performed. The patient refused the recommended pharmacological treatment.

After the clinical healing, the patient had a 7-month follow-up period but subsequently developed a visceral form of leishmaniasis characterized at onset by fever, diarrhea, hepatosplenomegaly, and lymphadenopathy. The patient was referred to the Infectious Diseases Department for treatment.

Case 3 – A 33-year-old man from Pakistan with a history of a 7-week swelling and soreness of the tongue with a difficulty in swallowing and 4-kg weight loss.

Physical examination revealed an ulcerated, soft-to-firm, and dome-shaped mass of the dorsum of the tongue. The largest diameter was approximately 3.5 cm.

No skin lesions were observed. The patient was treated with 20 intramuscular injections of meglumine antimoniate at a daily dose of 5 mg kg⁻¹ body weight.

After 1 month, the patient had a complete remission of the tongue lesion; after 13 months, the disease recurred.
with the onset of a cutaneous form of leishmaniasis. The patient was referred to the Dermatological Department for treatment.

Case 4 – A 41-year-old Caucasian man with a history of an 8-week asymptomatic lesion affecting the dorsum of the tongue.

The man was a farmer, and 4 weeks before the appearance of the lesion, he had undergone two courses of antibiotics for a prostatitis.

Physical examination showed a multinodular lesion affecting the posterior dorsum of the tongue, just anterior to the circumvallate papillae, with an increased consistency and a maximum diameter of 2 cm.

After excisional surgical treatment, the patient was treated with stibogluconate 20 mg kg\(^{-1}\) day\(^{-1}\) for 20 days and had a complete healing with a negative 12-month follow-up.

Case 5 – A 61-year-old Caucasian woman with a history of a 5-week diffuse and sore swelling of the upper lip.

The woman was from Ischia, a well-known endemic area of leishmaniasis in Italy. She was in good health except for a reported splenomegaly, confirmed by abdominal CT scan.

Physical examination showed a diffuse swelling of the upper lip measuring approximately 4 × 1.5 cm with ulcers and erosions extending to the anterior maxillary vestibule.

Two previous biopsies, performed in other hospitals, had been negative.

We performed a third biopsy that showed an inflammatory reaction and some parasites in the cytoplasm of the histiocytes, allowing us to establish the diagnosis.

Subsequently, the patient was treated with meglumine antimoniate at a daily dose of 5 mg kg\(^{-1}\) body weight. After 10 days, clinical improvement was present, and after 40 days, the patient had a complete healing.

After 2 years, she was referred to the Internal Medicine Department because of a para-ovarian abdominal mass: histopathology revealed a visceral form of leishmaniasis. The patient was referred to the Infectious Diseases Department for treatment.

Case 6 – A 66-year-old Caucasian man with a history of painful and tender lesion affecting the hard and soft palatal mucosa for several weeks. About 20 years before, he had been referred to the Department of Respiratory Diseases, Cotugno Hospital, Naples, Italy, because of a not specified pulmonary disease.

Physical examination showed a widespread, irregular, and granular plaque involving the hard palatal mucosa and extending to the soft palate, left maxillary gingiva, and alveolar mucosa. The lesion was also characterized by erythema, and multiple ulcerations mainly localized on the hard palatal mucosa.

The patient was treated with meglumine antimoniate at a daily dose of 5 mg kg\(^{-1}\) body weight for 6 weeks, and subsequently, the lesions healed. The follow-up period was 30 months, and the result was negative.

Case 7 – A 31-year-old Caucasian man with a history of a 4-week painful lesion affecting the buccal mucosa.

Physical examination revealed an exophytic and well-defined lesion of the left buccal mucosa.

This mucosal-colored plaque bled easily and had an increased consistency and a largest diameter of approximately 2.2 × 1.5 cm.

The patient was treated with meglumine antimoniate at a daily dose of 5 mg kg\(^{-1}\) body weight for 20 days. The follow-up period was 8 months, and the result was negative.

The case series from our retrospective study revealed seven patients with oral leishmaniasis, six of whom (85%) were men and one a woman with a mean (±SD) age at the time of diagnosis of the disease of 48 (±14) years.

The most commonly affected site was the tongue (four patients, 57%), followed by one case each on the lip, palatal mucosa, and buccal mucosa.

The clinical aspects presented were exophytic lesions in six patients (85%) and an ulcerated lesion in one patient (14%).

The analysis of the literature review revealed eleven articles published between 2005 and 2013, from five different countries, with a total of thirteen patients described. All the patients were male (100%), and their mean (±SD) age at the time of diagnosis of the disease was 56 (±14) years.

The largest number of cases was from Italy (six patients, 46%). Other patients came from Iran (three cases, 23%) and Brazil (two cases, 15%). The other two cases were from England and India. All the clinical results from the review are summarized in Tables 1–3.

The treatment was specified in only eleven of the thirteen cases. The most common treatment was amphotericin B (five cases, 45%), followed by meglumine antimoniate (three cases, 27%). One case each (9%) was treated with stibogluconate, miltefosine, and surgery. The healing times of the lesions were not available in detail, but the data on the outcomes, as well as the diagnostic criteria and follow-up, are summarized in Table 4.

Including our case series, the total number of Italian cases in literature has increased to 13.

No significant differences were found between our six cases (exclude Case 3 from Pakistan) and other cases from Italy found in the literature review in terms of gender, age, and response to treatment, except in relation to the affected sites.

In fact, in the review, there were no Italian cases of primary oral ML.

Discussion

The leishmaniases are a group of infectious diseases caused by obligate intracellular protozoan parasites of the genus *Leishmania* that continue to be an increasing worldwide threat. Approximately 58 000 cases of VL and 220 000 cases of CL are officially reported each year. Based on assessments of under-reporting, 0.2–0.4 million new cases of VL and 0.7–1.2 million new cases of CL are estimated to occur every year (Desjeux, 1996; Alvar et al., 2012). The WHO estimates that 350 million people are at risk of contracting leishmaniasis (World Health Organization, 2010). These data are troubling considering that the disease causes significant morbidity and mortality accounting for more than 57 000 deaths per year and an estimated
**Table 1** Data of 13 patients from pubmed search: epidemiology and predisposing factors

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Age</th>
<th>Sex</th>
<th>Underlying or past diseases</th>
<th>Predisposing risk factors</th>
<th>Local impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmeiro et al (2007)</td>
<td>Brazil</td>
<td>75</td>
<td>M</td>
<td>None</td>
<td>Endemic area</td>
<td>Farmer, Deficient oral hygiene,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hypertension (atenolol and hydrochlorothiazide treated)</td>
<td></td>
<td>poor tooth conservation</td>
</tr>
<tr>
<td>Cocuzza et al (2013)</td>
<td>Italy</td>
<td>64</td>
<td>M</td>
<td>(COPD) since 20 years. Hypersensitivity to non-steroidal treatment</td>
<td>Endemic area, Anti-inflammatory</td>
<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oryan (2013)</td>
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<td>42</td>
<td>M</td>
<td>None</td>
<td>Endemic area</td>
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<td>Hypertension (by ACE inhibitors treated)</td>
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<td>52</td>
<td>M</td>
<td>None</td>
<td>Endemic area, Shepherd</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ischemic cardiopathy (coronary by-pass surgery 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habibzadeh et al (2005)</td>
<td>Iran</td>
<td>40</td>
<td>M</td>
<td>None</td>
<td>Endemic area</td>
<td>--</td>
</tr>
<tr>
<td>Cocca et al (2005)</td>
<td>Italy</td>
<td>59</td>
<td>M</td>
<td>COPD, Low level of IgM (28.8 mg 100 ml⁻¹)</td>
<td>Endemic area</td>
<td>Smoker</td>
</tr>
<tr>
<td>Casolari (2005)</td>
<td>Italy</td>
<td>53</td>
<td>M</td>
<td>None</td>
<td>Endemic area</td>
<td>--</td>
</tr>
<tr>
<td>Tiseo et al (2008)</td>
<td>Italy</td>
<td>64</td>
<td>M</td>
<td>Well-compensated type 2 diabetes mellitus</td>
<td>Endemic area</td>
<td>--</td>
</tr>
<tr>
<td>Kassam et al (2013)</td>
<td>England</td>
<td>66</td>
<td>M</td>
<td>COPD (diagnosed in 2002) raised ESR (34 mm h⁻¹)</td>
<td>--</td>
<td>Former smoker (20 cig day⁻¹) for 40 years, recently had three courses of antibiotics and got salbutamol, steroid inhalers and an antihistamine for the COPD</td>
</tr>
</tbody>
</table>

- Not reported/unknown; ACE, angiotensin converting enzyme; COPD, chronic obstructive pulmonary disease; ESR, erythrocyte sedimentation rate.


The parasite resides intracellularly and is transmitted between hosts by the bite of the female sand fly (genus *Phlebotomus* in the Old World localities of Europe, Africa, and Asia, and *Lutzomyia* in the New World, the Americas, and Oceania). The primary hosts are vertebrates such as humans, domestic dogs and cats, opossums, the crab-eating fox, and the common black rat. Human infection is caused by more than 20 different species that infect mammals (Balasegaram et al, 2012; Montalvo et al, 2012; Stockdale and Newton, 2013).

*Leishmania* have a digenetic life cycle consisting of the morphologically distinct promastigote and amastigote stages. Promastigotes are flagellated, motile stage that then fuses with the lysosomes to generate an acidic compartment containing hydrolytic enzymes, where they further differentiate into amastigotes (Sacks et al, 1994; Murray et al, 2010).

Human VL is mainly caused by two species of *Leishmania* parasites, each having a characteristic regional distribution: *L. infantum* is the causative agent in the Mediterranean, Middle-east, Central Asia, China, and Central and South America; *L. donovani* in India and East Africa. VL may also be caused by *L. tropica* in the Old World and *L. amazonensis* in the New World and is fatal in 85–90% of untreated cases and up to 50% of treated cases (Gill and Beeching, 2009).

Visceral leishmaniasis is characterized by weight loss, fever, cytopenia, and hepatosplenomegaly (Carranza-Tamayo et al, 2010).

Approximately 90% of CL occurs in Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, the Islamic Republic of Iran, Brazil, and Peru (Kassi et al, 2008), with an incubation period ranging from 2 weeks up to several months (Grimaldi and Schotelli, 2001) and a wide spectrum of clinical presentations ranging from cutaneous ulceration to various degrees of mucosal involvement.

The symptoms and the extent and localization of the lesions depend both on the characteristics of the parasite and on the host immune response.

Cutaneous leishmaniasis is the most prevalent and is characterized by the presence of ulcers with a well-defined erythematous border and a central crust that is often hemorrhagic, located in exposed areas of the body and ranging in number from one to ten (Nogueira et al, 2008; Goto and Lindoso, 2010). MCL is the most severe form and presents clinically a few years after the manifestation of the cutaneous form, affecting the upper aerodigestive tract, with lesions mainly in the oral and nasal mucosa and occasionally in the laryngeal and pharyngeal mucosa (Palmeiro et al, 2007; Nogueira et al, 2008). These lesions are generally associated with pain, edema, halitosis, bleeding, and sialorrhea (Palmeiro et al, 2007).

Mucosal leishmaniasis of the upper respiratory tract is usually associated with the VL form or is found in immunosuppressed individuals like those with HIV infection or...
et al.

In immunocompetent patients, the primary and exclusive mucosal involvement of the head–neck region is very uncommon and the exclusive and localized oral mucosa leishmaniasis is an even more rare event. Most of the cases reported in literature, and excluded from our study, describe a mixed form of leishmaniasis, in which the mucosal lesions are contemporary with or secondary to the visceral or cutaneous forms. Diagnosis is very often a challenge with several reports in the literature of a significantly delayed diagnosis or even an erroneous clinical diagnosis of malignancy (Casolari et al., 2005; Mathur et al., 2006; Tiseo et al., 2008; Pellicioli et al., 2012; Cocuzza et al., 2013; Oryan et al., 2013). Differential diagnosis encompasses other infectious or non-infectious diseases such as fungal infections (blastosomyces), tertiary syphilis, paracoccidioidomycosis, histoplasmosis, tuberculosis, leprosy, lethal midline granuloma, pemphigus vulgaris, pemphigoid, plasmacytic gingivitis, (deficiencies) anemia, leukemia, anti-neutrophilic cytoplasmic antibody (ANCA)-associated (Wegener) granulomatosis, and squamous cell carcinoma (Casolari et al., 2005; Palmeiro et al., 2007; Pellicioli et al., 2012).

Table 2 Data of 13 patients from pubmed search: Clinical features

<table>
<thead>
<tr>
<th>Reference</th>
<th>Involved sites</th>
<th>Symptoms</th>
<th>Lesion description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmeiro et al.</td>
<td>Palatal mucosa (hard and soft palate)</td>
<td>Swallowing difficulties</td>
<td>Granular area with presence of coarse granulation</td>
</tr>
<tr>
<td></td>
<td>Uvula gingiva</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellicioli et al</td>
<td>Palatal mucosa (hard and soft palate)</td>
<td>Weight loss (approximately 8 kg),</td>
<td>Fine granulation</td>
</tr>
<tr>
<td></td>
<td>oropharynx</td>
<td>Slight pain and no bleeding upon palpation</td>
<td>Largest diameters measuring approximately 5 × 4 cm. Multiple ulcerated nodules, with granulomatous appearance and fibro-elastic consistency</td>
</tr>
<tr>
<td>Cocuzza et al.</td>
<td>True vocal cords</td>
<td>8 months history of hoarseness and discomfort</td>
<td>Two focal hard and whitish lesions</td>
</tr>
<tr>
<td>Oryan (2013)</td>
<td>Laryngeal mucosa</td>
<td>Several months history of dysphonia, dyspnea, hoarseness, and odynophagia</td>
<td>Single lesion</td>
</tr>
<tr>
<td>Oryan (2013)</td>
<td>Epiglottis, cricoarytenoid muscle, laryngeal mucosa</td>
<td>Several months history of dysphonia, dyspnea, hoarseness, odynophagia</td>
<td>Deep mucosal damaging processes</td>
</tr>
<tr>
<td>Pau et al. (2009)</td>
<td>Endonasal mucosa (anterior part of left cartilaginous septum)</td>
<td>Epistaxis</td>
<td>Bright red, 1 cm nodule bled easily, moderately infiltrated, covered by serohematic crusts</td>
</tr>
<tr>
<td>Pau et al. (2009)</td>
<td>Endonasal mucosa (left nasal vestibule)</td>
<td>Epistaxis, rhinorhoea, sense of obstruction, and respiratory difficulty.</td>
<td>Hard erythematous, polypoid, non-ulcerated and paintless, 1.5 cm diameter nodule</td>
</tr>
<tr>
<td>Habibzadeh et al. (2005)</td>
<td>Right side of the tongue (against the first molar tooth)</td>
<td>None</td>
<td>Fleshly mass, measuring 5 × 7 × 5 mm</td>
</tr>
<tr>
<td>Guido et al. (2005)</td>
<td>Larynx (subglottic mucosa)</td>
<td>Cough, exertional dyspnea, mucus production, bronchiectases</td>
<td>3 mm mucosal polypoid-like lesion</td>
</tr>
<tr>
<td>Casolari (2005)</td>
<td>Right epiglottis region</td>
<td>Dyspnea, Odynophagia</td>
<td>Whitish, fungating, swelling</td>
</tr>
<tr>
<td>Tiseo et al. (2008)</td>
<td>Left wall of the pharynx, vocal cords</td>
<td>Dysphonia</td>
<td>Lesion</td>
</tr>
<tr>
<td>Mathur et al. (2006)</td>
<td>Hemilarynx (bilaterally)</td>
<td>Hoarseness, cough and a history of fever for 18 months</td>
<td>Fixed ulcerative growth, With edematous surrounding areas</td>
</tr>
<tr>
<td>Kassam et al. (2013)</td>
<td>Tongue (left mid-dorsum)</td>
<td>3-month history of swallowing and soreness of the dorsum of the tongue because of painful ulceration, and some difficulty in swallowing</td>
<td>Lymphoid-like tissue swelling</td>
</tr>
</tbody>
</table>

Table 3 Comparative data on age, gender and sites of involvement

<table>
<thead>
<tr>
<th></th>
<th>Our cases (n = 7)</th>
<th>Review cases (n = 13)</th>
<th>Total (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall age (years)</td>
<td>31–66</td>
<td>32–75</td>
<td>31–75</td>
</tr>
<tr>
<td>Men</td>
<td>6</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>Women</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mean (±SD) age (years)</td>
<td>48 (±14)</td>
<td>56 (±14)</td>
<td>53 (±14)</td>
</tr>
<tr>
<td>Larynx</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Tongue</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Pharynx</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Palate</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Nasal mucosa</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Gingiva</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lip</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

SD, Standard deviation.

In immunocompetent patients, the primary and exclusive mucosal involvement of the head–neck region is very uncommon and the exclusive and localized oral mucosa leishmaniasis is an even more rare event. Most of the cases reported in literature, and excluded from our study, describe a mixed form of leishmaniasis, in which the mucosal lesions are contemporary with or secondary to the visceral or cutaneous forms. Diagnosis is very often a challenge with several reports in the literature of a significantly delayed diagnosis or even an erroneous clinical diagnosis of malignancy (Casolari et al., 2005; Mathur et al., 2006; Tiseo et al., 2008; Pellicioli et al., 2012; Cocuzza et al., 2013; Oryan et al., 2013). Differential diagnosis encompasses other infectious or non-infectious diseases such as fungal infections (blastosomyces), tertiary syphilis, paracoccidioidomycosis, histoplasmosis, tuberculosis, leprosy, lethal midline granuloma, pemphigus vulgaris, pemphigoid, plasmacytic gingivitis, (deficiencies) anemia, leukemia, anti-neutrophilic cytoplasmic antibody (ANCA)-associated (Wegener) granulomatosis, and squamous cell carcinoma (Casolari et al., 2005; Palmeiro et al., 2007; Pellicioli et al., 2012).
Table 4 Data of 13 patients from pubmed search: diagnosis and treatment

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diagnostic tests</th>
<th>Treatment</th>
<th>Follow-up (months)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serology (IIF)</td>
<td>(5 mg kg⁻¹ day⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histopathological (hematoxylin-eosin, Ziehl-Neelsen, Silver), Culture</td>
<td>i.m. for 20 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 Biopsies (inconclusive diagnoses)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histopathological (hematoxylin-eosin, Ziehl-Neelsen, Silver, Pas) Immunohistochemistry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cocuzza et al (2013)</td>
<td>Fibroscopy and laryngeal biopsy (inflammation and hyperplasia)</td>
<td>Amoxicillin</td>
<td>Lack of clinical improvement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Second laryngeal biopsy</td>
<td>Liposomal amphotericin B for 7 days, then (3 mg kg⁻¹ per once a week) for 5 weeks</td>
<td>Achieved complete recovery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd laryngeal biopsy</td>
<td>Liposomal amphotericin B for 7 days, then (3 mg kg⁻¹ per once a week) for 5 weeks</td>
<td>Resolution of symptoms and good health</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oryan (2013)</td>
<td>Laryngoscopy</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cytologic (Wright and Papanicolaou)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nested PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Facial X-ray</td>
<td>(300 mg ml⁻¹; intralesion infiltration (1 ml per weekly) for 4–5 weeks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histopathological (Giemsa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytological and histopathological (Giemsas)</td>
<td>(300 mg ml⁻¹; intral. infiltr. (1 ml per weekly) for 4 weeks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isoenzymatic characterization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habibzadeh et al (2005)</td>
<td>Indirect immunofluorescence</td>
<td>Surgical</td>
<td>48</td>
<td>Healing</td>
</tr>
<tr>
<td>Guddo et al (2005)</td>
<td>Bronchoscopy</td>
<td>Ciprofloxacine (without clinical improvement) then Amphotericin B: (0.5 mg kg⁻¹ day⁻¹)</td>
<td>-</td>
<td>Clinical improvement</td>
</tr>
<tr>
<td></td>
<td>Histopathological (hematoxylin-eosin, Ziehl-Neelsen, Gram, Grocott, Giemsas) PCR, ELISA test on serum Microbiological</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casolari et al (2005)</td>
<td>Laryngoscopy</td>
<td>Liposomal amphotericin B for 2 courses, 10 days apart, (3 mg kg⁻¹ day⁻¹) for 5 days</td>
<td>12</td>
<td>Healing</td>
</tr>
<tr>
<td></td>
<td>Multiple Histological (Giemsas), Culture Nested PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiseo et al (2008)</td>
<td>CT scan</td>
<td>Liposomal amphotericin B: starting with 0.5 mg kg⁻¹ day⁻¹, Overall dosage: 950 mg</td>
<td>2</td>
<td>Healing Lesion decrease</td>
</tr>
<tr>
<td>Mathur et al (2006)</td>
<td>Direct laryngoscopy</td>
<td>Sibogulonate (20 mg kg⁻¹ day⁻¹)</td>
<td>3</td>
<td>Significant symptom improvement</td>
</tr>
<tr>
<td></td>
<td>Histological (Giemsas)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kassam et al (2013)</td>
<td>Histopathological (hematoxylin-eosin, Giemsas) PCR</td>
<td>Miltefosine: 150 mg BD for 28 days</td>
<td>10</td>
<td>Healing</td>
</tr>
</tbody>
</table>

-, Not reported/unknown; IIF, indirect immunofluorescence; PCR, polymerase chain reaction; CT, computed tomography.

One further important aspect to be discussed is the difficulty of establishing the diagnosis of infectious diseases with a biopsy. Two cases from this literature review, and one in our case series, had previously been submitted to several earlier biopsy procedures, with inconclusive histopathological diagnoses. This is probably due to the similarity of all the granulomatous lesions in the histopathological analysis, the morphological differentiation of the parasites being the only way to make a histopathological diagnosis. This aspect highlights the need to complete the examination in all uncertain cases with a more specific diagnostic procedure like ELISA or PCR.

The literature shows a wide spectrum of diagnostic criteria which encompasses clinical signs, culture of smears of samples, and PCR. Serological testing has also been reported, such as the direct agglutination test, conventional ELISA test, and rk39 rapid diagnostic ELISA test (World Health Organization, 2010; Pellicioli et al, 2012; Masmoudi et al, 2013; Stockdale and Newton, 2013). Serology can be helpful when other diseases have to be considered in a differential diagnosis or if there are uncertain clinical or histopathological data. PCR can be used on skin mucosa biopsy samples or slit-skin specimens. The sensitivity of this test varies depending on the PCR methods.
and the clinical features of the lesions (World Health Organization, 2010; Oryan et al., 2013). For this reason, the identification of amastigotes in biopsy specimens from the skin or mucosa is the most common diagnostic tool (World Health Organization, 2010).

The cases described from our database confirm that the most common clinical presentation of primary ML in the head and neck region is an exophytic lesion found in 85% of our cases as compared to 69% in the literature review.

The most commonly involved oral site is the tongue, followed by the palatal mucosa.

Comparing our results with the data from the literature, we found an 8 year difference in the mean age of the patients, with the same standard deviation of 14 years. Our data confirm a near-exclusive dominance of the male gender.

Predisposing local conditions have to be highlighted: As reported in the review and in our case series, more than 50% of the patients had a local impairment, such as a smoking habit or inflammatory conditions such as chronic obstructive pulmonary disease, and recurrent respiratory tract infections (Ferlito et al., 1986; Benítez et al., 2001; Aliaga et al., 2003; Guddo et al., 2005; Tiseo et al., 2008; Teemu et al., 2013).

It is important to emphasize that, in our experience, localized ML can be considered in some cases as an aspect of a not yet detectable systemic infection. In fact, in our case series, during follow-up, two patients showed cutaneous forms of the disease and two patients a visceral form.

The relapse in some cases is probably due to undertreatment. We suppose that in these patients, there may remain a hidden reservoir of parasites in a quiescent state.

A limited number of drugs are available for the treatment of leishmaniasis, and these face challenges including the development of drug resistance, the limited efficacy for different strains and species, and the cost. The treatment depends on the causative Leishmania; however, species identification is fastidious, time-consuming, and not always available. In such cases, the choice of the drug should be inferred from the geographical setting of the patient, and the epidemiological data of Leishmania distribution as well as the clinical symptoms.

The treatment of choice for mucosal forms of leishmaniasis is based on the administration of pentavalent antimonial drugs, such as meglumine antimoniate, or stibogluconate (Masmoudi et al., 2013).

To conclude, leishmaniasis of the mucous membranes of the head–neck region must be considered in the differential diagnosis of mucosal lesions. It is becoming an emerging infectious disease not only in immunocompromised patients but also in those geographical settings where the parasite is endemic, because of the increasing modern habit of people to travel often abroad.

The diagnosis remains a challenge because of the varying clinical presentation and its ability to emulate different diseases. The dentist plays an important role in the early diagnosis of leishmaniasis which has systemic repercussions.

Any patient affected by leishmaniasis should be managed by a professional with expertise in this disease, preferably an infectious disease specialist. Furthermore, after a complete healing and a long follow-up period without signs or symptoms, the possibility of relapses has to be considered.

Author Contributions

Michele Davide Mignogna and Antonio Celentano have made the research design, drafting the paper and revising it critically. Stefania Leuci, Marco Cascone, Daniela Adamo and Elvira Ruoppi have made the acquisition of data. Michele Mignogna, Antonio Celentano and Gianfranco Favia have selected and classified patients. Michele Mignogna and Gianfranco Favia revised critically the paper.

References


Oral Diseases


**Grooved Tongue and Congenital Muscular Torticollis**

Antonio Celentano¹,²* and Michele Davide Mignogna¹

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A 5-year-old male presented with an asymptomatic grooved and atrophic right side of the tongue (Fig. 1A), noticed 8 months before by his pediatrician as "right mild lingual deviation".

At the time of birth, the child was in good health except for right-sided congenital muscular torticollis (CMT) without plagiocephaly and clinically evident facial asymmetry. Neurologic, radiologic (including cervical-spine 3D-CT-Scan), cardiologic, hematologic, ophthalmologic, and otorhinolaryngologic tests were negative, and the patient received only 8 months of physiotherapy started at the age of 18 months.

With the suspicion of an ipsilateral CMT sequela a differential diagnostic algorithm was started excluding a hypoglossus central dysfunction through Magnetic Resonance Imaging. Ultrasonography of the head and neck region showed the presence of a 5 mm median cyst in submental area (Fig. 1B) and asymmetry between the genioglossus muscles (right=2.8 mm; left=5.8 mm) (Fig. 1C).

Current treatment options for CMT encompass physiotherapy, surgery or other treatments like botulinum toxin. Conservative therapy includes stretching of the shortened SCM and is considered the first line of treatment. Early diagnosis and treatment are mandatory to prevent sequelae affecting the cervical range of motion and secondary musculoskeletal deformities (Jung et al., 2015; Lee et al., 2015; Nilesh and Mukherji, 2013).

In our patient we assumed that compressions of the neck anatomical structures due to CMT and to the right-sided head position could have impaired the normal growth of the genioglossus muscle acting directly or indirectly on mechanoreceptors or on the hypoglossus pre-lingual portion.

In addition, the initiation of physiotherapy was delayed, and the monitoring of the supraphyoid region muscles was insufficient.

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Therefore, it is likely that the soft-tissue compression has led to abnormalities of soft-tissue differentiation causing edema, degeneration of muscle fibers and fibrosis within the involved muscles (Nilesh and Mukherji 2013). Oral Medicine specialists should regularly monitor the appearance of any supra/infra-hyoid region asymmetry through Ultrasonography.

Fig. 1.
Conflict of Interest

None

Funding

None

References

