UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN

MEDICINA CLINICA E SPERIMENTALE

CURRICULUM IN SCIENZE ODONTOSTOMATOLOGICHE

XXIX Ciclo

Coordinatore: Prof. Gianni Marone

TESI DI DOTTORATO

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

TUTOR/RELATORE

Chiar. mo

Prof. Michele Davide Mignogna

CANDIDATO

Dott. Antonio Celentano

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

Dr. Antonio Celentano

January 2017

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 broad spectrum of metabolic processes. In addition to the adrenal-derived steroids, it is now recognised 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 adrenocorticotropin 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**

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to all the special people who have, in different ways, supported me and contributed to the completion of my thesis, among them are:

My mentor and supervisor, Professor Michele Davide Mignogna (Oral Medicine Unit, Department of Neuroscience, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Italy) for giving me this wonderful opportunity to study as a PhD student and introducing me to the world of research, for his invaluable guidance, advice, continuous support and encouragement in helping me along my Ph. D. path.

Professor Nicola Cirillo (Melbourne Dental School, The University of Melbourne, Australia), who believed in me and co-supervised the project, for his expert advice, priceless help and comments, and never-failing support for all the laboratory research project until the completion of this thesis. This project would not have been possible without his contribution. Thank you for enriching my life during this journey.

Professor Michael McCullough (Melbourne Dental School, The University of Melbourne, Australia), who is also my co-supervisor for his enthusiastic comments, continuous guidance and support for all the research projects and completion of my Ph. D., and for making my Australian experience memorable.

Professor Gilberto Sammartino, for his support on all my career choices and his willingness to allow me to go through "…the road less traveled by"….

Professor Gianni Marone, and Dr. Francescopaolo Granata, (Department of Translational Medical Sciences, University Federico II of Naples, Italy) for being by my side, and for their continuous support and guidance during the last three years of my life.

My friends, for their support during life adversity.

My devoted parents, my wonderful brothers, my gorgeous nephew Antonio, for their patience, love and continuous support, and for teaching me how life can be truly a daily miracle. You are my heroes. Thanks to you for always being my source of strength. None of this would have been possible without you, I love you.

My grandfather Giuseppe, for having inspired me since I was born.

My love, Tami, for constantly being by my side, holding my hand during this breath-taking journey.

Dedicated to my family

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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 coadjutant 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, 11bhydroxysteroid dehydrogenases (11b-HSD1/11b-HSD2), adrenocorticotropic hormone receptor (ACTHR/MC2R), and glucocorticoid receptor [3]. (**Fig. 1**)

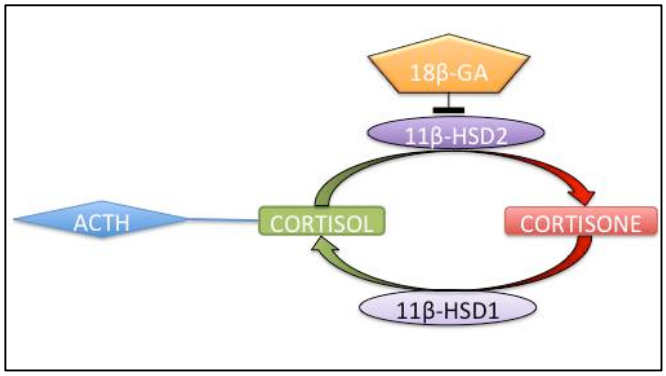


Fig. 1: *Non-adrenal steroideal system in human keratinocytes: local regulation and metabolic chain of cortisol.*

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 exerts 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 1: *Dysregulation of serum cortisol, 11b-HSDs, and glucocorticoid receptor in different types of cancers*

STUDY	**TYPE OF CANCER**	**DYSREGULATED MOLECULE**	**SPECIFIC FINDINGS**
Bernabé et al. (2012) [16]	Oral squamous cell carcinoma (SCC)	Cortisol	Elevated plasma and salivary cortisol levels in SCC and also higher levels in advanced stage compared to initial stage.
Rasmuson et al. (2001) [15]	Renal cell carcinoma (RCC)	Cortisol	Serum cortisol levels higher in RCC and positively correlated with tumor diameter and grade. Elevated cortisol levels also had worse prognosis.
Sephton et al. (2000) [45]	Metastatic breast cancer	Cortisol	Flat diurnal salivary cortisol circadian rhythm where elevated cortisol levels associated with early mortality.
Sephton et al. (2013) [46]	Lung cancer	Cortisol	Flattening of the diurnal cortisol rhythm associated with early death.
Terao et al. (2013) [47]	Skin SCC, basal cell carcinoma (BCC) and seborrheic keratosis (SK)	11b-HSD1 and 11b-HSD2 enzyme	Reduction in 11b-HSD1 expression with increased keratinocyte proliferation. 11b-HSD2 expression increased in basal cell proliferating conditions such as BCC and SK.
Parks et al. (1998) [48]	DMS-79 cells (cell line derived from an ACTH-producing small cell lung cancer).	11b-HSD2 enzyme	11b-HSD2 normally not found in healthy lung tissue, was expressed in cancerous lung tissue.
Temkin et al. (2006) [49]	Ovarian epithelial cancer	11b-HSD2 enzyme	11b-HSD2 enzyme not found in normal postmenopausal ovarian tissue was increased in cancer cells.
Bland et al. (1999) [50]	Osteosarcoma	11b-HSD2 enzyme	Overexpression of 11b-HSD2 enzyme in cancer, compared with predominant expression of 11b-HSD1 enzyme in normal human osteoblast cells.
Zbánková et al. (2004) [51]	Colorectal cancer	11b-HSD2 and 11b-HSD1 enzyme	Decrease in the abundance of 11b-HSD2 mRNA and enzyme activity in cancer tissue. Also demonstrated increase in 11b-HSD1 in some samples.
Cirillo et al. (2012) [52]	Oral squamous cell carcinoma (SCC)	11b-HSD2 enzyme	Decrease in 11b-HSD2 expression in cancer.
Lu et al. (2011) [53]	Breast cancer	11b-HSD1 enzyme	Decrease in 11b-HSD1 expression in cancer.
Budunova et al. (1997) [54]	Mouse epidermal papillomas and SCC.	Glucocorticoid receptor	In early skin papilloma GR expression is reduced but in late papilloma and in SCC, GR levels are similar or higher than normal.
Spiegelman et al. (1997) [27]	Mouse epidermal papillomas and SCC.	Glucocorticoid receptor	No significant changes in GR gene structure and expression in cancer but resistance to glucocorticoid fluocinolone acetonid more likely due to alterations in receptor function.
Waters et al. (2004) [55]	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Normal expression of GR, however its functionality altered by subtle changes in co-factors such as Nuclear co-repressor which was overexpressed in cancer.
Ray et al. (1996) [56]	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Mutations in GR gene structure lead to its impaired function.
Parks et al. (1998) [57]	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Abnormal splicing of the GR transcript causes GR resistance to GC stimulation in cancer cells.
Kay et al. (2011) [58]	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Increased DNA methylation of promoter c leads to decreased GR expression.
Li et al. (1996) [59]	Transformed A5 mouse lung cells	Glucocorticoid receptor	Transformed cells contain functional GR but express cjun which antagonizes glucocorticoids.

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 de-regulated 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.

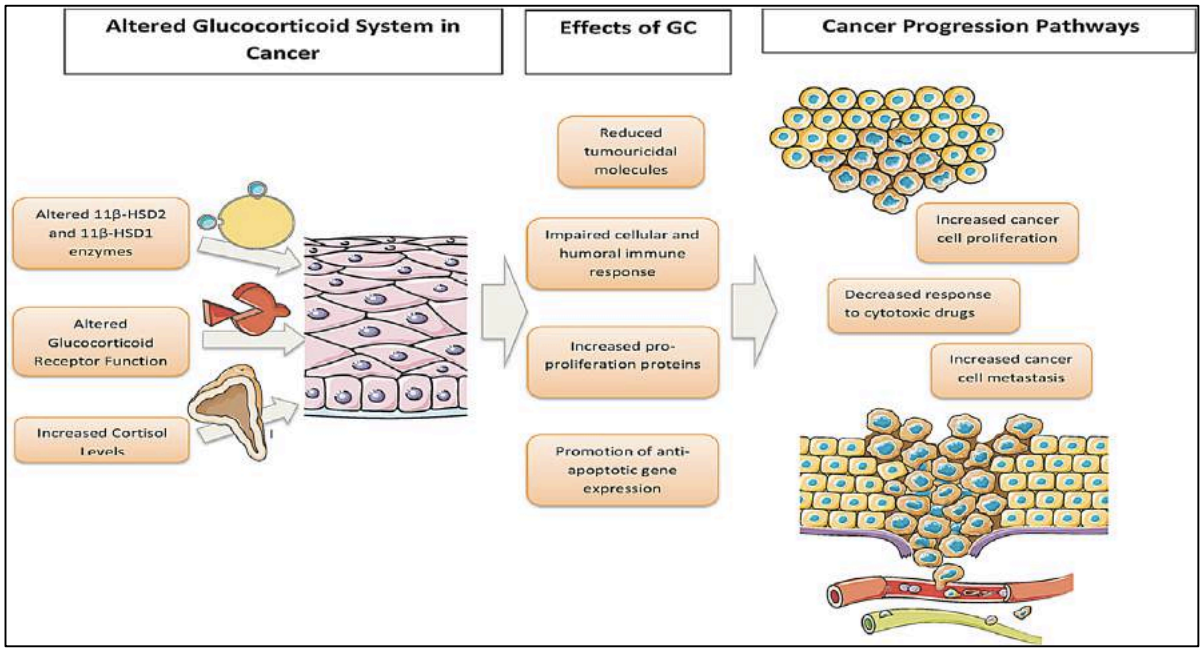


Fig. 2: *Schematic representation of cancer-promoting glucocorticoid effects*

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 splitted.

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-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).

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 vescicles 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)
    - Trypane blue assay (x1)

PLATE 1

T=0

	1 contr (H357)	2 (H357)	3 (H357)	4 (H357)
A t=12h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
B t=24h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
C t=48h	only medium)	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM

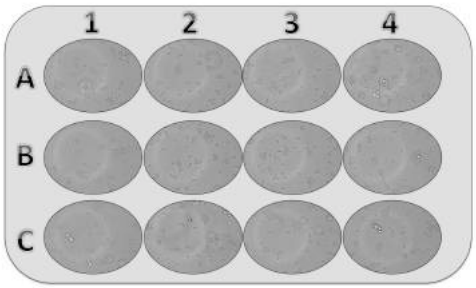


PLATE 2

T=0

	1 (H357)	2 contr (PMCC)	3 (PMCC)	4 (PMCC)
A t=12h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
B t=24h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
C t=48h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL

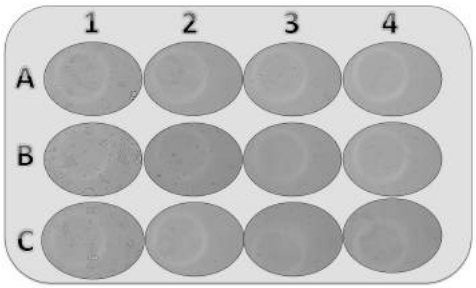


PLATE 1

T=12h

	1 contr (H357)	2 (H357)	3 (H357)	4 (H357)
A t=12h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
B t=24h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
C t=48h	only medium)	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM

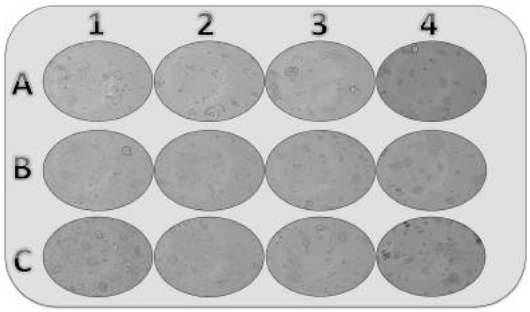


PLATE 2

T= 12h

	1 (H357)	2 contr (PMCC)	3 (PMCC)	4 (PMCC)
A t=12h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
B t=24h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
C t=48h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL

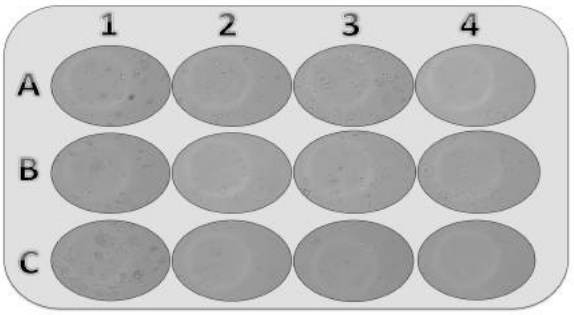


PLATE 1

T=24h

	1 contr (H357)	2 (H357)	3 (H357)	4 (H357)
A t=12h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
B t=24h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
C t=48h	only medium)	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM

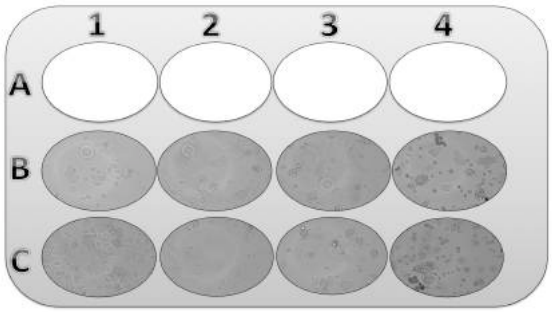


PLATE 2

T=24h

	1 (H357)	2 contr (PMCC)	3 (PMCC)	4 (PMCC)
A t=12h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
B t=24h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
C t=48h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL

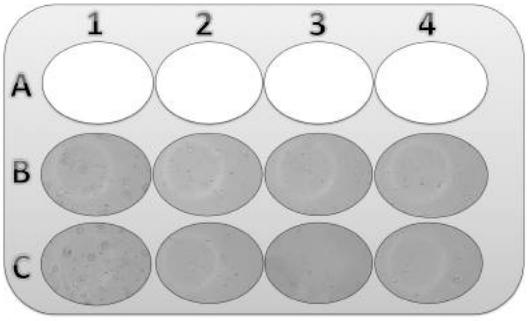


PLATE 1

	1	2	3	4
B	Result 25 of 100 August 28, 2015 - 4:07 PM Total cell count: 1.33x10^5 cells/ml August 28, 2015 - 4:07 PM Total cell count: 2.05x10^5 cells/ml Live cell count: 1.65x10^5 cells/ml Live cells: 80%	Result 27 of 100 August 28, 2015 - 4:08 PM Total cell count: 1.45x10^5 cells/ml August 28, 2015 - 4:08 PM Total cell count: 2.25x10^5 cells/ml Live cell count: 1.70x10^5 cells/ml Live cells: 76%	Result 29 of 100 August 28, 2015 - 4:09 PM Total cell count: 1.33x10^5 cells/ml August 28, 2015 - 4:09 PM Total cell count: 1.25x10^5 cells/ml Live cell count: 1.10x10^5 cells/ml Live cells: 88%	Result 31 of 100 August 28, 2015 - 4:10 PM Total cell count: 1.80x10^5 cells/ml August 28, 2015 - 4:10 PM Total cell count: 2.90x10^5 cells/ml Live cell count: 1.75x10^5 cells/ml Live cells: 60%

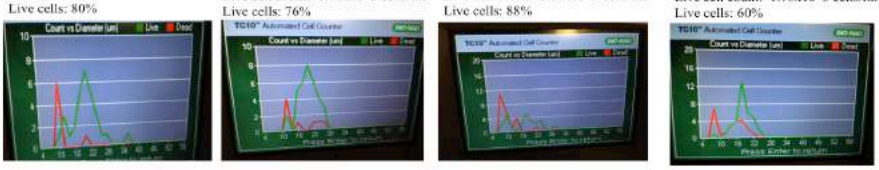


PLATE 2

	1	2	3	4
B	Result 36 of 100 August 28, 2015 - 4:17 PM Total cell count: 1.43x10^5 cells/ml August 28, 2015 - 4:18 PM Total cell count: 2.10x10^5 cells/ml Live cell count: 1.25x10^5 cells/ml Live cells: 60%	Result 38 of 100 August 28, 2015 - 4:18 PM Total cell count: 3.25x10^4 cells/ml August 28, 2015 - 4:19 PM Total cell count: 7.51x10^4 cells/ml Live cell count: 2.50x10^4 cells/ml Live cells: 33%	Result 41 of 100 August 28, 2015 - 4:20 PM Total cell count: 4.01x10^4 cells/ml August 28, 2015 - 4:25 PM Total cell count: 2.65x10^5 cells/ml Live cell count: 3.51x10^4 cells/ml Live cells: 13%	Result 54 of 100 August 28, 2015 - 4:28 PM Total cell count: 3.00x10^4 cells/ml August 28, 2015 - 4:29 PM Total cell count: 1.35x10^5 cells/ml Live cell count: 4.01x10^4 cells/ml Live cells: 30%

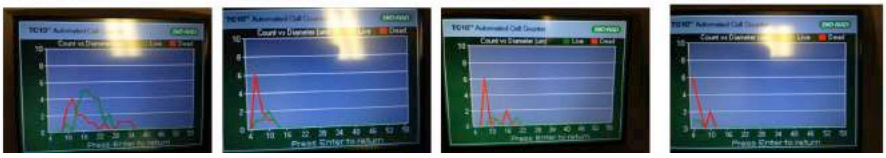


PLATE 1

T=48h

	1 contr (H357)	2 (H357)	3 (H357)	4 (H357)
A t=12h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
B t=24h	only medium	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM
C t=48h	only medium)	DOXORUB 1 µM	DOXORUB 10 µM	DOXORUB 50 µM

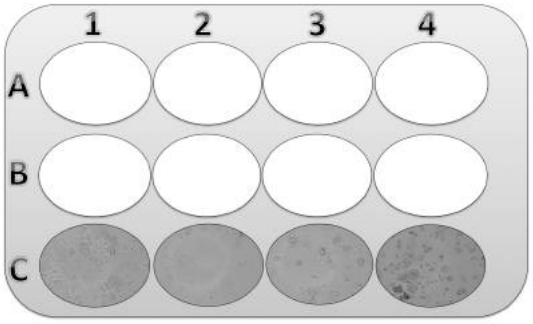
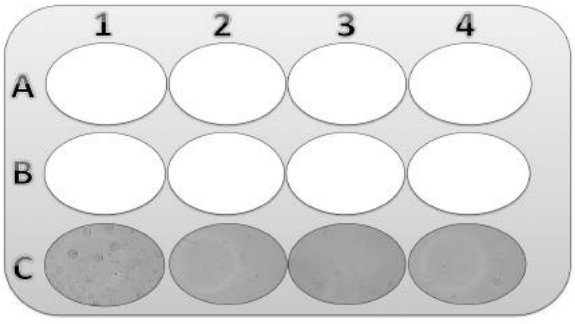


PLATE 2

T=48h

	1 (H357)	2 contr (PMCC)	3 (PMCC)	4 (PMCC)
A t=12h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
B t=24h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL
C t=48h	5FU 0,5 µg/mL	only medium	DOXORUB 10 µM	5FU 0,5 µg/mL



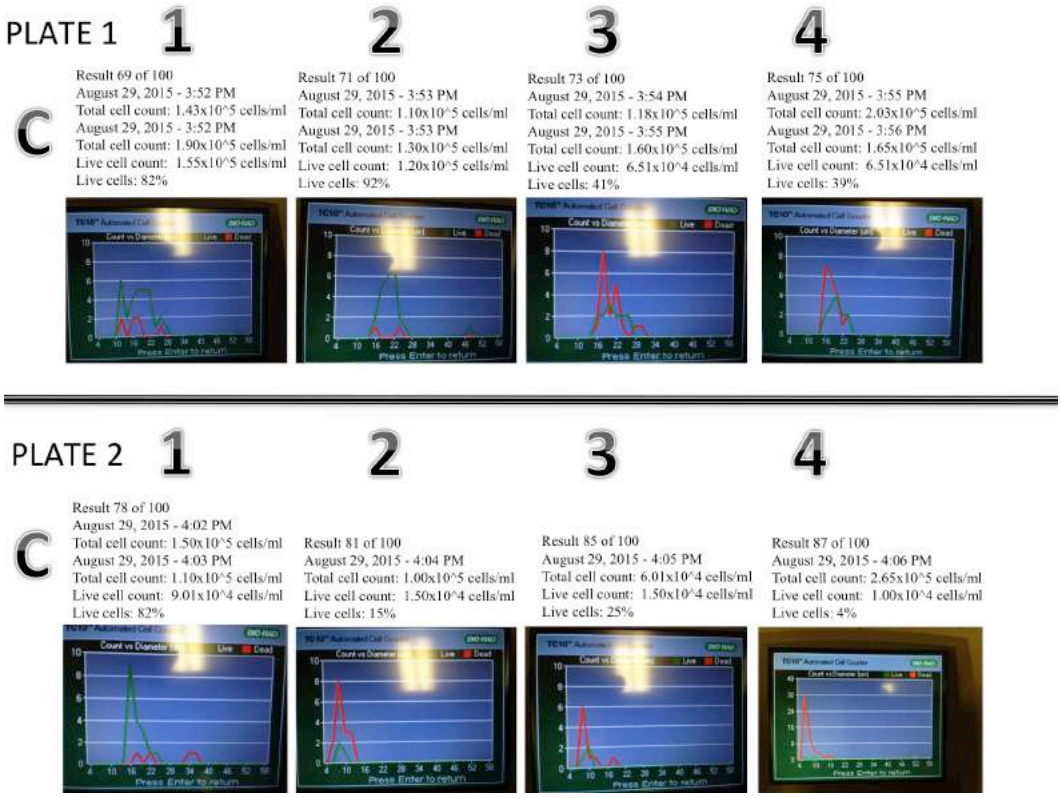
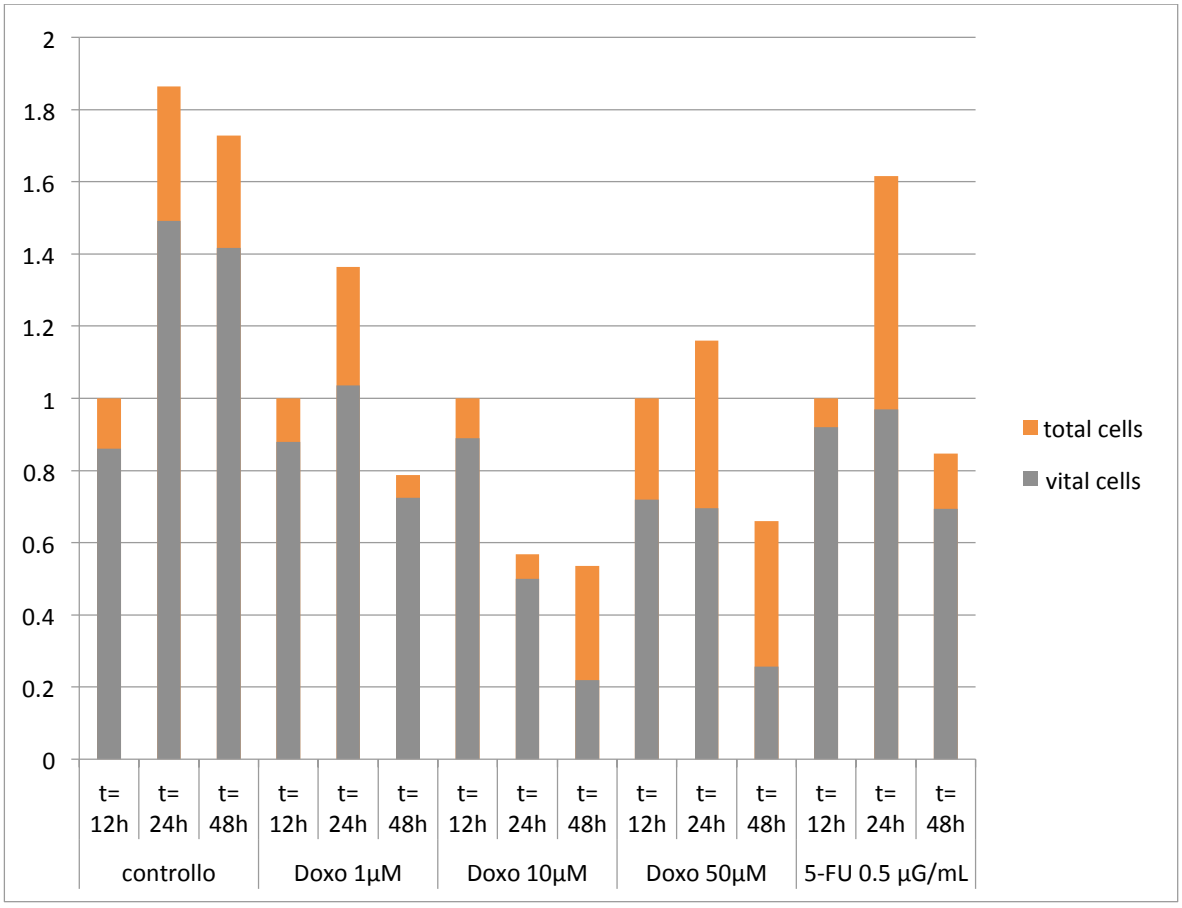


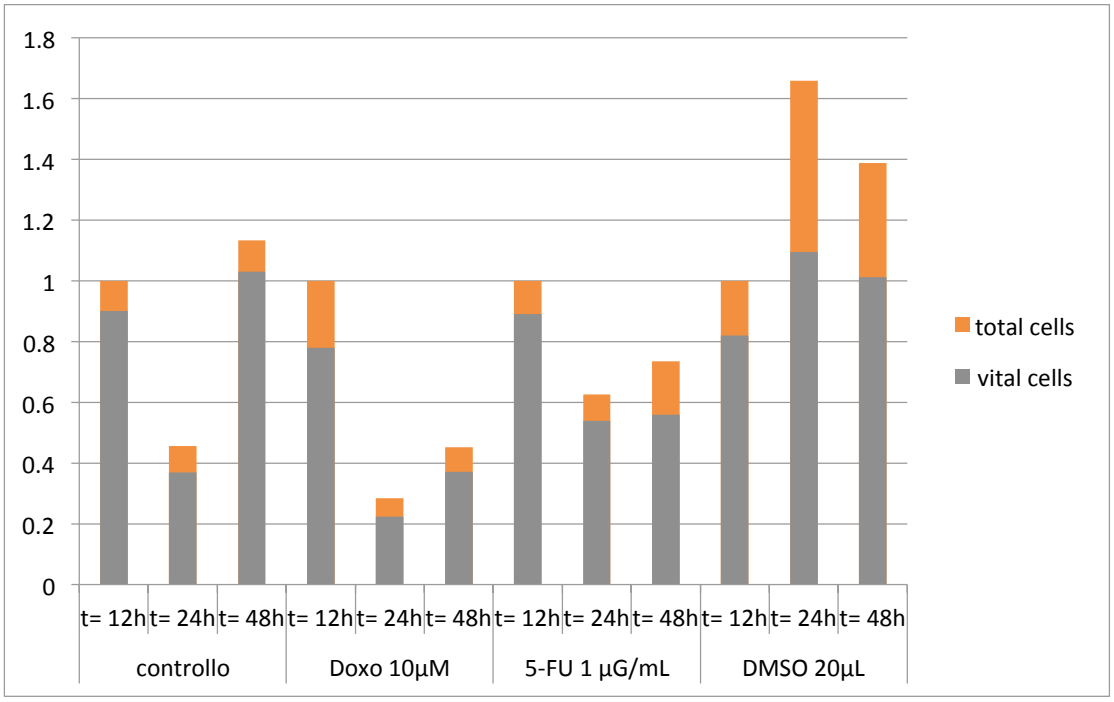
PLATE 1
1
2
3
4
C
Result 69 of 100
August 29, 2015 - 3:52 PM
Total cell count: 1.43x10^5 cells/ml
August 29, 2015 - 3:52 PM
Total cell count: 1.90x10^5 cells/ml
Live cell count: 1.55x10^5 cells/ml
Live cells: 82%
Result 71 of 100
August 29, 2015 - 3:53 PM
Total cell count: 1.10x10^5 cells/ml
August 29, 2015 - 3:53 PM
Total cell count: 1.30x10^5 cells/ml
Live cell count: 1.20x10^5 cells/ml
Live cells: 92%
Result 73 of 100
August 29, 2015 - 3:54 PM
Total cell count: 1.18x10^5 cells/ml
August 29, 2015 - 3:55 PM
Total cell count: 1.60x10^5 cells/ml
Live cell count: 6.51x10^4 cells/ml
Live cells: 41%
Result 75 of 100
August 29, 2015 - 3:55 PM
Total cell count: 2.03x10^5 cells/ml
August 29, 2015 - 3:56 PM
Total cell count: 1.65x10^5 cells/ml
Live cell count: 6.51x10^4 cells/ml
Live cells: 39%
PLATE 2
1
2
3
4
C
Result 78 of 100
August 29, 2015 - 4:02 PM
Total cell count: 1.50x10^5 cells/ml
August 29, 2015 - 4:03 PM
Total cell count: 1.10x10^5 cells/ml
Live cell count: 9.01x10^4 cells/ml
Live cells: 82%
Result 81 of 100
August 29, 2015 - 4:04 PM
Total cell count: 1.00x10^5 cells/ml
Live cell count: 1.50x10^4 cells/ml
Live cells: 15%
Result 85 of 100
August 29, 2015 - 4:05 PM
Total cell count: 6.01x10^4 cells/ml
Live cell count: 1.50x10^4 cells/ml
Live cells: 25%
Result 87 of 100
August 29, 2015 - 4:06 PM
Total cell count: 2.65x10^5 cells/ml
Live cell count: 1.00x10^4 cells/ml
Live cells: 4%


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
t= 12h
t= 24h
t= 48h
controllo
Doxo 1µM
Doxo 10µM
Doxo 50µM
5-FU 0.5 µG/mL
total cells
vital cells

4.2. Experiment 2: growth curve, 100% confluent cells

Protocol:

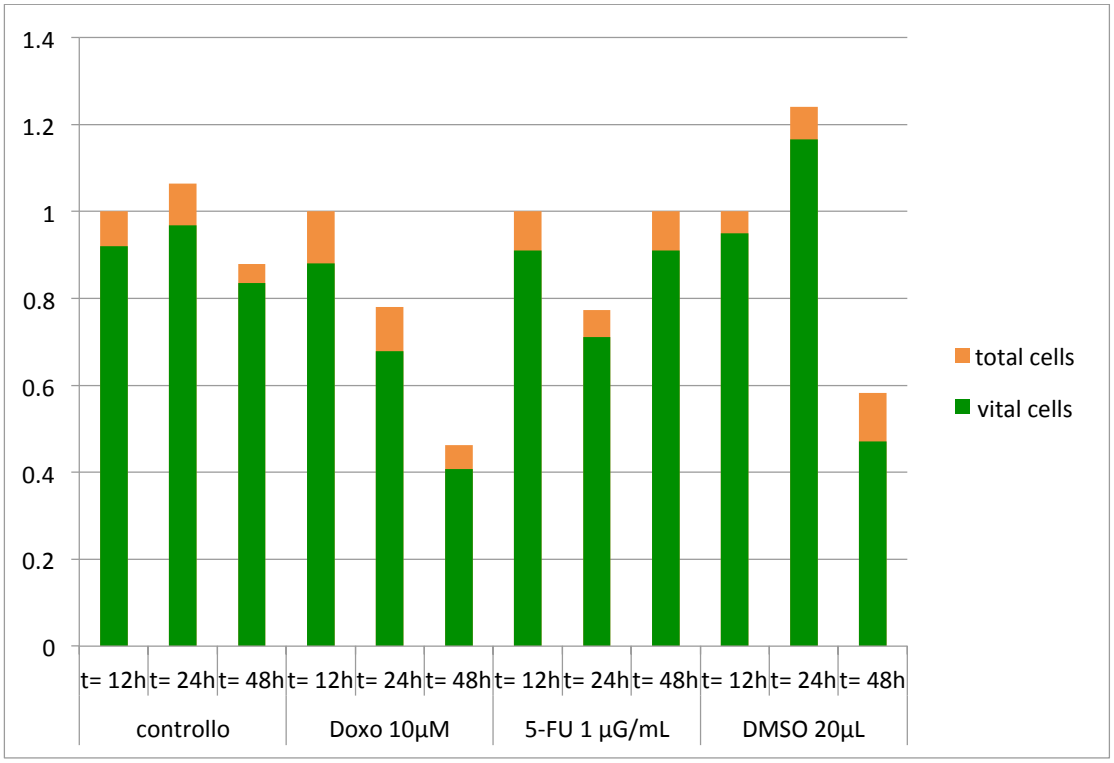
- Cell line: H357
- Cell culture 12-well Plate
- 100% confluent cells in a standard volume OF 1 mL
- Change medium (without washing)
- Add the chemetherapeutical agents all together
- At each timepoint:
    - Remove medium and put it in eppendorf
    - Centrifuge it at 800g x 4 min at 4°C
    - Discharge supernatant
    - In each well wash X2 with PBS
    - Pretreatment with µL 150 EDTA (10 mM) (8 min)
    - ADD 150 µL of 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)



4.3. 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 chemetherapeutical 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)

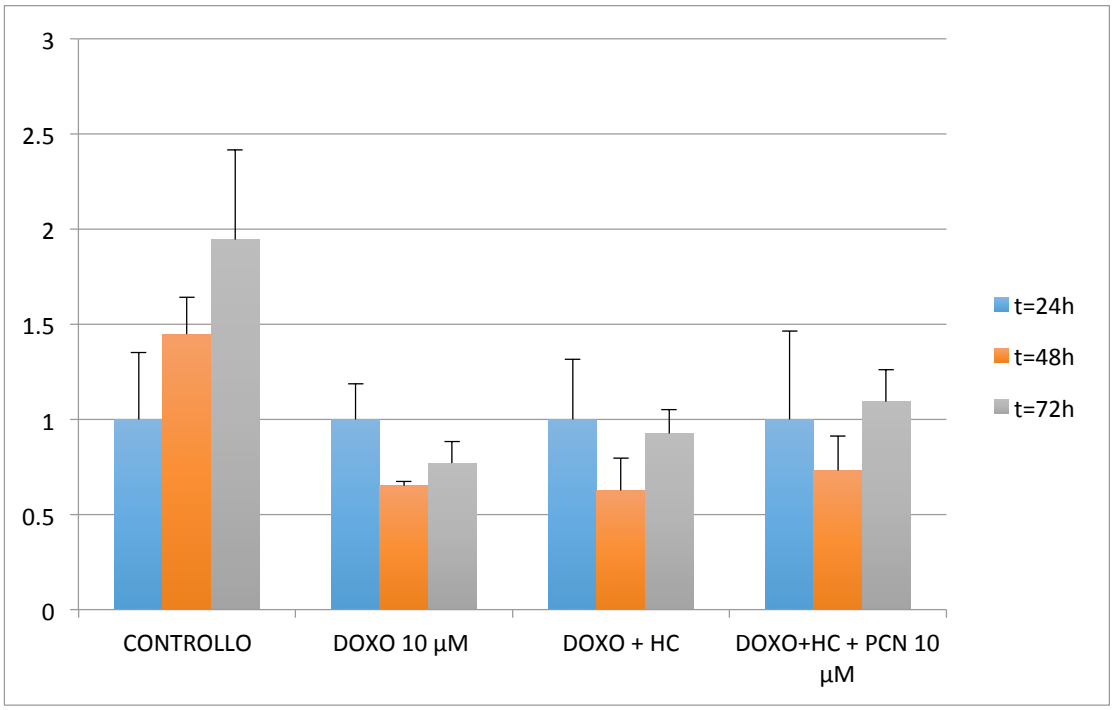


4.4. Experiment 7: DOXO , hydrocortisone, and PCN

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 500µL PBS
  - add all together the chemotherapeutic agents already diluted in1mL of fresh medium

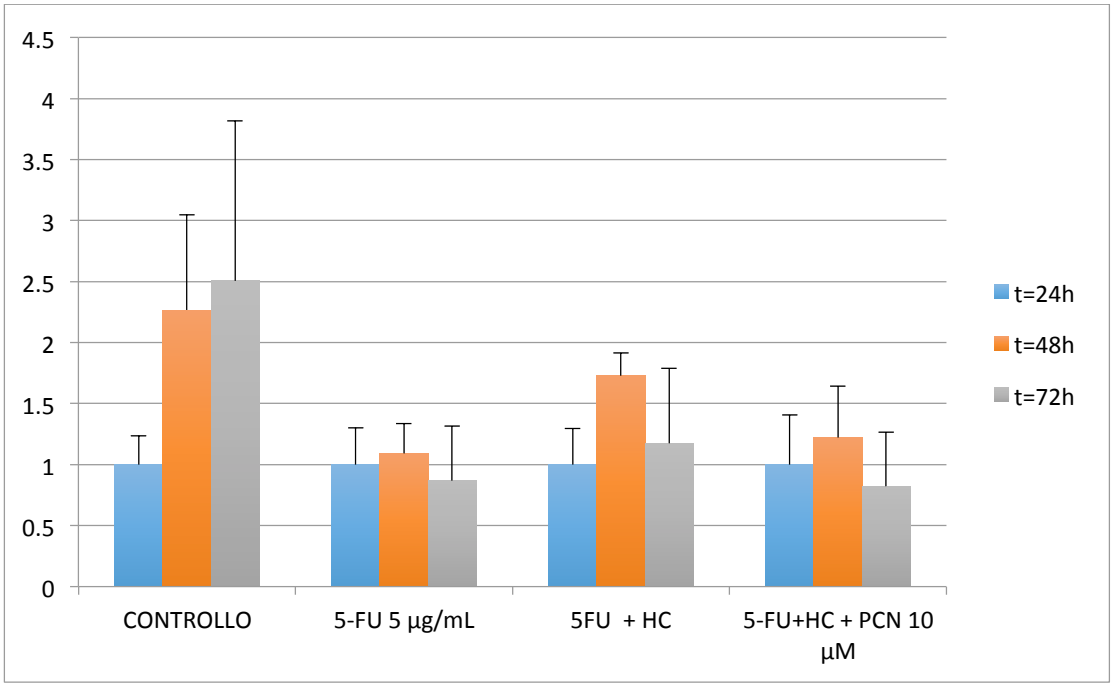
- At each timepoint in each well:
  - Take picture
  - Remove medium
  - wash X2 with 500 µL of PBS
  - Pretreatment with 150 µL of 10mM EDTA for 5 min
  - Discharge EDTA
  - Add 150 µL of trypsin-EDTA 0.25% for 5 min
  - Add 700 µL of medium
  - Cell counting with Trypane blue assay (x1) (10 readings each slide)



4.5. Experiment 8: 5-FU , hydrocortisone, and PCN

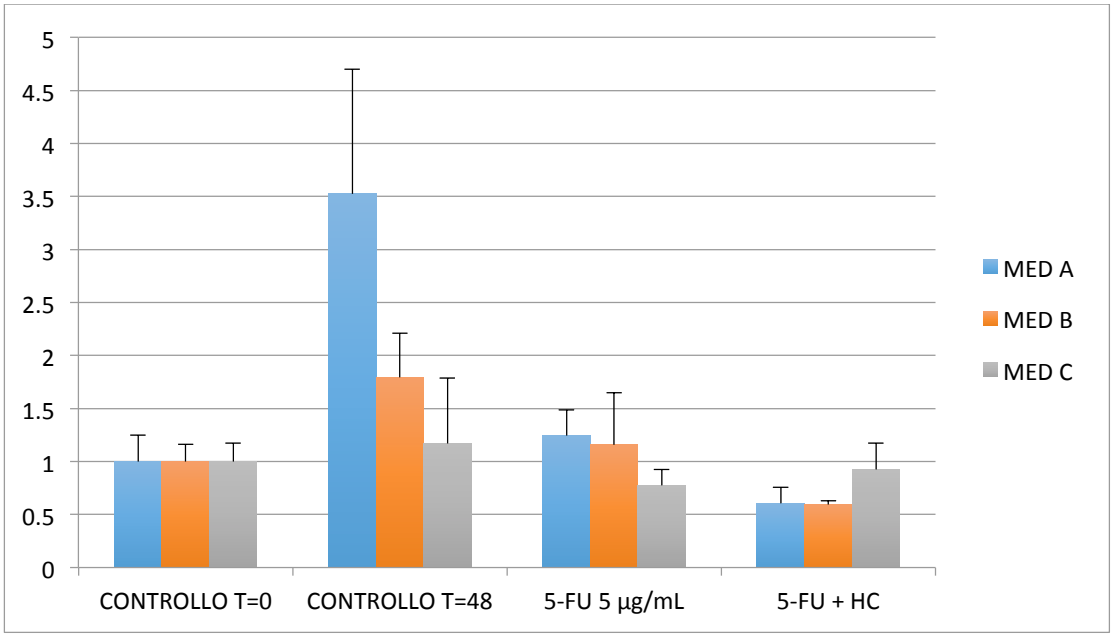
Protocol:

- 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)


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CONTROLLO
5-FU 5 µg/mL
5FU + HC
5-FU+HC + PCN 10 µM
t=24h
t=48h
t=72h

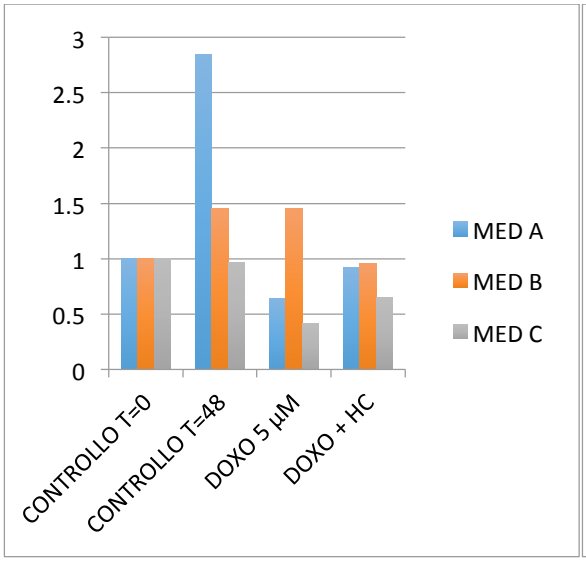
4.6. Experiment 9: 5-FU , hydrocortisone and 3 different experimental mediums

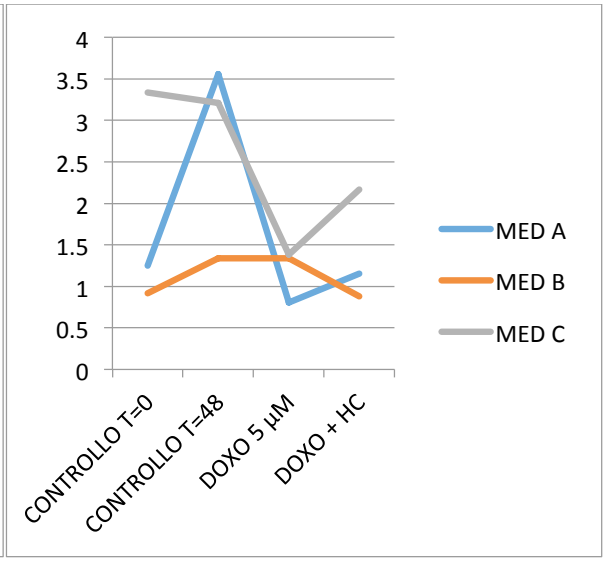
5-FU, HYDROCORTISONE, TRIPLICATE ON 10 READINGS
Medium A- DMEM F12 (1:1) + 1% Foetal Bovine Serum (FBS) +1% Pen/Strept
Medium B- DMEM F12 (1:1) + 10% Foetal Bovine Serum (FBS) +1% Pen/Strept
Medium C- DMEM F12 (3:1) + 10% Foetal Bovine Serum (FBS) +1% Pen/Strept
24 hours before switch the medium (without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

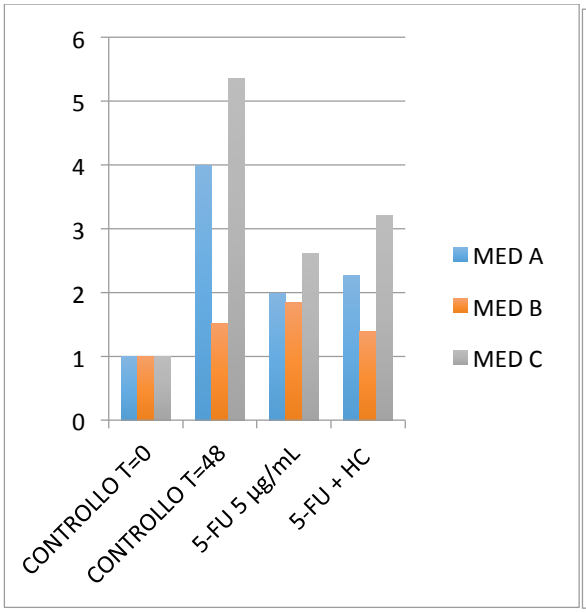


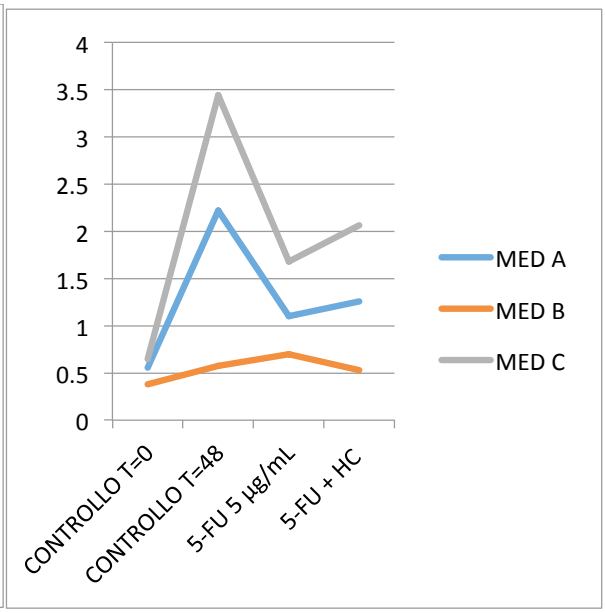
4.7. Experiment 10: 5-FU , hydrocortisone and 3 different experimental mediums

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: 1.5x105 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**).

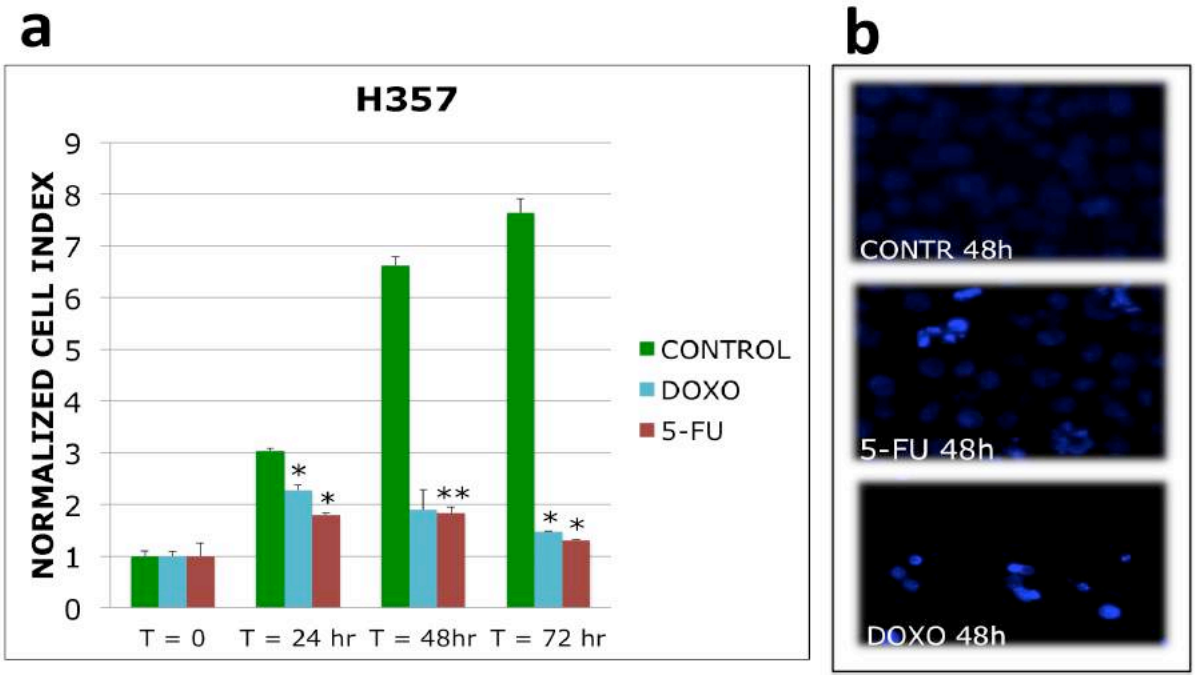


Fig. 3: a) *Effectiveness of chemotherapeutics agents on H357 cell line overtime;* ***b)*** *Hoechst fluorescence staining observed at microscopy at 48 hrs timepoint confirmed the induction of apoptosis in both Doxo and 5-FU groups. Statistical significance is given as follows: *p < 0.05; **p < 0.005.*

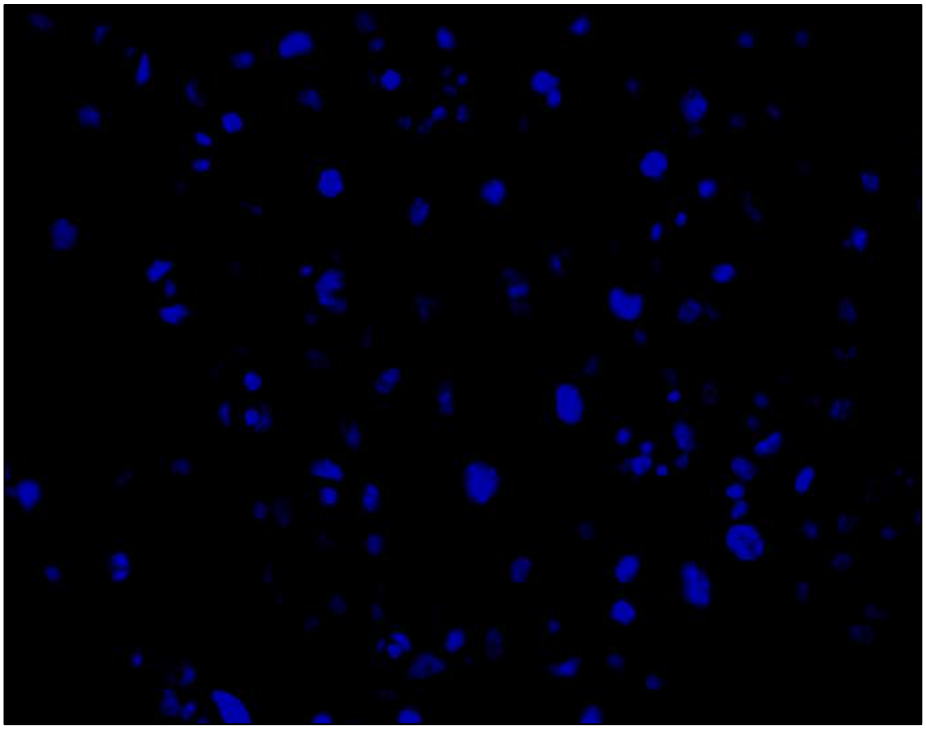


Fig. 4*: Fluorescence microscopy showing fragmented and shrinked DNA pattern induced by DOXO at 48 hrs on H357 cell line. (460X magnification)*

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 vescicles 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.

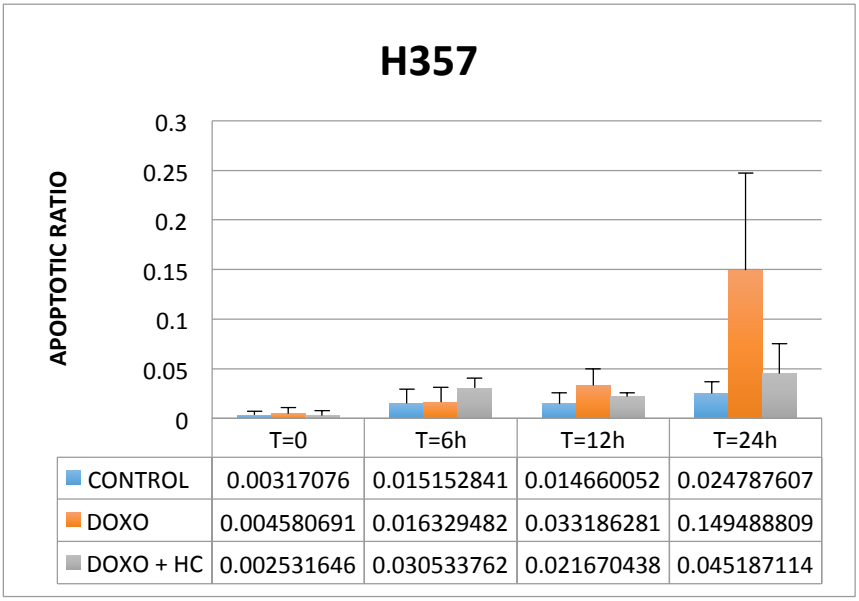
1 mM hydroxide peroxide for 1 hr was used as positive control at 0/12/24/48 hours timepoints.

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)**.

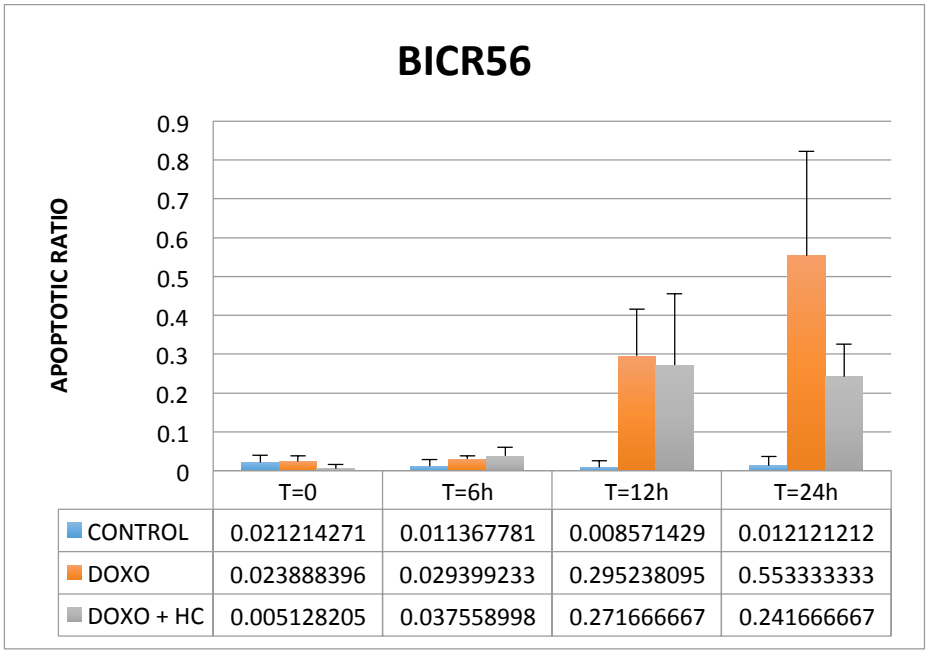


	T=0	T=6h	T=12h	T=24h
CONTROL	0.00317076	0.015152841	0.014660052	0.024787607
DOXO	0.004580691	0.016329482	0.033186281	0.149488809
DOXO + HC	0.002531646	0.030533762	0.021670438	0.045187114

***Fig. 5**: Annexin apoptosis assay on H357 cell line with DOXO, and DOXO+HC at 0/6/12/24 hours*

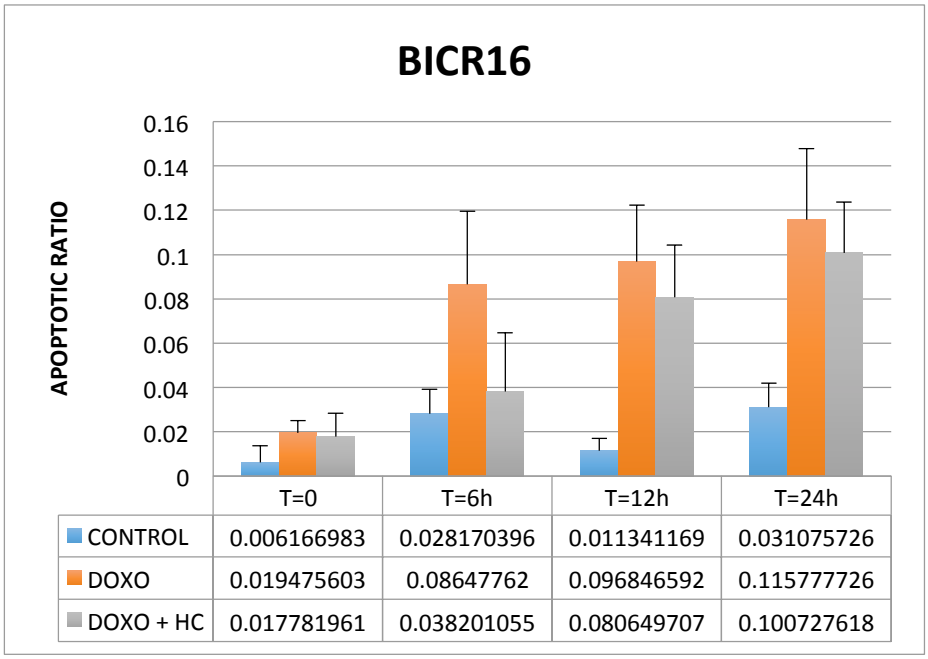
Conversely, an inverted trend has been found in H357 cells when the experimental medium was deprived of the serum (SFM). In this case in fact the effectiveness of DOXO was even enhanced by the addition of HC (**Fig. 8)**.

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**).



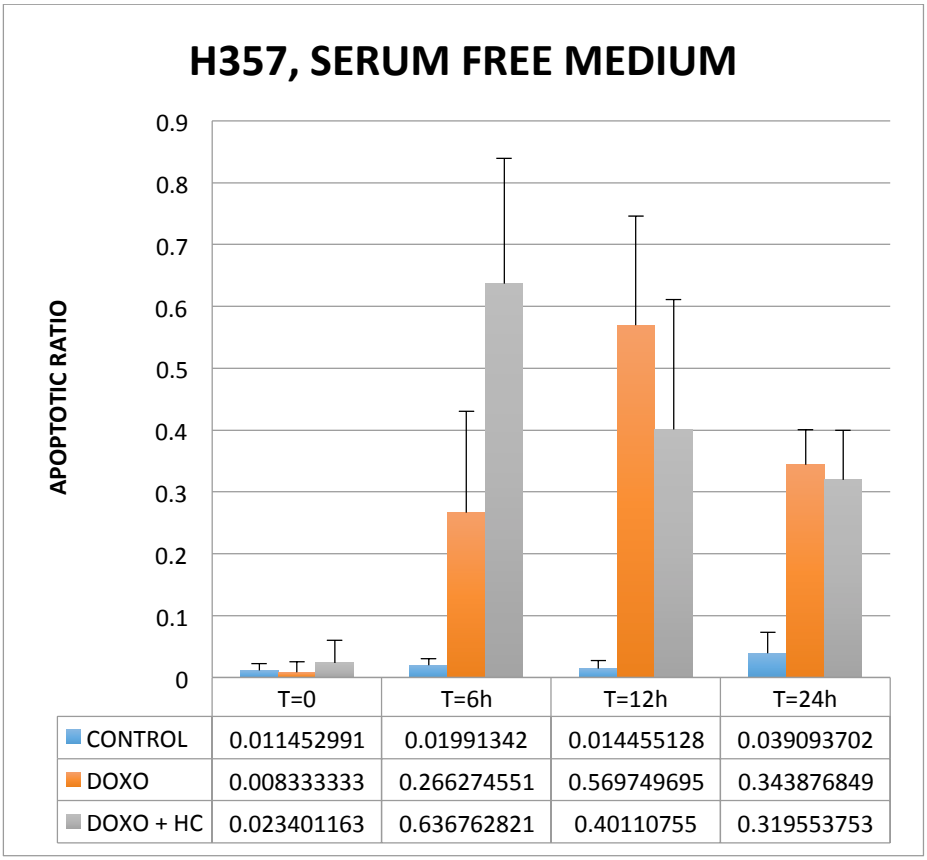
	T=0	T=6h	T=12h	T=24h
CONTROL	0.021214271	0.011367781	0.008571429	0.012121212
DOXO	0.023888396	0.029399233	0.295238095	0.553333333
DOXO + HC	0.005128205	0.037558998	0.271666667	0.241666667

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



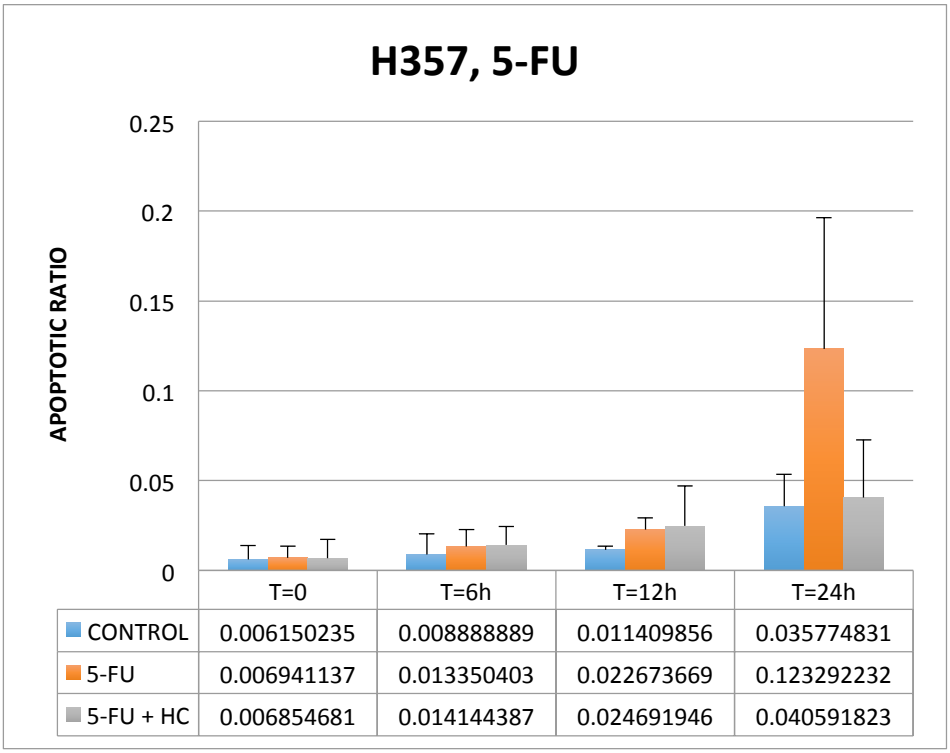
	T=0	T=6h	T=12h	T=24h
CONTROL	0.006166983	0.028170396	0.011341169	0.031075726
DOXO	0.019475603	0.08647762	0.096846592	0.115777726
DOXO + HC	0.017781961	0.038201055	0.080649707	0.100727618

***Fig. 7**: Annexin apoptosis assay on BICR16 cell line with DOXO, and DOXO+HC at 0/6/12/24 hours*



	T=0	T=6h	T=12h	T=24h
CONTROL	0.011452991	0.01991342	0.014455128	0.039093702
DOXO	0.008333333	0.266274551	0.569749695	0.343876849
DOXO + HC	0.023401163	0.636762821	0.40110755	0.319553753

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)*



	T=0	T=6h	T=12h	T=24h
CONTROL	0.006150235	0.008888889	0.011409856	0.035774831
5-FU	0.006941137	0.013350403	0.022673669	0.123292232
5-FU + HC	0.006854681	0.014144387	0.024691946	0.040591823

Fig. 9: *Annexin apoptosis assay on H357 cell line with 5-FU, and 5-FU+HC at 0/6/12/24 hours*

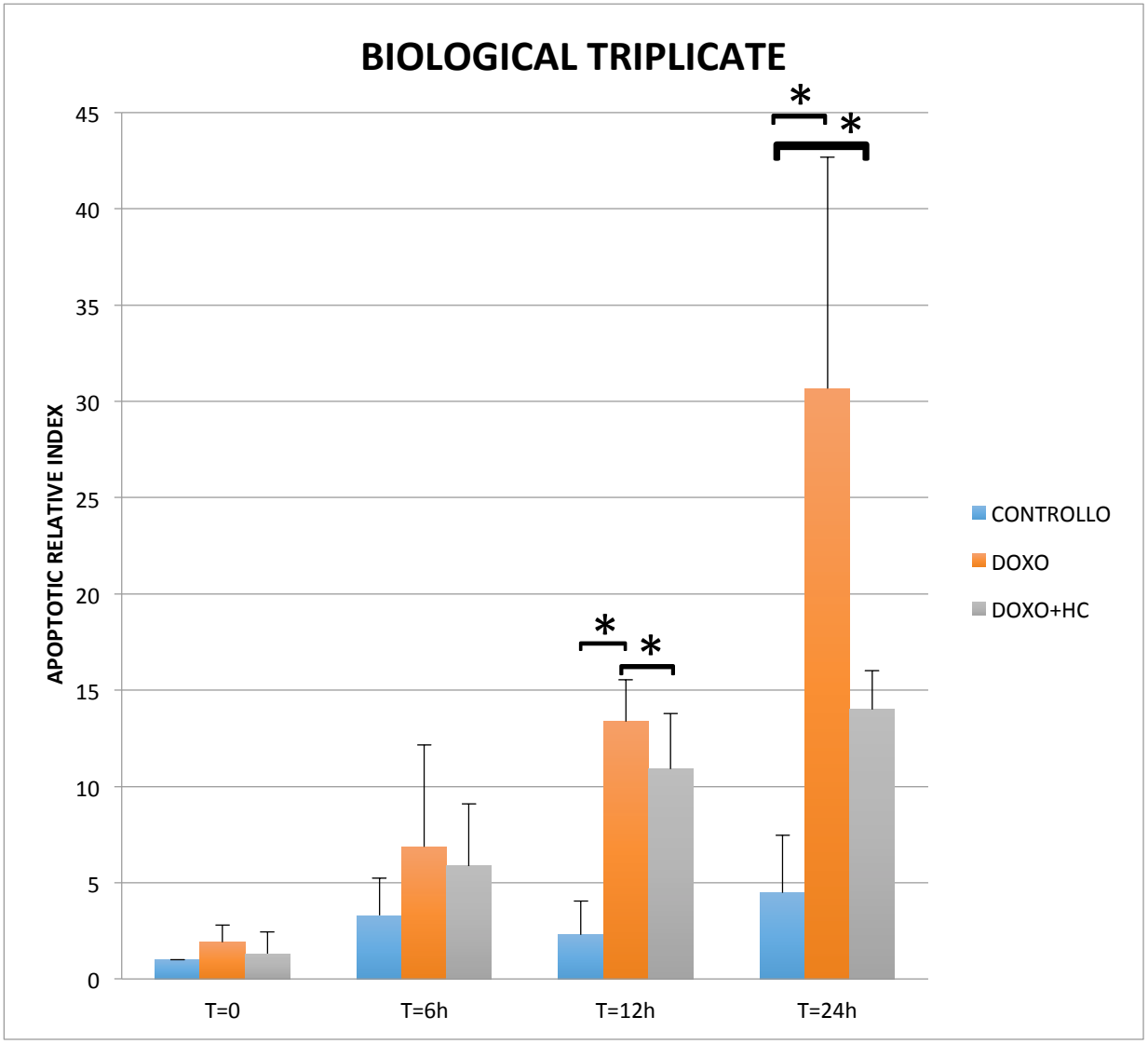


Fig. 10: *Annexin apoptosis assay. The results showed were calculated as the average (biological triplicate) of the results obtained with the single cell lines: H357, BICR16, BICR56. Statistical significance is given as follows: *p < 0.05; **p < 0.005*

Hydrogen peroxide is a well known inducer of apoptosis in vitro. The positve control was then performed with 1 mM hydroxgen peroxide at 0/6/12/24 hours. The results showed that 50% of the cells underwent apoptosis as early as 12 hours after H_2O_2 exposure, and after 24 hours the apoptotic cells were more than 90% of the total **(Fig. 11, Fig. 12)**.

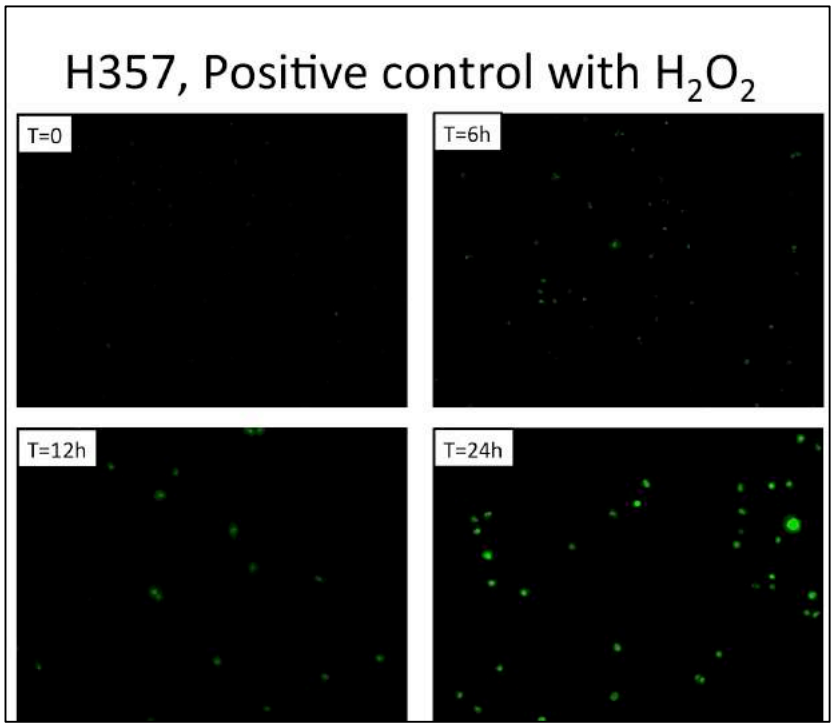


Fig. 11: *Positive control of Annexin apoptosis assay with 1 mM hydroxgen peroxide at 0/6/12/24 hours. The apoptotic cells showed strong green fluorescence signal increasing overtime at fluorescence microscopy.*

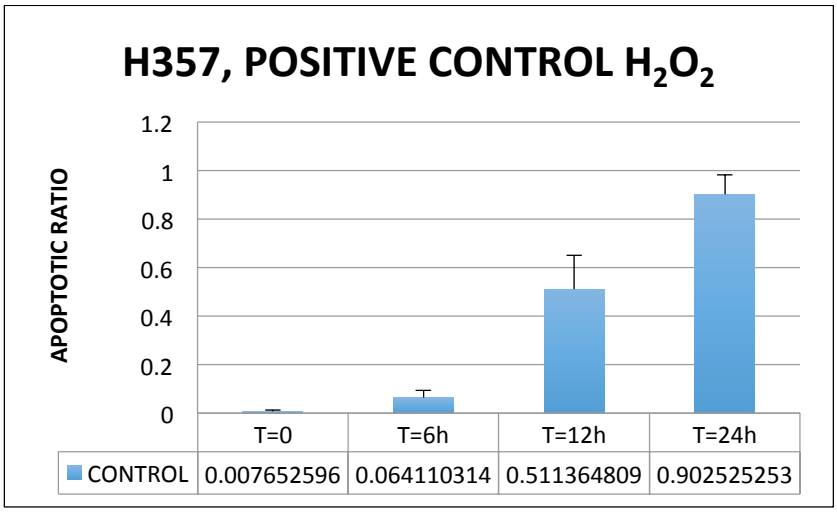


Fig. 12: *Positive control of Annexin apoptosis assay with 1 mM hydroxgen peroxide at 0/6/12/24 hours.*

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)**.

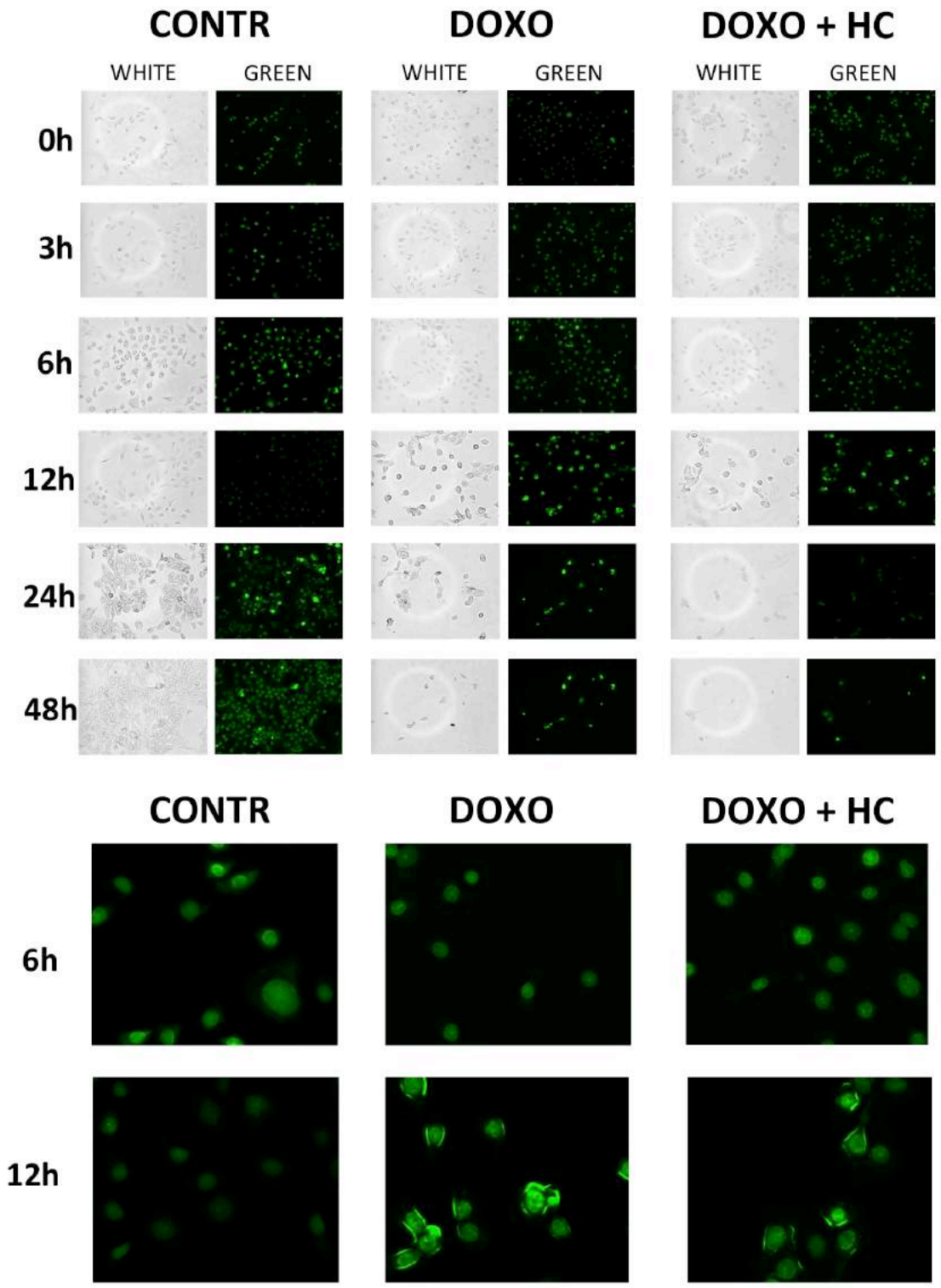


Fig. 13: *Phosphatidylserine exposure and trafficking overtime in the annexin apoptosis assay. The early apoptotic events were studied at 0/3/6/12/24/48 hours performing the cells fixation with 100% methanol inducing the permeabilization of the cell membranes.*

5.3. Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucose-dependent mechanisms

In this series of expriments 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 cemotherapeutic 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)**.

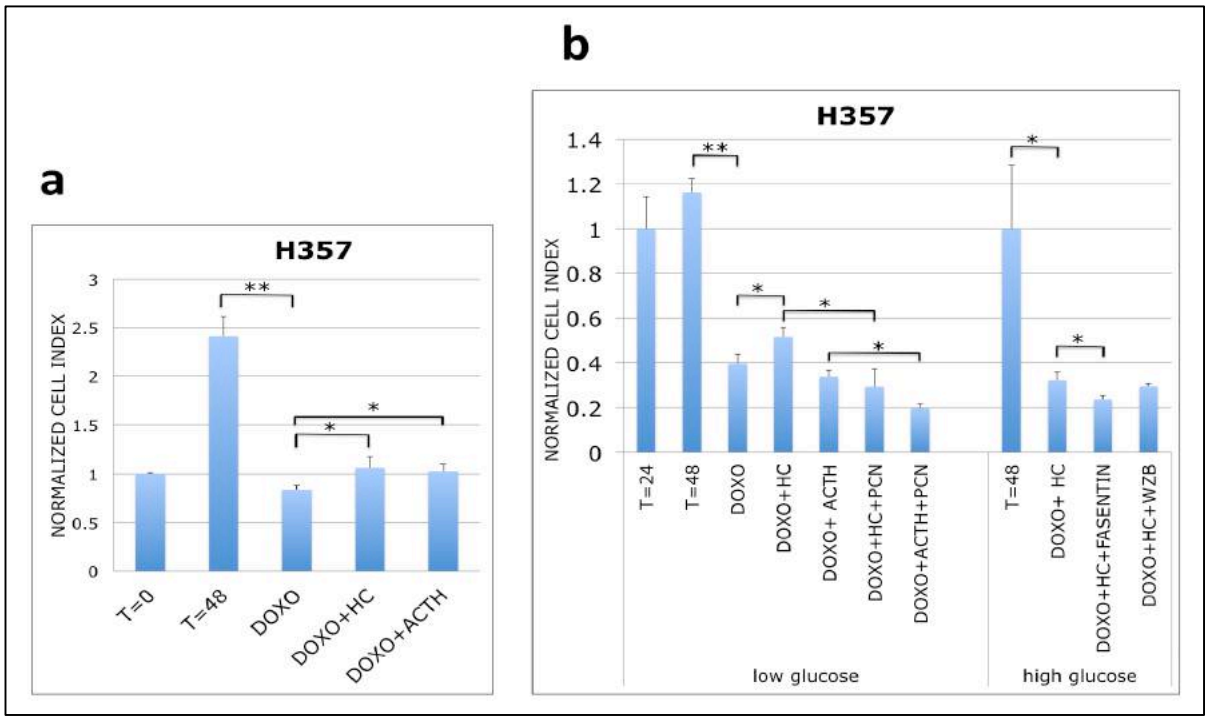


Fig. 14: *The effect of glucocorticoid system on the effectiveness of DOXO on H357 cell line* ***(a)****. The cell line was also tested with the GR inhibitor PCN, glucose uptake inhibitors, and different glucose dosages. Statistical significance is given as follows: *p < 0.05; **p < 0.005*

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 hypotesis (**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)**.

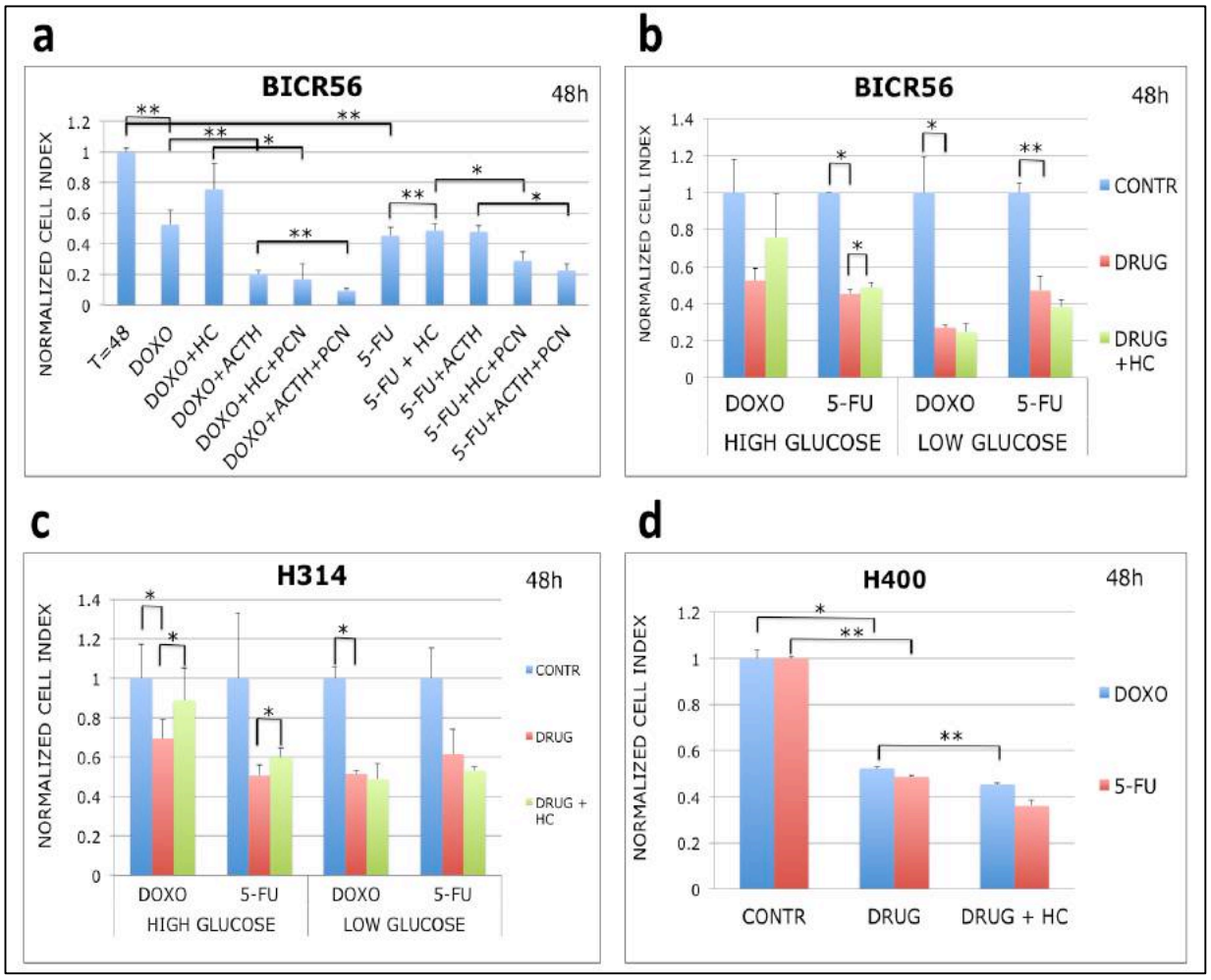


Fig. 15: *The effect of glucocorticoid system on the effectiveness of DOXO and 5-FU at 48 hours on BICR56* ***(a)****, BICR16* ***(b)****, H314* ***(c)****, and H400* ***(d)*** *cell lines. Statistical significance is given as follows: *p < 0.05; **p < 0.005*

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 effectivness 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**).

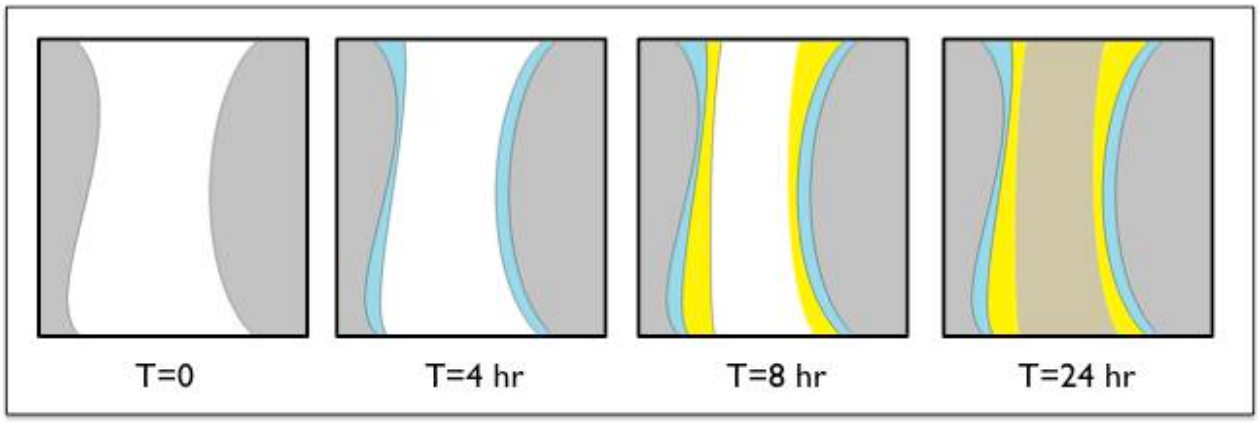


Fig. 16*: Schematic representation of wound healing assay: the grey areas represent the initial cell fronts (T=0). The blue (T=4hr) and yellow (T=8hr) areas represent the change in the size of the wound, as the cell fronts close. At T=24hr the wound is completely closed because of the cell migration.*

The Cells were grown to 100% confluency, 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).

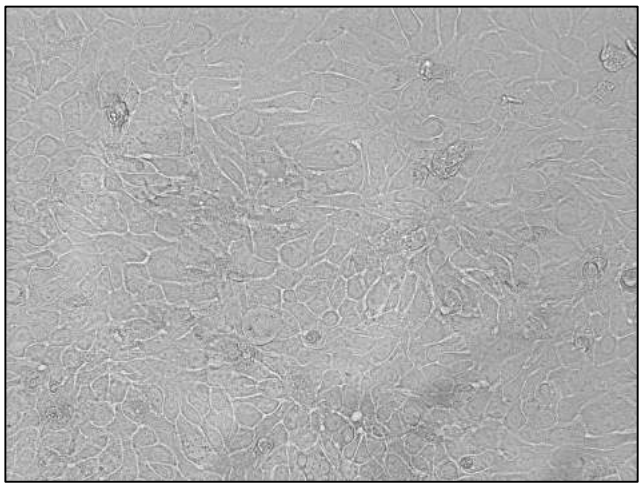


Fig. 17: *Aspect of the H357 cells at 100% confluency before being scratched for the wound healing assay (x460 magnification)*

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.

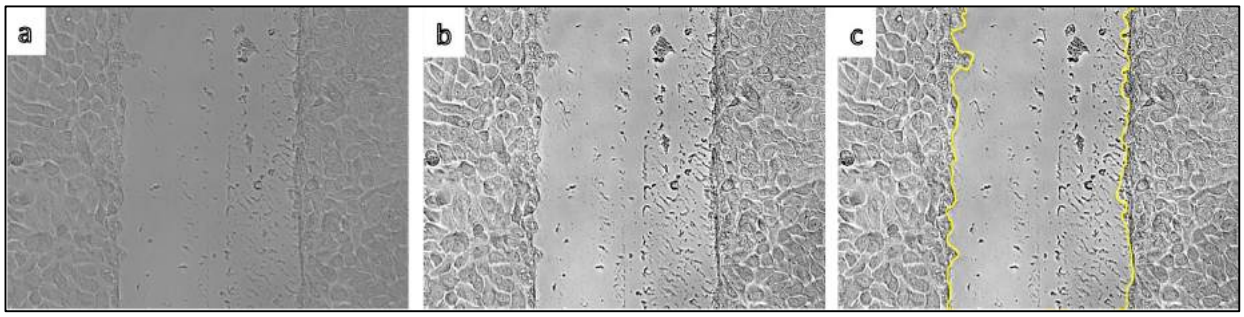


Fig. 18: *Example of image processing. a) aspect of the original picture (wound at T=0); b)the same image with enhanced contrast; c) the wound edges are detected and the wound area can be correctly measured. x460 magnification*

6.1.2. Results

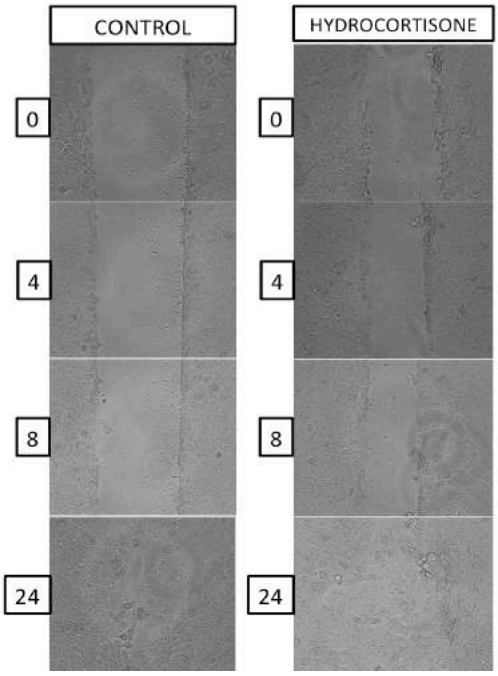


Fig. 19: *Aspect of the control and hydrocortisone groups wounds at 0/4/8/24 hours*

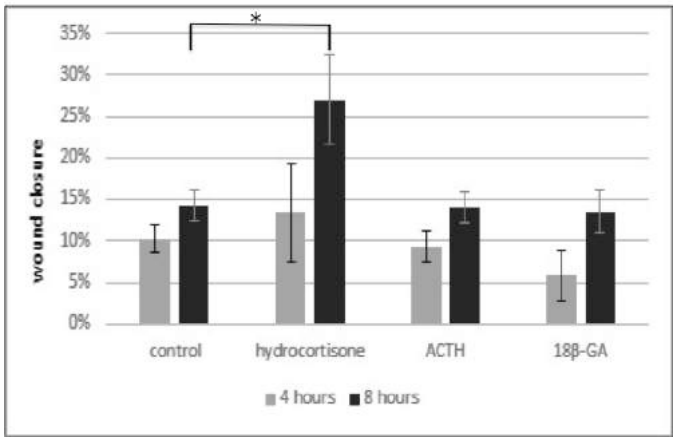


Fig. 20: *Wound closure percentage at 4 and 8 hours in the 4 study groups*

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% ± 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 glycyrrhetinic 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 completition 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 citotoxicity 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 trestment.

The chapter 5 also clearly established the role of exogenous corticosteroids in reducing the citotoxicity 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 in 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.

Obiviously, 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

1. John S, Johnson TA, Sung MH, Biddie SC, Trump S, Koch-Paiz CA, Davis SR, Walker R, Meltzer PS, Hager GL. Kinetic complexity of the global response to glucocorticoid receptor action. Endocrinology 2009 150:1766-74.
2. Slominski A, Zbytek B, Nikolakis G, Manna PR, Skobowiat C, Zmijewski M, Li W, Janjetovic Z, Postlethwaite A, Zouboulis CC, Tuckey RC. Steroidogenesis in the skin: implications for local immune functions. J Steroid Biochem Mol Biol 2013;137:107-23.
3. Cirillo N, Prime SS. Keratinocytes synthesize and activate cortisol. J Cell Biochem 2011 112:1499-150.
4. Azher S, Azami O, Amato C, McCullough M, Celentano A, Cirillo N. The Non-Conventional Effects of Glucocorticoids in Cancer. J Cell Physiol 2016 231:2368-73.
5. Herr I, Pfitzenmaier J. Glucocorticoid use in prostate cancer and other solid tumours: implications for effectiveness of cytotoxic treatment and metastases. Lancet Oncol. 2006 7:425-30.
6. Rivera C, Venegas B. Histological and molecular aspects of oral squamous cell carcinoma. Oncol Lett 2014 8:7–11.
7. Silverman S Jr. Demographics and occurrence of oral and pharyngeal cancers. The outcomes, the trends, the challenge. J Am Dent Assoc 2001 132:7S-11S.
8. Daniel FI, Fava M, da Rocha Hoffmann R, Cam MM, Yurgel LS. Main molecular markers of oral squamous cell carcinoma. Applied Cancer Research 2010 30:279-288.
9. Rivera C. Essentials of oral cancer. Int J Clin Exp Pathol 2015 8:11884-94.
10. Thorn C, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein T, Altman R. Doxorubicin pathways. Pharmacogenetics And Genomics 2011 21:440-446.
11. Tewey K, Rowe T, Yang L, Halligan B, Liu L. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 1984 226:466-468.

12. Abbasi MM, Jahanban-Esfahlan R, Monfaredan A, Seidi K, Hamishehkar H, Khiavi MM. Oral and IV Dosages of Doxorubicin-Methotrexate loaded-Nanoparticles Inhibit Progression of Oral Cancer by Down-Regulation of Matrix Methaloproteinase 2 Expression in Vivo. Asian Pacific Journal of Cancer Prevention 2014 15:10705-10711.
13. Schlossmacher G, Stevens A, White A. 2011. Glucocorticoid receptor-mediated apoptosis: Mechanisms of resistance in cancer cells. J Endocrinol 211:17–25.
14. Slominski AT, Manna PR, Tuckey RC. 2014. Cutaneous glucocorticosteroidogenesis: Securing local homeostasis and the skin integrity. Exp Dermatol 23:369–374.
15. Rasmuson T, Ljungberg B, Grankvist K, Jacobsen J, Olsson T. 2001. Increased serum cortisol levels are associated with high tumour grade in patients with renal cell carcinoma. Acta Oncologica 40:83–87
16. Bernabé DG, Tamae AC, Miyahara GI, Sundefeld MLM, Oliveira SP, Biasoli ÉR. 2012. Increased plasma and salivary cortisol levels in patients with oral cancer and their association with clinical stage. J Clin Pathol 65:934–939
17. Sidler D, Renzulli P, Schnoz C, Berger B, Schneider-Jakob S, Fl€uck C, Inderbitzin D, Corazza N, Candinas D, Brunner T. 2011. Colon cancer cells produce immunoregulatory glucocorticoids. Oncogene 30:2411–2419.
18. Feng X, Reini SA, Richards E, Wood CE, Keller-Wood M. 2013. Cortisol stimulates proliferation and apoptosis in the late gestation fetal heart: Differential effects of mineralocorticoid and glucocorticoid receptors. Am J Physiol Regul Integr Comp Physiol 305:R343–R350.
19. Itoi S, Terao M, Murota H, Katayama I. 2013. 11b-Hydroxysteroid dehydrogenase 1 contributes to the pro-inflammatory response of keratinocytes. Biochem Biophys Res Commun 440:265–270.

20. Romero LM, Raley-Susman KM, Redish DM, Brooke SM, Horner HC, Sapolsky RM. 1992. Possible mechanism by which stress accelerates growth of virally derived tumors. Proc Natl Acad Sci 89:11084–11087.
21. Deguchi M, Isobe Y, Matsukawa S, Yamaguchi A, Nakagawara G. 1998. Usefulness of metyrapone treatment to suppress cancer metastasis facilitated by surgical stress. Surgery 123:440–449.
22. Hogan MM, Vogel SN. 1988. Inhibition of macrophage tumoricidal activity by glucocorticoids. J Immunol 140:513–519.
23. Olnes MJ, Kotliarov Y, Biancotto A, Cheung F, Chen J, Shi R, Zhou H, Wang E, Tsang JS, Nussenblatt R; CHI Consortium. 2016. Effects of systemically administered hydrocortisone on the human immunome. Sci Rep 6:23002.
24. Feng Z, Liu L, Zhang C, Zheng T, Wang J, Lin M, Zhao Y, Wang X, Levine AJ, Hu W. 2012. Chronic restraint stress attenuates p53 function and promotes tumorigenesis. Proc Natl Acad Sci 109:7013–7018.
25. Guendisch S, Boeckeler E, Behrends U, Amtmann E, Ehrhardt H, Jeremias I. 2012. Glucocorticoids augment survival and proliferation of tumor cells. Anticancer Res 32:4251–4261.
26. Xing K, Gu B, Zhang P, Wu X. 2015. Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: An insight into the optimum application of glucocorticoids in anti-cancer therapy. BMC Immunol 16:39.
27. Spiegelman VS, Budunova IV, Carbajal S, Slaga TJ. 1997. Resistance of transformed mouse keratinocytes to growth inhibition by glucocorticoids. Mol Carcinogenesis 20:99–107.
28. Newton R. 2000. Molecular mechanisms of glucocorticoid action: What is important? Thorax 55:603–613.
29. Rabbitt E, Gittoes N, Stewart P, Hewison M. 2003. 11b-hydroxysteroid dehydrogenases, cell proliferation and malignancy. J Steroid Biochem Mol Biol 85:415–421.

30. Yuan HJ, Han X, He N, Wang GL, Gong S, Lin J, Gao M, Tan JH. 2016. Glucocorticoids impair oocyte developmental potential by triggering apoptosis of ovarian cells via activating the Fas system. Sci Rep 6:24036.
31. Bernabé DG, Tamae AC, Biasoli ÉR, Oliveira SH. 2011. Stress hormones increase cell proliferation and regulates interleukin-6 secretion in human oral squamous cell carcinoma cells. Brain Behav Immun 25:574–583.
32. Heikkilä K, Ebrahim S, Lawlor DA. 2008. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. Eur J Cancer 44:937–945.
33. Jobe NP, Rösel D, Dvoránková B, Kodet O, Lacina L, Mateu R, Smetana K, Brábek J. 2016. Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit CAF-induced human melanoma cell invasiveness. Histochem Cell Biol 144:1–13.
34. Duffy SA, Taylor JM, Terrell JE, Islam M, Li Y, Fowler KE, Wolf GT, Teknos TN. 2008. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. Cancer 113:750–757.
35. Nagata M, Fujita H, Ida H, Hoshina H, Inoue T, Seki Y, Ohnishi M, Ohyama T, Shingaki S, Kaji M. 2003. Identification of potential biomarkers of lymph node metastasis in oral squamous cell carcinoma by cDNA microarray analysis. Int J Cancer 106:683–689.
36. Kenouch S, Lombes M, Delahaye F, Eugene E, Bonvalet J-P, Farman N. 1994. Human skin as target for aldosterone: Coexpression of mineralocorticoid receptors and 11 betahydroxysteroid dehydrogenase. J Clin Endocrinol Metab 79:1334–1341.
37. Ren R, Oakley RH, Cruz-Topete D, Cidlowski JA. 2012. Dual role for glucocorticoids in cardiomyocyte hypertrophy and apoptosis. Endocrinology 153:5346–5360.
38. Stojadinovic O, Lee B, Vouthounis C, Vukelic S, Pastar I, Blumenberg M, Brem H, Tomic-Canic M. 2007. Novel genomic effects of glucocorticoids in epidermal keratinocytes inhibition of apoptosis, interferon-g pathway, and wound healing along with promotion of terminal differentiation. J Biol Chem 282:4021–4034.

39. Sanchis A, Alba L, Latorre V, Sevilla LM, Perez P. 2012. Keratinocyte-targeted overexpression of the glucocorticoid receptor delays cutaneous wound healing. PLoS ONE 7:e29701.

40. Wadsworth SJ, Nijmeh HS, Hall IP. 2006. Glucocorticoids increase repair potential in a novel in vitro human airway epithelial wounding model. J Clin Immunol 26:376–387.

41. Lanza A, Stellavato A, Heulfe I, Landi C, Gombos F, Cirillo N. 2009. Serum of patients with oral pemphigus vulgaris impairs keratinocyte wound repair in vitro: A time-lapse study on the efficacy of methylprednisolone and pyridostigmine bromide. Oral Dis 15:478–483.

42. Kennedy D, Pignatelli M, Hassona Y, Prime SS, Cirillo N. 2015. The role of the glucocorticoid system in anchorage-independence during progression of squamous cell carcinoma. Am J Oral Med 1:8–19.

43. Mazzoccoli G, Carughi S, De Cata A, La Viola M, Giuliani A, Tarquini R, Perfetto F. 2003. Neuroendocrine alterations in lung cancer patients. Neuroendocrinol Lett 24:77–82.

44. Tiganescu A, Tahrani AA, Morgan SA, Otranto M, Desmouli A, Abrahams L, Hassan- Smith Z, Walker EA, Rabbitt EH, Cooper MS. 2013. 11b-Hydroxysteroid dehydrogenase blockade prevents age-induced skin structure and function defects. J Clin Invest 123:3051–3060.

45. Sephton SE, Sapolsky RM, Kraemer HC, Spiegel D. 2000. Diurnal cortisol rhythm as a predictor of breast cancer survival. J Natl Cancer Inst 92:994–1000.

46. Sephton SE, Lush E, Dedert EA, Floyd AR, Rebholz WN, Dhabhar FS, Spiegel D, Salmon P. 2013. Diurnal cortisol rhythm as a predictor of lung cancer survival. Brain Behav Immun 30:S163–S170.

47. Terao M, Itoi S, Murota H, Katayama I. 2013. Expression profiles of cortisol-inactivating enzyme, 11b-hydroxysteroid dehydrogenase-2, in human epidermal tumors and its role in keratinocyte proliferation. Exp Dermatol 22:98–101.

48. Parks LL, Turney MK, Gaitan D, Kovacs WJ. 1998. Expression of 11b-hydroxysteroid dehydrogenase type 2 in an ACTH-producing small cell lung cancer. J Steroid Biochem Mol Biol 67:341–346.

49. Temkin S, Nacharaju VL, Hellman M, Lee Y-C, AbulafiaO. 2006. Type 2 11b-hydroxysteroid dehydrogenase activity in human ovarian cancer. Steroids 71:1019–1023.

50. Bland R, Worker C, Noble B, Eyre L, Bujalska I, Sheppard M, Stewart P, Hewison M. 1999. Characterization of 11beta-hydroxysteroid dehydrogenase activity and corticosteroid receptor expression in human osteosarcoma cell lines. J Endocrinol 161:455–464

51. Zbánková Š, Bryndová J, Kment M, Pàcha J. 2004. Expression of 11Z-hydroxysteroid dehydrogenase types 1 and 2 in colorectal cancer. Cancer Lett 210:95–100.

52. Cirillo N, Hassona Y, Pignatelli M, Gasparoto T, Morgan D, Prime S. 2012. Characterization of a novel oral glucocorticoid system and its possible role in disease. J Dental Res 91:97–103.

53. Lu L, Zhao G, Ouellet J, Fan Z, Labrie F, Pelletier G. 2011. Expression of 11b-hydroxysteroid dehydrogenase type 1 in breast cancer and adjacent non-malignant tissue. An immunocytochemical study. Pathol Oncol Res 17:627–632.

54. Budunova IV, Carbajal S, Kang Hi, Viaje A, Slaga TJ. 1997. Altered glucocorticoid receptor expression and function during mouse skin carcinogenesis. Mol Carcinogenesis 18:177–185.

55. Waters C, Stevens A, White A, Ray D. 2004. Analysis of co-factor function in a glucocorticoid-resistant small cell carcinoma cell line. J Endocrinol 183:375–383.

56. Ray DW, Davis JR, White A, Clark AJ. 1996. Glucocorticoid receptor structure and function in glucocorticoid-resistant small cell lung carcinoma cells. Cancer Res 56:3276–3280.

57. Parks LL, Turney MK, Detera-Wadleigh S, Kovacs WJ. 1998. An ACTH-producing small cell lung cancer expresses aberrant glucocorticoid receptor transcripts from a normal gene. Mol Cell Endocrinol 142:175–181.

58. Kay P, Schlossmacher G, Matthews L, Sommer P, Singh D, White A, Ray D. 2011. Loss of glucocorticoid receptor expression by DNA methylation prevents glucocorticoid induced apoptosis in human small cell lung cancer cells. PLoS ONE 6:e24839.

59. Li J, Johnson TA, Hanson LA, Beer DG. 1996. Loss of glucocorticoid-dependent growth inhibition in transformed mouse lung cells. Mol Carcinogenesis 16:213–220.

60. Sung J, Song Y-M, Lawlor DA, Smith GD, Ebrahim S. 2009. Height and site-specific cancer risk: A cohort study of a Korean adult population. Am J Epidemiol 170:53–64.
61. Oakley RH, Cidlowski JA. 2011. Cellular processing of the glucocorticoid receptor gene and protein: New mechanisms for generating tissue-specific actions of glucocorticoids. J Biol Chem 286:3177–3184.
62. Kesić L, Obradović R, Mihailović D, Radičević G, Stanković S, Todorović K. 2009. Incidence and treatment outcome of oral lichen planus in southeast Serbia in a 10-year period (1997–2007). Vojnosanitetski Pregled 66:434–439.
63. Kaplan I, Ventura-Sharabi Y, Gal G, Calderon S, Anavi Y. 2012. The dynamics of oral lichen planus: A retrospective clinicopathological study. Head Neck Pathol 6:178–183.
64. Ismail SB, Kumar SK, Zain RB. 2007. Oral lichen planus and lichenoid reactions: Etiopathogenesis, diagnosis, management and malignant transformation. J Oral Sci 49:89–106.
65. Scully C, Eisen D, Carrozzo M. 2000. Management of oral lichen planus. Am J Clin Dermatol 1:287–306.
66. Usatine RP, Tinitigan M. 2011. Diagnosis and treatment of lichen planus. Am Fam Phys 84:53–60.
67. Lodi G, Carrozzo M, Furness S, Thongprasom K. 2012. Interventions for treating oral lichen planus: A systematic review. Br J Dermatol 166:938–947.
68. Keith BD. 2008. Systematic review of the clinical effect of glucocorticoids on nonhematologic malignancy. BMC Cancer 8:1.
69. Hosking D, Stone M, Foote J. 1990. Potentiation of calcitonin by corticosteroids during the treatment of the hypercalcaemia of malignancy. Eur J Clin Pharmacol 38:37–41.
70. Nakane T, Szentendrei T, Stern L, Virmani M, Seely J, Kunos G. 1990. Effects of IL-1 and cortisol on beta-adrenergic receptors, cell proliferation, and differentiation in cultured human A549 lung tumor cells. J Immunol 145:260–266.

71. Zhang C, Beckermann B, Kallifatidis G, Liu Z, Rittgen W, Edler L, B€uchler P, Debatin K-M, Büchler MW, Friess H. 2006. Corticosteroids induce chemotherapy resistance in the majority of tumour cells from bone, brain, breast, cervix, melanoma and neuroblastoma. Int J Oncol 29:1295–1301.
72. Seyfried TN, Shelton LM, Mukherjee P. 2010. Does the existing standard of care increase glioblastoma energy metabolism? Lancet Oncol 11:811–813.
73. Herr I, Ucur E, Herzer K, Okouoyo S, Ridder R, Krammer PH, Doeberitz MVK, Debatin KM. 2003. Glucocorticoid co-treatment induces apoptosis resistance toward cancer therapy in carcinomas. Cancer Res 63:3112–3120.
74. Sui M, Chen F, Chen Z, Fan W. 2006. Glucocorticoids interfere with therapeutic efficacy of paclitaxel against human breast and ovarian xenograft tumors. Int J Cancer 119:712–717.
75. Zhang C, Mattern J, Haferkamp A, Pfitzenmaier J, Hohenfellner M, Rittgen W, Edler L, Debatin KM, Groene E, Herr I. 2006b. Corticosteroid-induced chemotherapy resistance in urological cancers. Cancer Biol Ther 5:59–64.
76. Prime SS, Nixon SV, Crane IJ, Stone A, Matthews JB, Maitland NJ, Remnant L, Powell SK, Game SM, Scully C. The behaviour of human oral squamous cell carcinoma in cell culture. J Pathol 1990;160:259-69.
77. Edington KG, Loughran OP, Berry IJ, Parkinson EK. Cellular immortality: a late event in the progression of human squamous cell carcinoma of the head and neck associated with p53 alteration and a high frequency of allele loss. Mol Carcinog 1995 13:254-65.
78. Niu G, Chen X. Apoptosis imaging: beyond Annexin V. J Nucl Med 2010 51:1659–1662.
79. Blankenberg FG, Katsikis PD, Tait JF, Davis RE, Naumovski L, Ohtsuki K, Kopiwoda S, Abrams MJ, Darkes M, Robbins RC, Maecker HT, Strauss HW. In vivo detection and imaging of phosphatidylserine expression during programmed cell death. Proc Natl Acad Sci U S A. 1998 May 26;95(11):6349-54.

80. Tait JF, Cerqueira MD, Dewhurst TA, Fujikawa K, Ritchie JL, Stratton JR. Evaluation of annexin V as a platelet-directed thrombus targeting agent. Thromb Res. 1994 Sep 1;75(5):491-501.

81. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky EK, Ang KK. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006;354(6):567–78.

82. Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, De Raucourt D, Bokemeyer C, Schueler A, Amellal N, Hitt R. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359(11):1116–27.

APPENDIX 1 – JOURNAL PUBLICATIONS

1: Cascone M, **Celentano A**, Adamo D, Leuci S, Ruoppo E, Mignogna MD. Oral lichen planus in childhood: a case series". Int J Dermatol, Accepted for publication (10-Jan-2017). DOI:10.1111/ijd.13571.

2: **Celentano A**, Mascolo M, Cirillo N, De Rosa G, Mignogna MD. Delayed Diagnosis of a Nasal Type Lymphoma Misdiagnosed as Persistent Sinusitis. J Adolesc Young Adult Oncol. 2017 Jan 6. doi: 10.1089/jayao.2016.0050. [Epub ahead of print]PubMed PMID: 28061034.

3: **Celentano A**, Mignogna MD, McCullough M, Cirillo N. Cover Image, Volume 232, Number 3, March 2017. J Cell Physiol. 2017 Mar;232(3):i. doi: 10.1002/jcp.25691. PubMed PMID: 27859301.

4: Marshall A, **Celentano A**, Cirillo N, Mirams M, McCullough M, Porter S. Immune receptors CD40 and CD86 in oral keratinocytes: implications for oral lichen planus. J Oral Sci. 5th nov 2016. Accepted for publication.

5: Cirillo N, Hassona Y, **Celentano A**, Lim KP, Manchella S, Parkinson EK, Prime SS. Cancer-associated fibroblasts regulate keratinocyte cell-cell adhesion via TGF-β-dependent pathways in genotype-specific oral cancer. Carcinogenesis. 2017 Jan;38(1):76-85. doi: 10.1093/carcin/bgw113. PubMed PMID: 27803052.

6: **Celentano A**, Mignogna MD, McCullough M, Cirillo N. Pathophysiology of the Desmo-Adhesome. J Cell Physiol. 2017 Mar;232(3):496-505. doi: 10.1002/jcp.25515. Review. PubMed PMID: 27505028.

7: **Celentano A**, Cirillo N. Desmosomes in disease: a guide for clinicians. Oral Dis. 2016 Jun 22. doi: 10.1111/odi.12527. [Epub ahead of print] Review. PubMed PMID: 27329525

8: Azher S, Azami O, Amato C, McCullough M, **Celentano A** and Cirillo N. The Non-Conventional Effects of Glucocorticoids in Cancer. Accepted manuscript online: 26 APR 2016 04:46PM EST | DOI:10.1002/jcp.25408.

9: Marshall A, **Celentano A**, Cirillo N, Mignogna MD, McCullough M, Porter S. Antimicrobial activity and regulation of CXCL9 and CXCL10 in oral keratinocytes. Eur J Oral Sci. 2016 Oct;124(5):433-439. doi: 10.1111/eos.12293. PubMed PMID: 27671889.

10: **Celentano A**, Tovaru S, Yap T, Adamo D, Aria M, Mignogna MD. In reply: Oral erythema multiforme: trends and clinical findings of a large retrospective: European case series. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016 Jun;121(6):681-2. doi: 10.1016/j.oooo.2016.02.011. Epub 2016 Mar 10. PubMed PMID: 27181445.

11: Nuzzolo P**, Celentano A**, Bucci P, Adamo D, Ruoppo E, Leuci S, Mignogna MD. Lichen planus of the lips: an intermediate disease between the skin and mucosa? Retrospective clinical study and review of the literature. Int J Dermatol. 2016 Mar 18. doi: 10.1111/ijd.13265. [Epub ahead of print] PubMed PMID: 26992292.

12: Moro A, DE Waure C, DI Nardo F, Spadari F, Mignogna MD, Giuliani M, Califano L, Giannì AB, Cardarelli L, **Celentano A**, Bombeccari G, Pelo S. The GOCCLES® medical device is effective in detecting oral cancer and dysplasia in dental clinical setting. Results from a multicentre clinical trial. Acta Otorhinolaryngol Ital. 2015 Dec;35(6):449-54. doi: 10.14639/0392-100X-922. Review. PubMed PMID: 26900252; PubMed Central PMCID: PMC4755053.

13: **Celentano A**, Tovaru S, Yap T, Adamo D, Aria M, Mignogna MD. Oral erythema multiforme: trends and clinical findings of a large retrospective European case series. Oral Surg Oral Med Oral Pathol Oral Radiol. 2015 Dec;120(6):707-16. doi: 10.1016/j.oooo.2015.08.010. Epub 2015 Aug 22. PubMed PMID: 26455287.

14: Adamo D, **Celentano A**, Ruoppo E, Cucciniello C, Pecoraro G, Aria M, Mignogna MD. The Relationship Between Sociodemographic Characteristics and Clinical Features in Burning Mouth Syndrome. Pain Med. 2015 Nov;16(11):2171-9. doi: 10.1111/pme.12808. Epub 2015 Aug 24. PubMed PMID: 26301724.

15: **Celentano A**, Mascolo M, De Rosa G, Mignogna MD. Nodular fasciitis of the tongue. Head Neck. 2016 Jan;38(1):E29-31. doi: 10.1002/hed.24088. Epub 2015 Jul 18. PubMed PMID: 25900798.

16: **Celentano A**, Ruoppo E, Mansueto G, Mignogna MD. Primary oral leishmaniasis mimicking oral cancer: a case report. Br J Oral Maxillofac Surg. 2015 Apr;53(4):396-8. doi:10.1016/j.bjoms.2015.01.021. Epub 2015 Feb 18. PubMed PMID: 25701438.

17: **Celentano A**, Adamo D, Leuci S, Mignogna MD. Oral manifestations of phosphatase and tensin homolog hamartoma tumor syndrome: a report of three cases. J Am Dent Assoc. 2014 Sep;145(9):950-4. doi: 10.14219/jada.2014.58. PubMed PMID: 25170002.

18: Mignogna MD, **Celentano A**, Leuci S, Cascone M, Adamo D, Ruoppo E, Favia G. Mucosal leishmaniasis with primary oral involvement: a case series and a review of the literature. Oral Dis. 2015 Jan;21(1):e70-8. doi: 10.1111/odi.12268. Epub 2014 Jul 12. PubMed PMID: 24939442.

19: **Celentano A**, Mignogna MD. Grooved tongue and congenital muscular torticollis. Am J of Oral Med. (2015) Vol. 1 No. 1 pp. 5-7. doi:10.7726/ajom.2015.1002.

APPENDIX 2 - ORAL PRESENTATIONS

1) Title : “Emerging Infectious diseases in oral medicine: what is changing?”. SENAME XI International Conference. Tirana, Albania. Date: 20-22/06/2014.

2) Title: “Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucose-dependent mechanisms”. XIII Biennal Congress of EAOM (European Academy of Oral Medicine). Turin, Italy. Date: 15-17th/09/2016.

APPENDIX 3 - PUBLISHED ABSTRACTS OF SCIENTIFIC MEETINGS

1) **Celentano A**, Adamo D, Ruoppo E, Leuci S, Mignogna MD. PTEN hamartoma tumor syndrome (PHTS) in oral medicine. Publication date: 2013/12. Proceedings del III Simposio Società Italiana di Patologia e Medicina Orale Torino, Scuola di Medicina Polo San Luigi, 17-18 Ottobre 2014,Volume 2, Pagine 18.

2) Adamo D, Ruoppo E, **Celentano A**, Leuci S, Mignogna MD. Stomatodynia in oral lichen planus: a case series data analysis of 28 patients. Publication date: 2013/12. Proceedings del III Simposio Società Italiana di Patologia e Medicina Orale Torino, Scuola di Medicina Polo San Luigi, 17-18 Ottobre 2014, Volume 2, Pagine 5.

3) Cascone M, Adamo D, **Celentano A**, Leuci S, Ruoppo E, Mignogna MD. Oral involvement in pediatric lichen planus. Publication date: 2013/12. Proceedings del III Simposio Società Italiana di Patologia e Medicina Orale Torino, Scuola di Medicina Polo San Luigi, 17-18 Ottobre 2014,Volume 2, Pagine 17.

4) **Celentano A**, Adamo D, Ruoppo E, et al. Oral erythema multiforme: a 20 years clinical experience. ORAL DISEASES, Volume: 20, Special Issue: SI Supplement, 2 Pages: 12-12, Meeting Abstract: 36 Published: SEP 2014.

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.

6) De Stefano M, Mangone C, Saturnino R, **Celentano A**, Ramaglia L. Reimplanting a traumatic avulsed tooth: case report. Publication date: 2014. MINERVA STOMATOLOGICA, Volume 63, Numero 4 (S1), Pagine 90.

7) Cascone M, **Celentano A**, Ruoppo E, Leuci S, Adamo D. Oral burning and psychological profile in patients with keratotic forms of oral lichen planus: a case control clinical study. Publication date: 2015/4/1. ANNALI DI STOMATOLOGIA,Volume 6, Numero 1, Pagine 22.

8) **Celentano A**, Mignogna MD, McCullough M, Cirillo N. Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucose dependent mechanisms". Oral Diseases (2016) 22 (Suppl. 2), 10–13 doi:10.1111/odi.12558.

APPENDIX 4 - RESEARCH PROCEEDINGS

1) **Celentano A**, Sadile G, Leuci S, Adamo D, Sammartino G, Mignogna MD, Ruoppo E. Title: "Clinical staging of drug-related osteonecrosis of the jaws: a new proposal". Xth SENAME & Vth ATORECD International Congress. Hammamet,Tunisia. Date: 27-29th/09/2013.

2) De Stefano M, Mangone C, Saturnino R, **Celentano A**, Ramaglia. Title: "Replanting of a lateral incisor after atraumatic avulsion: a case report". XXI National Congress of Italian Dentistry professors (Congresso Nazionale Collegio dei Docenti di Odontoiatria). Rome, Italy. Date: 10-12th/04/2014.

3) **Celentano A**, Adamo D, Ruoppo E, Leuci S, Tovaru S, Mignogna MD. Title: "Oral erythema multiforme: a 20 year clinical experience". XII Biennal Congress of EAOM (European Academy of Oral Medicine), Antalya, Turkey. Date: 11-14th/09/2014.

4) Adamo D, Ruoppo E, **Celentano A**, Leuci S, Mignogna MD. Title : "Socio-demographic characteristics and pain in 75 Burning Mouth Syndrome patients". XII Biennal Congress of EAOM (European Academy of Oral Medicine). Antalya, Turkey. Date: 11-14th/09/2014.

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.

6) Cascone M, **Celentano A**, Adamo D, Leuci S, Ruoppo E, Mignogna MD. Title: "Oral involvement in pediatric lichen planus". 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.

7) Cascone M, **Celentano A**, Ruoppo E, Leuci S, Adamo D. Title: "Oral burning and psychological profile in patients with keratotic forms of oral lichen planus: a case control clinical study". XIII National Congress of Italian Society of Oral Pathology ad Medicine (13° Congresso nazionale Società di Patologia e Medicina Orale) (SIPMO), Bologna, Italy. Date: 8-10th/10/2015.

8) Di Nardo F, Moro A, de Waure C, Spadari F, Mignogna MD, Giuliani M, Califano L, Giannì AB, CardarellI L, **Celentano A**, Bombeccari G, Pelo S. Title: "GOCCLES (Glasses for Oral Cancer – Curing Light Exposed – Screening). A new medical device for the prevention of oral cancer". 48° National Congress of Italian Society of Hygiene (48° Congresso nazionale Società Italiana di Igiene) (SITI), Milano, Italy. Date: 14-17th/10/2015.

9) **Celentano A**, Mignogna MD, McCullough M, Cirillo N Title: "Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucosedependent mechanisms". XIII Biennal Congress of EAOM (European Academy of Oral Medicine), Turin, Italy. Date: 15-17th/09/2016.

10) Adamo D, Ruoppo E, **Celentano A**, Aria M, Leuci S, Mignogna M. Title: "Antipsychotics in the treatment of Burning Mouth Syndrome". XIII Biennal Congress of EAOM (European Academy of Oral Medicine), Turin, Italy. Date: 15-17th/09/2016.

11) Leuci S, Adamo D, **Celentano A**, Ruoppo E, Mignogna MD. Title: "A 3-year e-learning program to early detection/prevention of oral cancer". XIII Biennal Congress of EAOM (European Academy of Oral Medicine), Turin, Italy. Date: 15-17th/09/2016.

APPENDIX 5– HONOURS AND AWARDS

Date	Honour / Prize	Awarding body	Subject/ Title
2016	EAOM 2016 Award (Best Oral Research Presentation)	EAOM (European Association of Oral Medicine)	"Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucose dependent mechanisms"
2016	Journal Cover Page (DOI: 10.1002/jcp.25691)	Journal of Cellular Physiology Board	Cover Image, Volume 232, Number 3, March 2017. J Cell Physiol. 2017 Mar;232(3):i. doi: 10.1002/jcp.25691. PubMed PMID: 27859301.
2016	MTC: Melbourne teaching certificate	The University of Melbourne, Australia	

LIST OF ABBREVIATIONS

5-FU: 5-fluorouracil

11b-HSD:11b-Hydroxysteroid dehydrogenase

11b-HSD1/11b-HSD2: 11bhydroxysteroid dehydrogenases

18ß-GA: 18β-Glycyrrhetinic acid

ACTH: adrenocorticotropin 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

International Journal of Dermatology

Oral lichen planus in childhood: a case series

Journal:	*International Journal of Dermatology*
Manuscript ID	IJD-2015-1220.R4
Manuscript Type:	Report
Date Submitted by the Author:	09-Nov-2016
Complete List of Authors:	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
Keywords:	oral diseases, lichen planus, pediatric dermatology

Title: "Oral lichen planus in childhood: a case series"

Authors:

Cascone M, DDS University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences

Celentano A, DDS University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences

Adamo D, DDS University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences

Leuci S, DDS, PhD University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences

Ruoppo E, DDS, PhD University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences

Mignogna MD, MD, DMD University Federico II of Naples, Department of Neurosciences, Reproductive and Odontostomatological Sciences

Corresponding Author:

Marco Cascone Address: via Pansini 5, 80131 Naples, Italy Phone: 00393933259564

email:marcocascone.unina@gmail.com

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.[8]

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.[9,10]

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 multiforme, allergic gingivostomatitis, and gluten sensitivity enteropathy.[10–12]

In challenging cases more sensitive diagnostic techniques could be useful to achieve diagnosis such as direct and indirect immunofluorescence.[13]

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 Table2.

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%[17] compared with 1% to 2% of the general population.[2]

The present case series confirms the epidemiological data previously collected concerning the pediatric LP population with a balanced M:F ratio[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.[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.[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.[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.[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 cases[6] 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[6] 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[25] even if the prognosis seems to be more favorable. [19]

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

1 Patel S, Yeoman CM, Murphy R. Oral lichen planus in childhood: a report of three cases. Int J Paediatr Dent 2005;15:118–22.

2 Munde AD, Karle RR, Wankhede PK, Shaikh SS, Kulkurni M. Demographic and clinical profile of oral lichen planus: A retrospective study. Contemp Clin Dent 2013;4:181–5.

3 Pandhi D, Singal A, Bhattacharya SN. Lichen planus in childhood: a series of 316 patients. Pediatr Dermatol 2014;31:59–67.

4 Madke B, Gutte R, Doshi B, Khopkar U. Hyperkeratotic Palmoplantar Lichen Planus in a child. Indian J Dermatol 2013;58:405.

5 Sharma A, Białynicki-Birula R, Schwartz RA, Janniger CK. Lichen planus: an update and review. Cutis 2012;90:17–23.

6 Woo VL, Manchanda-Gera A, Park DS, Yoon AJ, Zegarelli DJ. Juvenile oral lichen planus: a report of 2 cases. Pediatr Dent 2007;29:525–30.

7 De Moraes PC, Teixeira RG, Tacchelli DP, Bönecker M, Junqueira JLC, Oliveira LB. Atypical case of oral lichen planus in a pediatric patient: clinical presentation and management. Pediatr Dent 2011;33:445–7.

8 Andreasen JO. Oral lichen planus. 1. A clinical evaluation of 115 cases. Oral Surg Oral Med Oral Pathol 1968;25:31–42.

9 GunaShekhar M, Sudhakar R, Shahul M, Tenny J, Ravikanth M, Manikyakumar N. Oral lichen planus in childhood: A rare case report. Dermatol Online J 2010;16:9.

10 Laeijendecker R, Van Joost T, Tank B, Oranje AP, Neumann H a M. Oral lichen planus in childhood. Pediatr Dermatol 2005;22:299–304.

11 Pendyala G, Joshi S, Kalburge J, Joshi M, Tejnani A. Oral lichen planus: a report and review of an autoimmune-mediated condition in gingiva. Compend Contin Educ Dent 2012;33:e102–8.

12 Sanjaya P, Hallikeri K, Angadi P. Disseminated form of childhood lichen planus. East J Med 2013;16:72–5.

13 Van der Meij EH, van der Waal I. Lack of clinicopathologic correlation in the diagnosis of oral lichen planus based on the presently available diagnostic criteria and suggestions for modifications. J Oral Pathol Med 2003;32:507–12.

14 Chandna P, Adlakha VK, Singal G, Sharma R. Pediatric oral lichen planus: review and case report. Curr Pediatr Rev 2014;10:292–6.

15 Khandelwal V, Nayak PA, Nayak UA, Gupta A. Oral lichen planus in a young Indian child. BMJ Case Rep 2013;2013.

16 Moger G, Thippanna CK, Kenchappa M, Puttalingaiah VD. Erosive oral lichen planus with cutaneous involvement in a 7-year-old girl: a rare case report. J Indian Soc Pedod Prev Dent 2013;31:197–200.

17 Padmini C, Bai KY, Chaitanya V, Reddy MS. Ulcerative lichen planus in childhood. Case Rep Dent 2013;2013:874895.

18 Chaitra TR, Telgi RL, Kishor A, Kulkarni AU. Juvenile oral lichen planus: a clinical rarity. BMJ Case Rep 2012;2012.

19 Anuradha C, Reddy GS, Nandan SRK, Kumar SR, Reddy BVR. Oral mucosal lichen planus in nine-year-old child. N Y State Dent J 2011;77:28–30.

20 Mohan Das U, Jp B. Oral lichen planus in children. Int J Clin Pediatr Dent 2009;2:49–51.

21 Singal A. Familial mucosal lichen planus in three successive generations. Int J Dermatol 2005;44:81–2.

22 Alam F, Hamburger J. Oral mucosal lichen planus in children. Int J Paediatr Dent 2001;11:209–14.

23 Scully C, de Almeida OP, Welbury R. Oral lichen planus in childhood. Br J Dermatol 1994;130:131–3.

24 Borrego Hernando L, Vanaclocha Sebastián F, Hergueta Sánchez J, Ortiz Romero P, Iglesias Diez L. Lichen planus pemphigoides in a 10-year-old girl. J Am Acad Dermatol 1992;26:124–5.

25 Chatterjee K, Bhattacharya S, Mukherjee CG, Mazumdar A. A retrospective study of oral lichen planus in paediatric population. J Oral Maxillofac Pathol 2012;16:363–7.

26 Mignogna MD, Fedele S, Lo Russo L, Ruoppo E, Lo Muzio L. Polyglandular autoimmune syndrome type II associated with oral lichen planus. Int J Dermatol 2002;41:244–6.

27 Handa S, Sahoo B. Childhood lichen planus: a study of 87 cases. Int J Dermatol 2002;41:423–7.

28 Lodi G, Carrozzo M, Furness S, Thongprasom K. Interventions for treating oral lichen planus: a systematic review. Br J Dermatol 2012;166:938–47.

29 Barbosa NG, Silveira ÉJD, Lima EN de A, Oliveira PT, Soares MSM, de Medeiros AMC. Factors associated with clinical characteristics and symptoms in a case series of oral lichen planus. Int J Dermatol 2015;54:e1–6.

30 Carbone M, Arduino PG, Carrozzo M, et al. Course of oral lichen planus: a retrospective study of 808 northern Italian patients. Oral Dis 2009;15:235–43.

Table 1

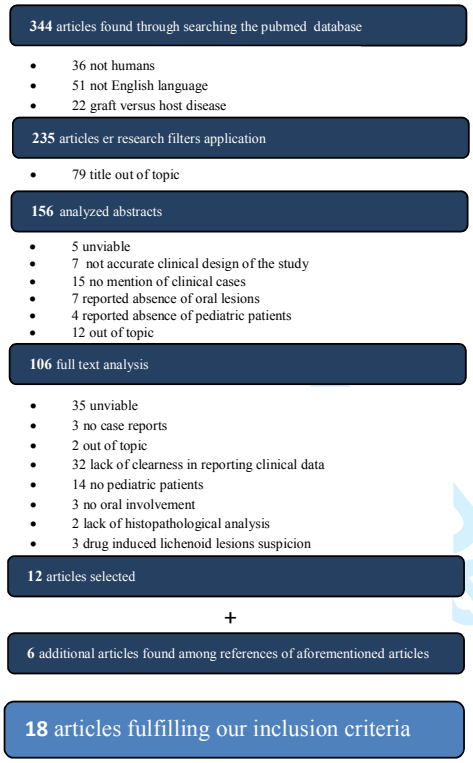


Table 2

Case	Age	Sex	Preexisting Medical conditions	Family history	HBV vaccination status	Drugs	Concomitant oral factors	Confirmatory histology	Clinical pattern	Oral sites	Oral symptoms	Extra-oral sites	Treatment	Resolution of oral lesions
1	17	F	allergy to NSAIDs †	atopy (mother)	**vaccinated**	none	parafunction	HE ‡	plaque-like + erosive plaque-like + erosive	margins of tongue bilaterally ventrum of tongue bilaterally	**burning with spicy foods**	none	topical steroids topical antimycotics	none
2	17	M	atopy, ↓ Hb §	OLP (father)	**vaccinated**	none	none	HE	reticular plaque-like	hard palate retromolar fossae bilaterally	**dryness**	none	topical antimycotics	none
3	14	F	none	none	**vaccinated**	none	none	HE	plaque-like plaque-like + atrophic plaque-like + atrophic plaque-like + pigmentation	buccal mucosa right side margins of tongue bilaterally ventrum of tongue bilaterally lower lip	**none**	none	topical antimycotics	**complete**
4	14	M	Hg (NH_2) C patch test ++ IgG-EBV ¶ 159 U/l erythema discromicum perstans	none	**vaccinated**	none	orthodontics	HE	reticular reticular reticular + atrophic reticular + atrophic	buccal mucosae bilaterally gingiva dorsum of tongue bilaterally margins of tongue bilaterally	**none**	none	topical antimycotics	none
5	15	M	autoimmune thyroiditis atopy	autoimmune thyroiditis (mother)	**vaccinated**	none	none	HE	reticular reticular	buccal mucosae bilaterally ventrum of tongue bilaterally	**none**	none	topical antimycotics	complete
6	9	F	APECED††	N/A ‡‡	**N/A**	N/A	none	HE	bullous + atrophic + reticular bullous + atrophic + reticular reticular bullous + atrophic + reticular	dorsum of tongue margins of tongue bilaterally gingival buccal mucosae bilaterally	**pain**	none	topical steroids topical antimycotics	none
7	11	F	atopy	OLP (father)	**vaccinated**	none	parafunction	HE	reticular	gingiva	**none**	none	topical antimycotics	none
8	11	M	atopy immune carrier for Mediterranean anemia	Chron's disease rheumatoid arthritis	**vaccinated**	none	none	HE	reticular + atrophic	dorsum of tongue	**roughness**	none	topical steroids topical antimycotics	none

Table 3

Authors	Age	Sex	Preexisting Medical conditions	Family history	Drugs	Concomitant oral factors	Confirmatory histology	Clinical pattern	Oral sites	Oral symptoms	Extra-oral sites	Treatment	Resolution of oral lesions
Chandna et al. [14] (2014)	7	F	none	none	none	none	HE ‡ (oral)	reticular papular + reticular + pigmentation	buccal mucosae bilaterally upper and lower lips	**none**	skin	oral hygiene topical steroids	**partial**
Khandelwal et al. [13] (2013)	10	F	↓ Hb § (10.2 g)	none	none	N/A ‡‡	HE + DIF §§ (oral) (oral)	reticular + pigmentation reticular + pigmentation	buccal mucosae bilaterally retromolar fossae bilaterally	**burning with spicy foods**	none	topical retinoids	none
Moger et al.[16] (2013)	7	F	none	none	none	caries at 36,63,65,74	HE + DIF (oral) (oral)	reticular+ atrophic + erosive + papular + pigmentation	buccal mucosae bilaterally	**burning with spicy and hot foods**	skin	topical antimycotic topical steroids topical anesthetic	partial
Padmini et al.[17] (2013)	12	M	none	N/A	none	none	HE (oral)	erosive	dorsum of tongue	**burning with spicy foods**	none	topical steroids topical anesthetic topical antimycotic	complete
Chaitra et al. [18] (2012)	9	F	none	N/A	none	none	HE‡ (oral)	reticular + erosive + pigmentation	buccal mucosae bilaterally	**burning with spicy foods**	N/A	topical steroids topical anesthetic	none
Pendyala et al.[11] (2012)	17	M	none	none	none	none	HE (oral)	bullous	gingiva	**burning exacerbated by acid and spicy foods**	none	topical steroids topical NSAIDs † antioxidants Chlorhexidine	complete
De Moraes et al.[7] (2011)	7	F	none	N/A	none	parafunction	2 X HE (oral)	reticular reticular + atrophic	buccal mucosae bilaterally upper lip	**discomfort (upper lip)**	N/A	topical steroids topical CNIs ¶¶	complete
Anuradha et al.[19] (2011)	9	F	none	none	N/A	N/A	HE + DIF (oral) (oral)	reticular reticular	buccal mucosae bilaterally retromolar fossae bilaterally	**burning with spicy foods**	none	N/A	none
Sanjaya et al.[12] (2011)	9	M	none	none	none	N/A	2 X HE (oral + skin)	reticular reticular reticular reticular	buccal mucosae bilaterally hard palate lower and upper lips tongue	**burning with spicy foods**	skin	systemic steroids	complete
GunaShekar et al.[9] (2010)	7	M	none	none	none	caries at 36,37	HE (oral)	reticular + atrophic reticular + atrophic reticular + atrophic reticular + atrophic reticular + pigmentation	buccal mucosa right side margins of tongue bilaterally dorsum of tongue floor of mouth upper and lower lips	**soreness with spicy foods (tongue)**	none	chlorhexidine topical steroids	none
Mohan Das & Jp[20] (2009)	12	F	↓ Hb (9 g)	none	none	none	HE (oral)	reticular + erosive + pigmentation	buccal mucosae bilaterally	**burning on consuming food**	none	topical retinoids	none
Woo et al.[8] (2007)	9	F	ANA + †††	none	none	none	HE (oral)	reticular + papular + erosive reticular + papular + erosive	margins of tongue bilaterally ventrum of tongue	**dryness**	none	topical steroids systemic steroids	N/A
Woo et al.[6] (2007)	11	F	none	none	none	orthodontics	HE (oral)	reticular reticular	buccal mucosae bilaterally ventrum of tongue bilaterally	**none**	none	orthodontics removal	none
Amgal [21] (2005)	11	M	N/A	OLP (father) OLP (grandmother)	N/A	N/A	HE (oral)	plaque-like + pigmentation	dorsum of tongue	**burning with hot and spicy foods**	none	topical steroids	complete
Laeijendecker et al. [10] (2005)	11	F	fragrance mix 8% pet patch test ++	none	none	four amalgam fillings	HE (oral)	reticular	buccal mucosae bilaterally	**none**	none	none	complete
Laeijendecker et al. [10] (2005)	16	M	none	none	none	eight amalgam fillings, poor oral hygiene	HE + DIF (oral)	erosive erosive	buccal mucosae bilaterally gingiva	**pain and stinging**	none	topical steroids topical anesthetic topical CNIs systemic steroids systemic CNIs	complete
Laeijendecker et al. [10] (2005)	14	F	atopy nickel sulfate 5% pet patch test ++	none	cetirizine	orthodontics	yes	reticular reticular	buccal mucosae bilaterally margins of tongue bilaterally	**soreness**	none	systemic steroids systemic CNIs	complete
Patel et al. [5] (2005)	15	F	↓ferritin (8µg/L) ↓IgA ¶¶¶ (0.60 g/L) hypothyroidism	none	none	none	HE (N/A)	erosive + reticular erosive + reticular erosive + reticular	margins of tongue bilaterally dorsum of tongue floor of mouth	**pain**	skin	topical steroids	none
Patel et al. [5] (2005)	6	M	autism	none	none	N/A	HE (oral)	plaque-like	dorsum of tongue	**none**	none	none	none
Allam & Hamburger [22] (2001)	6	M	none	none	none	N/A	HE (oral)	reticular reticular	buccal mucosa right side dorsum of tongue	**N/A**	none	none	complete
Allam & Hamburger [22] (2001)	7	M	none	none	none	poor oral hygiene	HE (oral)	reticular + atrophic erosive	buccal mucosae bilaterally gingiva	**soreness**	none	chlorhexidine topical steroids	none
Allam & Hamburger [22] (2001)	14	M	asthma	N/A	salbutamol and beclomethasone inhaler	N/A	HE (oral)	reticular plaque-like	buccal mucosae bilaterally margins of tongue bilaterally	**pain with spicy foods**	N/A	none	complete
Allam & Hamburger [22] (2001)	14	M	none	none	none	N/A	HE (oral)	reticular + atrophic + pigmentation	buccal mucosa left side	**none**	N/A	none	none
Scully et al. [23] (1994)	11	F	none	none	none	N/A	HE (oral)	erosive	margin of tongue	**soreness**	none	topical steroids	none
Scully et al. [23] (1994)	10	F	none	none	none	N/A	HE (oral)	erosive erosive erosive	buccal mucosae bilaterally ventrum of tongue floor of mouth	**N/A**	none	topical steroids topical CNIs	complete
Crego Hernando et al. [24] (1992)	10	F	IgG anti-BMZ ‡‡‡ 1:80 LPP §§§	N/A	N/A	N/A	HE + DIF (skin)	reticular	buccal mucosae bilaterally	**N/A**	skin, vulvar mucosae	systemic steroids	complete

Table 4

	CASE SERIES (n=8)		REVIEW CASES (n=26)		TOTAL (n=34)	
AGE						
overall age range (years)	9 - 17		6 – 17		6 – 17	
mean (± sd)age	13.5	(± 2.73)	10.41	(± 3.24)	11.18	(± 3.40)
GENDER						
males	4	(50%)	11	(42.3%)	15	(46.8%)
females	4	(50%)	15	(57.7%)	19	(55.8%)
FAMILIAL OLP	3	(37.5%)	1	(3.8%)	4	(11.7%)
SYSTEMIC CONDITIONS						
PREEXISTING MEDICAL CONDITIONS	7	(87.5%)	9	(34.6%)	16	(47%)
IMMUNOLOGICAL DISORDERS	7	(87.5%)	5	(19.2%)	12	(35.2%)
CONFIRMATORY HISTOLOGY	8	(100%)	26	(100%)	34	(100%)
CONCOMITANT ORAL FACTORS	3	(37.5%)	8	(30.7%)	11	(32.3%)
OLP CLINICAL PATTERN						
reticular	5	(62.5%)	19	(79.1%)	24	(70.5%)
papular	0		3	(11.5%)	3	(8.8%)
plaque-like	3	(37.5%)	3	(11.5 %)	6	(17.6%)
atrophic	3	(37.5%)	5	(19.2%)	8	(23.5%)
erosive	1	(12.5%)	10	(38.4%)	11	(32.3%)
bullous	1	(12.5%)	1	(3.8%)	2	(5.8%)
mixed	5	(62.5%)	10	(38.4%)	15	(44.1%)
OLP SITE INVOLVEMENT						
tongue	6	(75%)	13	(50%)	19	(55.8%)
buccal mucosae	4	(50%)	19	(73%)	23	(76.4%)
gingiva	3	(37.5%)	3	(11.5%)	6	(17.6%)
retromolar fossae	1	(12.5%)	2	(7.6%)	3	(8.8%)
lips	1	(12.5%)	4	(15.4%)	5	(14.7%)
palate	1	(12.5%)	1	(3.8%)	2	(5.8%)
floor of mouth	0		3	(11.5%)	3	(8.8%)
EXTRA-ORAL INVOLVEMENT	0		5	(19.2%)	5	(14.7%)
SYMPTOMS REFERRAL	4	(50%)	18	(69.2%)	22	(64.7%)
COMPLETE RESOLUTION OF ORAL LESIONS	2	(25%)	11	(42.3%)	13	(38.2%)

Table 5

topic	childhood	adulthood
OLP frequency	0.03% [17]	1-2% [2]
familial OLP	1-4% [3]	1.5% [21]
most common clinical pattern	reticular erosive	reticular (83.5%) [2] erosive (15-39%) [2,6]
most common oral sites involved	buccal mucosae and tongue [25]	buccal mucosae (88%) tongue and gingiva (18.7%). [2]
involvement of both skin and oral mucosae	12.6% [27]	20-34%[2]
histology	no dysplasia, basal cell degeneration and band-like subepithelial lymphocytic infiltration [25]	no dysplasia, basal cell degeneration and band-like subepithelial lymphocytic infiltration [13]
treatment	symptomatic[6]	symptomatic [28]
resolution	more frequent than in adults[17]	2-5% [29,30]
malignant transformation of OLP	never reported[10]	0.4-5.3% [2]

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.

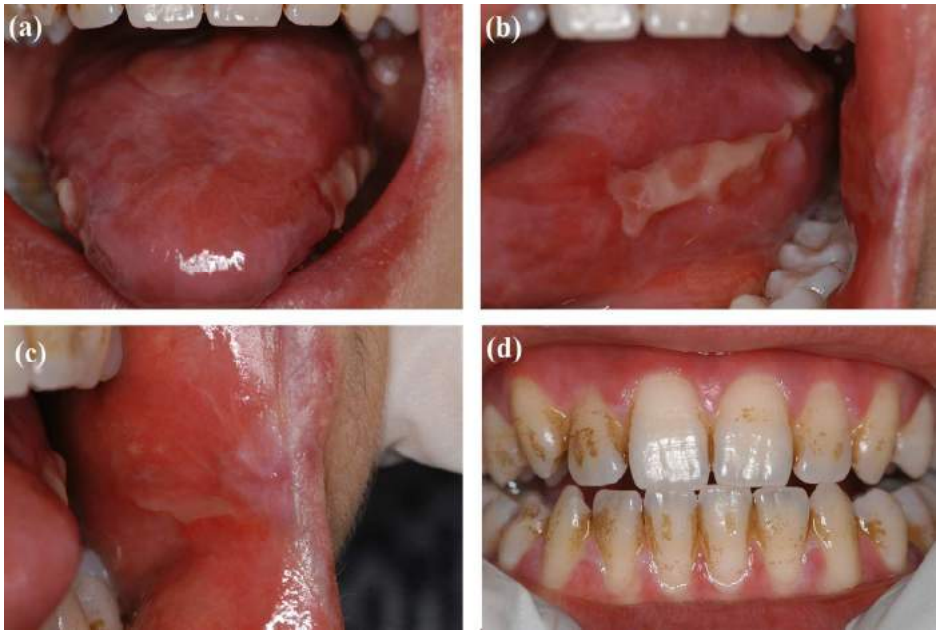
‡:hemotoxylin eosin §:hemoglobin ‡‡:not available §§:direct immunofluorescence †:non-steroidal anti-inflammatory drugs ¶¶:calcineurin inhibitors †††: antinuclear antibodies ¶¶¶:Immunoglobulin A‡‡‡:basal membrane zone §§§:lichen planus pemphigoides

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.
Fig. 1
129x86mm (300 x 300 DPI)

JOURNAL OF ADOLESCENT AND YOUNG ADULT ONCOLOGY
Volume 00, Number 00, 2017

DOI: 10.1089/jayao.2016.0050

Case Study

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, DMD[1]

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 time the clinical status of the patient 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 Because of their highly aggressive behavior, we suggest that early therapy of T/NK-cell lymphomas may be started before completing the specific diagnostic investigations.

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 in 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.[1,2]

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.[1]

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.[2]

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.[3] 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%).[3–6]

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.[4,5,7]

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, blastic or monomorphic NK cell lymphoma/leukemia, CD56-positive peripheral T-cell lymphoma, and enteropathy-associated T-cell lymphoma.[4]

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.[4]

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.

[1]Department of Neuroscience, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Naples, Italy.
[2]The University of Melbourne, Melbourne Dental School and Oral Health CRC, Melbourne, Australia.
[3]Pathology Section, Department of Advanced Biomedical Sciences, University Federico II of Naples, Naples, Italy.

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

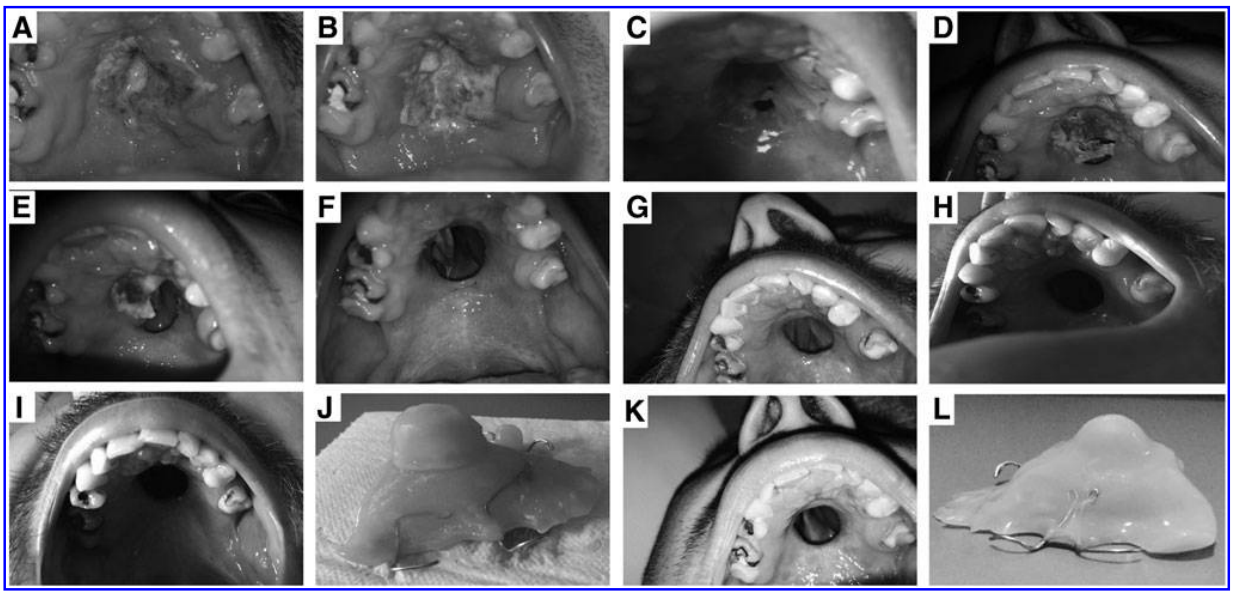


FIG. 1. Intraoral clinical aspect at initial presentation in Oral Medicine. The hard palate and the alveolar process presented with an ulcerative and destructive lesion with an extensive zone of necrosis (**A** = 10 weeks, **B** = 12 weeks, **C** = 14 weeks). Progressive disintegration of the bony support with development of a wide oronasal fistula during chemotherapy (**D** = 4 months, **E** = 5 months, **F** = 6 months). Clinical aspect of the fistula at 10 months during the radiotherapy (**G**), at 1 year (**H**), at 18 months (**I**) and at 2 years (**K**). Examples of palatal obturator prostheses used at 1 year (**J**), and 2 years (**L**).

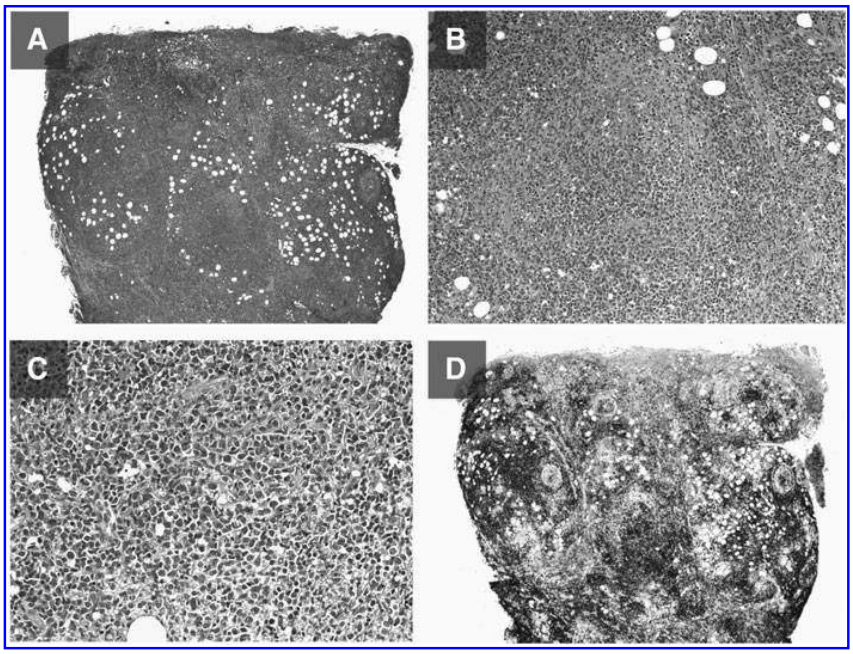


FIG. 2. Extranodal NK/T-cell lymphoma, nasal type. **(A)** Low magnification showing a dense and diffuse infiltrate of pleomorphic lymphocytes within the entire chorion with an angiocentric pattern of infiltration (hematoxylin and eosin, ×25); **(B)** prominent tumor necrosis (hematoxylin and eosin, ×100); **(C)** medium-sized pleomorphic lymphocytes predominate (hematoxylin and eosin, ×200); and **(D)** most cells strongly express CD2 (CD2, ×25). NK, natural killer.

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 Hematoncology 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/m^2 IV, Dexamethasone 40 mg IV + leucovorin 15 mg ×4 doses/day IV + ifosfamide 1500 mg/m^2 IV + etoposide 100 mg/m^2 IV, and L-asparaginase 6000 U/m^2 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 in our department occurred. 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.[8]

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.[9–11] 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 lymphoma and metastatic cancer. Furthermore, fine needle aspiration cytology (FNAC) is a simple and safe procedure and is proved to be accurate in the diagnosis of reactive hyperplasia, infections, granulomatous lymphadenopathies, lymphomas, and metastatic malignancies. The accuracy of image-guided lymph nodes biopsy by FNAC in diagnosing lymphoma and metastatic cancer has been reported to be in the range of 76%–100% and 82%–96%, respectively.[13] Further examinations such as maxillofacial CT scan could have been useful to have initial staging of the disease since the first visits.

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

No competing financial interests exist.

References

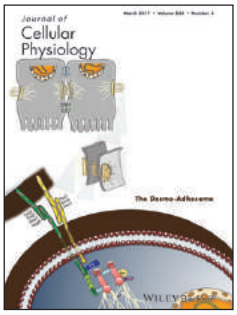
1. Kato N, Yasukawa K, Onozuka T, Kikuta H. Nasal and nasal-type T/NK-cell lymphoma with cutaneous involvement. J Am Acad Dermatol. 1999;40:850–6.
2. Cotta CV, Hsi ED. Pathobiology of mature T-cell lymphomas. Clin Lymphoma Myeloma. 2008;8:168–179.
3. Rodriguez J, Romaguera JE, Manning J, et al. Nasal-type T/NK lymphomas: a clinicopathologic study of 13 cases. Leuk Lymphoma. 2000;39:139–44.
4. Jaffe ES, Chan JK, Su IJ, et al. Report of the workshop on nasal and related extranodal angiocentric T/natural killer cell lymphomas. Definitions, differential diagnosis, and epidemiology. Am J Surg Pathol. 1996;20:103–11.
5. Yoon TY, Lee HT, Chang SH. Nasal-type T/natural killer cell angiocentric lymphoma, Epstein-Barr virus-associated, and showing clonal T-cell receptor gamma gene rearrangement. Br J Dermatol. 1999;140:505–8.
6. Takata K, Hong ME, Sitthinamsuwan P, et al. Primary cutaneous NK/T-cell lymphoma, nasal type and CD56-positive peripheral T-cell lymphoma: a cellular lineage and clinicopathologic study of 60 patients from Asia. Am J Surg Pathol. 2015;39:1–12.
7. Ohshima K, Suzumiya J, Shimazaki K, et al. NasalT/NK cell lymphomas commonly express perforin and Fas ligand: important mediators of tissue damage. Histopathology. 1997;31:444–50.
8. Ohshima K. Pathological diagnosis of mature T/NK cell lymphoma. Nihon Rinsho. 2014;72:445–9.
9. Boros A, Michot JM, Hoang-Xuan K, Mazeron R. Role of radiotherapy in the treatment of NK/T-cell nasal type and primary cerebral lymphomas. Cancer Radiother. 2016;20:535–42.
10. Rong QL, Li YX, Zhu Y, et al. Treatment Outcome for Patients With Stage II Extranodal Nasal-Type NK/T-Cell Lymphoma: a Multicenter Study. Int J Radiat Oncol Biol Phys. 2016;96(2S):E489.
11. Tse E, Kwong YL. Diagnosis and management of extranodal NK/T cell lymphoma nasal type. Expert Rev Hematol. 2016;9:861–71.
12. Hsi ED, Said J, Macon WR, et al. Diagnostic accuracy of a defined immunophenotypic and molecular genetic approach for peripheral T/NK-cell lymphomas. A North American PTCL study group project. Am J SurgPathol. 2014;38:768–75.
13. Mohseni S, Shojaiefard A, Khorgami Z, et al. Peripheral lymphadenopathy: approach and diagnostic tools. Iran J Med Sci. 2014;39:158–70.

Address correspondence to:
Antonio Celentano, DDS
Department of Neuroscience
Reproductive and Odontostomatological Sciences
University Federico II of Naples
Via Pansini 5
Naples 80131
Italy

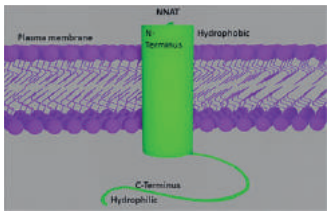
E-mail: antony.celentano@gmail.com,
antonio.celentano@unina.it,
antonio.celentano@unimelb.edu.au

Journal of
Cellular Physiology

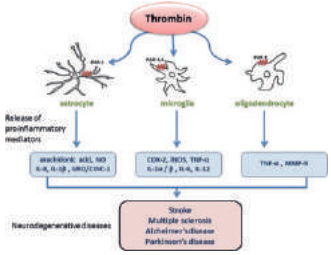
VOL. 232 MARCH 2017 NO. 3



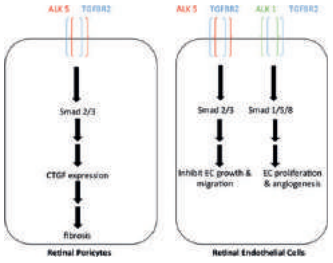
Contents



page 478



page 483

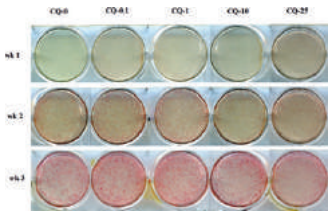


page 487

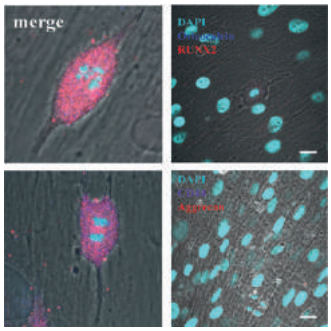
page 494

Cover: The cover image, by Celentano et al., is based on the Review *Article Pathophysiology of the Desmo-Adhesome*, DOI: 10.1002/jcp.25515

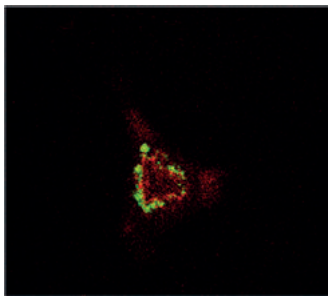
Contents *Continued...*



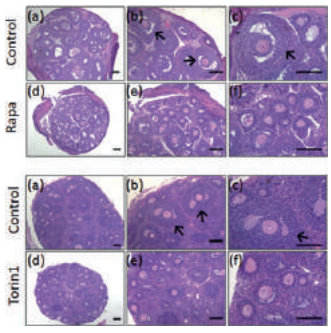
page 544



page 552



page 561



page 591

ORIGINAL RESEARCH ARTICLES

Contents *Continued...*

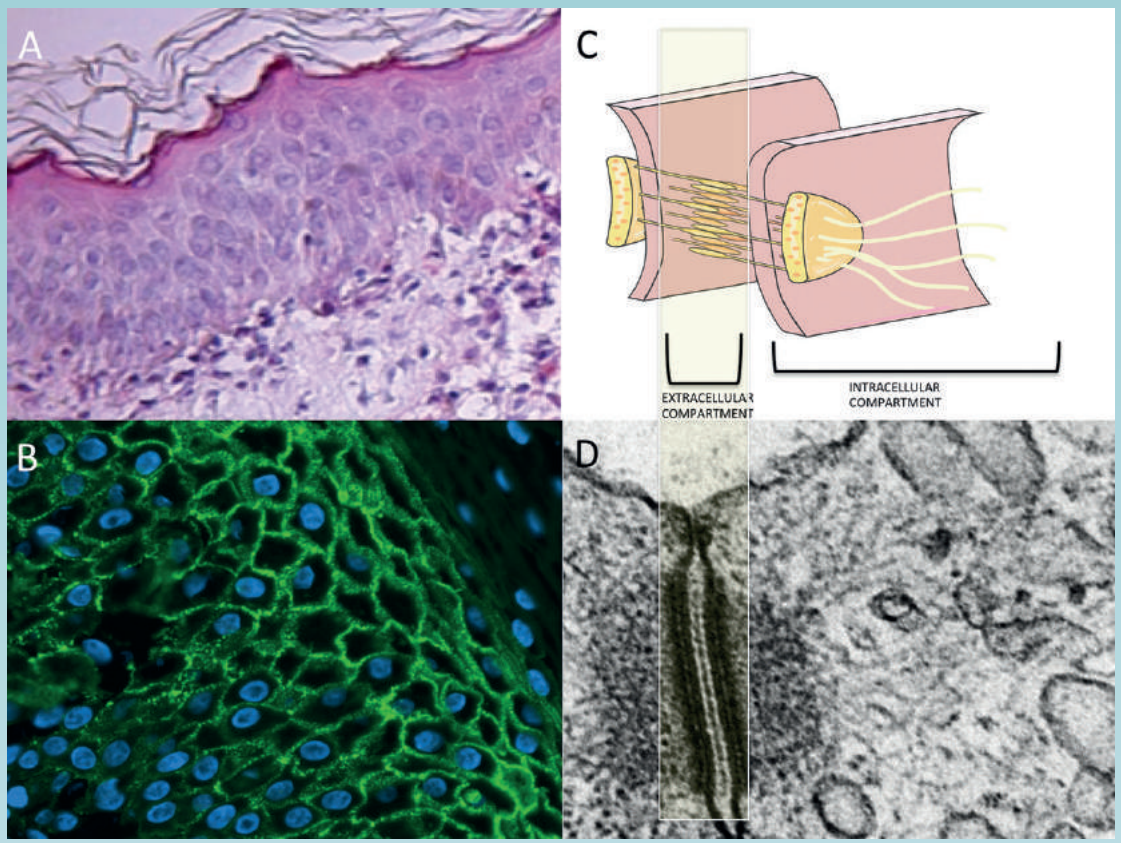
EDITOR'S CHOICE

VOL. 232 MARCH 2017 NO. 3

ANTONIO CELENTANO, MICHELE DAVIDE MIGNOGNA, MICHAEL MCCULLOUGH, AND NICOLA CIRILLO

496 **Pathophysiology of the Desmo-Adhesome**

Accepted manuscript online in Wiley Online Library, 9 August 2016



page 498

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.

HIGHLIGHTS

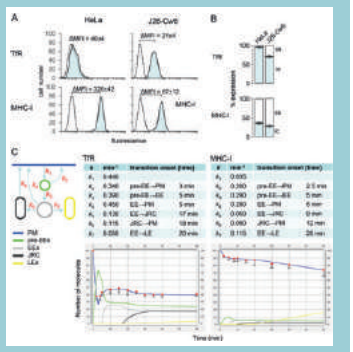
VOL. 232 MARCH 2017 NO. 3

GORDANA BLAGOJEVIĆ ZAGORAC, HANA MAHMUTEFENDIĆ, SENKA MAĆEŠIĆ, LJERKA KARLEUŠA, AND PERO LUČIN

463 **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.



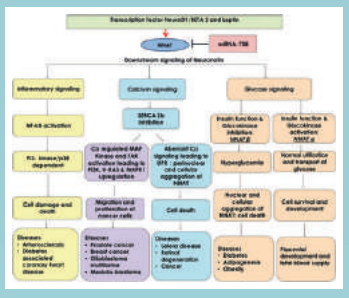
page 467

PRIYAMVADA M. PITALE, WAYNE HOWSE, AND MARINA GORBATYUK

477 **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.

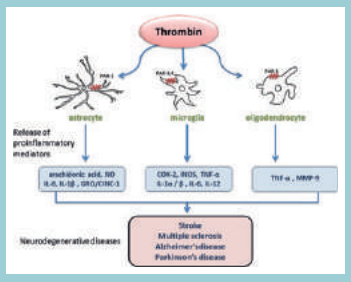


page 479

Journal of
Cellular
Physiology

SAFIEH EBRAHIMI, NAJME JABERI, AMIR AVAN, MIKHAIL RYZHIKOV, MOHAMMAD REZA KERAMATI, MOHAMMAD REZA PARIZADEH, AND SEYED MAHDI HASSANIAN

Accepted manuscript online in Wiley Online Library, 26 July 2016

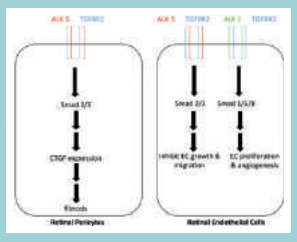


Proinflammatory signaling function of thrombin increases secretion of proinflammatory cytokines and chemokines, triggers vascular permeability, promotes leukocyte migration, and induces adhesion molecule expression.
This review summarizes the role of thrombin in the pathogenesis of central nervous system (CNS) inflammatory diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), promoting greater understanding and clinical management of these diseases.

page 483

SARAH E. WHEELER AND NAM Y. LEE

Accepted manuscript online in Wiley Online Library, 29 July 2016

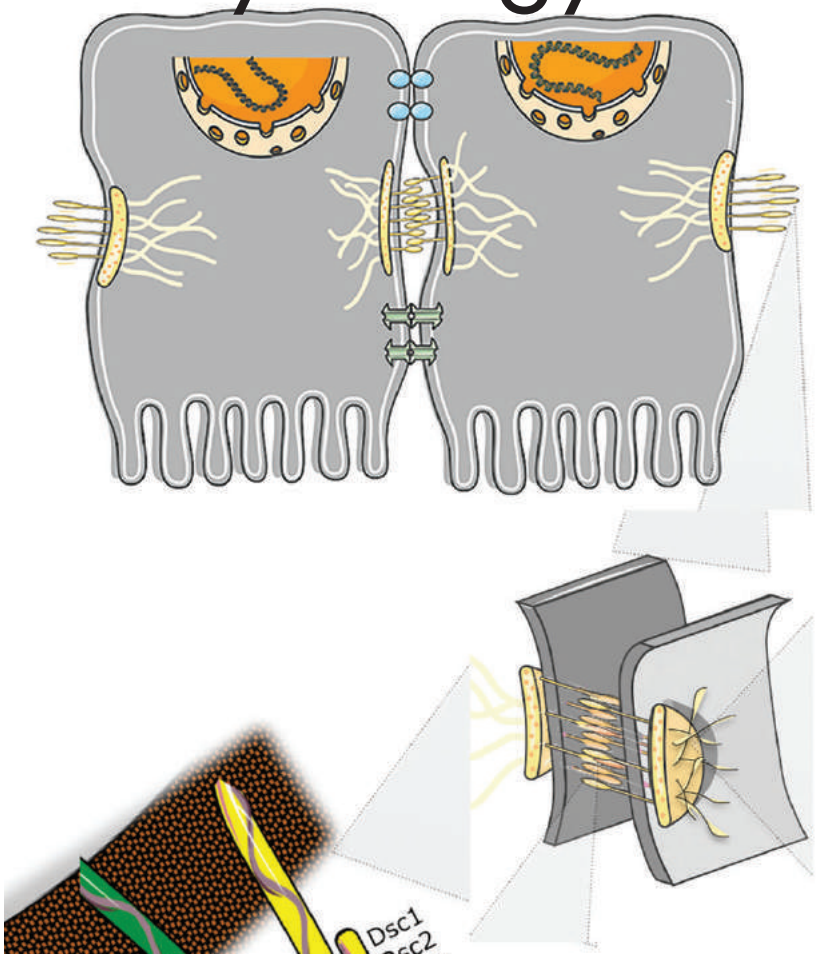


Transforming growth factor beta (TGF-beta) has a clear role in the pathogenesis of diabetic retinopathy, although the underlying mechanisms are poorly established. Here, our current but limited understanding of how signaling by TGF-beta superfamily ligands contributes to the pathogenesis and progression of diabetic retinopathy are discussed.

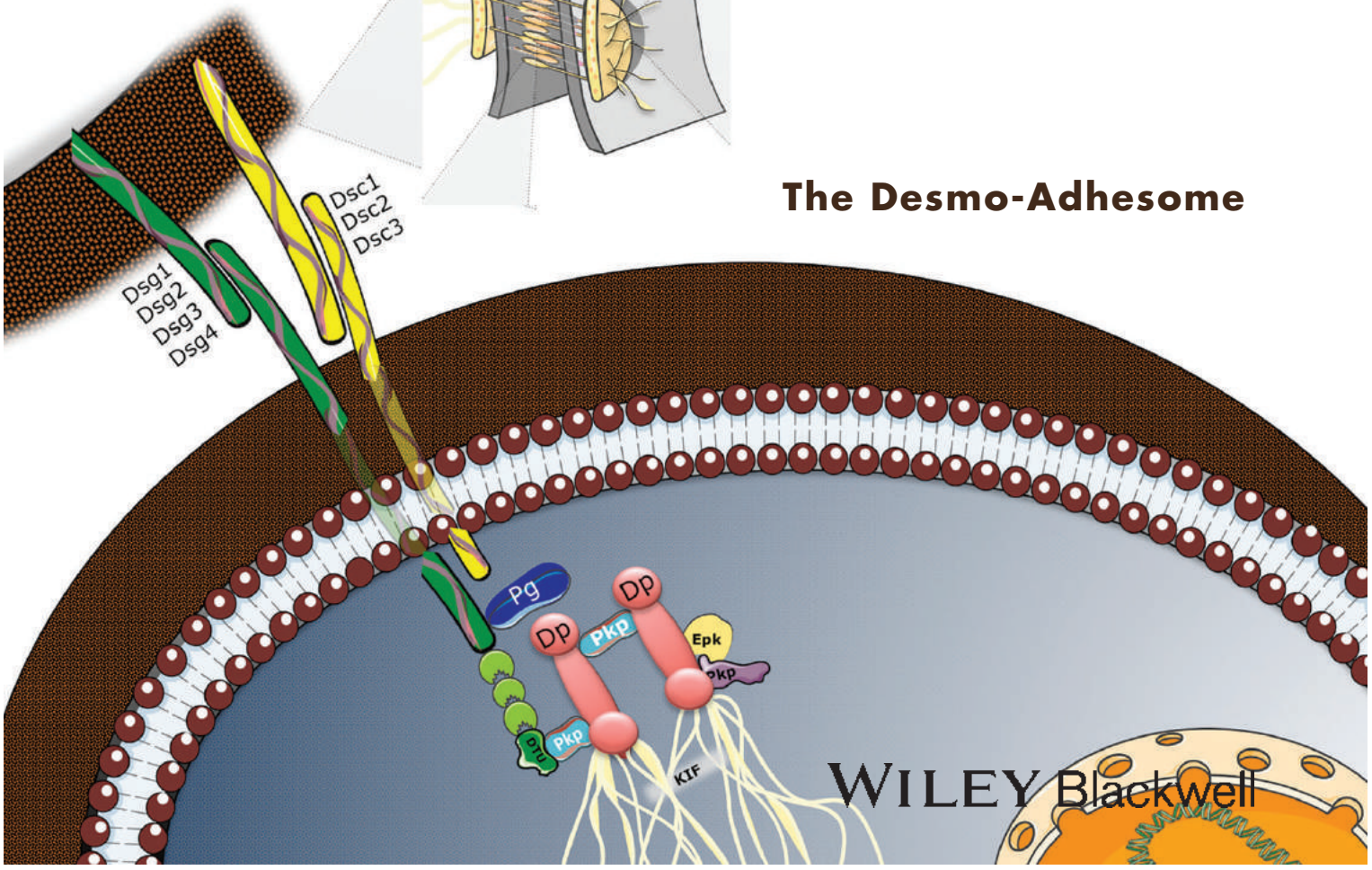
page 487

March 2017 • Volume 232 • Number 3

Journal of Cellular Physiology



The Desmo-Adhesome



WILEY Blackwell

JOS Journal of Oral Science NUSD Nihon University School of Dentistry

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

Journal:	*Journal of Oral Science*
Manuscript ID	JOS-16-0334.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	26-Oct-2016
Complete List of Authors:	Marshall, Alison; University College London Eastman Dental Institute Celentano, Antonio; University Federico II of Naples, Department of Neuroscience, Reproductive and Odontostomatological sciences; University of Melbourne Faculty of the VCA and MCM, Melbourne Dental School & Oral Health CRC Cirillo, Nicola; University of Melbourne Faculty of the VCA and MCM, Melbourne Dental School & Oral Health CRC Mirams, Michiko; University of Melbourne Faculty of the VCA and MCM, Melbourne Dental School & Oral Health CRC McCullough, Michael; University of Melbourne Faculty of the VCA and MCM, Melbourne Dental School & Oral Health CRC Porter, Stephen; University College London Eastman Dental Institute
Keywords:	CD40, CD86, oral lichen planus, Regulatory T Cells, oral inflammation, mucosa

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

Authors: Alison Marshall[1], Antonio Celentano[23]*, Nicola Cirillo[3], Michiko Mirams[3], Michael McCullough[3] and Stephen Porter[1]

1: University College London, UCL Eastman Dental Institute, 256 Gray's Inn Rd, London, WC1X 8LD, United Kingdom.

2: University Federico II of Naples, Department of Neuroscience, Reproductive and Odontostomatological Sciences, Via Pansini n.5, Naples, 80131, Italy.

3: The University of Melbourne, Melbourne Dental School and Oral Health CRC, 720 Swanston street, Melbourne, 3053, Victoria, Australia.

***CORRESPONDENCE**: Dr. Antonio Celentano, Department of Neuroscience, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Via Pansini 5, Naples, 80131, Italy; Melbourne Dental School, Oral Health CRC, The University of Melbourne, 720 Swanston street, 3053, Melbourne, Victoria, Australia. antony.celentano@gmail.com, antonio.celentano@unina.it, acelentano@unimelb.edu.au

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 $1mm^3$ pieces and culturing at 37^oC /5% CO_2 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 TTGGCCAAATCCA -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 dH_2O 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 guanidine-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

Cabinet (AlphaInnotech Corp., Cannock, UK) and digital images acquired and stored using AlphaImager Software (AlphaInnotech Corp., Cannock, UK). The primers utilised for the study of housekeeping expression encoded a region of 18S ribosomal RNA (5'- tttcggaactgaggccatga-3', 5'- gcatgccagagtctcgttcg -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

Archivial 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% H_2O_2 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 immunogenicity 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 tolergenic 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

1) Hassan GS, Stagg J, Mourad W (2015) Role of CD154 in cancer pathogenesis and immunotherapy. Cancer Treat Rev 41, 431-440.

2) Iwata M, Soya K, Sawa M, Sakimoto T, Hwang DG (2002) CD40 expression in normal human cornea and regulation of CD40 in cultured human corneal epithelial and stromal cells. Invest Ophthalmol Vis Sci 43, 348-57.

3) Gallagher NJ, Eliopoulos AG, Agathangelo A, Oates J, Crocker J, Young LS (2002) CD40 activation in epithelial ovarian carcinoma cells modulates growth, apoptosis, and cytokine secretion. Mol Pathol 55, 110-20.

4) Dimitriou ID, Kapsogeorgou EK, Moutsopoulos HM, Manoussakis MN (2002) CD40 on salivary gland epithelial cells: high constitutive expression by cultured cells from Sjögren's syndrome patients indicating their intrinsic activation. Clin Exp Immunol 127, 386-92.

5) Tanaka H, Maeda K, Nakamura Y, Azuma M, Yanagawa H, Sone S (2001) CD40 and IFN-gamma dependent T cell activation by human bronchial epithelial cells. J Med Invest 48, 109-17.

6) Portillo JA, Greene JA, Schwartz I, Subauste MC, Subauste CS (2015) Blockade of CD40-TRAF2,3 or CD40-TRAF6 is sufficient to inhibit pro-inflammatory responses in non-haematopoietic cells. Immunology 144, 21-33.

7) Caproni M, Torchia D, Antiga E, Giomi B, Mercuri M, Volpi W, Fabbri P (2007) The CD40/CD40 ligand system in the skin of patients with subacute cutaneous lupus erythematosus. J Rheumatol 34, 2412-6.

8) Farmer I, Freysdottir J, Dalghous AM, Fortune F (2001) Expression of adhesion and activation molecules in human buccal epithelial cell lines and normal human buccal epithelium in situ. J Oral Pathol Med 30, 113-20.

9) Neppelberg E, Loro LL, Oijordsbakken G, Johannessen AC (2007) Altered CD40 and E-cadherin expression--putative role in oral lichen planus. J Oral Pathol Med 36, 153-60.

10) Loro LL, Ohlsson M, Vintermyr OK, Liavaag PG, Jonsson R, Johannessen AC (2001) Maintained CD40 and loss of polarised CD40 ligand expression in oral squamous cell carcinoma. Anticancer Res 21, 113-7.

11) Villarroel Dorrego M, Whawell SA, Speight PM, Barrett AW (2006) Transfection of CD40 in a human oral squamous cell carcinoma keratinocyte line upregulates immune potency and costimulatory molecules. Br J Dermatol 154, 231-8.

12) Toubi E, Shoenfeld Y (2004) The role of CD40-CD154 interactions in autoimmunity and the benefit of disrupting this pathway. Autoimmunity 37, 457-64.

13) Di Sabatino A, Rovedatti L, Vetrano S, Vidali F, Biancheri P, Rescigno M, Danese S, Macdonald TT, Corazza GR (2011) Involvement of CD40-CD40 ligand in uncomplicated and refractory celiac disease. Am J Gastroenterol 106, 519-27.

14) McAdam AJ, Schweitzer AN, Sharpe AH (1998) The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. Immunol Rev 165, 231-47.

15) Vella AT, Mitchell T, Groth B, Linsley PS, Green JM, Thompson CB, Kappler JW, Marrack P (1997) CD28 engagement and proinflammatory cytokines contribute to T cell expansion and long-term survival in vivo. J Immunol 158, 4714-20.

16) Lanzavecchia A, Sallusto F (2001) Antigen decoding by T lymphocytes: from synapses to fate determination. Nat Immunol 2, 487-92.

17) Augustin M, Dietrich A, Niedner R, Kapp A, Schöpf E, Ledbetter JA, Brady W, Linsley PS, Simon JC (1993) Phorbol-12-myristate-13-acetate-treated human keratinocytes express B7-like molecules that serve a costimulatory role in T-cell activation. J Invest Dermatol 100, 275-81.

18) Fleming TE, Mirando WS, Trefzer U, Tubesing KA, Elmets CA (1993) In situ expression of a B7-like adhesion molecule on keratinocytes from human epidermis. J Invest Dermatol 101, 754-8.

19) Nasir A, Ferbel B, Gaspari AA (1995) Human keratinocytes regulate their expression of B7/BB-1 antigen by a unique, calcium-dependent mechanism. J Invest Dermatol 104, 763-7.

20)Lang S, Vujanovic NL, Wollenberg B, Whiteside TL (1998) Absence of B7.1-CD28/CTLA-4-mediated co-stimulation in human NK cells. Eur J Immunol 28, 780-6.

21) Villarroel-Dorrego M, Speight PM, Barrett AW (2005) Expression of major histocompatibility complex class II and costimulatory molecules in oral carcinomas in vitro. Med Oral Patol Oral Cir Bucal 10, 188-95.

22) Thomas GR, Chen Z, Oechsli MN, Hendler FJ, Van Waes C (1999) Decreased expression of CD80 is a marker for increased tumorigenicity in a new murine model of oral squamous-cell carcinoma. Int J Cancer 82, 377-84.

23) Lang S, Atarashi Y, Nishioka Y, Stanson J, Meidenbauer N, Whiteside TL (2000) B7.1 on human carcinomas: costimulation of T cells and enhanced tumor-induced T-cell death. Cell Immunol 201, 132-43.

24) Vonderheide RH, Butler MO, Liu JF, Battle TE, Hirano N, Gribben JG, Frank DA, Schultze JL, Nadler LM (2001) CD40 activation of carcinoma cells increases expression of adhesion and major histocompatibility molecules but fails to induce either CD80/CD86 expression or T cell alloreactivity. Int J Oncol 19, 791-8.

25) Prime SS, Nixon SV, Crane IJ, Stone A, Matthews JB, Maitland NJ, Remnant L, Powell SK, Game SM, Scully C (1990) The behaviour of human oral squamous cell carcinoma in cell culture. J Pathol 160, 259-69.

26) Altenburg A, Baldus SE, Smola H, Pfister H, Hess S (1999) CD40 ligand-CD40 interaction induces chemokines in cervical carcinoma cells in synergism with IFN-gamma. J Immunol 162, 4140-7.

27) Ford GS, Barnhart B, Shone S, Covey LR (1999) Regulation of CD154 (CD40 ligand) mRNA stability during T cell activation. J Immunol 162, 4037-4044.

28) Weiler M, Kachko L, Chaimovitz C, Van Kooten C, Douvdevani A (2001) CD40 ligation enhances IL-15 production by tubular epithelial cells. J Am Soc Nephrol 12, 80-7.

29) Li W, Buzoni-Gatel D, Debbabi H, Hu MS, Mennechet FJ, Durell BG, Noelle RJ, Kasper LH (2002) CD40/CD154 ligation is required for the development of acute ileitis following oral infection with an intracellular pathogen in mice. Gastroenterology 122, 762-73.

30) Li J, Farthing PM, Thornhill MH (2000) Oral and skin keratinocytes are stimulated to secrete monocyte chemoattractant protein-1 by tumour necrosis factor-alpha and interferon-gamma. J Oral Pathol Med 29, 438-44.

31) Propst SM, Denson R, Rothstein E, Estell K, Schwiebert LM (2000) Proinflammatory and Th2-derived cytokines modulate CD40-mediated expression of inflammatory mediators in airway epithelia: implications for the role of epithelial CD40 in airway inflammation. J Immunol 165, 2214-21.

32) Little MC, Griffiths CE, Watson RE, Pemberton MN, Thornhill MH (2003) Oral mucosal keratinocytes express RANTES and ICAM-1, but not interleukin-8, in oral lichen planus and oral lichenoid reactions induced by amalgam fillings. Clin Exp Dermatol 28, 64-69.

33) Woltman AM, de Haij S, Boonstra JG, Gobin SJ, Daha MR, van Kooten C (2000) Interleukin-17 and CD40-ligand synergistically enhance cytokine and chemokine production by renal epithelial cells. J Am Soc Nephrol 11, 2044-55.

34) Fuller BW, Nishimura T, Noell RJ (2002) The selective triggering of CD40 on keratinocytes in vivo enhances cell-mediated immunity. Eur J Immunol 32, 895-902.

35) Williams IR, Ort RJ, Kupper TS (1994) Keratinocyte expression of B7-1 in transgenic mice amplifies the primary immune response to cutaneous antigens. Proceedings of the National Academy of Science USA 91, 12780-84

36) Gaspari AA, Burns RP Jr, Kondo S, Nasir A, Kurup A, Mlodynia D, Sauder D, Barth RK (1998) Characterization of the altered cutaneous reactivity of transgenic mice whose keratinocytes overexpress B7-1. Clin Immunol Immunopathol 86, 259-70.

37) Secchiero P, Zella D, Curreli S, Mirandola P, Capitani S, Gallo RG, Zauli G (2000) Engagement of CD28 modulates CXC chemokine receptor 4 surface expression in both resting and CD3-stimulated CD4+ T cells. J Immunol 164, 4018-4024.

38) Simon JC, Dietrich A, Mielke V, Wuttig C, Vansheidt W, Linsley PS, Schopf E, Sterry W (1994) Expression of the B7/BB1 activation antigen and its ligand CD28 in T-cell-mediated skin diseases. J Invest Dermatol 103, 439-543.

39) Freeman GJ, Cardoso AA, Boussiotis VA Anumanthan A, Groves RW, Kupper TS, Clark EA, Nadler LM (1998) The BB1 monoclonal antibody recognizes both cell surface CD74 (MHC class II-associated invariant chain) as well as B7-1 (CD80), resolving the question regarding a third CD28/ CTLA counterreceptor. J Immunol 161, 2708-15.

40) Salomon B, Bluestone JA (2001) Complexities of CD28 / B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol 19, 225-252.

41) Thornhill MH (2001) Immune mechanisms in oral lichen planus. Acta Odontol Scand 59, 174-7.

42) Albanesi C, Cavani A, Giampiero G (1998) Interferon-gamma stimulated human keratinocytes express the genes necessary for the production of peptide-loaded MHC class II molecules. J Invest Dermatol 110, 138-142.

43) Walker LSK, Abbas AK (2002) The enemy within: Keeping self-reactive T cells at bay in the periphery. Nature Reviews Immunology 2, 11-19.

44) Nakazawa A, Watanabe M, Kanai T, Yajima T, Yamazaki M, Ogata H, Ishii H, Azuma M, Hibi T (1999) Functional expression of costimulatory molecule CD86 on epithelial cells in the inflamed colonic mucosa. Gastroenterology 117, 536-45.

45) Hersberg RM, Mayer LF (2000) Antigen processing and presentation by intestinal epithelial cells – polarity and complexity. Immunol Today 21, 123-129.

46) Byrne B, Madrigal-Estabas L, McEvoy A, Carton J, Doherty DG, Whelan A, Feighery C, O'Donoghue DP, O'Farrelly C (2002) Human duodenal epithelial cells constitutively express molecular components of antigen presentation but not costimulatory molecules. Hum Immunol 63, 977-986.

47) Medzhitov R, Janeway CA (2002) Decoding the patterns of self and nonself by the innate immune system. Science 296, 298-300.

48) Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kB pathway. Int Immunol 12, 1539-1546.

49) Curry JL, Qin J-Z, Bonish B, Carrick BA, Bacon P, Panella J, Robinson J, Nickoloff BJ (2003) Innate immune-related receptors in normal and psoriatic skin. Arch Pathol Lab Med 127, 178-186.

50) Chaiyarit P, Kafrawy AH, Miles DA, Zunt SL, Van Dis ML, Gregory RL (1999) Oral lichen planus: an immunohistochemical study of heat shock proteins (HSPs) and cytokeratins (CKs) and a unifying hypothesis of pathogenesis. J Oral Pathol Med 28, 210-5.

51): Sinon SH, Rich AM, Parachuru VP, Firth FA, Milne T, Seymour GJ (2016) Downregulation of toll-like receptor-mediated signalling pathways in oral lichen planus. J Oral Pathol Med 45, 28-34.

52) García-García V, Bascones-Martínez A, García-Kass AI, Martinelli-Kläy CP, Küffer R, Álvarez-Fernández E, Lombardi T (2013) Analysis of the expression of heat-shock protein 27 in patients with oral lichen planus. Oral Dis 19, 65-72.

53) Perez VL (1997) Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity 6, 411-417.

54) Karandikar NJ, Eagar TN, Vanderlugt CL, Bluestone JA, Miller SD (2000) CTLA-4 downregulates epitope spreading and mediates remission in relapsing experimental autoimmune encephalomyelitis. J Neuroimmunol 109, 173-80.

55) Alaibac M, Fortina AB, Poletti A, Vandenberghe P, Marino F, Tarantello M, Peserico A (2000) In situ expression of the CTLA-4 receptor in T-cell-mediated inflammatory and neoplastic skin diseases. Arch Dermatol Res 292, 472-474.

56) Kapsogeorgou EK, Moutsopoulos HM, Manoussakis MN (2001) Functional expression of a costimulatory B7.2 (CD86) protein on human salivary gland epithelial cells that interacts with the CD28 receptor, but has reduced binding to CTLA-4. J Immunol 166, 3107-3113.

57) Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2, 389-400.

58) Hasséus B, Jontell M, Brune M, Johansson P, Dahlgren UI (2001) Langerhans cells and T cells in oral graft versus host disease and oral lichen planus. Scand J Immunol 54, 516-24.

59) Belkaid Y, Hoffmann KF, Mendez S, Kamhawi S, Udey MC, Wynn TA, Sacks DL (2001) The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med. 194, 1497-506.

60) Croft M, Bradley LM, Swain SL (1994) Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen presenting cell types including resting B cells. J Immunol 152, 2675–85.

61) Katz-Levy Y, Neville KL, Girvin AM, Vanderlugt CL, Pope JG, Tan LJ, Miller SD (1999) Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus-infected mice. J Clin Invest 104, 599-610.

62) Ohashi PS (2002) T-cell signalling and autoimmunity: molecular mechanisms of disease. Nat Rev Immunol 2, 427-38.

63) Vanderlugt CL, Miller SD (2002) Epitope spreading in immune-mediated diseases: implications for immunotherapy. Nature Reviews Immunology 2, 85-95.

64) Vanderlugt CL, Neville KL, Nikcevich KM, Eagar TN, Bluestone JA, Miller SD (2000) Pathologic role and temporal appearance of newly emerging autoepitopes in relapsing experimental autoimmune encephalomyelitis. J. Immunol 164, 670–678.

65) Howard LM, Miga AJ, Vanderlugt CL, Dal Canto MC, Laman JD, Noelle RJ, Miller SD (1999) Mechanisms of immunotherapeutic intervention by anti-CD40L (CD154) antibody in an animal model of multiple sclerosis. J. Clin. Invest. 103, 281–290.

66) Ito D, Ogasawara K, Iwabuchi K, Inuyama Y, Onoé K (2000) Induction of CTL responses by simultaneous administration of liposomal peptide vaccine with anti-CD40 and anti-CTLA-4 mAb. J Immunol 164, 1230-5.

67) Lefrançois L, Altman JD, Williams K, Olson S (2000) Soluble antigen and CD40 triggering are sufficient to induce primary and memory cytotoxic T cells. J Immunol, 164, 725-32.

68) Masopust D, Vezys V, Marzo AL, Lefrancois L (2001) Preferential localization of effector memory cells in nonlymphoid tissue. Science 291, 2413-7.

69) Heath WR, Carbone FR (2001) Cross-presentation in viral immunity and self-tolerance. Nature Reviews Immunology 1, 126-135.

70) Huang F-P, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, MacPherson GG (2000) A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. J Exp Med 191, 535-443.

71) Parr MB, Kepple L, Parr EL (1991) Langerhans cells phagocytose vaginal epithelial cells undergoing apoptosis during the murine estrous cycle. Biol Reprod 45, 252-260.

72) Vezys V, Lefrançois L (2002) Cutting edge: inflammatory signals drive organ-specific autoimmunity to normally cross-tolerizing endogenous antigen. J Immunol 169, 6677-80.

73) Rafii-Tabar E, Czitrom AA (1986) Ontogeny of priming of cytotoxic T cells to minor alloantigens: the development of direct priming precedes that of cross-priming. Eur J Immunol 16, 1025-27.

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 house keeping. 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).

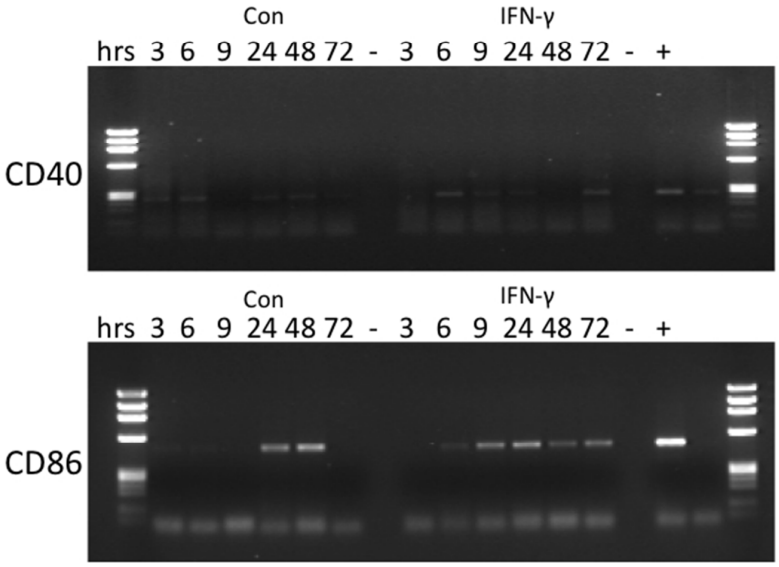


Fig. 1

254x190mm (72 x 72 DPI)

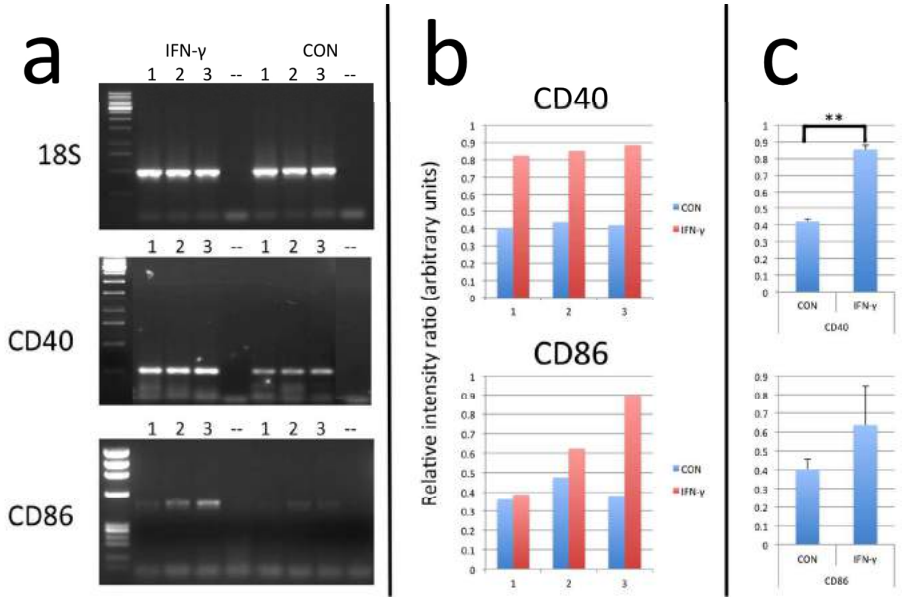


Figure 2

595x378mm (72 x 72 DPI)

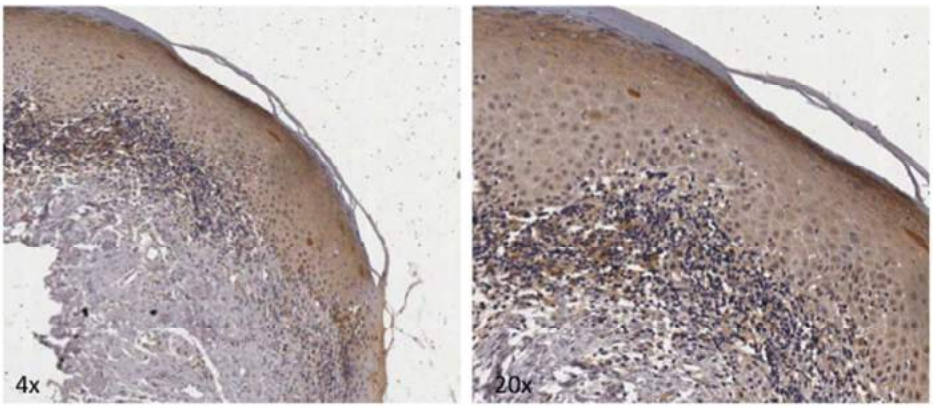


Fig. 3

226x98mm (72 x 72 DPI)

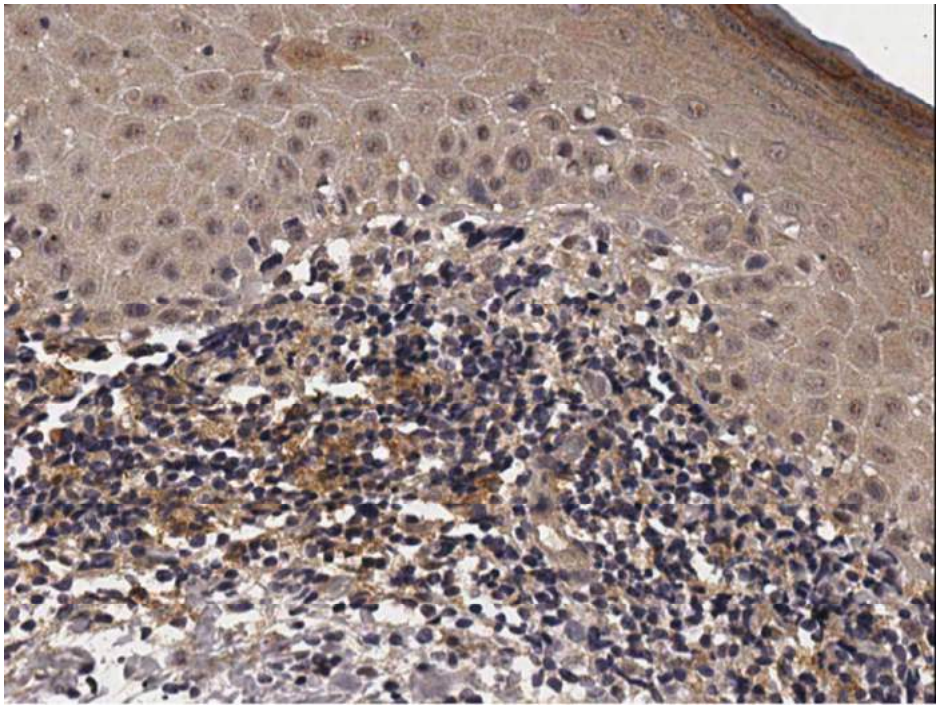


Fig. 4

254x190mm (72 x 72 DPI)

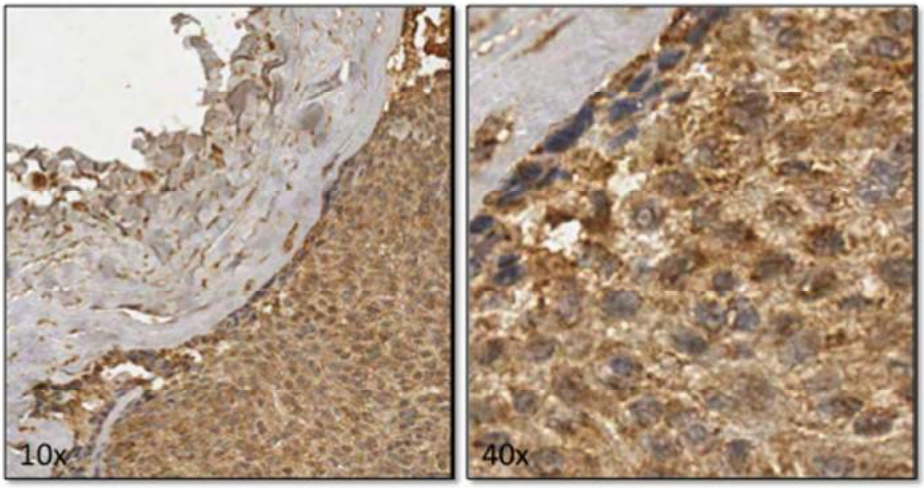
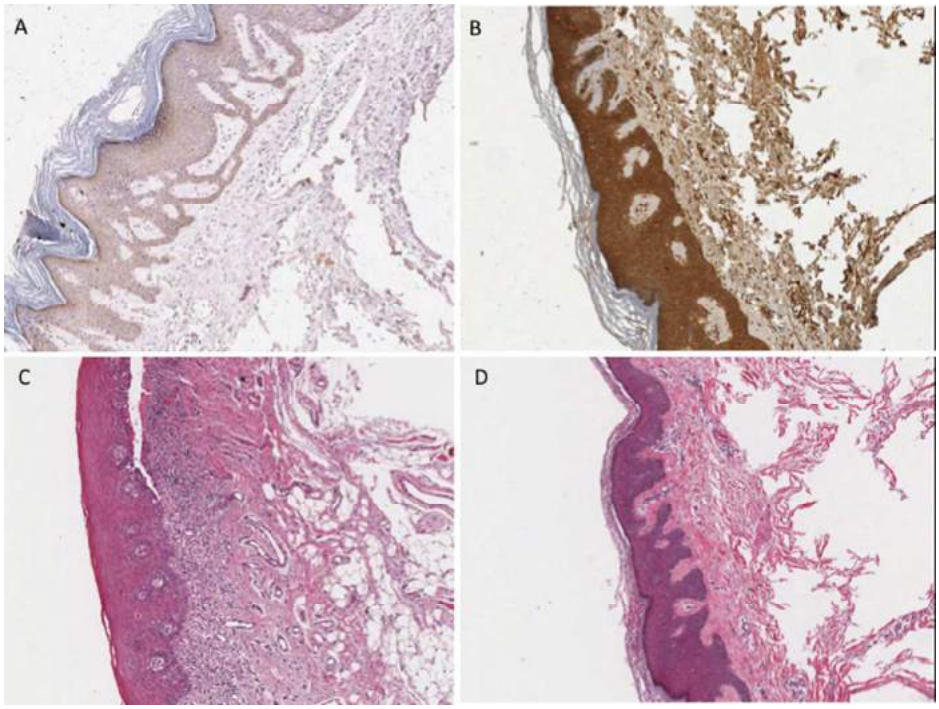


Fig. 5

177x94mm (72 x 72 DPI)



Suppl Fig. 1

254x190mm (72 x 72 DPI)

Carcinogenesis, 2017, Vol. 38, No. 1, 76–85

doi:10.1093/carcin/bgw113
Advance Access publication November 21, 2016
Original Manuscript

ORIGINAL MANUSCRIPT

Cancer-associated fibroblasts regulate keratinocyte cell–cell adhesion via TGF-β-dependent pathways in genotype-specific oral cancer

N. Cirillo[1,*], Y. Hassona[2], A. Celentano[1,3], K.P. Lim[4], S. Manchella[1], E.K. Parkinson[5] and S.S. Prime[5]

[1]Melbourne Dental School and Oral Health CRC, University of Melbourne, 3053 Carlton, Victoria, Australia, [2]Department of Dentistry, University of Jordan, Amman, Jordan, [3]Department of Neurosciences, Reproductive and Odontostomatological Sciences, Federico II University of Naples, Italy, [4]Cancer Research Initiatives Foundation, Sime Darby, Subang Jaya Medical Centre, 47500 Subang Jaya, Selangor, Malaysia, [5]Centre for Clinical and Diagnostic Oral Sciences, Institute of Dentistry, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK

*To whom correspondence should be addressed. Tel: +61 3 9341 1473; Email: nicola.cirillo@unimelb.edu.au

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/p16^{INK4A}* dysfunction, telomerase activation, frequent

Received: March 25, 2016; **Revised:** September 11, 2016; **Accepted:** October 14, 2016

Abbreviations

BSA	bovine serum albumin
CAFs	cancer-associated fibroblasts
CM	conditioned media
DTG	differentially transcribed genes
EMT	epithelial–mesenchymal transition
FC	fold change
GU-OSCC	genetically unstable oral squamous cell carcinomas
GS-OSCC	genetically stable oral squamous cell carcinomas
H2O2	hydrogen peroxide
huSM	highly up-regulated secretory molecule
muSM	mildly up-regulated secretory molecules
OSCC	oral squamous cell carcinoma
PPI	protein–protein interaction
TGF-β	transforming growth factor-β

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/p16*^{INK4A} deletions are detectable *in vivo* 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 pivotal 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 seceretory 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-β-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 (H 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 OSCCs were human papillomavirus negative. Keratinocytes were derived from OSCC (H357, H103), SCC (HaCaT-II3), 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-30F, BICR-59F, BICR-66F, BICR-69F, BICR-70F, BICR-71F, BICR-73F, BICR-80F) and GU-OSCC (H314F, H357F, H413F, BICR-3F, BICR-18F, BICR-31F, BICR-63F, BICR-68F, BICR-78F, BICR-82F). The mesenchymal origin of the fibroblast strains was confirmed by positive and negative labelling with vimentin and pan-cytokeratin/keratin 14 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 vivo*, 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 cultured in Dulbecco's modified Eagle's medium-F12 supplemented with 10% (v/v) fetal bovine serum and 0.5 μg/ml hydrocortisone; cells were grown under the same standard conditions (5% carbon dioxide, 37°C).

In certain experiments, 1×10^4 normal fibroblasts (NHOF1) were seeded into 60 mm culture dishes, grown until 60% confluent and then treated with 600 μM hydrogen peroxide (H_2O_2; BDH) for 2 h each day for 5 days. The cells were recovered in fresh culture media for 5 days before use. 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 of conditioned media

Cells were grown in 75 cm 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 normalized for 0.5×10^6 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 1μg/ml, respectively, and incubated for 30 min at 37°C. CM was used immediately.

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 (8) 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 2 < 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 × 10^4 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). Negative controls included omission of primary antibody. The readings of protein expression in each well were normalized according to the readings of the negative control.

Enzyme-linked immunosorbent assay

CM from fibroblasts were collected as described previously. The production of TGF-β1 and TGF-β2 was assessed with a TGF-β Parameter Assay kits (R&D Systems, Minneapolis, MN) and quantified at 415 nm with the EL×808 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 (Nunc™ 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 Hoetstch staining (H6024, 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/CDH1 antibody (FITC conjugate, A15757, Thermo Fisher Scientific) at a 1/100 dilution in 2% BSA-PBS, and with anti-vimentin antibody (Ab154207, Abcam's RabMAb®) at a 1/1000 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 × 10^4 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 (2×). Then, the keratinocytes 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 10μl 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

Table 1. Primary antibodies used for Western blotting, concentrations and suppliers

Primary antibody	Origin	Concentration	Supplier	Cat. no
Alpha-tubulin	Mouse monoclonal	1:10000	Sigma	T5168
Beta-catenin	Rabbit polyclonal	1:250	Santa Cruz	Sc-7199
Desmoplakin (I/II)	Rabbit polyclonal	1:200	Santa Cruz	Sc-33555
DSC3	Rabbit polyclonal	1:200	Santa Cruz	Sc-48750
DSG1	Rabbit polyclonal	1:250	Santa Cruz	Sc-20114
DSG3	Rabbit polyclonal	1:200	Santa Cruz	Sc-20116
E-Cadherin	Rabbit polyclonal	1:200	Santa Cruz	Sc-7870
Vimentin	Mouse monoclonal	1:200	Santa Cruz	Sc-73259

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; H_2O_2-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

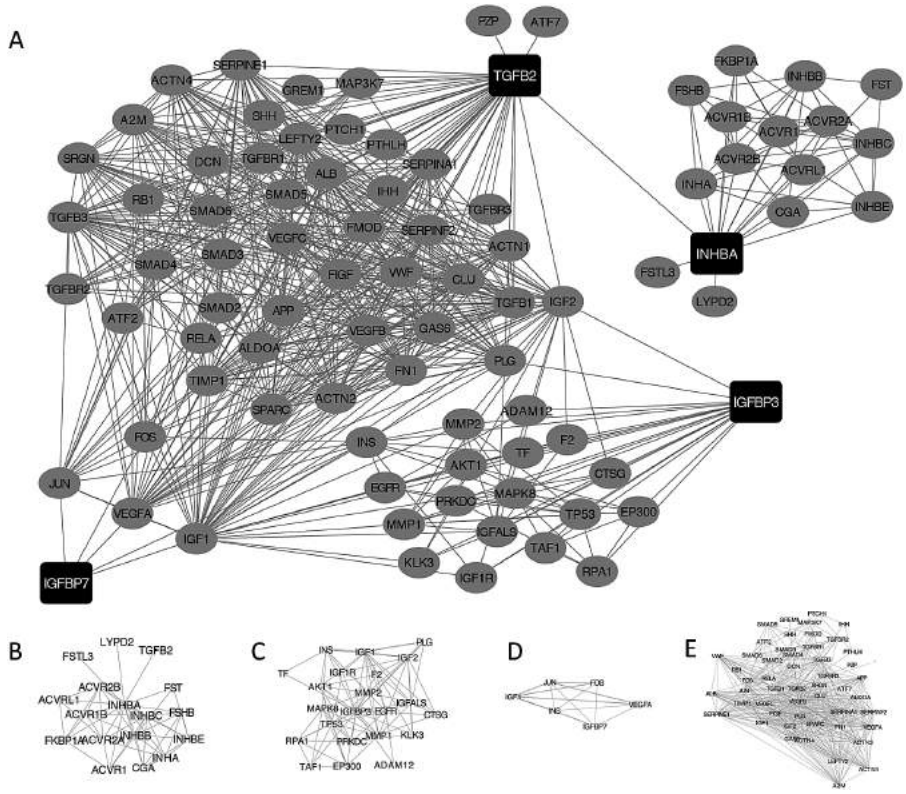


Figure 1. Interactome formed by four huSM and their first neighbours. (**A**) First-degree connections and binding interactions, i.e. edges (non-directional) are represented in light grey, whereas the four main nodes (huSM) are depicted in dark grey. The four individual subnetworks are shown in the small panel, namely INHBA (**B**), IGFBP3 (**C**), IGFBP7 (**D**) and TGFB2 (**E**).

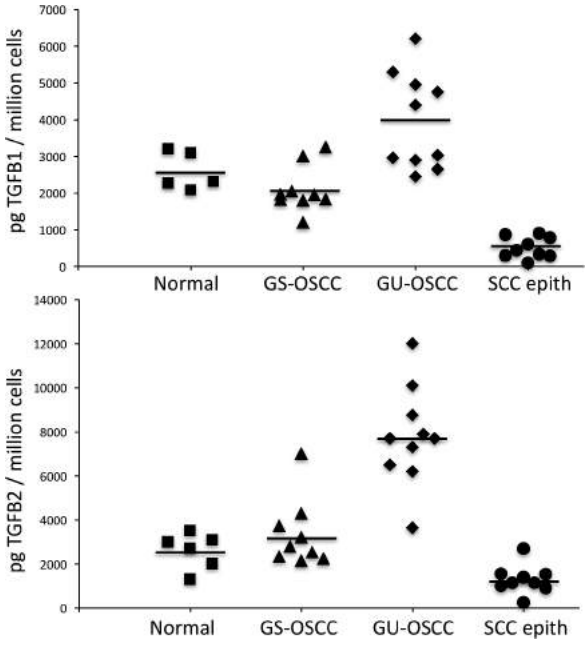


Figure 2. TGF-β1 (A) and TGF-β2 (B) protein expression in CM from fibroblasts derived from GS-OSCC and GU-OSCC, together with fibroblasts from normal oral mucosa and malignant keratinocyte cell lines. Cells were grown to 70% confluence, rinsed in serum-free medium and incubated in serum-free medium for 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.

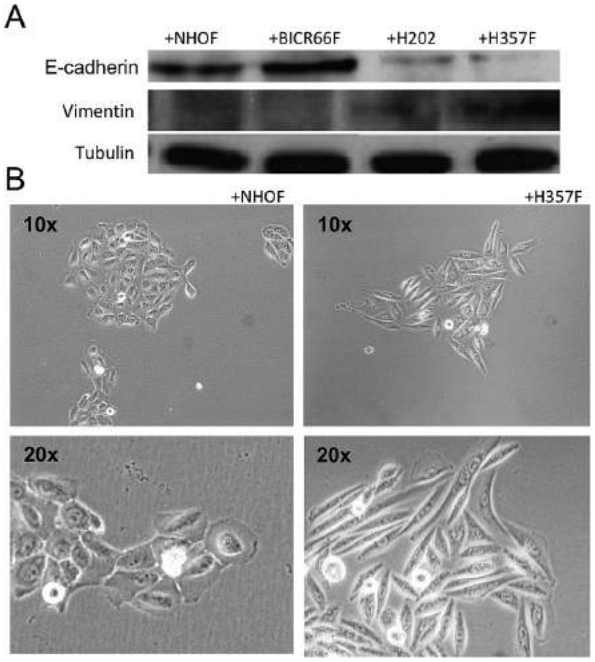


Figure 3. Senescent CAFs induce EMT. **(A)** Western blot analysis showing CM from GU-OSCC CAFs (H357F), but not GS-OSCC CAFs (BICR66F), caused depletion of E-cadherin and up-regulation of vimentin in OSCC keratinocytes (H357). H_2O_2-treated NHOF1 and untreated NHOF1 were used as positive and negative controls. (**B**) Treatment of H357 keratinocytes with CM from GU-OSCC CAFs (H357F), but not NHOF1, caused cells to adopt a spindle cell morphology indicative of EMT.

(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). H_2O_2-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 DGS3), 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-II3) 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-tumourigenic 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; BICR73F), 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-β mediates the effect of CAFs on keratinocyte adhesion and invasion

Since our *in silico* data indicated that TGF-β2 was an important secretory molecule of CAFs and both TGF-β1 and TGF-β2 were overexpressed by CAFs, we wanted to examine whether TGF-β

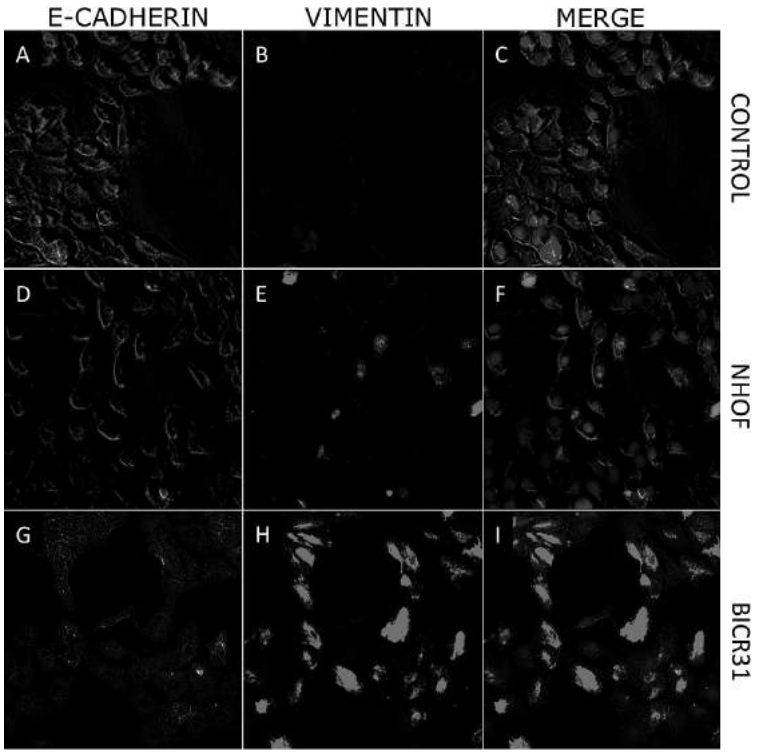


Figure 4. Expression of E-cadherin and vimentin in malignant oral keratinocytes. Representative E-cadherin and vimentin immunofluorescent staining of H357 cells 48 h with Dulbecco's modified Eagle's medium (serum-free medium, panels **A**, **B**, **C**), CM from normal human oral fibroblasts (NHOF, panels **D**, **E**, **F**) and CM from GU-OSCC CAFs (BICR31F) (**G**, **H**, **I**). Fluorescence indicates the presence of E-cadherin (A,D,G) and vimentin (B,E,H). In the merge column, Hoechst-stained nuclei are shown (C,F,I).

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 discohesion (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 discohesion 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 H357 keratinocytes into collagen gels following stimulation by GU-OSCC CAFs (H357F; BICR82F; BICR18F; 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, TGFB2, 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 a subunit of both activin and inhibin, two closely related glycoproteins with opposing biological effects that belong to the TGF-β superfamily and that function through the Smad 2/3 canonical signal transduction pathway (20). Recent data have shown that 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

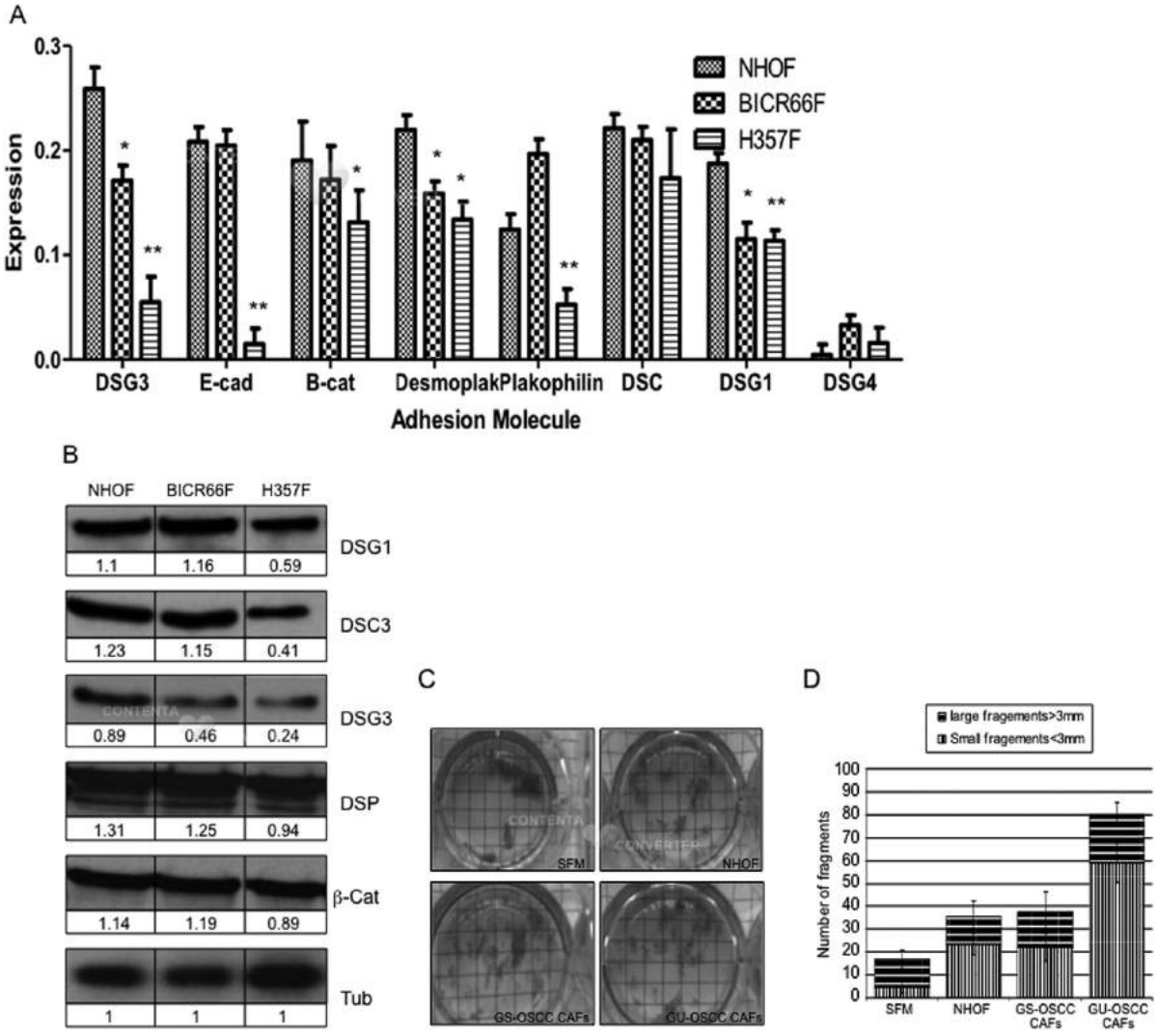


Figure 5. CAFs induce epithelial discohesion *in vitro*. Treatment of H357 keratinocytes with CM from GU-OSCC CAFs (H357F) caused decreased expression of E-cadherin, DSG1 and 3, DSP, β-catenin and plakophilin, whereas CM from GS-OSCC CAFs (BICR66F) caused decreased expression of DSG3, DSP and DSG1 relative to treatment of keratinocytes with CM from NHOF1. Three independent experiments were undertaken, and the data points reflect the mean ± standard deviation (**A**). Canonical western blot analysis showing decreased expression the cadherins and DSP in H357 keratinocytes treated with CM from GU-OSCC CAFs (H357F); the lanes illustrated are from the same blot and CM from NHOF1 was used as a control (**B**). Treatment of H357 keratinocytes with CM from GU-OSCC CAFs (H357F), but not GS-OSCC CAFs (BICR66F) or normal fibroblasts (NHOF1), resulted in epithelial dis-cohesion, as demonstrated by the number of dissociated fragments in a dispase dissociation assay (**C**, **D**). Serum-free media and CM from normal fibroblasts (NHOF1 were used as controls. *$P < 0.05$; **$P < 0.01$.

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

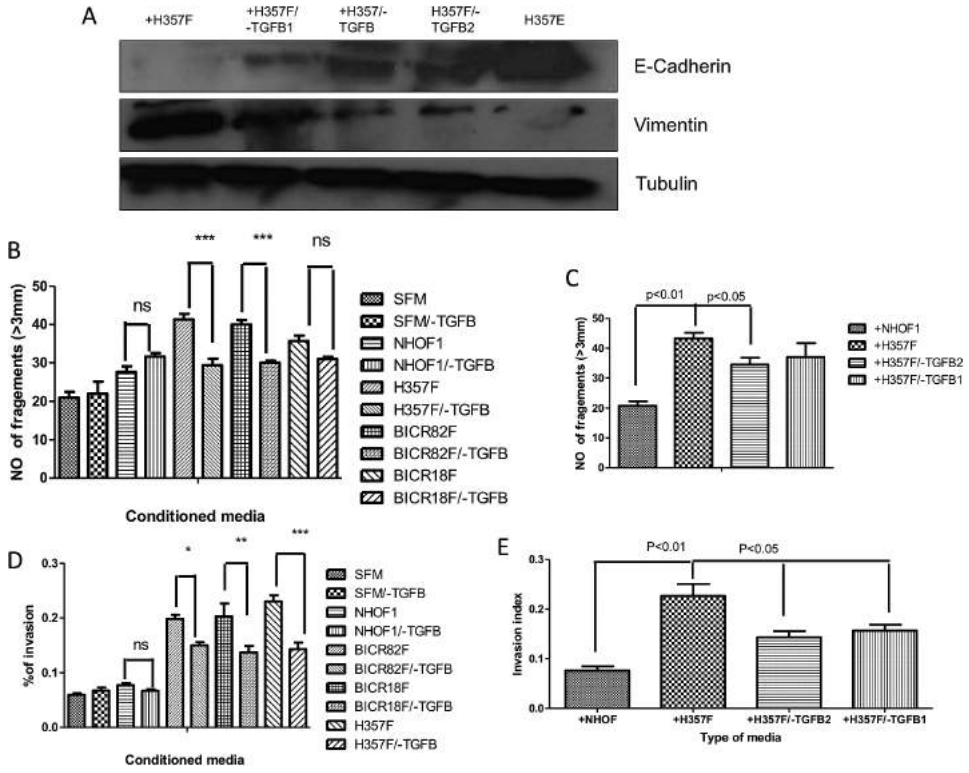


Figure 6. TGF-β acts as an intermediary in fibroblast–epithelial interactions. Western blot analysis demonstrating attenuation of EMT in H357 keratinocytes by GU-OSCC CAFs (H357F) using pan-TGF-β (5 μg/ml), anti-TGF-β1 (0.05 μg/ml) and anti-TGF-β2 (1 μg/ml) antibodies (**A**). Loss of epithelial cohesion 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**).

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/II 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, PG and 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). These 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. The 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

interactome (14) and have been reported to cleave desmosomal protein during apoptosis (52,53); hence, it is likely that MMP-2 weakens cell–cell adhesion by proteolytic targeting of desmosomes. It is also possible, however, that TGF-β signalling down-regulates the expression of cell adhesion molecules *via* transcriptional or translational mechanisms. In addition to intercellular cohesion, matrix metalloproteinases can also modulate cell–extracellular matrix adhesion by degrading integrins and components of the basement membrane. Therefore, the ability TGF-β members to work in concert with matrix metalloproteinases raises the possibility that these two classes of molecules operate synergistically to promote the disruption of the basement membrane and subsequent invasion of connective tissues by cancer cells. Further studies are needed to confirm this hypothesis.

In summary, the results of this study show that TGF-β family members are secreted by CAFs from GU-OSCC and have biological relevance. TGF-β1 and TGF-β2 are overexpressed in non-proliferating, senescent CAFs from GU-OSCC, but not in proliferating, non-senescent CAFs from GS-OSCC or normal fibroblasts. CAFs from GU-OSCC induced EMT and down-regulated a broad spectrum of epithelial cell adhesion molecules resulting in epithelial discohesion and invasion *in vitro* in a TGF-β dependent manner.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1 to 4 can be found at http://carcin.oxfordjournals.org/

Funding

University of Bristol, UK grant no. CMM2010 (N.C.).

Acknowledgements

Y.H. was in receipt of a clinical Fellowship from the University of Jordan. N.C., A.C. and S.M. gratefully acknowledge the support of Melbourne Dental School, the University of Melbourne.
Conflict of Interest Statement: None declared.

References

1. Tselepis, C. et al. (1998) Desmosomal adhesion inhibits invasive behavior. Proc. Natl. Acad. Sci. USA, 95, 8064–8069.
2. Fanjul-Fernández, M. et al. (2013) Cell-cell adhesion genes CTNNA2 and CTNNA3 are tumour suppressors frequently mutated in laryngeal carcinomas. Nat. Commun., 4, 2531.
3. Tang, R. et al. (2004) Colorectal cancer without high microsatellite instability and chromosomal instability—an alternative genetic pathway to human colorectal cancer. Carcinogenesis, 25, 841–846.
4. Edington, K.G. et al. (1995) Cellular immortality: a late event in the progression of human squamous cell carcinoma of the head and neck associated with p53 alteration and a high frequency of allele loss. Mol. Carcinog., 13, 254–265.
5. Hunter, K.D. et al. (2006) Divergent routes to oral cancer. Cancer Res., 66, 7405–7413.
6. Reed, A.L. et al. (1996) High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res., 56, 3630–3633.
7. Kalluri, R. et al. (2009) The basics of epithelial-mesenchymal transition. J. Clin. Invest., 119, 1420–1428.
8. Lim, K.P. et al. (2011) Fibroblast gene expression profile reflects the stage of tumour progression in oral squamous cell carcinoma. J. Pathol., 223, 459–469.
9. Hassona, Y. et al. (2013) Progression of genotype-specific oral cancer leads to senescence of cancer-associated fibroblasts and is mediated by oxidative stress and TGF-β. Carcinogenesis, 34, 1286–1295.
10. Coppé, J.P. et al. (2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol., 6, 2853–2868.
11. Krtolica, A. et al. (2001) Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc. Natl. Acad. Sci. USA, 98, 12072–12077.
12. Shannon, P. et al. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res., 13, 2498–2504.
13. Pieroni, E. et al. (2008) Protein networking: insights into global functional organization of proteomes. Proteomics, 8, 799–816.
14. Cirillo, N. et al. (2009) Desmosomal interactome in keratinocytes: a systems biology approach leading to an understanding of the pathogenesis of skin disease. Cell. Mol. Life Sci., 66, 3517–3533.
15. Thurlow, J.K. et al. (2010) Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma. J. Clin. Oncol., 28, 2881–2888.
16. Cirillo, N. et al. (2010) Induction of hyper-adhesion attenuates autoimmune-induced keratinocyte cell-cell detachment and processing of adhesion molecules via mechanisms that involve PKC. Exp. Cell Res., 316, 580–592.
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.
18. Celentano, A. et al (2016) Pathophysiology of the desmo-adhesome. J. Cell. Physiol, doi: 10.1002/jcp.25515
19. Zaidel-Bar, R. et al. (2007) Functional atlas of the integrin adhesome. Nat. Cell Biol., 9, 858–867.
20. Shi, Y. et al. (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell, 113, 685–700.
21. Acosta, J.C. et al. (2013) A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nat. Cell Biol., 15, 978–990.
22. Seder, C.W. et al. (2009) Upregulated INHBA expression may promote cell proliferation and is associated with poor survival in lung adenocarcinoma. Neoplasia, 11, 388–396.
23. Wang, Q. et al. (2012) Upregulated INHBA expression is associated with poor survival in gastric cancer. Med. Oncol., 29, 77–83.
24. Okano, M. et al. (2013) Significance of INHBA expression in human colorectal cancer. Oncol. Rep., 30, 2903–2908.
25. Pesson, M. et al. (2014) A gene expression and pre-mRNA splicing signature that marks the adenoma-adenocarcinoma progression in colorectal cancer. PLoS One, 9, e87761.
26. Shimizu, S. et al. (2007) Identification of molecular targets in head and neck squamous cell carcinomas based on genome-wide gene expression profiling. Oncol. Rep., 18, 1489–1497.
27. Ye, H. et al. (2008) Transcriptomic dissection of tongue squamous cell carcinoma. BMC Genomics, 9, 69.
28. Gold, L.I. et al. (2000) TGF-beta isoforms are differentially expressed in increasing malignant grades of HaCaT keratinocytes, suggesting separate roles in skin carcinogenesis. J. Pathol., 190, 579–588.
29. Chambers, K.F. et al. (2011) Stroma regulates increased epithelial lateral cell adhesion in 3D culture: a role for actin/cadherin dynamics. PLoS One, 6, e18796.
30. Zhang, C. et al. (2009) Transforming growth factor-beta2 is a molecular determinant for site-specific melanoma metastasis in the brain. Cancer Res., 69, 828–835.
31. Dieterich, L.C. et al. (2012) Transcriptional profiling of human glioblastoma vessels indicates a key role of VEGF-A and TGFβ2 in vascular abnormalization. J. Pathol., 228, 378–390.
32. Fahey, M.S. et al. (1996) Dysregulation of autocrine TGF-beta isoform production and ligand responses in human tumour-derived and Ha-ras-transfected keratinocytes and fibroblasts. Br. J. Cancer, 74, 1074–1080.
33. Jakowlew, S.B. (2006) Transforming growth factor-beta in cancer and metastasis. Cancer Metastasis Rev., 25, 435–457.
34. Rosenthal, E. et al. (2004) Elevated expression of TGF-beta1 in head and neck cancer-associated fibroblasts. Mol. Carcinog., 40, 116–121.
35. Kim, S.A. et al. (2015) Co-culture of 3D tumor spheroids with fibroblasts as a model for epithelial-mesenchymal transition in vitro. Exp. Cell Res., 335, 187–196.

36. Prime, S.S. et al. (1994) TGF-beta receptor regulation mediates the response to exogenous ligand but is independent of the degree of cellular differentiation in human oral keratinocytes. Int. J. Cancer, 56, 406–412.
37. Davies, M. et al. (2012) Transforming growth factor-β enhances invasion and metastasis in Ras-transfected human malignant epidermal keratinocytes. Int. J. Exp. Pathol., 93, 148–156.
38. Mani, S.A. et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell, 133, 704–715.
39. Hasegawa, T. et al. (2014) Cancer-associated fibroblasts might sustain the stemness of scirrhous gastric cancer cells via transforming growth factor-β signaling. Int. J. Cancer, 134, 1785–1795.
40. Teh, M.T. et al. (2011) A molecular study of desmosomes identifies a demoglein isoform switch in head and neck squamous cell carcinoma. J. Oral Pathol. Med, 40, 67–76.
41. Chen, Y.J. et al. (2007) DSG3 is overexpressed in head neck cancer and is a potential molecular target for inhibition of oncogenesis. Oncogene, 26, 467–476.
42. Brown, L. et al. (2014) Desmoglein 3 promotes cancer cell migration and invasion by regulating activator protein 1 and protein kinase C-dependent-Ezrin activation. Oncogene, 33, 2363–2374.
43. Agrawal, N. et al. (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science, 333, 1154–1157.
44. Stransky, N. et al. (2011) The mutational landscape of head and neck squamous cell carcinoma. Science, 333, 1157–1160.
45. Parrinello, S. et al. (2005) Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. J. Cell Sci., 118(Pt 3), 485–496.
46. Martinez-Outschoorn, U.E. et al. (2010) Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: a new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. Cell Cycle, 9, 3256–3276.
47. Cheifetz, S. et al. (1987) The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. Cell, 48, 409–415.
48. Yu, Y. et al. (2014) Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-β signalling. Br. J. Cancer, 110, 724–732.
49. Pickup, M. et al. (2013) The roles of TGFβ in the tumour microenvironment. Nat. Rev. Cancer, 13, 788–799.
50. Giampieri, S. et al. (2009) Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. Nat. Cell Biol., 11, 1287–1296.
51. Hassona, Y. et al. (2014) Senescent cancer-associated fibroblasts secrete active MMP-2 that promotes keratinocyte dis-cohesion and invasion. Br. J. Cancer, 111, 1230–1237.
52. Dusek, R.L. et al. (2006) The differentiation-dependent desmosomal cadherin desmoglein 1 is a novel caspase-3 target that regulates apoptosis in keratinocytes. J. Biol. Chem., 281, 3614–3624.
53. Cirillo, N. et al. (2007) Metalloproteinase 9 is the outer executioner of desmoglein 3 in apoptotic keratinocytes. Oral Dis., 13, 341–345.

Journal of Cellular Physiology

Pathophysiology of the Desmo-Adhesome

ANTONIO CELENTANO,[1,2]* MICHELE DAVIDE MIGNOGNA,[1] MICHAEL MCCULLOUGH,[2,3] AND NICOLA CIRILLO[2,3]**

[1]*Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Naples, Italy*

[2]*Melbourne Dental School, University of Melbourne, Carlton, Victoria, Australia*

[3]*Oral Health Cooperative Research Centre (CRC), University of Melbourne, Carlton, Victoria, Australia*

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.

J. Cell. Physiol. 9999: 1–10, 2016.

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 adhesion 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 desmosome 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 difficultly 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).

*Correspondence to: Antonio Celentano, Melbourne Dental School, University of Melbourne, 720 Swanston Street, 3053 Carlton, VIC, Australia. E-mail: antonio.celentano@unimelb.edu.au
**Correspondence to: Nicola Cirillo, Melbourne Dental School and Oral Health CRC, University of Melbourne, 720 Swanston Street, 3053 Carlton, VIC, Australia.
E-mail: nicola.cirillo@unimelb.edu.au

Manuscript Received: 5 August 2016
Manuscript Accepted: 8 August 2016

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 00 Month 2015.
DOI: 10.1002/jcp.25515

TABLE 1. Comprehensive summary of the diseases that target desmosome components

Molecular target Abbrev (molec. weight)	Tissue distribution	Disease			
		Cancer type (regulation)	Autoimmune	Infectious	Genetic
Desmoglein 1 Dsg1 (160 kDa)	Restricted to stratified squamous epithelia	SCC (down); Pancreatic AC (down)	PF PV PNP IAP (i-e)	SSSS BI	SPPK SAM
Desmoglein 2 Dsg2 (122 kDa)	All desmosome-bearing epithelia	Familial and gastric (down); Pancreatic AC (down); Melanoma, Prostate, SCC (up)	None known	Respiratory and UT infections (adenovirus serotypes 3, 7, 11, and 14)	ARVD/C
Desmoglein 3 Dsg3 (130 kDa)	Restricted to stratified squamous epithelia	SCC (down); Oral SCC (down); Head and neck (up)	PV PNP IAP (i-e)	None known	None known
Desmoglein 4 Dsg4 (114 kDa)	Highly differentiated layers of the epidermidis: Hair follicle epithelia. salivary gland, testis, prostate, and skin	None known	Cross- reactivity of anti-DSG1 IgG	None	Localized autosomal recessive hypotrichosis; Recessive monilethrix
Desmocollin 1 Dsc1 (100 kDa)	Stratified epithelia, hair follicle epithelia	Anal SCC (down); Lung (down)	IAP (s-c) PV PF (Brazilian endemic patients)	None known	None known
Desmocollin 2 Dsc2 (120 kDa)	Simple and stratified epithelia, myocardium	Pancreatic ductal AC (down); Oral SCC (down); Lung (down)	PV PNP PF (Brazilian endemic pts)	Giardiasis	ARVD/C; Wooly hair; keratoderma
Desmocollin 3 Dsc3 (105 kDa)	Stratified epithelia	Lung (down)	PV PNP	Giardiasis	None known
Plakoglobin Pg (86 kDa)	All desmosome-bearing epithelia, myocardium	Oral SCC, Prostate (down); SCC (abnormal)	PNP	*Bacillus anthracis*	Naxos disease Lethal congenital epidermolysis bullosa
Plakophilin 1 Pkp1 (75 kDa)	Suprabasal cells of the epidermis	Prostate AC (down); Oral SCC (down)	PNP	None known	EDSFS, PPK
Plakophilin 2 Pkp2 (93–97 kDa)	Stratified epithelia	Gastric (down)	PNP	None known	ARVD/C, Brugada syndrome
Plakophilin 3 Pkp3 (87 kDa)	Stratified epithelia	Colorectal Ca (down); Lung, Prostate AC, Breast (up)	PNP	None known	None known
P0071 Plakophilin 4 (Pkp4) (134 kDa)		Oropharynx SCC			ARVD/C rare (Xu et al., 2010; Gandjbakhch et al., 2013)
Desmoplakin 1 Dsp1 (250 kDa)	All desmosome-bearing epithelia, myocardium	Lung (down),	PNP	*B. anthracis*	Carvajal syndrome SAM Naxos-like
Desmoplakin 2 Dsp2 (220 kDa)		SCC (abnormal)	EM SJS		disease SPPK ARVD/C LA-EB LDAC
Envoplakin EVP (210 kDa)	Stratified epithelia and myocardium	None known	PNP PF	None known	None known
Periplakin PPL (195 kDa)	Stratified epithelia and myocardium	Esophageal SCC (down)	PNP PF	None known	None known
Plectin (500 kDa)	Basal surface of basal keratinocytes (hemidesmosomes); In striated muscle, (as a component of the z-line)	None known	PNP, BP	None known	None known

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 (s-c), IgA pemphigus (subcorneal pustular dermatosis); LA-EB Lethal acantholytic epidermolysis bullosa; LDAC left dominant arrythmogenic cardiomyopathy MC, Mucocutaneous type; MD, Mucosal dominant type; PF, Pemphigus foliaceus; PG, Plakoglobin; PNP, paraneoplastic pemphigus; PPK, palmoplantar keratoderma; PV, pemphigus vulgaris; SAM, Skin dermatitis multiple allergies and metabolic wasting syndrome; SCC, squamous cell carcinoma; SJS, Stevens-Johnson syndrome; SPPK, striate palmoplantar keratoderma; SSSS, staphylococcal scalded skin syndrome.

Focusing on its composition, the structure of the desmosome 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 1) (Collins et al., 1991; King et al., 1997; Bazzi et al., 2006). At the intracellular level, their extremities interact with plakoglobin (Pg) and plakophilins (Pkps) (Bass-Zubek et al., 2009), which in turn bind to the desmoplakin (Dp) a plakin

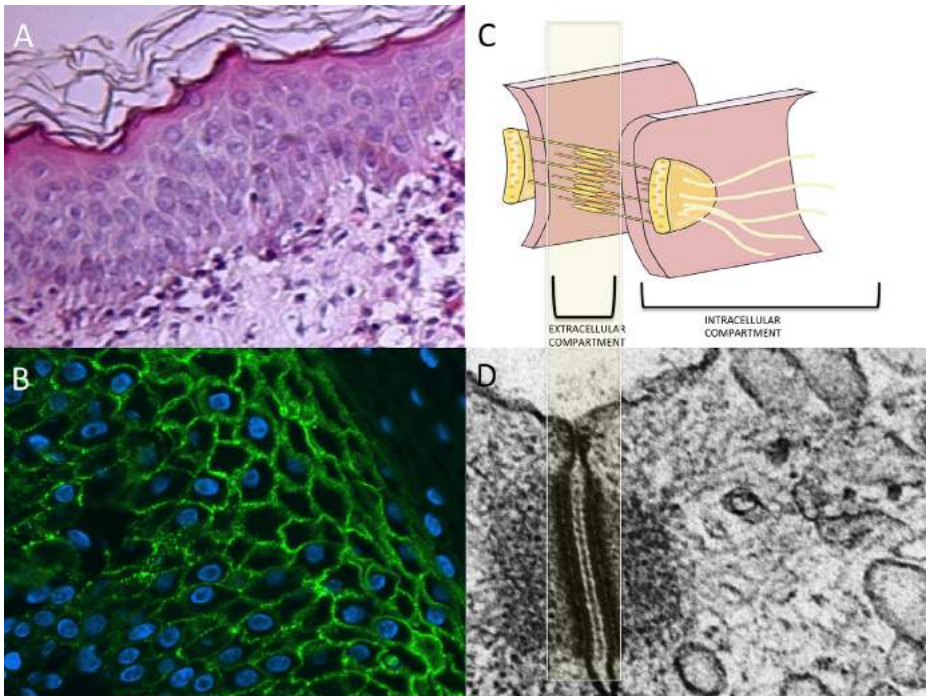


Fig. 1. Desmosomes are particularly abundant in tissues subject to high mechanical stress such as stratified squamous epithelia (A). Desmosomal molecules localise at the cell-cell border and their presence may be revealed by several techniques, including IF (B). Panel B depicts the classical IF staining 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.

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 a single transmembrane proteic 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.

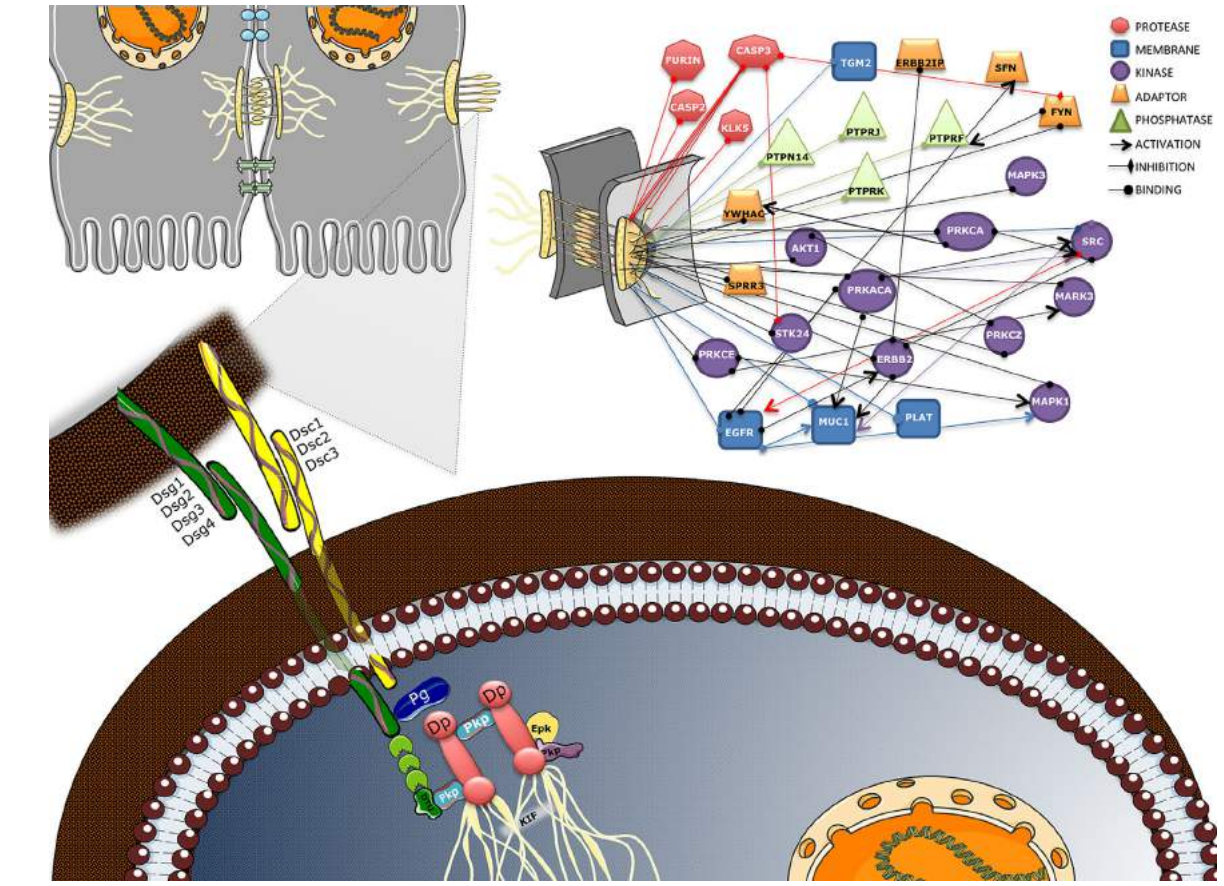


Fig. 2. Schematic representation of desmosome and its components, including accessory components of the desmo-adhesome.

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 pathomechanisms 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 Pg, 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. Aside 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, autoantibodies 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 clinicopathological 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. Prognosis 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 Foliaceus (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-Brigagão 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 (Kljuic 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 hyperkeratosis 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 Foliaceus* (PF) and *Paraneoplastic Pemphigus* (PNP) 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., 1997; 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 inclue a typically polymorphous 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 profiles 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 (Schepens 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* (EM) and *Stevens-Johnson syndrome* (SJS). EM and SJS are two acute immune-mediated disorders that can be triggered by numerous factors (Celentano et al., 2015). The skin and mucous membranes can be affected 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 (Foedinger et al., 1995; Johnson et al., 1999; Fukiwake et al., 2007; Cozzani et al., 2011; Ellis and Sidhu, 2014). So far, the pathogenic 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.

Infectious. Desmosomal plaque disruption and anomalies have been linked to only one infectious agent, *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 Palmoplantar Keratoderma in its striated (SPPK) (MIM 148700) (Hunt et al., 2001; Kljuic et al., 2003b; Barber et al., 2007; Sakiyama and Kubo, 2016) and simple variants (PPK) (Milingou et al., 2006). It is an autosomal dominant skin disease characterized by linear and focal hyperkeratosis of the palms, hands, and soles of the feet, probably due to haplo-insufficiency of Dsg1. Histological and ultrastructural analyses show reduction in the number of desmosomes, accompanied by widening of intercellular spaces between suprabasal keratinocytes, and retracted keratin intermediate filaments (KIF). In the simple variant, the hyperkeratinisation affects sites exposed to mechanical trauma, such as knees, ankles, and finger knuckles. There may be mild nail dystrophy (Armstrong et al., 1999; Whittock et al., 1999).

Another clinical entity recently identified through familial studies, is

– *Severe skin dermatitis, multiple allergies and metabolic wasting (SAM) syndrome* found to be linked to two homozygous mutations in Dsg1 (Samuelov et al., 2013; Has et al., 2015; Schlipf et al., 2016), a novel homozygous splicing mutation (Cheng et al., 2016) and, in one case, to heterozygous missense mutation in Dsp gene (McAleer et al., 2015). The clinical features of this syndrome include recurrent infections, severe metabolic wasting, severe PPK, severe erythroderma, severe dermatitis, skin erosion and scaling, ichthyosis, nail dystrophy, diffuse hypotrichosis, macrocephaly, a widespread superficial pustulosis, development delay, nystagmus, multiple allergies, atopya, persistent eosinophilia, increased IgE levels, hard and curly hair. The severity of the symptoms is generally determined by the position of mutation loci.
– *Arrhythmogenic right ventricular dysplasia/cardiomyopathy* (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 atrophy 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 arrythmogenic cardiomyopathy*) and truncating mutations in desmoplakin are consistently associated with these aggressive phenotypes (López-Ayala et al., 2014).
– *Woolly hair* is a clinical feature shared syndromically with palmoplantar hyperkeratosis 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 (EDSFS)* is an autosomal recessive dermatosis that has been associated with at least eleven different recessively inherited mutations in the Pkp1 gene. It is characterized by skin fragility (with trauma-induced erosions and blistering), non-cicatricial alopecia, palmoplantar keratoderma (PPK), onichodystrophy, and in some cases hypohidrosis (McGrath et al., 1999; Hernández-Martín 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 leading to the formation of a truncated protein, 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 (Jonkman et al., 2005).
– *Lethal congenital epidermolysis bullosa* is a recently described phenotype of the epidermolysis bullosa diseases 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 nodes, 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; Sibilia 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; Skålhegg 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 (164730) 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, hypsarrhythmia, severe developmental delay, and more clinical features have been associated with specific deletions in the YWHAG gene that encodes for 14-3-3 protein gamma, an accessory protein of the desmosome-associated regulatory network (Komoike et al., 2010; Ramocki et al., 2010; Röthlisberger 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 (Tselepis 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., 2012), head, and neck (Chen et al., 2007), lung (Cui et al., 2012), breast (Klus et al., 2001; Oshiro et al., 2005), prostate (Barber et al., 2014), cervix (Alazawi et al., 2003), etc. Paradoxically, the expression of desmosomal cadherins has been reported to be increased 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; Cui et al., 2012), gastric (Yashiro et al., 2006) and colon cancer (Khan et al., 2006; Knösel et al., 2012; Schüle et al., 2014), pancreatic ductal adenocarcinoma (Hamidov et al., 2011), SCC of the sinonasal cavity (Huang et al., 2010), cutaneous SCC (Tada et al., 2000; Kurzen et al., 2003), esophageal adenocarcinoma (Wang et al., 2014), and not least OSSC (Xin et al., 2014). Interestingly, cell cycle control and triggers for apoptosis can be indirectly influenced by altered functional signaling 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; Stahley and Kowalczyk, 2015), and provide more detail in Table 1.

Concluding Remarks

The desmo-adhesome 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-Dehaghi et al., 2013; Otten et al., 2014). This study has also suggests 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 will unveil novel disease mechanisms that will ultimately be targeted for patients' benefit.

Literature Cited

Adam RD. 2001. Biology of giardia lamblia. Clin Microbiol Rev 14:447–475.

Al-Amoudi A, Castañ o-Diez D, Devos DP, Russell RB, Johnson GT, Frangakis AS. 2011. The three-dimensional molecular structure of the desmosomal plaque. Proc Natl Acad Sci 108:6480–6485.

Alazawi WO, Morris LS, Stanley MA, Garrod DR, Coleman N. 2003. Altered expression of desmosomal components in high-grade squamous intraepithelial lesions of the cervix. Virchows Arch 443:51–56.

Allen CM, Camisa C. 2000. Paraneoplastic pemphigus: A review of the literature. Oral Dis 6:208–214.

Amagai M, Stanley JR. 2012. Desmoglein as a target in skin disease and beyond. J Invest Dermatol 132:776–784.

Amagai M, Matsuyoshi N, Wang ZH, Andl C, Stanley JR. 2000. Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. Nat Med 6:1275–1277.

Amagai M. 2010. Autoimmune and infectious skin diseases that target desmogleins. Proc Jpn Acad Ser B Phys Biol Sci 86:524–537.

Anhalt GJ, Kim SC, Stanley JR, Korman NJ, Jabs DA, Kory M, Izumi H, Ratrie H, 3rd, Mutasim D, Ariss-Abdo L, Labib RS. 1990. Paraneoplastic pemphigus. An autoimmune mucocutaneous disease associated with neoplasia. N Engl J Med 323:1729–1735.

Anhalt GJ. 2004. Paraneoplastic pemphigus. J Investig Dermatol Symp Proc 9:29–33.

Armstrong DK, McKenna KE, Purkis PE, Green KJ, Eady RA, Leigh IM, Hughes AE. 1999. Haploinsufficiency of desmoplakin causes a striate subtype of palmoplantar keratoderma. Hum Mol Genet 8:143–148.

Au AC, Hernandez PA, Lieber E, Nadroo AM, Shen YM, Kelley KA, Gelb BD, Diaz GA. 2010. Protein tyrosine phosphatase PTPN14 is a regulator of lymphatic function and choanal development in humans. Am J Hum Genet 87:436–444.

Awad MM, Dalal D, Cho E, Amat-Alarcon N, James C, Tichnell C, Tucker A, Russell SD, Bluemke DA, Dietz HC, Calkins H, Judge DP. 2006. DSG2 mutations contribute to arrhythmogenic right ventricular dysplasia/cardiomyopathy. Am J Hum Genet 79:136–142.

Barber AG, Wajid M, Columbo M, Lubetkin J, Christiano AM. 2007. Striate palmoplantar keratoderma resulting from a frameshift mutation in the desmoglein 1 gene. J Dermatol Sci 45:161–166.

Barber AG, Castillo-Martin M, Bonal DM, Rybicki BA, Christiano AM, Cordon-Cardo C. 2014. Characterization of desmoglein expression in the normal prostatic gland. Desmoglein 2 is an independent prognostic factor for aggressive prostate cancer. PLoS ONE 9:e98786.

Bass-Zubek AE, Godsel LM, Delmar M, Green KJ. 2009. Plakophilins: Multifunctional scaffolds for adhesion and signaling. Curr Opin Cell Biol 21:708–716.

Bazzi H, Getz A, Mahoney MG, Ishida-Yamamoto A, Langbein L, Wahl JK, 3rd, Christiano AM. 2006. Desmoglein 4 is expressed in highly differentiated keratinocytes and trichocytes in human epidermis and hair follicle. Differentiation 74:129–140.

Bergeron L, Perez GI, Macdonald G, Shi L, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JC, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL, Yuan J. 1998. Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev 12:1304–1314.

Bernard P. 2008. Management of common bacterial infections of the skin. Curr Opin Infect Dis 21:122–128.

Bonné S, Gilbert B, Hatzfeld M, Chen X, Green KJ, van Roy F. 2003. Defining desmosomal plakophilin-3 interactions. J Cell Biol 161:403–416.

Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, Kimball TF, Lorenz JN, Nairn AC, Liggett SB, Bodi I, Wang S, Schwartz A, Lakatta EG, DePaoli-Roach AA, Robbins J, Hewett TE, Bibb JA, Westfall MV, Kranias EG, Molkentin JD. 2004. PKC-alpha regulates cardiac contractility and propensity toward heart failure. Nat Med 10:248–254.

Breault JE, Shiina H, Igawa M, Ribeiro-Filho LA, Deguchi M, Enokida H, Urakami S, Terashima M, Nakagawa M, Kane CJ, Carroll PR, Dahiya R. 2005. Methylation of the γ-catenin gene is associated with poor prognosis of renal cell carcinoma. Clin Cancer Res 11:557–564.

Campbell P, Morton PE, Takeichi T, Salam A, Roberts N, Proudfoot LE, Mellerio JE, Aminu K, Wellington C, Patil SN, Akiyama M, Liu L, McMillan JR, Aristodemou S, Ishida-Yamamoto A, Abdul-Wahab A, Petrof G, Fong K, Harnchoowong S, Stone KL, Harper JI, McLean WH, Simpson MA, Parsons M, McGrath JA. 2014. Epithelial inflammation resulting from an inherited loss-of-function mutation in EGFR. J Invest Dermatol 134:2570–2578.

Campuzano O, Fernández-Falgueras A, Iglesias A, Brugada R. 2016. Brugada syndrome and PKP2: Evidences and uncertainties. Int J Cardiol 214:403–405.

Celentano A, Tovaru S, Yap T, Adamo D, Aria M, Mignogna MD. 2015. Oral erythema multiforme: Trends and clinical findings of a large retrospective European case series. Oral Surg Oral Med Oral Pathol Oral Radiol 6:707–716.

Chan R, Hardy WR, Laing MA, Hardy SE, Muller WJ. 2002. The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development. Mol Cell Biol 22:1073–1078.

Chen YJ, Chang JT, Lee L, Wang HM, Liao CT, Chiu CC, Chen PJ, Cheng AJ. 2007. DSG3 is overexpressed in head neck cancer and is a potential molecular target for inhibition of oncogenesis. Oncogene 26:467–476.

Chen J, Shea OC, Fitzpatrick JE, Koster MI, Koch PJ. 2012. Loss of Desmocollin 3 in skin tumor development and progression. Mol Carcinog 51:535–545.

Cheng R, Yan M, Ni C, Zhang J, Li M, Yao Z. 2016. Report of Chinese family with severe dermatitis, multiple allergies and metabolic wasting syndrome caused by novel homozygous desmoglein-1 gene mutation. J Dermatol. doi: 10.1111/1346-8138.13431 Epub ahead of print. PMID: 27154412.

Cirillo N, Prime SS. 2009. Desmosomal interactome in keratinocytes: A systems biology approach leading to an understanding of the pathogenesis of skin disease. Cell Mol Life Sci 66:3517–3533.

Cirillo N, Gombos F, Lanza A. 2007. Pemphigus vulgaris immunoglobulin G can recognize a 130 000MW antigen other than desmoglein 3 on peripheral blood mononuclear cell surface. Immunology 121:377–382.

Cirillo N. 2014. 150th anniversary series: Desmosomes in physiology and disease. Cell Commun Adhes 21:85–88.

Cirillo N. 2016. Desmosome assembly, homeostasis, and desmosomal disease. Cell Health Cytoskelet 8:9–23.

Coates JC. 2003. Armadillo repeat proteins: Beyond the animal kingdom. Trends Cell Biol 12:463–471.

Collins JE, Legan PK, Kenny TP, MacGarvie J, Holton JL, Garrod DR. 1991. Cloning and sequence analysis of desmosomal glycoproteins 2 and 3 (desmocollins): Cadherin-like desmosomal adhesion molecules with heterogeneous cytoplasmic domains. J Cell Biol 113:381–391.

Cowin P, Kapprell HP, Franke WW, Tamkun J, Hynes RO. 1986. Plakoglobin: A protein common to different kinds of intercellular adhering junctions. Cell 46:1063–1073.

Cozzani E, Di Zenzo G, Calabresi V, Caproni M, Schena D, Quaglino P, Marzano AV, Fabbri P, Rebora A, Parodi A. 2011. Anti-desmoplakin antibodies in erythema multiforme and Stevens-Johnson syndrome sera: Pathogenic or epiphenomenon? Eur J Dermatol 21:32–36.

Cribier B, Piemont Y, Grosshans E. 1994. Staphylococcal scalded skin syndrome in adults. A clinical review illustrated with a new case. J Am Acad Dermatol 30:319–324.

Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross J, Jr, Chien KR, Lee KF. 2002. ErbB2 is essential in the prevention of dilated cardiomyopathy. Nat Med 8:459–465.

Cui T, Chen Y, Yang L, Mireskandari M, Knösel T, Zhang Q, Kohler LH, Kunze A, Presselt N, Petersen I. 2012. Diagnostic and prognostic impact of desmocollins in human lung cancer. J Clin Pathol 65:1100–1106.

Dietrich P, Dragatsis I, Xuan S, Zeitlin S, Efstratiadis A. 2000. Conditional mutagenesis in mice with heat shock promoter-driven cre transgenes. Mamm Genome 11:196–205.

Dmochowski M, Hashimoto T, Garrod DR, Nishikawa T. 1993. Desmocollins I and II are recognized by certain sera from patients with various types of pemphigus, particularly Brazilian pemphigus foliaceus. J Invest Dermatol 100:380–384.

Dusek RL, Godsel LM, Green KJ. 2007. Discriminating roles of desmosomal cadherins: Beyond desmosomal adhesion. J Dermatol Sci 45:7–21.

Ellis E, Sidhu S. 2014. Circulating plakin autoantibodies in a patient with erythema multiforme major: Are they pathogenic or a manifestation of epitope spreading? Australas J Dermatol 55:266–269.

Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frantz G, Bauer M, Lu LH, Moore MW. 1997. ErbB3 is required for normal cerebellar and cardiac development: A comparison with ErbB2-and heregulin-deficient mice. Development 124:4999–5011.

Eyre RW, Stanley JR. 1988. Identification of pemphigus vulgaris antigen extracted from normal human epidermis and comparison with pemphigus foliaceus antigen. J Clin Invest 81:807–812.
Foedinger D, Anhalt GJ, Boecskoer B, Elbe A, Wolff K, Rappersberger K. 1995. Autoantibodies to desmoplakin I and II in patients with erythema multiforme. J Exp Med 181:169–179.
Fukiwake N, Moroi Y, Urabe K, Ishii N, Hashimoto T, Furue M. 2007. Detection of autoantibodies to desmoplakin in a patient with oral erythema multiforme. Eur J Dermatol 17:238–241.
Gandjbakhch E, Vite A, Gary F, Fressart V, Donal E, Simon F, Hidden-Lucet F, Komajda M, Charron P, Villard E. 2013. Screening of genes encoding junctional candidates in arrhythmogenic right ventricular cardiomyopathy/dysplasia. Europace 15:1522–1525.
Green KJ, Simpson CL. 2007. Desmosomes: New perspectives on a classic. J Invest Dermatol 127:2499–2515.
Hamidov Z, Altendorf-Hofmann A, Chen Y, Settmacher U, Petersen I, Knösel T. 2011. Reduced expression of desmocollin 2 is an independent prognostic biomarker for shorter patients survival in pancreatic ductal adenocarcinoma. J Clin Pathol 64:990–994.
Handler MZ, Schwartz RA. 2014. Staphylococcal scalded skin syndrome: Diagnosis and management in children and adults. J Eur Acad Dermatol Venereol 28:1418–1423.
Hara T, Saito Y, Hirai T, Nakamura K, Nakao K, Katsuki M, Chida K. 2005. Deficiency of protein kinase Calpha in mice results in impairment of epidermal hyperplasia and enhancement of tumor formation in two-stage skin carcinogenesis. Cancer Res 65:7356–7362.
Hartman-Adams H, Banvard C, Juckett G. 2014. Impetigo: Diagnosis and treatment. Am Fam Physician 90:229–235.
Has C, Jakob T, He Y, Kiritsi D, Hausser I, Bruckner-Tuderman L. 2015. Loss of desmoglein 1 associated with palmoplantar keratoderma, dermatitis and multiple allergies. Br J Dermatol 172:257–261.
Hashimoto T, Amagai M, Watanabe K, Dmochowski M, Chidgey MA, Yue KK, Garrod DR, Nishikawa T. 1995. A case of pemphigus vulgaris showing reactivity with pemphigus antigens (Dsg1 and Dsg3) and desmocollins. J Invest Dermatol 104:541–544.
Hata T, Nishimoto S, Nagao K, Takahashi H, Yoshida K, Ohyama M, Yamada T, Asano K, Amagai M. 2013. Ectopic expression of epidermal antigens renders the lung a target organ in paraneoplastic pemphigus. J Immunol 191:83–90.
Hatano N, Mori Y, Oh-hora M, Kosugi A, Fujikawa T, Nakai N, Niwa H, Miyazaki J, Hamaoka T, Ogata M. 2003. Essential role for ERK2 mitogen-activated protein kinase in placental development. Genes CellsNov 8:847–856.
Hatzfeld M. 2007. Plakophilins: Multifunctional proteins or just regulators of desmosomal adhesion? Biochim Biophys Acta 1773:69–77.
He W, Cowin P, Stokes DL. 2003. Untangling desmosomal knots with electron tomography. Science 302:109–113.
Hernández-Martín A, Torrelo A, Ciria S, Colmenero I, Aguilar A, Grimalt R, González-Sarmiento R. 2013. Ectodermal dysplasia-skin fragility syndrome: A novel mutation in the PKP1 gene. Clin Exp Dermatol 38:787–790.
Huang CC, Lee TJ, Chang PH, Lee YS, Chuang CC, Jhang YJ, Chen YW, Chen CW, Tsai CN. 2010. Desmoglein 3 is overexpressed in inverted papilloma and squamous cell carcinoma of sinonasal cavity. Laryngoscope 120:26–29.
Huber O, Petersen I. 2015. 150th anniversary series: Desmosomes and the hallmarks of cancer. Cell Commun Adhes 22:15–28.
Hunt DM, Rickman L, Whittock NV, Eady RA, Simrak D, Dopping-Hepenstal PJ, Stevens HP, Armstrong DK, Hennies HC, Küster W, Hughes AE, Arnemann J, Leigh IM, McGrath JA, Kelsell DP, Buxton RS. 2001. Spectrum of dominant mutations in the desmosomal cadherin desmoglein 1, causing the skin disease striate palmoplantar keratoderma. Eur J Hum Genet 9:197–203.
Johnson SM, Smoller BR, Horn TD. 1999. Erythema multiforme associated human autoantibodies against desmoplakin I and II. J Invest Dermatol 112:395–396.
Joly P, Litrowski N. 2011. Pemphigus group (vulgaris, vegetans, foliaceus, herpetiformis, brasiliensis). Clin Dermatol 29:432–436.
Jonkman MF, Pasmooij AM, Pasmans SG, van den Berg MP, Ter Horst HJ, Timmer A, Pas HH. 2005. Loss of desmoplakin tail causes lethal acantholytic epidermolysis bullosa. Am J Hum Genet 77:653–660.
Kalantari-Dehaghi M, Anhalt GJ, Camilleri MJ, Chernyavsky AI, Chun S, Felgner PL, Jasinskas A, Leiferman KM, Liang L, Marchenko S, Nakajima-Sasaki R, Pittelkow MR, Zone JJ, Grando SA. 2013. Pemphigus vulgaris autoantibody profiling by proteomic technique. PLoS ONE 8:e57587.
Khan N, Asif AR. 2015. Transcriptional regulators of claudins in epithelial tight junctions. Mediators Inflamm 2015:219843.
Khan K, Hardy R, Haq A, Ogunbiyi O, Morton D, Chidgey M. 2006. Desmocollin switching in colorectal cancer. Br J Cancer 95:1367–1370.
King IA, Angst BD, Hunt DM, Kruger M, Arnemann J, Buxton RS. 1997. Hierarchical expression of desmosomal cadherins during stratified epithelial morphogenesis in the mouse. Differentiation 62:83–96.
Kitajima Y. 2014. 150(th) anniversary series: Desmosomes and autoimmune disease, perspective of dynamic desmosome remodeling and its impairments in pemphigus. Cell Commun Adhes 21:269–280.
Kljuic A, Bazzi H, Sundberg JP, Martinez-Mir A, O'Shaughnessy R, Mahoney MG, Levy M, Montagutelli X, Ahmad W, Aita VM, Gordon D, Uitto J, Whiting D, Ott J, Fischer S, Gilliam TC, Jahoda CA, Morris RJ, Panteleyev AA, Nguyen VT, Christiano AM. 2003a. Desmoglein 4 in hair follicle differentiation and epidermal adhesion: Evidence from inherited hypotrichosis and acquired pemphigus vulgaris. Cell 113:249–260.
Kljuic A, Gilead L, Martinez-Mir A, Frank J, Christiano AM, Zlotogorski A. 2003b. A nonsense mutation in the desmoglein 1 gene underlies striate keratoderma. Exp Dermatol 12:523–527.
Klus GT, Rokaeus N, Bittner ML, Chen Y, Korz DM, Sukumar S, Schick A, Szallasi Z. 2001. Down-regulation of the desmosomal cadherin desmocollin 3 in human breast cancer. Int J Oncol 19:169–174.
Knösel T, Chen Y, Hotovy S, Settmacher U, Altendorf-Hofmann A, Petersen I. 2012. Loss of desmocollin 1-3 and homeobox genes PITX1 and CDX2 are associated with tumor progression and survival in colorectal carcinoma. Int J Colorectal Dis 27:1391–1399.
Kneisel A, Hertl M. 2011a. Autoimmune bullous skin diseases. part 1. clinical manifestations. J Dtsch Dermatol Ges 9:844–856.
Kneisel A, Hertl M. 2011b. Autoimmune bullous skin diseases. Part 2: Diagnosis and therapy. J Dtsch Dermatol Ges 9:927–947.
Komoike Y, Fujii K, Nishimura A, Hiraki Y, Hayashidani M, Shimojima K, Nishizawa T, Higashi K, Yasukawa K, Saitsu H, Miyake N, Mizuguchi T, Matsumoto N, Osawa M, Kohno Y, Higashinakagawa T, Yamamoto T. 2010. Zebrafish gene knockdowns imply roles for human YWHAG in infantile spasms and cardiomegaly. Genesis 48:233–243.
Korman NJ, Eyre RW, Klaus-Kovtun V, Stanley JR. 1989. Demonstration of an adhering-junction molecule (plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. N Engl J Med 321:631–635.
Kowalczyk AP, Green KJ. 2013. Structure, function, and regulation of desmosomes. Prog Mol Biol Transl Sci 116:95–118.
Kowalczyk AP, Hatzfeld M, Bornslaeger EA, Kopp DS, Borgwardt JE, Corcoran CM, Settler A, Green KJ. 1999. The head domain of plakophilin-1 binds to desmoplakin and enhances its recruitment to desmosomes. Implications for cutaneous disease. J Biol Chem 274:18145–18148.
Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA. 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384:368–372.
Kurzen H, Münzing I, Hartschuh W. 2003. Expression of desmosomal proteins in squamous cell carcinomas of the skin. J Cutan Pathol 30:621–630.
López-Ayala JM, Gómez-Milanés I, Sánchez Muñoz JJ, Ruiz-Espejo F, Ortíz M, González-Carrillo J, López-Cuenca D, Oliva-Sandoval MJ, Monserrat L, Valdés M, Gimeno JR. 2014. Desmoplakin truncations and arrhythmogenic left ventricular cardiomyopathy: Characterizing a phenotype. Europace 16:1838–1846.
Ladhani S, Evans RW. 1998. Staphylococcal scalded skin syndrome. Arch Dis Child 78:85–88.
Langer M, Duggan ES, Booth JL, Patel VI, Zander RA, Silasi-Mansat R, Ramani V, Veres TZ, Prenzler F, Sewald K, Williams DM, Coggeshall KM, Awasthi S, Lupu F, Burian D, Ballard JD, Braun A, Metcalf JP. 2012. *Bacillus anthracis* lethal toxin reduces human alveolar epithelial barrier function. Infect Immun 80:4374–4387.
Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 378:394–398.
Leitges M, Plomann M, Standaert ML, Bandyopadhyay G, Sajan MP, Kanoh Y, Farese RV. 2002. Knockout of PKC alpha enhances insulin signaling through PI3K. Mol Endocrinol 16:847–858.
Leung CL, Green KJ, Liem RK. 2002. Plakins: A family of versatile cytolinker proteins. Trends Cell Biol 12:37–45.
Lindhurst MJ, Sapp JC, Teer JK, Johnston JJ, Finn EM, Peters K, Turner J, Cannons JL, Bick D, Blakemore L, Blumhorst C, Brockmann K, Calder P, Cherman N, Deardorff MA, Everman DB, Golas G, Greenstein RM, Kato BM, Keppler-Noreuil KM, Kuznetsov SA, Miyamoto RT, Newman K, Ng D, O'Brien K, Rothenberg S, Schwartzentruber DJ, Singhal V, Tirabosco R, Upton J, Wientroub S, Zackai EH, Hoag K, Whitewood-Neal T, Robey PG, Schwartzberg PL, Darling TN, Tosi LL, Mullikin JC, Biesecker LG. 2011. A mosaic activating mutation in AKT1 associated with the proteus syndrome. N Engl J Med 365:611–619.
Maia-Brigagão C, Morgado-Díaz JA, De Souza W. 2012. Giardia disrupts the arrangement of tight, adherens and desmosomal junction proteins of intestinal cells. Parasitol Int 61:280–287.
Mao X, Nagler AR, Farber SA, Choi EJ, Jackson LH, Leiferman KM, Ishii N, Hashimoto T, Amagai M, Zone JJ, Payne AS. 2010. Autoimmunity to desmocollin 3 in pemphigus vulgaris. Am J Pathol 177:2724–2730.
Marchiando AM, Graham WV, Turner JR. 2010. Epithelial barriers in homeostasis and disease. Annu Rev Pathol 5:119–144.
McAleer MA, Pohler E, Smith FJ, Wilson NJ, Cole C, MacGowan S, Koetsier JL, Godsel LM, Harmon RM, Gruber R, Crumrine D, Elias PM, McDermott M, Butler K, Broderick A, Sarig O, Sprecher E, Green KJ, McLean WH, Irvine AD. 2015. Severe dermatitis, multiple allergies, and metabolic wasting syndrome caused by a novel mutation in the N-terminal plakin domain of desmoplakin. J Allergy Clin Immunol 136:1268–1276.
McGrath JA, Hoeger PH, Christiano AM, McMillan JR, Mellerio JE, Ashton GH, Dopping-Hepenstal PJ, Lake BD, Leigh IM, Harper JI, Eady RA. 1999. Skin fragility and hypohidrotic ectodermal dysplasia resulting from ablation of plakophilin 1. Br J Dermatol 140:297–307.
Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, Derynck R. 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature 376:337–341.
Milingou M, Wood P, Masouyé I, McLean WH, Borradori L. 2006. Focal palmoplantar keratoderma caused by an autosomal dominant inherited mutation in the desmoglein 1 gene. Dermatology 212:117–122.
Mimouni D, Foedinger D, Kouba DJ, Orlow SJ, Rappersberger K, Sciubba JJ, Nikolskaia OV, Cohen BA, Anhalt GJ, Nousari CH. 2004. Mucosal dominant pemphigus vulgaris with anti-desmoplakin autoantibodies. J Am Acad Dermatol 51:62–67.
Mockenhaupt M, Idzko M, Grosber M, Schöpf E, Norgauer J. 2005. Epidemiology of staphylococcal scalded skin syndrome in Germany. J Invest Dermatol 124:700–703.
Moss C, Martinez-Mir A, Lam H, Tadin-Strapps M, Kljuic A, Christiano AM. 2004. A recurrent intragenic deletion in the desmoglein 4 gene underlies localized autosomal recessive hypotrichosis. J Invest Dermatol 123:607–610.
Nagaoka I, Matsui K, Ueyama T, Kanemoto M, Wu J, Shimizu A, Matsuzaki M, Horie M. 2006. Novel mutation of plakophilin-2 associated with arrhythmogenic right ventricular cardiomyopathy. Circ J 70:933–935.
Natarajan A, Wagner B, Sibilia M. 2007. The EGF receptor is required for efficient liver regeneration. Proc Natl Acad Sci USA 104:17081–17086.
Nei H, Saito T, Tobioka H, Itoh E, Mori M, Kudo R. 1996. Expression of component desmosomal proteins in uterine endometrial carcinoma and their relation to cellular differentiation. Cancer 78:461–470.
Nguyen VT, Ndoye A, Shultz LD, Pittelkow MR, Grando SA. 2000. Antibodies against keratinocyte antigens other than desmogleins 1 and 3 can induce pemphigus vulgaris-like lesions. J Clin Invest 106:1467–1479.
Nolan MA, Babcock DF, Wennemuth G, Brown W, Burton KA, McKnight GS. 2004. Sperm-specific protein kinase A catalytic subunit Calpha2 orchestrates cAMP signaling for male fertility. Proc Natl Acad Sci USA 101:13483–13488.
Nollet F, Kools P, van Roy F. 2000. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. J Mol Biol 299:551–572.
North AJ, Bardsley WG, Hyam J, Bornslaeger EA, Cordingley HC, Trinnaman B, Hatzfeld M, Green KJ, Magee AI, Garrod DR. 1999. Molecular map of the desmosomal plaque. J Cell Sci 112:4325–4336.
Orloff MS, He X, Peterson C, Chen F, Chen J-L, Mester JL, Eng C. 2013. Germline PIK3CA and AKT1 mutations in cowden and cowden-like syndromes. Am J Hum Genet 92:76–80.
Oshiro MM, Kim CJ, Wozniak RJ, Junk DJ, Muñoz-Rodríguez JL, Burr JA, Fitzgerald M, Pawar SC, Cress AE, Domann FE, Futscher BW. 2005. Epigenetic silencing of DSC3 is a common event in human breast cancer. Breast Cancer Res 7:R669–R680.
Ossoff R, Giunta JL. 1975. The staphylococcal scalded-skin syndrome versus erythema multiforme. Oral Surg Oral Med Oral Pathol 40:126–129.
Otten JV, Hashimoto T, Hertl M, Payne AS, Sitaru C. 2014. Molecular diagnosis in autoimmune skin blistering conditions. Curr Mol Med 14:69–95.

Pan J, Chen Y, Mo C, Wang D, Chen J, Mao X, Guo S, Zhuang J, Qiu S. 2014. Association of DSC3 mRNA down-regulation in prostate cancer with promoter hypermethylation and poor prognosis. PLoS ONE 9:e92815.
Paris L, Bazzoni G. 2008. The protein interaction network of the epithelial junctional complex: A system-level analysis. Mol Biol Cell 19:5409–5421.
Patel GK. 2004. Treatment of staphylococcal scalded skin syndrome. Expert Rev Anti Infect Ther 2:575–587.
Pigors M, Kiritsi D, Krümpelmann S, Wagner N, He Y, Podda M, Kohlhase J, Hausser I, Bruckner-Tuderman L, Has C. 2011. Lack of plakoglobin leads to lethal congenital epidermolysis bullosa: A novel clinico-genetic entity. Hum Mol Genet 20:1811–1819.
Pilichou K, Nava A, Basso C, Beffagna G, Bauce B, Lorenzon A, Frigo G, Vettori A, Valente M, Towbin J, Thiene G, Danieli GA, Rampazzo A. 2006. Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy. Circulation 113:1171–1179.
Röthlisberger B, Hoigné I, Huber AR, Brunschwiler W, Capone Mori A. 2010. Deletion of 7q11.21-q11.23 and infantile spasms without deletion of MAGI2. Am J Med Genet 152:434–437.
Rafei D, Müller R, Ishii N, Llamazares M, Hashimoto T, Hertl M, Eming R. 2011. IgG autoantibodies against desmocollin 3 in pemphigus sera induce loss of keratinocyte adhesion. Am J Pathol 178:718–723.
Ramocki MB, Bartnik M, Szafranski P, Kołodziejska KE, Xia Z, Bravo J, Miller GS, Rodriguez DL, Williams CA, Bader PI, Szczepanik E, Mazurczak T, Antczak-Marach D, Coldwell JG, Akman CI, McAlmon K, Cohen MP, McGrath J, Roeder E, Mueller J, Kang SH, Bacino CA, Patel A, Bocian E, Shaw CA, Cheung SW, Mazurczak T, Stankiewicz P. 2010. Recurrent distal 7q11.23 deletion including HIP1 and YWHAG identified in patients with intellectual disabilities, epilepsy, and neurobehavioral problems. Am J Hum Genet 87:857–865.
Rocha-Alvarez R, Ortega-Loayza AG, Friedman H, Campbell I, Aoki V, Rivitti EA, Dasher D, Li N, Diaz LA. 2007. Cooperative group on fogo selvagem research. endemic pemphigus vulgaris. Arch Dermatol 143:895–899.
Ruocco V, Ruocco E, Lo Schiavo A, Brunetti G, Guerrera LP, Wolf R. 2013. Pemphigus: Etiology, pathogenesis, and inducing or triggering factors: Facts and controversies. Clin Dermatol 31:374–381.
Sakiyama T, Kubo A. 2016. Hereditary palmoplantar keratoderma "clinical and genetic differential diagnosis". J Dermatol 43:264–274.
Samuelov L, Sarig O, Harmon RM, Rapaport D, Ishida-Yamamoto A, Isakov O, Koetsier JL, Gat A, Goldberg I, Bergman R, Spiegel R, Eytan O, Geller S, Peleg S, Shomron N, Goh CS, Wilson NJ, Smith FJ, Pohler E, Simpson MA, McLean WH, Irvine AD, Horowitz M, McGrath JA, Green KJ, Sprecher E. 2013. Desmoglein 1 deficiency results in severe dermatitis, multiple allergies and metabolic wasting. Nat Genet 45:1244–1248.
Sato H, Matsumori Y, Tanabe T, Saito H, Shimizu A, Kawano J. 1994. A new type of staphylococcal exfoliative toxin from a *Staphylococcus aureus* strain isolated from a horse with phlegmon. Infect Immun 62:3780–3785.
Savci-Heijink CD, Kosari F, Aubry MC, Caron BL, Sun Z, Yang P, Vasmatzis G. 2009. The role of desmoglein-3 in the diagnosis of squamous cell carcinoma of the lung. Am J Pathol 174:1629–1637.
Schüle S, Neuhäuser C, Rauchfuß F, Knösel T, Settmacher U, Altendorf-Hofmann A. 2014. The influence of desmocollin 1-3 expression on prognosis after curative resection of colorectal liver metastases. Int J Colorectal Dis 29:9–14.
Schaffer JV, Bazzi H, Vitebsky A, Witkiewicz A, Kovich OI, Kamino H, Shapiro LS, Amin SP, Orlow SJ, Christiano AM. 2006. Mutations in the desmoglein 4 gene underlie localized autosomal recessive hypotrichosis with monilethrix hairs and congenital scalp erosions. J Invest Dermatol 126:1286–1291.
Schepens I, Jaunin F, Begre N, Läderach U, Marcus K, Hashimoto T, Favre B, Borradori L. 2010. The protease inhibitor alpha-2-macroglobulin-like-1 is the p170 antigen recognized by paraneoplastic pemphigus autoantibodies in human. PLoS ONE 5:e12250.
Schlipf NA, Vahlquist A, Teigen N, Virtanen M, Dragomir A, Fismen S, Barenboim M, Manke T, Rösler B, Zimmer A, Fischer J. 2016. Whole-exome sequencing identifies novel autosomal recessive DSG1 mutations associated with mild SAM syndrome. Br J Dermatol 174:444–448.
Schmidt E, Zillikens D. 2010. Modern diagnosis of autoimmune blistering skin diseases. Autoimmun Rev 10:84–89.
Schweizer J. 2006. More than one gene involved in monilethrix: Intracellular but also extracellular players. J Invest Dermatol 126:1216–1219.
Sharma P, Mao X, Payne AS. 2007. Beyond steric hindrance: The role of adhesion signaling pathways in the pathogenesis of pemphigus. J Dermatol Sci 48:1–14.
Sibilia M, Wagner EF. 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 269:234–238.
Skålhegg BS, Huang Y, Su T, Idzerda RL, McKnight GS, Burton KA. 2002. Mutation of the Calpha subunit of PKA leads to growth retardation and sperm dysfunction. Mol Endocrinol 16:630–639.
Sonnenberg A, Liem RK. 2007. Plakins in development and disease. Exp Cell Res 313:2189–2203.
Soriano P, Montgomery C, Geske R, Bradley A. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 64:693–702.
Spicer AP, Rowse GJ, Lidner TK, Gendler SJ. 1995. Delayed mammary tumor progression in Muc-1 null mice. J Biol Chem 270:30093–30101.
Spindler V, Heupel WM, Efthymiadis A, Schmidt E, Eming R, Rankl C, Hinterdorfer P, Müller T, Drenckhahn D, Waschke J. 2009. Desmocollin 3-mediated binding is crucial for keratinocyte cohesion and is impaired in pemphigus. J Biol Chem 284:30556–30564.
Sprecher E. 2015. Paraneoplastic pemphigus: An entity still in search of an identity? Br J Dermatol 173:1363–1364.
Stahley SN, Kowalczyk AP. 2015. Desmosomes in acquired disease. Cell Tissue Res 360:439–456.
Syrris P, Ward D, Asimaki A, Sen-Chowdhry S, Ebrahim HY, Evans A, Hitomi N, Norman M, Pantazis A, Shaw AL, Elliott PM, McKenna WJ. 2006. Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy. Circulation 113:356–364.
Syrris P, Ward D, Asimaki A, Evans A, Sen-Chowdhry S, Hughes SE, McKenna WJ. 2007. Desmoglein-2 mutations in arrhythmogenic right ventricular cardiomyopathy: A genotype-phenotype characterization of familial disease. Eur Heart J 28:581–588.
Tada H, Hatoko M, Tanaka A, Kuwahara M, Muramatsu T. 2000. Expression of desmoglein I and plakoglobin in skin carcinomas. J Cutan Pathol 27:24–29.
Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, Barnard JA, Yuspa SH, Coffey RJ, Magnuson T. 1995. Targeted disruption of mouse EGF receptor: Effect of genetic background on mutant phenotype. Science 269:230–234.
Toosi S, Collins JW, Lohse CM, Wolz MM, Wieland CN, Camilleri MJ, Bruce AJ, McEvoy MT, Lehman JS. 2016. Clinicopathologic features of IgG/IgA pemphigus in comparison with classic (IgG) and IgA pemphigus. Int J Dermatol 55:e184–e190.
Tselepis C, Chidgey M, North A, Garrod D. 1998. Desmosomal adhesion inhibits invasive behavior. Proc Natl Acad Sci USA 95:8064–8069.
Tsuruta D, Ishii N, Hamada T, Ohyama B, Fukuda S, Koga H, Imamura K, Kobayashi H, Karashima T, Nakama T, Dainichi T, Hashimoto T. 2011. IgA pemphigus. Clin Dermatol 29:437–442.
Vasioukhin V, Bowers E, Bauer C, Degenstein L, Fuchs E. 2001. Desmoplakin is essential in epidermal sheet formation. Nat Cell Biol 3:1076–1085.
Wang Q, Peng D, Zhu S, Chen Z, Hu T, Soutto M, Saad R, Zhang S, Ei-Rifai W. 2014. Regulation of desmocollin3 expression by promoter hypermethylation is associated with advanced esophageal adenocarcinomas. J Cancer 5:457–464.
Wang H, Ducournau C, Saydaminova K, Richter M, Yumul R, Ho M, Carter D, Zubieta C, Fender P, Lieber A. 2015. Intracellular signaling and desmoglein 2 shedding triggered by human adenoviruses ad3, ad14, and Ad14P1. J Virol 89: 10841–10859.
Wang JM, Xiao YJ, Liang YH. 2015b. Novel D323G mutation of DSG4 gene in a girl with localized autosomal recessive hypotrichosis clinically overlapped with monilethrix. Int J Dermatol 54:1163–1168.
Whittock NV, Bower C. 2003. Genetic evidence for a novel human desmosomal cadherin, desmoglein 4. J Invest Dermatol 120:523–530.
Whittock NV, Ashton GH, Dopping-Hepenstal PJ, Gratian MJ, Keane FM, Eady RA, McGrath JA. 1999. Striate palmoplantar keratoderma resulting from desmoplakin haploinsufficiency. J Invest Dermatol 113:940–946.
Wiley BB, Rogolsky M. 1977. Molecular and serological differentiation of staphylococcal exfoliative toxin synthesized under chromosomal and plasmid control. Infect Immun 18:487–494.
Wong MP, Cheang M, Yorida E, Coldman A, Gilks CB, Huntsman D, Berean K. 2008. Loss of desmoglein 1 expression associated with worse prognosis in head and neck squamous cell carcinoma patients. Pathology 40:611–616.
Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kägi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev 12:806–819.
Xin Z, Yamaguchi A, Sakamoto K. 2014. Aberrant expression and altered cellular localization of desmosomal and hemidesmosomal proteins are associated with aggressive clinicopathological features of oral squamous cell carcinoma. Virchows Arch 465:35–47.
Xu T, Yang Z, Vatta M, Rampazzo A, Beffagna G, Pilichou K, Scherer SE, Saffitz J, Kravitz J, Zareba W, Danieli GA, Lorenzon A, Nava A, Bauce B, Thiene G, Basso C, Calkins H, Gear K, Marcus F, Towbin JA. 2010. Multidisciplinary study of right ventricular dysplasia investigators. Compound and digenic heterozygosity contributes to arrhythmogenic right ventricular cardiomyopathy. J Am Coll Cardiol 55:587–597. Erratum in: J Am Coll Cardiol. 2010 Mar 30;55(13):1401. Pillichou, Kalliopi [corrected to Pilichou, Kalliopi]. PMID: 20152563; PubMed Central PMCID: PMC2852685.
Yagi T, Aizawa S, Tokunaga T, Shigetani Y, Takeda N, Ikawa Y. 1993. A role for Fyn tyrosine kinase in the suckling behaviour of neonatal mice. Nature 366:742–745.
Yamaguchi T, Nishifuji K, Sasaki M, Fudaba Y, Aepfelbacher M, Takata T, Ohara M, Komatsuzawa H, Amagai M, Sugai M. 2002. Identification of the *Staphylococcus aureus* etd pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. Infect Immun 70:5835–5845.
Yashiro M, Nishioka N, Hirakawa K. 2006. Decreased expression of the adhesion molecule desmoglein-2 is associated with diffuse-type gastric carcinoma. Eur J Cancer 42:2397–2403.
Yong AA, Tey HL. 2013. Paraneoplastic pemphigus. Australas J Dermatol 54:241–250.
Zhou S, Ferguson DJ, Allen J, Wojnarowska F. 1997. The location of binding sites of pemphigus vulgaris and pemphigus foliaceus autoantibodies: A post-embedding immunoelectron microscopic study. Br J Dermatol 136:878–883.

ORAL DISEASES
Leading in Oral, Maxillofacial, Head & Neck Medicine

Oral Diseases (2016) doi:10.1111/odi.12527

www.wiley.com

INVITED CONCISE REVIEW

Desmosomes in disease: a guide for clinicians

A Celentano[1,2], N Cirillo[1]

[1]*Melbourne Dental School and Oral Health CRC, University of Melbourne, Melbourne, Vic., Australia;* [2]*Department of Neurosciences, Reproductive and Odontostomatological Sciences, Federico II University of Naples, Naples, Italy*

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.

Oral Diseases (2016) doi: 10.1111/odi.12527

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

Correspondence: Nicola Cirillo, Level 5, Melbourne Dental School, University of Melbourne, 3053 Carlton, Vic, Australia. Tel/Fax: +61 3 9341 1473, E-mail: Nicola.cirillo@unimelb.edu.au
Received 17 June 2016; accepted 18 June 2016

Table 1 Comprehensive summary of the diseases that target desmosome components

Type	*Disease*	*Molecular Target(S)*	*Manifestations*		*Histology*	*Immunofluorescence*	*Additional Diagnostic Tools*
			Intraoral	*Extraoral*			
Autoimmune	PV	Dsg1, Dsg3, Dsc1, Dsc2, Dsc3	Painful refractory erosions and ulcers of the oral mucosa	Flaccid bullae and erosions on skin. Lesions of: lips, pharynx, larynx, esophagus, eyelid conjunctiva and vagina.	Suprabasal acantholysis; 'row of tombstones' pattern of basal keratinocytes; sparse inflammatory infiltrate in the dermis with eosinophils	DIF: Intra-epidermal intercellular deposition of IgG and/or C3, in a 'cobblestone' or 'fishnet' pattern IIF: Intercellular deposition of IgG	ELISA: Dsg1 and Dsg3 autoantibodies Protein microarrays: additional non-Dsg IgGs
	PF	Dsg1, Dsc1, Dsc2, EVP, PPL	Mucosal involvement absent	Fragile blisters, shallow erosions, erythematous patches, crusts most frequently affecting head, face, chest and back	Subcorneal or granular layer acantholysis; mixed inflammatory infiltrate in the superficial dermis	DIF: Intercellular deposition of IgG IIF: Intercellular deposition of IgG	ELISA: Dsg1 autoantibodies
	PNP	Dsg1, Dsg3, Dsc2, Dsc3, Pg, Pkp1, Pkp2, Pkp3, Dsp1, Dsp2, EVP, PPL, Plectin, epiplakin, BP230, A2ML1	Severe and extensive mucosal erosions and ulcerations crusting of vermillion border	Variable cutaneous findings, for example blisters, erosions, lichenoid lesions, bronchiolitis obliterans; severe mucous membrane involvement	Variable findings: suprabasal acantholysis, keratinocyte necrosis, and lichenoid interface dermatitis are most common	DIF: Intercellular and/or basement membrane zone deposition of IgG and/or C3 IIF: Intercellular deposition of IgG	ELISA: IgG autoantibodies to Dsg1, Dsg3, Dsc1, Dsc3, EVP, PPL, Dsp1, Dsp2, plectin, and BP230
	IAP (i-e)	Dsg1, Dsg3, Dsc1	Mucosal involvement usually absent	Vesicles, pustules, crusts on skin; annular, circinate, or herpetiform morphology	Intra-epidermal pustules; minimal acantholysis; mixed infiltrate in dermis	DIF: Intercellular deposition of IgA[a] IIF: Intercellular deposits of IgA[a]	ELISA: Dsg1 and Dsg3 autoantibodies have been reported in some patients
	IAP (s-c)	Dsc1			Subcorneal clefts and pustules; minimal acantholysis; mixed infiltrate in dermis		ELISA: Dsc1 autoantibodies[b]
	EM/SJS	Dsp	Variable: erosive/ bullous lesions. The buccal mucosa, vermilion border, and labial mucosa are the most commonly affected sites	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 >10% of BSA	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	DIF: Strongly positive linear staining for C3 and weakly positive immunoglobulin G IIF: Can be positive	ELISA: Peptide-specific Dp Autoantibodies (PMID: 16086758) PCR: to asses HSV and M. Pneumoniae presence.

(continued)

Table 1 (continued)

Type	*Disease*	*Molecular Target(S)*	*Manifestations*		*Histology*	*Immunofluorescence*	*Additional Diagnostic Tools*
			Intraoral	*Extraoral*			
Infectious	BI	Dsg1	Flaking and fissuring of the lips	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.	Intra-epidermal cleavage, beneath or within the granular layers; the underlying dermis contains mixed infiltrates of neutrophils and lymphocytes	Not usually indicated	ELISA: No apparent IgM reactivity against Dsg1 or Dsg3 Gram stain and culture: of pus or exudate is recommended
	SSSS	Dsg1	Lack of mucous membrane involvement; Perioral manifestation: Flaking and fissuring of the lips	Diffuse erythema, fever, positive Nikolsky's sign. Rash that evolves in lesions ranging from localized blisters to severe exfoliation of >90% of BSA	Subcorneal cleavage, Presence of only a stratum corneum with a single epidermal cell layer; absence of necrotic keratinocytes.	Not usually indicated	ELISA: low titers of anti-Dsg1 IgG autoantibodies Gram stain and culture: swab of the skin, orificial areas, and mucous membranes
	Respiratory and urinary tract infections (ad 3,7,11 & 14),	Dsg2	Exudative tonsillitis	Pharyngitis and coryza, acute respiratory disease, bronchitis, pneumonia, pharyngoconjunctival fever, conjunctivitis, laryngotracheitis, fever, malaise, headache, myalgia, abdominal pain, otitis media, acute	Not usually indicated	Not usually indicated	ELISA: adenovirus-specific ELISA kit is commercially available. Viral culture: of the nasopharynx or throat PCR and real-time PCR adenovirus detection
	GD	Dsc2, Dsc3	50% of patients clear the infection in absence of clinical symptoms 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%) 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		Histopathological exam require a lot of effort and experienced staff lead to false results in 10–50% of the cases. Diagnosis is made with direct observation of the cysts or trophozoites microscopically in the stool or duodenal fluid or examination of small intestinal samples	DIF: fluorescent monoclonal antibodies binding specifically to G. intestinalis cysts are used (sensitivity 100%, specificity 100%)	ELISA: Monoclonal ELISA detect G. intestinalis cyst antigens in the stool (sensitivity 88–99%, specificity 100%). Various nucleic acid amplification techniques: (NAATs), MICROSPHERES (Luminex), or LAMP (loop-mediated isothermal amplification)
	BA-LT (Anthrax)	Pg, Dsp	Oropharyngeal: Pseudomembranous ulcers and necrosis reported to be localized at base and dorsal surface of the tongue, hard palate, soft palate, uvula, and tonsils	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: Ulcerations can occur in the stomach, esophagus, and duodenum Respiratory: hemorrhagic necrosis of the thoracic lymph nodes draining the lungs	Bacilli presence easily identifiable. Focal hemorrhage, necrosis, edema, and congestion. Infiltration by neutrophils and prominent immunoblasts, various degrees of mononuclear inflammation, cyst formation	DIF: intracellular distribution and fine structure of ZO-1, E-cadherin, and β-catenin are altered by lethal toxin in alveolar epithelial cells	ELISA: detection of antiprotective antigen (PA) immunoglobulin (Ig)G Standard culture and Susceptibility testing IHC: The organism can be identified by direct observation through immunohistochemical staining. PCR: LRN, Standard or RT PCR performed on a variety of specimen types

(continued)

Table 1 (continued)

Type	*Disease*	*Molecular Target(S)*	*Manifestations*		*Histology*	*Immunofluorescence*	*Additional Diagnostic Tools*
			Intraoral	*Extraoral*			
Genetic	PPK	Dsc2, Pkp1	Perioral hyperkeratotic plaques with severe pruritus, hypodontia and dental defects, periodontitis, leukokeratoses, and leukoplakia can be present	Thick, mild, or severe diffuse hyperkeratosis, hyperhidrosis	Epidermolytic or non-epidermolytic hyperkeratosis. Enlarged intercellular spaces and partial separation of keratinocytes	Not indicated	Genetic test: responsible gene: Dsg1, KRT1, KRT6c, KRT9, KRT16, SERPINB7, AQP5, SLURP1, TRPV3, AAGAB, COOL14A1
	SPPK	Dsg1, Dsp		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	Reduction in the number of desmosomes, accompanied by widening of intercellular spaces between suprabasal keratinocytes, and retracted KIFs	Not indicated	Genetic test: responsible gene: Dsg1 (MIM no. 148700), Dsp (MIM no. 612908), and KRT1 (MIM no. 607654)
	SAM	Dsg1	Limited oral involvement	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	Psoriasiform dermatitis, with focal intracellular and intercellular edema and acantholysis within the spinous and granular layers	Not indicated	Genetic test: two homozygous mutations in Dsg1 gene
		Dsp	Marked hypodontia, poor periodontal health	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	Irregular psoriasiform hyperplasia, hyperkeratosis and parakeratosis, and a mild superficial dermal inflammatory infiltrate, florid subcorneal pustular dermatosis		

(continued)

Table 1 (continued)

Type	*Disease*	*Molecular Target(S)*	*Manifestations*		*Histology*	*Immunofluorescence*	*Additional Diagnostic Tools*
			Intraoral	*Extraoral*			
	ARVD/C	Dsg2, Dsc2, Pkp2, Pkp4, Dsp	Oral involvement usually absent	Myocardial atrophy and fibrofatty replacement of cardiac myocytes, ventricular arrhythmias, sudden cardiac death, syncope, and end-stage heart failure, typically affecting the right ventricle	Endomyocardial biopsy (EMB) is not recommended	Not indicated	Genetic test: truncating mutations in Dsp ECG Echocardiogram Cardiac magnetic resonance
	Left dominant arrythmogenic cardiomyopathy	Dsp	Oral involvement usually absent	Palpitations, less frequent syncope, chest pain, dyspnea, and, rarely, sudden cardiac death	Endomyocardial biopsy (EMB) is not recommended	Not indicated	Genetic test: truncating mutations in Dsp ECG Echocardiogram Cardiac magnetic resonance
	LAH	Dsg4	Oral involvement usually absent	Hypotrichosis of the scalp, chest, arms and legs	Histology of scalp skin revealed thin and atrophic Hair Follicles and hair shafts that often coil up within the skin	Not indicated	Genetic test: several mutations in the Dsg4 gene (18q12.1; 3q27.3; 13q14.11;13q21.32)
	Recessive monilethrix	Dsg4	Oral involvement usually absent	Moniliform hairs, pili torti, trichoschisis, trichorrhexis nodosa-like defects, tapered hairs	Curled ingrown hair shafts within the hair follicle	Not indicated	Genetic test: mutations in three type II hair cortex keratins ($KRT8_1$,$KRT8_3$, $KRT8_6$), and Dsg4
	Wooly hair	Dsc2	Oral involvement usually absent	Hair with: elliptical cross section, variations in caliber, axis rotation, kinked formation, and non-homogeneous keratinization	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	Not indicated	Genetic test: can be carried out when the gene locus is known Hair examination: is carried out by light and electron microscopy Trichogram
	Naxos disease	Pg, Dsp	Leukokeratoses, and hypo/oligodontia can be present	Familial wooly hair, diffuse non-epidermolytic PPK, ARVC with heart-rhythm disruptions	Not usually indicated	Not indicated	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
	Carvajal syndrome	Dsp	Hypo/oligodontia can be present	Dilated left ventricular Cardiomyopathy, wooly hair, keratoderma	Not usually indicated	Not indicated	Genetic test: utosomal-dominant mutations in the DSP gene

(continued)

Table 1 (continued)

Type	*Disease*	*Molecular Target(S)*	*Manifestations*		*Histology*	*Immunofluorescence*	*Additional Diagnostic Tools*
			Intraoral	*Extraoral*			
	EDFSF	Pkp1	Perioral fissuring	Widespread skin fragility, non-cicatricial alopecia, PPK, nail dystrophy, hypohidrosis	Orthokeratosis, hypergranulosis and wide intercellular spaces between keratinocytes,	Not indicated	Genetic test: At least eleven different recessively inherited mutations in the Pkp1 gene (chromosome 1q32) Electron Microscopy
	Lethal congenital epidermolysis bullosa	Pg	Tongue mobility reduction	Severe skin fragility, generalized epidermolysis, massive transcutaneous fluid loss, esophageal stenosis, Pyloric atresia	Pronounced acantholysis, and cleavage within the epidermis, with the loss of upper spinous, granular and horny layers.	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.	Genetic test: homozygous nonsense JUP mutation, c.1615C>T, p.Q539X, that leads to the lack of Pg. Electron Microscopy
	Lethal acantholytic epidermolysis bullosa	Dsp	Possible neonatal teeth	Complete alopecia, nail loss, cardiomyopathy, ears malformation, extensive neonatal epithelial shedding (>50% BSA), syndactyly, neonatal death	Acantholytic cell-poor intra-epidermal blister with a single row of preserved but partially necrotic basal cells on the blister floor	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	Genetic test: genomic DNA (gDNA) can be extracted from peripheral blood leukocytes, or from formalin-fixed paraffin-embedded (FFPE) skin tissue Electron Microscopy
	Brugada syndrome	Pkp2	Absence of oral involvement	Life-threatening ventricular arrhythmias, sudden cardiac arrest, atrial fibrillation, syncope, Nocturnal agonal respiration	From autopsy: Absence of active myocardial inflammation, moderate myocardial hypertrophy, moderate fibrosis and fatty replacement of the myocardium (predominantly in the septum)	Not indicated	Genetic test: sequencing SCN5A gene commercially available Case by case evaluation of: drug challenge (e.g., sodium channel blockers), signal-average ECG invasive electrophysiology testing

ARVD/C, 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.

[a]Indirect immunofluorescence is negative in around 50 percent of patients with IgA pemphigus.

[b]Test 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 spinous 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 (Schepens *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 acantholysis 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 been also 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 *(SPPK) 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 (McAleer *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* (EDFSF) 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/cardiomyopathy (ARVD/C) and left dominant arrythmogenic cardiomyopathy* 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 on 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 fluorescin-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 substrate is the monkey esophagus. In general, is it 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-c type. (Kneisel and Hertl, 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 immunoserological 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

Abasq C, Mouquet H, Gilbert D *et al* (2009). ELISA testing of anti-desmoglein 1 and 3 antibodies in the management of pemphigus. *Arch Dermatol* **145**: 529–535.

Adam RD (2001). Biology of Giardia lamblia. *Clin Microbiol Rev* **14**: 447–475.

Amagai M (2010). Autoimmune and infectious skin diseases that target desmogleins. *Proc Jpn Acad Ser B Phys Biol Sci* **86**: 524–537.

Amagai M, Stanley JR (2012). Desmoglein as a target in skin disease and beyond. *J Invest Dermatol* **132**: 776–784.

Amagai M, Matsuyoshi N, Wang ZH, Andl C, Stanley JR (2000). Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. *Nat Med* **6**: 1275–1277.

Awad MM, Dalal D, Cho E *et al* (2006). DSG2 mutations contribute to arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Am J Hum Genet* **79**: 136–142.

Bass-Zubek AE, Godsel LM, Delmar M, Green KJ (2009). Plakophilins: multifunctional scaffolds for adhesion and signaling. *Curr Opin Cell Biol* **21**: 708–716.

Bizzozero G (1864). Delle cellule cigliate, del reticolo Malpighiano dell'epidermide. *Ann Univ Med* **190**: 110–118.

Chiorean R, Mahler M, Sitaru C (2014). Molecular diagnosis of autoimmune skin diseases. *Rom J Morphol Embryol* **55** (Suppl): 1019–1033.

Cirillo N (2016). Desmosome assembly, homeostasis, and desmosomal disease. *Cell Health Cytoskelet* **8**: 9–23.

Cirillo N, Prime SS (2009). Desmosomal interactome in keratinocytes: a systems biology approach leading to an understanding of the pathogenesis of skin disease. *Cell Mol Life Sci* **66**: 3517–3533.

Cirillo N, Gombos F, Lanza A (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.

Cirillo N, Cozzani E, Carrozzo M, Grando SA (2012). Urban legends: pemphigus vulgaris. *Oral Dis* **18**: 442–458.

Ellis E, Sidhu S (2014). Circulating plakin autoantibodies in a patient with erythema multiforme major: are they pathogenic or a manifestation of epitope spreading? *Australas J Dermatol* **55**: 266–269.

Hata T, Nishimoto S, Nagao K *et al* (2013). Ectopic expression of epidermal antigens renders the lung a target organ in paraneoplastic pemphigus. *J Immunol* **191**: 83–90.

Huber O, Petersen I (2015). 150th Anniversary series: desmosomes and the hallmarks of cancer. *Cell Commun Adhes* **22**: 15–28.

Johnson SM, Smoller BR, Horn TD (1999). Erythema multiforme associated human autoantibodies against desmoplakin I and II. *J Invest Dermatol* **112**: 395–396.

Joly P, Litrowski N (2011). Pemphigus group (vulgaris, vegetans, foliaceus, herpetiformis, brasiliensis). *Clin Dermatol* **29**: 432–436.

Kalantari-Dehaghi M, Anhalt GJ, Camilleri MJ *et al* (2013). Pemphigus vulgaris autoantibody profiling by proteomic technique. *PLoS One* **8**: e57587.

Kitajima Y (2014). 150(th) anniversary series: desmosomes and autoimmune disease, perspective of dynamic desmosome remodeling and its impairments in pemphigus. *Cell Commun Adhes* **21**: 269–280.

Kneisel A, Hertl M (2011). Autoimmune bullous skin diseases. Part 2: diagnosis and therapy. *J Dtsch Dermatol Ges* **9**: 927–947.

Langer M, Duggan ES, Booth JL *et al* (2012). Bacillus anthracis lethal toxin reduces human alveolar epithelial barrier function. *Infect Immun* **80**: 4374–4387.

McAleer MA, Pohler E, Smith FJ *et al* (2015). Severe dermatitis, multiple allergies, and metabolic wasting syndrome caused by a novel mutation in the N-terminal plakin domain of desmoplakin. *J Allergy Clin Immunol* **136**: 1268–1276.

Mignogna MD, Fortuna G, Leuci S, Ruoppo E (2010). Oropharyngeal pemphigus vulgaris and clinical remission: a long-term, longitudinal study. *Am J Clin Dermatol* **11**: 137–145.

Moss C, Martinez-Mir A, Lam H, Tadin-Strapps M, Kljuic A, Christiano AM (2004). A recurrent intragenic deletion in the desmoglein 4 gene underlies localized autosomal recessive hypotrichosis. *J Invest Dermatol* **123**: 607–610.

Nagaoka I, Matsui K, Ueyama T *et al* (2006). Novel mutation of plakophilin-2 associated with arrhythmogenic right ventricular cardiomyopathy. *Circ J* **70**: 933–935.

Otten JV, Hashimoto T, Hertl M, Payne AS, Sitaru C (2014). Molecular diagnosis in autoimmune skin blistering conditions. *Curr Mol Med* **14**: 69–95.

Payne AS, Stanley JR (2012). Pemphigus. In: Goldsmith LA, Katz SI, Gilchrest BA *et al* (eds). *Fitzpatrick's Dermatology in General Medicine*. **Vol 1**, 8th edn. McGraw Hill: New York, pp. 586.

Ruocco V, Ruocco E, Lo Schiavo A, Brunetti G, Guerrera LP, Wolf R (2013). Pemphigus: etiology, pathogenesis, and inducing or triggering factors: facts and controversies. *Clin Dermatol* **31**: 374–381.

Samuelov L, Sarig O, Harmon RM *et al* (2013). Desmoglein 1 deficiency results in severe dermatitis, multiple allergies and metabolic wasting. *Nat Genet* **45**: 1244–1248.

Schepens I, Jaunin F, Begre N *et al* (2010). The protease inhibitor alpha-2-macroglobulin-like-1 is the p170 antigen recognized by paraneoplastic pemphigus autoantibodies in human. *PLoS One* **5**: e12250.

Stahley SN, Kowalczyk AP (2015). Desmosomes in acquired disease. *Cell Tissue Res* **360**: 439–456.

Syrris P, Ward D, Asimaki A *et al* (2006). Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy. *Circulation* **113**: 356–364.

Toosi S, Collins JW, Lohse CM *et al* (2016). Clinicopathologic features of IgG/IgA pemphigus in comparison with classic (IgG) and IgA pemphigus. *Int J Dermatol* **55**: e184–e190.

Tselepis C, Chidgey M, North A, Garrod D (1998). Desmosomal adhesion inhibits invasive behavior. *Proc Natl Acad Sci U S A* **95**: 8064–8069.

Vasioukhin V, Bowers E, Bauer C, Degenstein L, Fuchs E (2001). Desmoplakin is essential in epidermal sheet formation. *Nat Cell Biol* **3**: 1076–1085.

Vodegel RM, de Jong MC, Meijer HJ, Weytingh MB, Pas HH, Jonkman MF (2004). Enhanced diagnostic immunofluorescence using biopsies transported in saline. *BMC Dermatol* **4**: 10.

Yong AA, Tey HL (2013). Paraneoplastic pemphigus. *Australas J Dermatol* **54**: 241–250.

Zone JJ (2009). The value of desmoglein 1 and 3 antibody ELISA testing in patients with pemphigus. *Arch Dermatol* **145**: 585–587.

Journal of Cellular Physiology

The Non-Conventional Effects of Glucocorticoids in Cancer

SIMRA AZHER,[1] OMID AZAMI,[1] CATERINA AMATO,[1] MICHAEL MCCULLOUGH,[1,2] ANTONIO CELENTANO,[1,3] AND NICOLA CIRILLO[1,2]*

[1]*Melbourne Dental School, University of Melbourne, Carlton, Victoria, Australia*

[2]*Oral Health Cooperative Research Centre (CRC), University of Melbourne, Carlton, Victoria, Australia*

[3]*Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Naples, Italy*

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. We have recently shown that epithelial tissues, 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 induce effects that are more complex and controversial than previously acknowledged. Published studies clearly demonstrate that this class of molecules influences pathophysiological processes that are strictly related to malignancy, providing the rationale for further investigation.

J. Cell. Physiol. 231: 2368–2373, 2016.

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 (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 Pfitzenmaier, 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, 11b-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 Pfitzenmaier, 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).

*Correspondence to: Nicola Cirillo, Melbourne Dental School and Oral Health CRC, University of Melbourne, 720 Swanston Street, 3053 Carlton, VIC, Australia. E-mail: nicola.cirillo@unimelb.edu.au

Manuscript Received: 25 April 2016
Manuscript Accepted: 25 April 2016

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 26 April 2016.
DOI: 10.1002/jcp.25408

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 tumorigenesis (Feng et al., 2012). 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 (Heikkilä 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 documented 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, 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 (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

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 (Lanza et al., 2009). 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/non-tumorigenic (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 (Kennedy et al., 2015). 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.

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 (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 (Schlossmacher 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 Cidlowski, 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 (Kesić 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 Tinitigan, 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 glycolysis-dependent tumors and also

TABLE 1. Dysregulation of serum cortisol, 11β-HSDs, and glucocorticoid receptor in different types of cancers

Study	Type of cancer	Dysregulated molecule	Specific findings
Bernabé et al. (2012)	Oral squamous cell carcinoma (SCC)	Cortisol	Elevated plasma and salivary cortisol levels in SCC and also higher levels in advanced stage compared to initial stage.
Rasmuson et al. (2001)	Renal cell carcinoma (RCC)	Cortisol	Serum cortisol levels higher in RCC and positively correlated with tumor diameter and grade. Elevated cortisol levels also had worse prognosis.
Sephton et al. (2000)	Metastatic breast cancer	Cortisol	Flat diurnal salivary cortisol circadian rhythm where elevated cortisol levels associated with early mortality.
Sephton et al. (2013)	Lung cancer	Cortisol	Flattening of the diurnal cortisol rhythm associated with early death.
Terao et al. (2013)	Skin SCC, basal cell carcinoma (BCC) and seborrheic keratosis (SK)	11β-HSD1 and 11β-HSD2 enzyme	Reduction in 11β-HSD1 expression with increased keratinocyte proliferation. 11β-HSD2 expression increased in basal cell proliferating conditions such as BCC and SK.
Parks et al. (1998b)	DMS-79 cells (cell line derived from an ACTH-producing small cell lung cancer).	11β-HSD2 enzyme	11β-HSD2 normally not found in healthy lung tissue, was expressed in cancerous lung tissue.
Temkin et al. (2006)	Ovarian epithelial cancer	11β-HSD2 enzyme	11β-HSD2 enzyme not found in normal post-menopausal ovarian tissue was increased in cancer cells.
Bland et al. (1999)	Osteosarcoma	11β-HSD2 enzyme	Overexpression of 11β-HSD2 enzyme in cancer, compared with predominant expression of 11β-HSD1 enzyme in normal human osteoblast cells.
Žbánková et al. (2004)	Colorectal cancer	11β-HSD2 and 11β-HSD1 enzyme	Decrease in the abundance of 11β-HSD2 mRNA and enzyme activity in cancer tissue. Also demonstrated increase in 11β-HSD1 in some samples.
Cirillo et al. (2012)	Oral squamous cell carcinoma (SCC)	11β-HSD2 enzyme	Decrease in 11β-HSD2 expression in cancer.
Lu et al. (2011)	Breast cancer	11β-HSD1 enzyme	Decrease in 11β-HSD1 expression in cancer.
Budunova et al. (1997)	Mouse epidermal papillomas and SCC.	Glucocorticoid receptor	In early skin papilloma GR expression is reduced but in late papilloma and in SCC, GR levels are similar or higher than normal.
Spiegelman et al. (1997)	Mouse epidermal papillomas and SCC.	Glucocorticoid receptor	No significant changes in GR gene structure and expression in cancer but resistance to glucocorticoid fluocinolone acetonid more likely due to alterations in receptor function.
Waters et al. (2004)	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Normal expression of GR, however its functionality altered by subtle changes in co-factors such as Nuclear co-repressor which was overexpressed in cancer.
Ray et al. (1996)	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Mutations in GR gene structure lead to its impaired function.
Parks et al. (1998a)	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Abnormal splicing of the GR transcript causes GR resistance to GC stimulation in cancer cells.
Kay et al. (2011)	Small cell lung carcinoma (SCLC)	Glucocorticoid receptor	Increased DNA methylation of promoter c leads to decreased GR expression.
Li et al. (1996)	Transformed A5 mouse lung cells	Glucocorticoid receptor	Transformed cells contain functional GR but express cjun which antagonizes glucocorticoids.

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

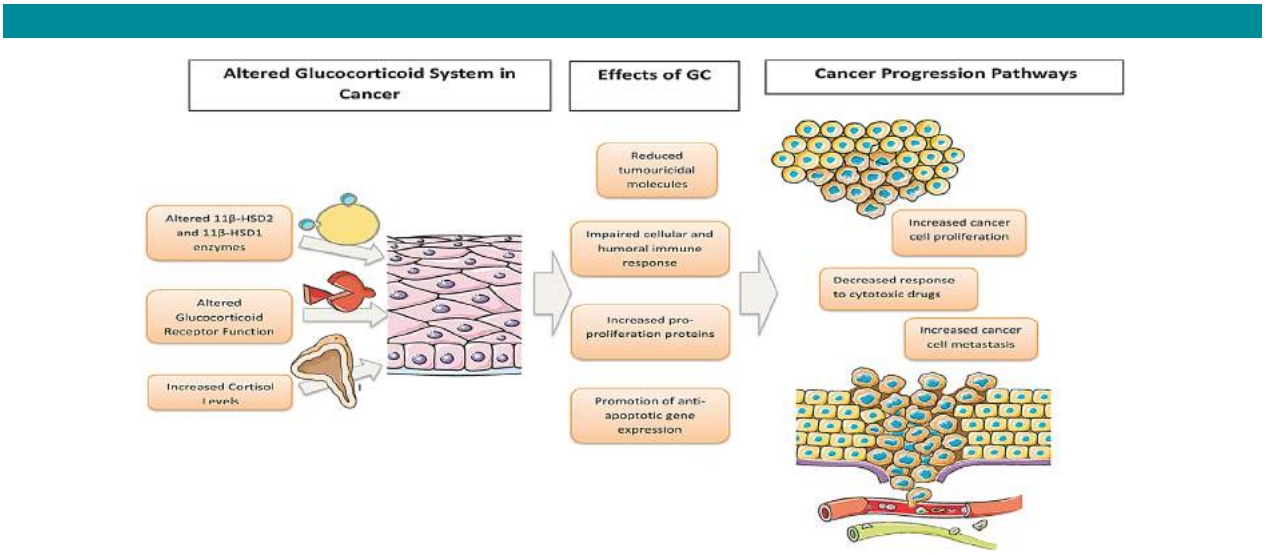


Fig. 1. Schematic representation of cancer-promoting glucocorticoid effects.

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.

Literature Cited

Bernabé DG, Tamae AC, Biasoli ÉR, Oliveira SH. 2011. Stress hormones increase cell proliferation and regulates interleukin-6 secretion in human oral squamous cell carcinoma cells. Brain Behav Immun 25:574–583.

Bernabé DG, Tamae AC, Miyahara GI, Sundefeld MLM, Oliveira SP, Biasoli ÉR. 2012. Increased plasma and salivary cortisol levels in patients with oral cancer and their association with clinical stage. J Clin Pathol 65:934–939.

Bland R, Worker C, Noble B, Eyre L, Bujalska I, Sheppard M, Stewart P, Hewison M. 1999. Characterization of 11beta-hydroxysteroid dehydrogenase activity and corticosteroid receptor expression in human osteosarcoma cell lines. J Endocrinol 161:455–464.

Budunova IV, Carbajal S, Kang Hi, Viaje A, Slaga TJ. 1997. Altered glucocorticoid receptor expression and function during mouse skin carcinogenesis. Mol Carcinogenesis 18:177–185.

Cirillo N, Hassona Y, Pignatelli M, Gasparoto T, Morgan D, Prime S. 2012. Characterization of a novel oral glucocorticoid system and its possible role in disease. J Dental Res 91:97–103.

Cirillo N, Prime SS. 2011. Keratinocytes synthesize and activate cortisol. J Cell Biochem 112:1499–1505.

Deguchi M, Isobe Y, Matsukawa S, Yamaguchi A, Nakagawara G. 1998. Usefulness of metyrapone treatment to suppress cancer metastasis facilitated by surgical stress. Surgery 123:440–449.

Duffy SA, Taylor JM, Terrell JE, Islam M, Li Y, Fowler KE, Wolf GT, Teknos TN. 2008. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. Cancer 113:750–757.

Feng X, Reini SA, Richards E, Wood CE, Keller-Wood M. 2013. Cortisol stimulates proliferation and apoptosis in the late gestation fetal heart: Differential effects of mineralocorticoid and glucocorticoid receptors. Am J Physiol Regul Integr Comp Physiol 305:R343–R350.

Feng Z, Liu L, Zhang C, Zheng T, Wang J, Lin M, Zhao Y, Wang X, Levine AJ, Hu W. 2012. Chronic restraint stress attenuates p53 function and promotes tumorigenesis. Proc Natl Acad Sci 109:7013–7018.

Guendisch S, Boeckeler E, Behrends U, Amtmann E, Ehrhardt H, Jeremias I. 2012. Glucocorticoids augment survival and proliferation of tumor cells. Anticancer Res 32:4251–4261.

Heikkilä K, Ebrahim S, Lawlor DA. 2008. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. Eur J Cancer 44:937–945.

Herr I, Ucur E, Herzer K, Okouoyo S, Ridder R, Krammer PH, Doeberitz MVK, Debatin KM. 2003. Glucocorticoid co-treatment induces apoptosis resistance toward cancer therapy in carcinomas. Cancer Res 63:3112–3120.

Herr I, Pfitzenmaier J. 2006. Glucocorticoid use in prostate cancer and other solid tumours: Implications for effectiveness of cytotoxic treatment and metastases. Lancet Oncol 7:425–430.

Hogan MM, Vogel SN. 1988. Inhibition of macrophage tumoricidal activity by glucocorticoids. J Immunol 140:513–519.

Hosking D, Stone M, Foote J. 1990. Potentiation of calcitonin by corticosteroids during the treatment of the hypercalcaemia of malignancy. Eur J Clin Pharmacol 38:37–41.

Ismail SB, Kumar SK, Zain RB. 2007. Oral lichen planus and lichenoid reactions: Etiopathogenesis, diagnosis, management and malignant transformation. J Oral Sci 49:89–106.

Itoi S, Terao M, Murota H, Katayama I. 2013. 11β-Hydroxysteroid dehydrogenase 1 contributes to the pro-inflammatory response of keratinocytes. Biochem Biophys Res Commun 440:265–270.

Jobe NP, Rösel D, Dvořánková B, Kodet O, Lacina L, Mateu R, Smetana K, Brábek J. 2016. Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit CAF-induced human melanoma cell invasiveness. Histochem Cell Biol 144:1–13.

Kaplan I, Ventura-Sharabi Y, Gal G, Calderon S, Anavi Y. 2012. The dynamics of oral lichen planus: A retrospective clinicopathological study. Head Neck Pathol 6:178–183.

Kay P, Schlossmacher G, Matthews L, Sommer P, Singh D, White A, Ray D. 2011. Loss of glucocorticoid receptor expression by DNA methylation prevents glucocorticoid induced apoptosis in human small cell lung cancer cells. PLoS ONE 6:e24839.

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

Kennedy D, Pignatelli M, Hassona Y, Prime SS, Cirillo N. 2015. The role of the glucocorticoid system in anchorage-independence during progression of squamous cell carcinoma. Am J Oral Med 1:8–19.

Kenouch S, Lombes M, Delahaye F, Eugene E, Bonvalet J-P, Farman N. 1994. Human skin as target for aldosterone: Coexpression of mineralocorticoid receptors and 11 beta-hydroxysteroid dehydrogenase. J Clin Endocrinol Metab 79:1334–1341.

Kesić L, Obradović R, Mihailović D, Radičević G, Stanković S, Todorović K. 2009. Incidence and treatment outcome of oral lichen planus in southeast Serbia in a 10-year period (1997–2007). Vojnosanitetski Pregled 66:434–439.

Lanza A, Stellavato A, Heulfe I, Landi C, Gombos F, Cirillo N. 2009. Serum of patients with oral pemphigus vulgaris impairs keratinocyte wound repair in vitro: A time-lapse study on the efficacy of methylprednisolone and pyridostigmine bromide. Oral Dis 15:478–483.

Li J, Johnson TA, Hanson LA, Beer DG. 1996. Loss of glucocorticoid-dependent growth inhibition in transformed mouse lung cells. Mol Carcinogenesis 16:213–220.

Lodi G, Carrozzo M, Furness S, Thongprasom K. 2012. Interventions for treating oral lichen planus: A systematic review. Br J Dermatol 166:938–947.

Lu L, Zhao G, Ouellet J, Fan Z, Labrie F, Pelletier G. 2011. Expression of 11β-hydroxysteroid dehydrogenase type 1 in breast cancer and adjacent non-malignant tissue. An immunocytochemical study. Pathol Oncol Res 17:627–632.

Mazzoccoli G, Carughi S, De Cata A, La Viola M, Giuliani A, Tarquini R, Perfetto F. 2003. Neuroendocrine alterations in lung cancer patients. Neuroendocrinol Lett 24:77–82.

Nagata M, Fujita H, Ida H, Hoshina H, Inoue T, Seki Y, Ohnishi M, Ohyama T, Shingaki S, Kaji M. 2003. Identification of potential biomarkers of lymph node metastasis in oral squamous cell carcinoma by cDNA microarray analysis. Int J Cancer 106:683–689.

Nakane T, Szentendrei T, Stern L, Virmani M, Seely J, Kunos G. 1990. Effects of IL-1 and cortisol on beta-adrenergic receptors, cell proliferation, and differentiation in cultured human A549 lung tumor cells. J Immunol 145:260–266.

Newton R. 2000. Molecular mechanisms of glucocorticoid action: What is important? Thorax 55:603–613.

Oakley RH, Cidlowski JA. 2011. Cellular processing of the glucocorticoid receptor gene and protein: New mechanisms for generating tissue-specific actions of glucocorticoids. J Biol Chem 286:3177–3184.

Olnes MJ, Kotliarov Y, Biancotto A, Cheung F, Chen J, Shi R, Zhou H, Wang E, Tsang JS, Nussenblatt R; CHI Consortium. 2016. Effects of systemically administered hydrocortisone on the human immunome. Sci Rep 6:23002.

Parks LL, Turney MK, Detera-Wadleigh S, Kovacs WJ. 1998a. An ACTH-producing small cell lung cancer expresses aberrant glucocorticoid receptor transcripts from a normal gene. Mol Cell Endocrinol 142:175–181.

Parks LL, Turney MK, Gaitan D, Kovacs WJ. 1998b. Expression of 11β-hydroxysteroid dehydrogenase type 2 in an ACTH-producing small cell lung cancer. J Steroid Biochem Mol Biol 67:341–346.
Rabbitt E, Gittoes N, Stewart P, Hewison M. 2003. 11β-hydroxysteroid dehydrogenases, cell proliferation and malignancy. J Steroid Biochem Mol Biol 85:415–421.
Rasmuson T, Ljungberg B, Grankvist K, Jacobsen J, Olsson T. 2001. Increased serum cortisol levels are associated with high tumour grade in patients with renal cell carcinoma. Acta Oncologica 40:83–87.
Ray DW, Davis JR, White A, Clark AJ. 1996. Glucocorticoid receptor structure and function in glucocorticoid-resistant small cell lung carcinoma cells. Cancer Res 56:3276–3280.
Ren R, Oakley RH, Cruz-Topete D, Cidlowski JA. 2012. Dual role for glucocorticoids in cardiomyocyte hypertrophy and apoptosis. Endocrinology 153:5346–5360.
Romero LM, Raley-Susman KM, Redish DM, Brooke SM, Horner HC, Sapolsky RM. 1992. Possible mechanism by which stress accelerates growth of virally derived tumors. Proc Natl Acad Sci 89:11084–11087.
Sanchis A, Alba L, Latorre V, Sevilla LM, Pérez P. 2012. Keratinocyte-targeted overexpression of the glucocorticoid receptor delays cutaneous wound healing. PLoS ONE 7:e29701.
Schlossmacher G, Stevens A, White A. 2011. Glucocorticoid receptor-mediated apoptosis: Mechanisms of resistance in cancer cells. J Endocrinol 211:17–25.
Scully C, Eisen D, Carrozzo M. 2000. Management of oral lichen planus. Am J Clin Dermatol 1:287–306.
Sephton SE, Lush E, Dedert EA, Floyd AR, Rebholz WN, Dhabhar FS, Spiegel D, Salmon P. 2013. Diurnal cortisol rhythm as a predictor of lung cancer survival. Brain Behav Immun 30:S163–S170.
Sephton SE, Sapolsky RM, Kraemer HC, Spiegel D. 2000. Diurnal cortisol rhythm as a predictor of breast cancer survival. J Natl Cancer Inst 92:994–1000.
Seyfried TN, Shelton LM, Mukherjee P. 2010. Does the existing standard of care increase glioblastoma energy metabolism? Lancet Oncol 11:811–813.
Sidler D, Renzulli P, Schnoz C, Berger B, Schneider-Jakob S, Flück C, Inderbitzin D, Corazza N, Candinas D, Brunner T. 2011. Colon cancer cells produce immunoregulatory glucocorticoids. Oncogene 30:2411–2419.
Slominski AT, Manna PR, Tuckey RC. 2014. Cutaneous glucocorticosteroidogenesis: Securing local homeostasis and the skin integrity. Exp Dermatol 23:369–374.
Spiegelman VS, Budunova IV, Carbajal S, Slaga TJ. 1997. Resistance of transformed mouse keratinocytes to growth inhibition by glucocorticoids. Mol Carcinogenesis 20:99–107.
Stojadinovic O, Lee B, Vouthounis C, Vukelic S, Pastar I, Blumenberg M, Brem H, Tomic-Canic M. 2007. Novel genomic effects of glucocorticoids in epidermal keratinocytes inhibition of apoptosis, interferon-γ pathway, and wound healing along with promotion of terminal differentiation. J Biol Chem 282:4021–4034.
Sui M, Chen F, Chen Z, Fan W. 2006. Glucocorticoids interfere with therapeutic efficacy of paclitaxel against human breast and ovarian xenograft tumors. Int J Cancer 119:712–717.
Sung J, Song Y-M, Lawlor DA, Smith GD, Ebrahim S. 2009. Height and site-specific cancer risk: A cohort study of a Korean adult population. Am J Epidemiol 170:53–64.
Temkin S, Nacharaju VL, Hellman M, Lee Y-C, Abulafia O. 2006. Type 2 11β-hydroxysteroid dehydrogenase activity in human ovarian cancer. Steroids 71:1019–1023.
Terao M, Itoi S, Murota H, Katayama I. 2013. Expression profiles of cortisol-inactivating enzyme, 11β-hydroxysteroid dehydrogenase-2, in human epidermal tumors and its role in keratinocyte proliferation. Exp Dermatol 22:98–101.
Tiganescu A, Tahrani AA, Morgan SA, Otranto M, Desmoulière A, Abrahams L, Hassan-Smith Z, Walker EA, Rabbitt EH, Cooper MS. 2013. 11β-Hydroxysteroid dehydrogenase blockade prevents age-induced skin structure and function defects. J Clin Invest 123:3051–3060.
Usatine RP, Tinitigan M. 2011. Diagnosis and treatment of lichen planus. Am Fam Phys 84:53–60.
Wadsworth SJ, Nijmeh HS, Hall IP. 2006. Glucocorticoids increase repair potential in a novel in vitro human airway epithelial wounding model. J Clin Immunol 26:376–387.
Waters C, Stevens A, White A, Ray D. 2004. Analysis of co-factor function in a glucocorticoid-resistant small cell carcinoma cell line. J Endocrinol 183:375–383.
Xing K, Gu B, Zhang P, Wu X. 2015. Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: An insight into the optimum application of glucocorticoids in anti-cancer therapy. BMC Immunol 16:39.
Yuan HJ, Han X, He N, Wang GL, Gong S, Lin J, Gao M, Tan JH. 2016. Glucocorticoids impair oocyte developmental potential by triggering apoptosis of ovarian cells via activating the Fas system. Sci Rep 6:24036.
Žbánková Š, Bryndová J, Kment M, Pácha J. 2004. Expression of 11Ž-hydroxysteroid dehydrogenase types 1 and 2 in colorectal cancer. Cancer Lett 210:95–100.
Zhang C, Beckermann B, Kallifatidis G, Liu Z, Rittgen W, Edler L, Büchler P, Debatin K-M, Büchler MW, Friess H. 2006a. Corticosteroids induce chemotherapy resistance in the majority of tumour cells from bone, brain, breast, cervix, melanoma and neuroblastoma. Int J Oncol 29:1295–1301.
Zhang C, Mattern J, Haferkamp A, Pfitzenmaier J, Hohenfellner M, Rittgen W, Edler L, Debatin KM, Groene E, Herr I. 2006b. Corticosteroid-induced chemotherapy resistance in urological cancers. Cancer Biol Ther 5:59–64.

European Journal of Oral Sciences

Eur J Oral Sci 2016; 124: 433–439
DOI: 10.1111/eos.12293

European Journal of Oral Sciences

Antimicrobial activity and regulation of CXCL9 and CXCL10 in oral keratinocytes

Alison Marshall[1], Antonio Celentano[2,3], Nicola Cirillo[3], Michele D. Mignogna[2], Michael McCullough[3], Stephen Porter[1]

[1]University College London, UCL Eastman Dental Institute, London, UK; [2]University Federico II of Naples, Department of Neuroscience, Reproductive and Odontostomatological Sciences, Naples, Italy; [3]The University of Melbourne, Melbourne Dental School and Oral Health CRC, Melbourne, Vic., Australia

Marshall A, Celentano A, Cirillo N, Mignogna MD, McCullough M, Porter S. Antimicrobial activity and regulation of CXCL9 and CXCL10 in oral keratinocytes. Eur J Oral Sci 2016; 124: 433–439. © 2016 Eur J Oral Sci

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.

Antonio Celentano, Department of Neuroscience, Reproductive and Odontostomatological Sciences, University Federico II of Naples Via Pansini 5, Naples 80131, Italy.

E-mail: antonio.celentano@unina.it

Key words: CCL28; chemokines; CXCL10; CXCL9; *Streptococcus sanguinis*

Accepted for publication July 2016

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 mm^3 and cultured at 37°C/5% CO_2 in keratinocyte basal medium-2 containing the recommended growth supplements (Biowhittaker, 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/strains were derived before 2001 and therefore were not 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 Prime *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 3% TSB. They were grown for 48 h at 37°C/5% CO_2 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 underlay 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 8×10^4 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 H_2O (dH_2O). Then, 1 ml of RNAaseIN (Ambion), 2 ml of 10× MuLVRT 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 MgCl in each reaction, and dH_2O 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 staining with 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 AlphaImager software (AlphaInnotech).

The *CXCL9* (forward: 5′-ccaacaccccacagaagtgc-3′; reverse: 5′-gccagcacctgctctgagac-3′), *CXCL10* (forward: 5′-gccaatttt gtccacgtgttg-3′; reverse: 5′-aaagaatttgggccccttgg-3′), and *18S* ribosomal RNA (forward: 5′-tttcggaactgaggccatga-3′; reverse: 5′-gcatgccagagtctcgttcg-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 AlphaImager 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.

Production of *CXCL9* and *CXCL10* mRNA transcripts in an oral epithelial cell line in response to LPS

CXCL10 mRNA was expressed in control cells (no exposure to LPS), but was enhanced by stimulation with LPS, 2, 4, and 6 h after exposure. This expression subsequently decreased after 8 h of stimulation (Fig. 3). In contrast, *CXCL9* mRNA was not expressed in either control or LPS-stimulated cells at any of the time-points tested.

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

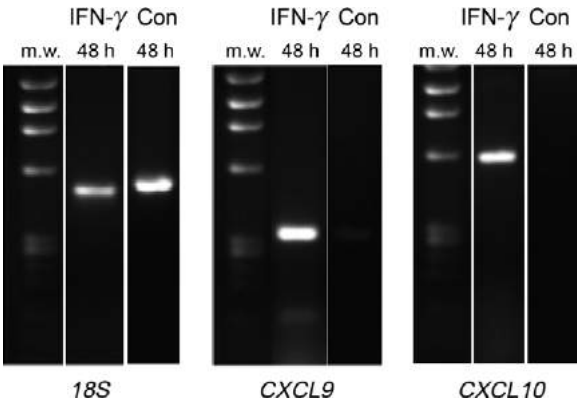


Fig. 1. Expression of *18S*, chemokine CXCL9, and chemokine (C-X-C motif) ligand 10 (*CXCL10*) mRNAs in the H357 cell line, either treated with interferon-γ for 48 h (IFN) or untreated for 48 h (CON). The left column 'm.w.' indicates the molecular weight. The molecular weight of CXCL9 expression is located at 351 bp, CXCL10 expression is at 601 bp.

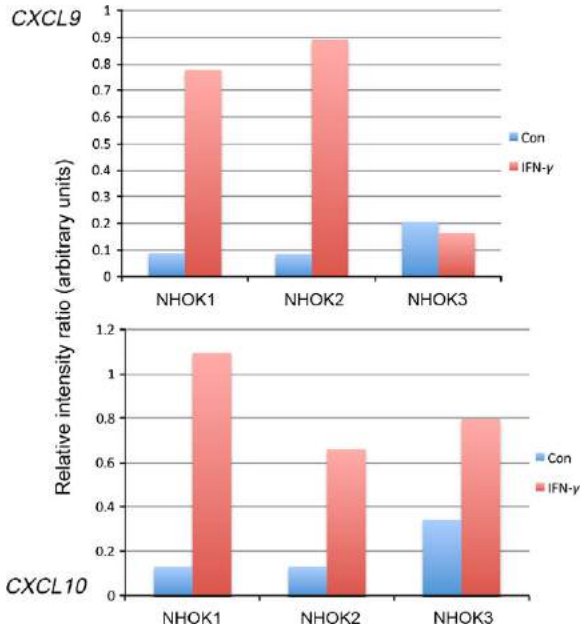


Fig. 2. Expression of chemokine (C-X-C motif) ligand 9 (*CXCL9*) and chemokine (C-X-C motif) ligand 10 (*CXCL10*) mRNAs in three different strains of normal human oral keratinocytes (NHOK1, NHOK2, and NHOK3), either treated with interferon-γ for 48 h (IFN-γ) or untreated for 48 h (Con). The values were normalized relative to expression of *18S* mRNA, used as a housekeeping mRNA.

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.

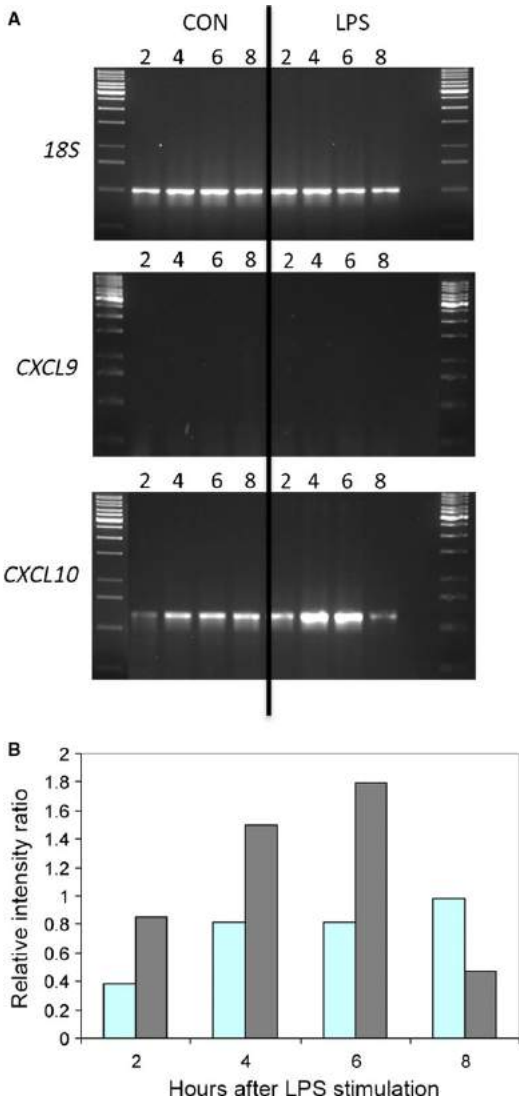


Fig. 3. (A) Expression of *CXCL9*, *CXCL10*, and *18S* mRNAs by H357 cells, unstimulated (CON) or stimulated with lipopolysaccharide (LPS) for 2, 4, 6, or 8 h. (B) Densitometric analysis of *CXCL10* mRNA relative to *18S* mRNA, with (grey) and without (blue) LPS treatment.

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

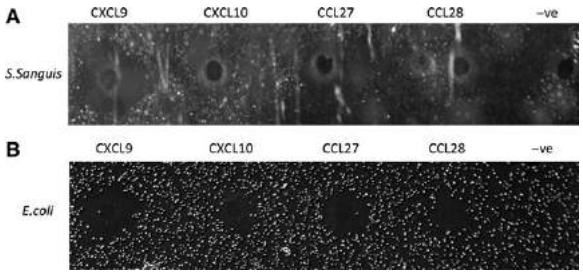


Fig. 4. Radial diffusion gels demonstrating the antimicrobial activity of 100 μg ml^{-1} of human recombinant chemokine CXCL9, CXCL10, chemokine (C-C motif) ligand (CCL)27, and CCL28 on *Streptococcus sanguinis* (A) and *Escherichia coli* (B). The negative control (-ve) contains 0.01% acetic acid only. The diameter of the cleared zone around the well containing chemokine represents the antimicrobial properties of the chemokine.

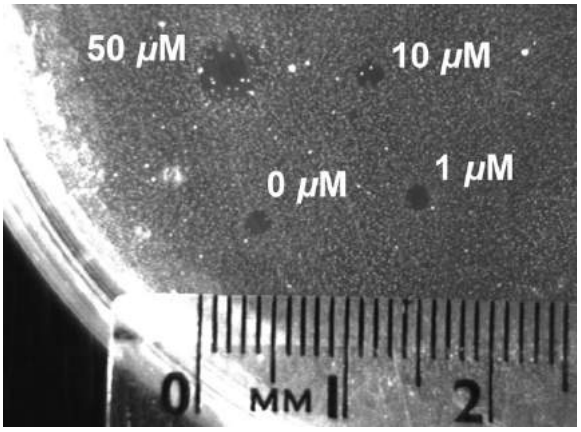


Fig. 5. Antimicrobial activity of 50, 10, and 1 μM concentrations of CXCL9 on *Escherichia coli* JM22; 0 μM contains 0.01% acetic acid only.

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

Antimicrobial activity of 100 μg *ml^{-1} of human recombinant chemokine CXCL9, CXCL10, chemokine CCL27 and CCL28 on* Streptococcus sanguinis *and* Escherichia coli

Bacterial species	CXCL9	CXCL10	CCL27	CCL28
E. coli	34.6 ± 6.80	14.9 ± 5.65	20.6 ± 0.90	15.4 ± 6.04
S. sanguis	44.4 ± 14.05	8.4 ± 3.52	10.0 ± 5.45	21.0 ± 5.70

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 Berri *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

1. Meyer DH, Sreenivasan PK, Rives-Taylor PM. Evidence for invasion of a human oral cell line by Actinobacillus actinomycetemcomitans. *Infect Immun* 1991; **59**: 2719–2726.
2. Henderson B, Poole S, Wilson M, eds. Bacteria-Cytokine Interactions In: *Health and Disease*. London: Portland Press, 1998; 167–209.
3. Erridge C, Bennett-Guerrero E, Poxton IR. Structure and function of lipopolysaccharides. *Microbes Infect* 2002; **4**: 837–851.
4. Percoco G, Merle C, Jaouen T, Ramdani Y, Bénard M, Hillion M, Mijouin L, Lati E, Feuilloley M, Lefeuvre L, Driouich A, Follet-Gueye ML. Antimicrobial peptides and pro-inflammatory cytokines are differentially regulated across epidermal layers following bacterial stimuli. *Exp Dermatol* 2013; **22**: 800–806.
5. Cox MA, Jenh CH, Gonsiorek W, Fine J, Narula SK, Zavodny PJ, Hipkin RW. Human interferon-inducible 10kDa protein and human interferon-inducible T cell αlphaa chemoattractant are allotropic ligands for human CXCR3: differential binding to receptor states. *Mol Pharmacol* 2001; **59**: 707–715.
6. Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol* 2001; **167**: 623–627.
7. Hieshima K, Ohtani H, Shibano M, Izawa D, Nakayma T, Kawasaki Y, Shiba F, Shiota M, Katou F, Saito T, Yoshie O. CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. *J Immunol* 2003; **170**: 1452–1461.
8. Tang YQ, Yeaman MR, Selsted ME. Antimicrobial peptides from human platelets. *Infect Immun* 2002; **70**: 6524–6533.
9. Ichimura M, Hiratsuka K, Ogura N, Utsunomiya T, Sakamaki H, Kondoh T, Abiko Y, Otake S, Yamamoto M. Expression profile of chemokines and chemokine receptors in epithelial cell layers of oral lichen planus. *J Oral Pathol* 2013; **35**: 167–174.
10. Ohta K, Shigeishi H, Taki M, Nishi H, Higashikawa K, Takechi M, Kamata N. Regulation of CXCL9/10/11 in oral keratinocytes and fibroblasts. *J Dent Res* 2008; **87**: 1160–5.
11. Kocgozlu L, Elkaim R, Tenenbaum H, Werner S. Variable cell responses to P. gingivalis lipopolysaccharide. *J Dent Res* 2009; **88**: 741–745.
12. Meyer M, Hensbergen PJ, van der Raaij-Helmer EM, Brandacher G, Margreiter R, Heufler C, Koch F, Narumi S, Werner ER, Colvin R, Luster AD, Tensen CP, Werner-Felmayer G. Cross reactivity of three T cell attracting murine chemokines stimulating the CXC chemokine receptor CXCR3 and their induction in cultured cells and during allograft rejection. *Eur J Immunol* 2001; **31**: 2521–2527.
13. Lauw FN, Simpson AJH, Prins JM, van Deventer SJH, Chaowagul W, White NJ, van der Poll T. The CXC chemokines gamma interferon (IFN-γ)-inducible protein 10 and monokine induced by IFN-γ are released during severe melioidosis. *Infect Immun* 2000; **68**: 3888–3893.
14. Rouabhia M, Ross G, Pagè N, Chakir J. Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to Candida albicans or lipopolysaccharide stimulation. *Infect Immun* 2002; **70**: 7073–7080.
15. Ryu OH, Choi SJ, Linares AM, Song IS, Kim YJ, Jang KT, Hart T. Gingival epithelial cell expression of macrophage

inflammatory protein-1alpha induced by interleukin-1beta and lipopolysaccharide. *J Periodontol* 2007; **78**: 1627–1634.
16. Suga T, Mitani A, Mogi M, Kikuchi T, Fujimura T, Takeda H, Hishikawa T, Yamamoto G, Hayashi J, Ishihara Y, Noguchi T. Aggregatibacter actinomycetemcomitans lipopolysaccharide stimulated epithelial cells produce interleukin-15 that regulates T cell activation. *Arch Oral Biol* 2013; **58**: 1541–1548.
17. Yin L, Chino T, Horst OV, Hacker BM, Clark EA, Dale BA, Chung WO. Differential and coordinated expression of defensins and cytokines by gingival epithelial cells and dendritic cells in response to oral bacteria. *BMC Immunol* 2010; **11**: 37.
18. Hassona Y, Cirillo N, Lim KP, Herman A, Mellone M, Thomas GJ, Pitiyage GN, Parkinson EK, Prime SS. Progression of genotype-specific oral cancer leads to senescence of cancer-associated fibroblasts and is mediated by oxidative stress and TGF-β. *Carcinogenesis* 2013; **34**: 1286–1295.
19. Prime SS, Nixon SV, Crane IJ, Stone A, Matthews JB, Maitland NJ, Remnant L, Powell SK, Game SM, Scully C. The behaviour of human oral squamous cell carcinoma in cell culture. *J Pathol* 1990; **160**: 259–269.
20. Altenburg A, Baldus SE, Smola H, Pfister H, Hess S. CD40 ligand-CD40 interaction induces chemokines in cervical carcinoma cells in synergism with IFN-gamma. *J Immunol* 1999; **162**: 4140–4147.
21. Sun H, Kundu N, Dorsey R, Jackson MJ, Fulton AM. Expression of the chemokines IP-10 and MIG in IL-10 transduced tumors. *J Immunother* 2001; **24**: 138–143.
22. Kopydlowski KM, Salkowski CA, Cody MJ, van Rooijen N, Major J, Hamilton TA, Vogel SN. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J Immunol* 1999; **163**: 1537–1544.
23. Eskan MA, Rose BG, Benakanakere MR, Lee MJ, Kinane DF. Sphingosine 1 phosphate 1 and TLR4 mediate IFN-beta expression in human gingival epithelial cells. *J Immunol* 2008; **180**: 1818–1825.
24. Boorsma DM, Flier J, Sampat S, Ottevanger C, de Haan P, Hooft L, Willemze R, Tensen CP, Stoof TJ. Chemokine IP-10 expression in cultured human keratinocytes. *Arch Dermatol Res* 1998; **290**: 335–341.
25. Widney DP, Xia Y-R, Lusis AJ, Smith JB. The murine chemokine CXCL11 (IFN-inducible T cell α attractant is an IFN-γ and lipopolysaccharide-inducible glucocorticoid-attenuated response gene expressed in lung and other tissues during endotoxemia. *J Immunol* 2000; **164**: 6322–6331.
26. Dan H, Liu W, Wang J, Wang Z, Wu R, Chen Q, Zeng X, Zhou Y. Elevated IL-10 concentrations in serum and saliva from patients with oral lichen planus. *Quintessence Int* 2011; **42**: 157–163.
27. Uehara A, Sugawara S, Takada H. Priming of human oral epithelail cells by interferon-γ to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans. *J Med Microbiol* 2002; **51**: 626–635.
28. Kawai K, Shimura H, Minagawa M, Ito A, Tomiyama K, Ito M. Expression of functional toll-like receptor 2 on human epidermal keratinocytes. *J Dermatol Sci* 2002; **30**: 185–194.
29. Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, Williams BR, Major J, Hamilton TA, Fenton MJ, Vogel SN. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol* 2002; **3**: 392–398.
30. Re F, Strominger JL. Toll-like receptor (TLR2) and TLR4 differentially activate human dendritic cells. *J Biol Chem* 2001; **276**: 37692–37699.
31. Lee SH. Antagonistic effect of peptidoglycan of Streptococcus sanguinis on lipopolysaccharide of major periodontal pathogens. *J Microbiol* 2015; **53**: 553–560.
32. Ma S, Li H, Yan C, Wang D, Li H, Xia X, Dong X, Zhao Y, Sun T, Hu P, Guan W. Antagonistic effect of protein extracts from Streptococcus sanguinis on pathogenic bacteria and fungi of the oral cavity. *Exp Ther Med* 2014; **7**: 1486–1494.
33. Peyret-Lacombe A, Brunel G, Watts M, Charveron M, Duplan H. TLR2 sensing of F. nucleatum and S. sanguinis distinctly triggered gingival innate response. *Cytokine* 2009; **46**: 201–210.
34. Berri M, Virlogeux-Payant I, Chevaleyre C, Melo S, Zanello G, Salmon H, Meurens F. CCL28 involvement in mucosal tissues protection as a chemokine and as an antibacterial peptide. *Dev Comp Immunol* 2014; **44**: 286–290.

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.

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

Antonio Celentano, DDS,[a,b] Serban Tovaru, MD,[c] Tami Yap, BDSc, FRACDS,[d] Daniela Adamo, DDS,[e] Massimo Aria, PhD,[f] and Michele Davide Mignogna, MD, DMD[e]

Objective. Erythema multiforme (EM) continues to be an underestimated disease with a lack of strict classification and diagnostic criteria. We present the analysis of a case series of 60 oral EM patients from 2 centers and illustrate the range of oral clinical presentations.

Study Design. Clinical data from 60 EM patients with oral involvement, diagnosed and treated between 1982 and 2014, were retrospectively collected from the archives of 2 independent hospitals. Statistical analyses of the data were performed using the Pearson χ-squared test and the Mann-Whitney U test.

Result. Thirty-one patients (51.7%) were male and 29 (48.3%) were female, with a mean (±SD) age of 37.9 years (±18.1). The frequency of previous occurrences ranged from 0 to 10 (mean ± SD: 1.4 ± 2.0). Twenty-nine patients (48%) had no previous occurrence. Medications (particularly antipyretics, food additives, and antibiotics) were the suspected precipitants in 28 patients (46.7%), whereas herpes simplex virus infection was suspected in 18 (30.0%). All but 1 patient had involvement of multiple oral sites, with the buccal mucosa being the most commonly involved oral site (75%), followed by the vermillion border (71.7%).

Conclusions. Patients with EM may present initially to oral health care workers. Medications and herpes simplex virus continue to be the most typically involved precipitating factors. Our data highlight the additional role of food-derived antigens. Although laboratory tests can provide support diagnostically, EM diagnosis continues to be based on clinical features. A medication and food diary should be encouraged particularly in patients with recurrent forms. (Oral Surg Oral Med Oral Pathol Oral Radiol 2015;120:707-716)

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, *Mycobaterium leprae*, and varicella 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.

The study was approved by the Ethics Committee of the University "Federico II" of Naples in June 2014.

[a]PhD student at the Department of Neurosciences, Reproductive, and Odontostomatological Sciences, University Federico II of Naples, Naples, Italy.
[b]Melbourne Dental School and Oral Health CRC, University of Melbourne, Victoria, Australia.
[c]Department of Oral Medicine, Faculty of Dental Medicine, University of Medicine and Pharmacy Carol Davila, Bucharest, Romania.
[d]PhD student at Melbourne Dental School, University of Melbourne, Victoria, Australia.
[e]Department of Neurosciences, Reproductive, and Odontostomatological Sciences, University Federico II of Naples, Naples, Italy.
[f]Department of Economics and Statistics, University of Naples Federico II, Monte Sant' Angelo, Naples, Italy.

Received for publication Apr 2, 2015; returned for revision Aug 8, 2015; accepted for publication Aug 13, 2015.

2212-4403/$ - see front matter
http://dx.doi.org/10.1016/j.oooo.2015.08.010

eruption, bullous pemphigoid, paraneoplastic pemphigus, Sweet syndrome, Rowell syndrome, polymorphous light eruption, and cutaneous small vessel vasculitis. To improve the diagnostic accuracy, histopathologic analyses and others laboratory tests can be used.[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.[1,14] Both SJS and TEN can be fatal, with a reported mortality rate of 1% to 5% and 25% to 35%, respectively.[34]

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."[35,36]

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.[14,35] Daily antiviral therapy has been used successfully to control the disease in patients with recurrent EM.[37]

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,[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.[3] The analysis of site distribution was performed with the Pearson χ-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 ($\pm$SD) age of 37.9 years ($\pm$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 $\pm$ SD $= 1.4 \pm 2.0$). 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

						Involved oral sites								
No.	*HSV infection*	*Other infections*	*Drug antecedents/other precipitants*	*Other affected mucosae*	*Skin lesions*	*B*	*L*	*V*	*T*	*A*	*F*	*P*	*TP*	*Classification*
1	No	No	Sulfonamide	No	Yes: Arms symmetrically	X	X	X	X	X				EMm
2	No	No	Sulfonamide (Sumetrolim)	No	No	X	X							EMm
3	No	No	No	No	Yes: Lips symmetrically		X	X						EMm
4	No	No	Sulfonamide (Sumetrolim)	No	No	X			X					EMm
5	Yes	No	No	No	No					X		X		EMm
6	No	No	Food allergen	No	Yes: Lips	X	X	X	X					EMm
7	No	Respiratory virosis	No	No	No	X	X					X		EMm
8	No	Tonsilar infection	No	No	Yes: Perioral			X		X		X		EMm
9	No	No	Phenazone	No	Yes: Perioral	X	X	X			X	X		EMm
10	Yes	No	Phenazone	No	Yes: Perioral	X			X			X		EMm
11	Yes	Flu	Phenazone	No	Yes: Perioral	X	X	X						EMm
12	No	No	Food allergen	No	No	X	X	X	X					EMm
13	Yes	No	No	No	Yes: Perioral	X	X	X	X	X	X	X		EMm
14	No	No	Paracetamol	No	No			X	X			X		EMm
15	No	No	Phenazone	Yes: ocular	No	X		X			X	X		EMM
16	No	No	Fluanxol, haloperidol	No	No	X		X	X					EMm
17	No	No	Phenazone	No	Yes: Arms, palms			X		X				EMm
18	No	No	No	No	Yes: Knees, elbows, feet,	X		X	X					EMm
19	No	No	No	No	No	X								EMm
20	No	No	Amoxicillin, paracetamol	Yes: ocular	No	X	X	X	X			X		EMM
21	No	HCV	No	No	No			X						EMm
22	No	No	Food conservants	No	No	X	X				X	X	X	EMm
23	No	Pericoronarytis of 48	No	No	Yes: Perioral		X	X	X					EMm
24	Yes	No	No	No	No	X		X						EMm
25	No	Urinary Infection (2005)	Augumentin, ciprofloxacin, fluoconazol	No	No			X						EMm
26	No	No	Food antigens	No	No		X							EMm
27	No	Urinary infection	Nystatin, bioparox, local antiseptics, local propolis	No	Yes: Palms	X	X	X			X			EMm
28	No	Pneumonia	No	No	No		X	X						EMm
29	No	No	Doxicyclin	No	Yes: Fingers symmetrically	X	X	X				X		EMm
30	No	Tubes infection (genital)	Triregol, ciprofloxacin, sumetrolin, ampicillin, birth control pills	No	No	X	X	X				X		EMm
31	Yes	No	No	No	No	X		X					X	EMm
32	Yes	No	No	No	No	X			X					EMm
33	No	No	Diclofenac, diflucan	No	No	X	X	X	X	X	X	X		EMm
34	No	No	Amoxicillin, metronidazol, food allergens	No	No	X			X	X		X		EMm
35	Yes	No	Acyclovir	No	Yes: Arms, perioral	X		X				X		EMm
36	No	No	Paracetamol	No	Yes: Arms	X	X		X				X	EMm

(*continued on next page*)

Table I. Continued

No.	*HSV infection*	*Other infections*	*Drug antecedents/other precipitants*	*Other affected mucosae*	*Skin lesions*	*Involved oral sites*								*Classification*
						B	*L*	*V*	*T*	*A*	*F*	*P*	*TP*	
37	Yes	No	Acyclovir	No	Yes: Arms, palpebral	X	X	X	X					EMm
38	Yes	No	No	Yes: genital	No	X	X	X	X	X	X	X		EMM
39	No	HBV (2009)	No	No	No	X	X	X	X					EMm
40	No	No	Food antigens	No	No	X	X							EMm
41	Yes	No	No	No	Yes	X	X							EMm
42	Yes	No	No	No	No		X							EMm
43	No	No	Phenazone	No	No	X		X					X	EMm
44	No	Pneumonia	Phenazone	No	No		X	X	X				X	EMm
45	No	HCV	No	No	No	X	X	X	X			X		EMm
46	No	No	Food antigens	No	No			X	X					EMm
47	Yes	No	No	Yes: Genital	Yes: Elbows, palms	X	X	X						EMM
48	No	No	Phenazone	Yes: Ocular, Genital	Yes: Acral	X	X		X					EMM
49	No	Mycoplasma	No	No	Yes: Widespread				X		X			EMM/mild SJS
50	Yes	No	No	No	Yes: Acral	X	X	X	X	X	X	X		EMm
51	No	No	Salycilate	No	Yes:Acral	X		X	X	X				EMm
52	No	No	K sigma 1 year before	Yes: Genital	No	X	X	X	X		X		X	EMM/mild SJS
53	Yes	No	No	No	Yes: Perioral	X	X	X	X	X	X			EMm
54	Yes	No	No	No	No	X	X	X	X			X		EMm
55	No	No	Ketoprofen	No	Yes: Acral	X			X	X		X		EMm
56	No	No	Ampicillin	No	No	X	X	X	X					EMm
57	No	Adenovirus (pharynx)	No	No	No	X	X	X	X	X				EMm
58	Yes	No	Paracetamol	No	No	X		X	X	X		X		EMm
59	Yes	No	No	Yes: Nasal	No	X	X	X	X	X	X	X	X	EMM/mild SJS
60	No	No	Mefloquine	Yes: Genital	Yes: Acral	X	X	X	X	X	X			EMM

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.

Table II. Distribution of site of lesions by gender

Site	*Gender*		*Total*	*P value*[*]
	Female	*Male*		
B	Yes 79.3%	Yes 71.0%	Yes 75.0%	.556
	No 20.7%	No 29.0%	No 25.0%	
L	Yes 72.4%	Yes 51.6%	Yes 61.7%	.098
	No 27.6%	No 48.4%	No 38.3%	
V	Yes 79.3%	Yes 64.5%	Yes 71.7%	.204
	No 20.7%	No 35.5%	No 28.3%	
T	Yes 62.1%	Yes 51.6%	Yes 56.7%	.414
	No 37.9%	No 48.4%	No 43.3%	
A	Yes 27.6%	Yes 25.8%	Yes 26.7%	.876
	No 72.4%	No 74.2%	No 73.3%	
F	Yes 37.9%	Yes 6.5%	Yes 21.7%	.003[†]
	No 62.1%	No 93.5%	No 78.3%	
P	Yes 34.5%	Yes 38.7%	Yes 36.7%	.734
	No 65.5%	No 61.3%	No 63.3%	
TP	Yes 13.8%	Yes 9.7%	Yes 11.7%	.620
	No 86.2%	No 90.3%	No 88.3%	
Number of sites with lesions	Median−IQR 4.03.0	Median−IQR 3.0 2.0	Median−IQR 3.5−3.0	.095

IQR, Interquartile range. The significance difference between conditional distributions was measured by the Pearson χ-squared test. The significance difference between medians was measured by the Mann-Whitney U test.
*Significant $P < .01$ to $\leq .05$.
†Significant $P \leq .01$.

Table III. Dependence analysis between drug antecedents and precipitators

	Precipitators	*Median−IQR*	*P value*[*]
No. of drug antecedents	Herpes		.002[†]
	Yes	0.0−1.0	
	No	1.0−1.0	
	Infection		.418
	Yes	0.0−2.0	
	No	1.0−1.0	
	Classification		.929
	EM minor	1.0−2.0	
	EM major	1.0−1.0	

IQR is the interquartile range. The significance difference between medians was measured by Mann-Whitney U test.
*Significant $.01 < P \leq .05$.
†Significant $P \leq .01$.

Table IV. Dependence analysis between demographic characteristics and precipitators

Precipitators	*Gender*		*P value*[*]
	Female	*Male*	
Herpes simplex virus	Yes 34.52%	Yes 25.8%	0.464
	No 65.5%	No 74.2%	
Other Infections	Yes 17.2%	Yes 29.0%	0.281
	No 82.8%	No 71.0%	
Classification	EM major 24.1%	EM major 6.5%	.045[†]
	EM minor 75.9%	EM minor 93.5%	
	Age Median−IQR		
HSV	Yes 29−17		.628
	No 30−33		
Other Infections	Yes 29−34		.993
	No 29−27		
Classification	EM major 39−40		.045[†]
	EM minor 29−27		

IQR is the inter-quartile range. The significance difference between conditional distributions was measured by the Pearson χ-squared test. The significance difference between medians was measured by the Mann-Whitney U test.
*Significant $P < .01$ to $\leq .05$.
†Significant $P \leq .01$.

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 (46.66%). All but 1 patient had involvement of

multiple oral sites. The buccal mucosa was the most commonly involved oral site (75%) followed by the vermillion 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.[3,39] 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 more frequent in males. Clinical forms were additionally significantly related to age (see Table IV).

The most commonly reported triggers are infections agents 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.[47] 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-α 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

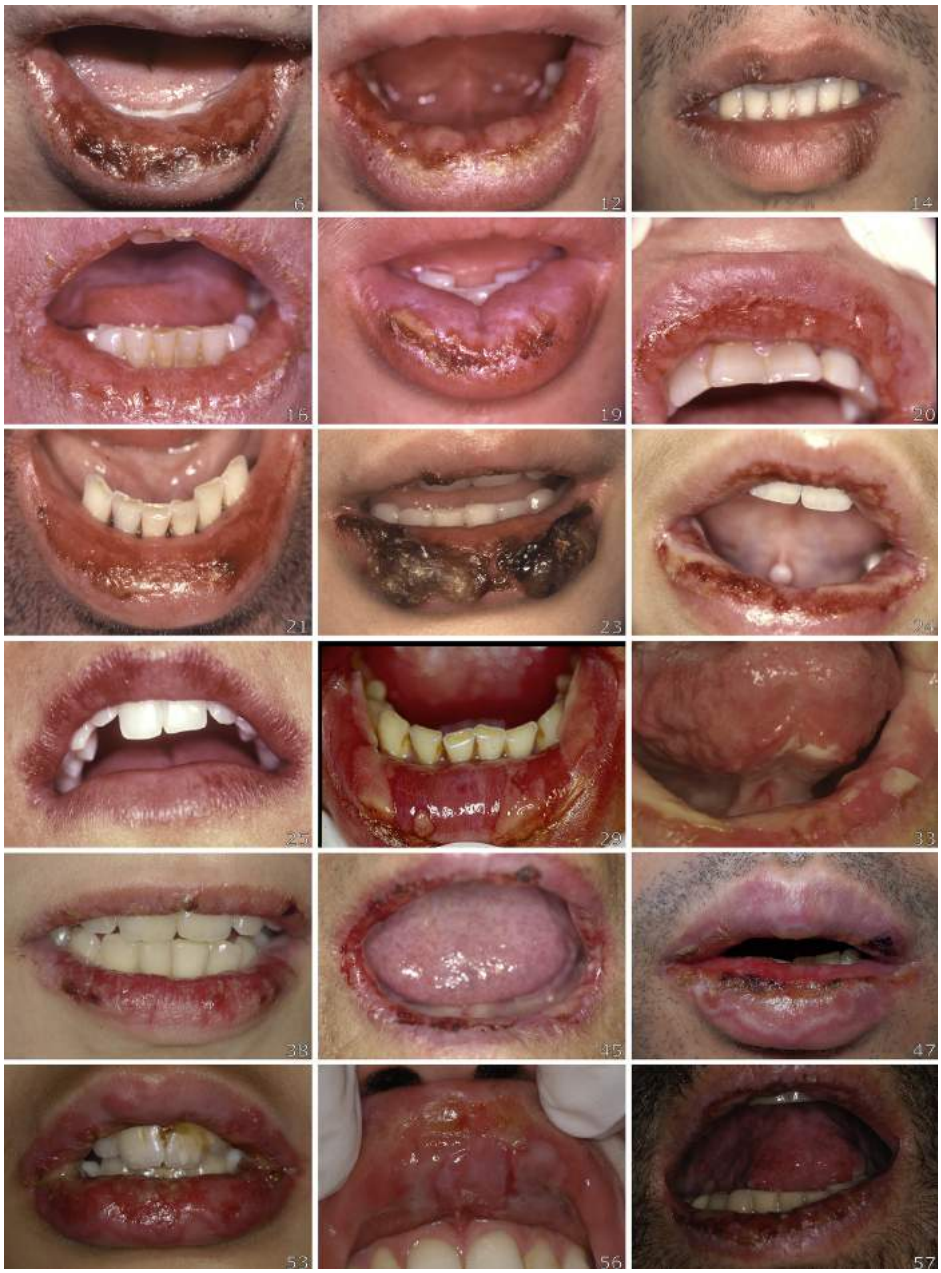


Fig. 1. The presenting labial clinical features were dominated by an erosive/bullous pattern and crusting. The number found in the lower right corner corresponds to the patient number as reported in Table I.

represent a distinct syndrome from EM and SJS.[67] An established association with *M. pneumoniae* was clearly reported in only 1 of our 3 pneumonia case, 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.[38] 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.[14,15]

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.[1,2] 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

predisposed to by 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.[3] 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 variably defined by either the presence or absence of mucous membrane involvement or the extent or number of mucosal sites involved.[3,14,46,68] In our study, we utilized the classification system described by Al-Johani et al (2007), which distinguishes EMm and EMM as involvement of 1 or more than 1 mucosal sites, respectively.[3] 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.[3] 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%).[69]

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

1. Scully C, Bagán JV. Oral mucosal diseases: erythema multiforme. *Br J Oral Maxillofac Surg*. 2008;46:90-95.
2. Wetter DA, Davis MDP. Recurrent erythema multiforme: clinical characteristics, etiologic associations, and treatment in a series of 48 patients at Mayo Clinic, 2000 to 2007. *J Am Acad Dermatol*. 2010;62:45-53.
3. Al-Johani KA, Fedele S, Porter SR. Erytema multiforme and related disorders. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2007;103:642-654.
4. Osterne RL, Matos Brito RG, Pacheco IA, Alves AP, Sousa FB. Management of erythema multiforme associated with recurrent herpes infection: a case report. *J Can Dent Assoc*. 2009;75:597-601.
5. Letko E, Papaliodis DN, Papaliodis GN, Daoud YJ, Ahmed AR, Foster CS. Stevens-Johnson syndrome and toxic epidermal necrolysis: a review of the literature. *Ann Allergy Asthma Immunol*. 2005;94:419-436.
6. Auquier-Dunant A, Mockenhaupt M, Naldi L, Correia O, Schröder W, Roujeau JC; SCAR Study Group. Severe cutaneous adverse reactions. Correlations between clinical patterns and causes of erythema multiforme majus, Stevens-Johnson syndrome, and toxic epidermal necrolysis: results of an international prospective study. *Arch Dermatol*. 2002;138:1019-1024.
7. Assier H, Bastuji-Garin S, Revuz J, Roujeau JC. Erythema multiforme with mucous membrane involvement and Stevens-Johnson syndrome are clinically different disorders with distinct causes. *Arch Dermatol*. 1995;131:539-543.
8. Weston WL. Herpes-associated erythema multiforme. *J Invest Dermatol*. 2005;124:xv-xvi.
9. Mockenhaupt M. Stevens-Johnson syndrome and toxic epidermal necrolysis: clinical patterns, diagnostic considerations, etiology, and therapeutic management. *Semin Cutan Med Surg*. 2014;33:10-16.
10. Chrysomali E, Lozada-Nur F, Dekker NP, Papanicolaou SI, Regezi JA. Apoptosis in oral erythema multiforme. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 1997;83:272-280.
11. Sawada T, Suehiro M. Erythema multiforme associated with *Chlamydophila pneumoniae* infection: a report of two cases and a mini-literature review. *J Dermatol*. 2015;42:336-337.
12. Imashuku S, Kudo N. *Chlamydia pneumoniae* infection-associated erythema multiforme. *Pediatr Rep*. 2013;5:35-37.
13. Hosokawa R, Kobayashi T, Higashino T, et al. Two cases of erythema exsudativum multiforme associated with *Chlamydia pneumoniae* infection. *J Dermatol*. 2012;39:306-308.
14. Sokumbi O, Wetter DA. Clinical features, diagnosis, and treatment of erythema multiforme: a review for the practicing dermatologist. *Int J Dermatol*. 2012;51:889-902.
15. Turnbull N, Hawkins D, Atkins M, Francis N, Roberts N. Persistent erythema multiforme associated with Epstein-Barr virus infection. *Clin Exp Dermatol*. 2014;39:154-157.

16. Özdemir PG, Yazicioğlu M, Aylanç H, Ozkayin N. EBV-induced erythema multiforme: a case report. *Allergol Immunopathol (Madr)*. 2011;39:116-118.
17. Joseph RH, Haddad FA, Matthews AL, Maroufi A, Monroe B, Reynolds M. Erythema multiforme after Orf virus infection: a report of two cases and literature review. *Epidemiol Infect*. 2015;143:385-390.
18. Ozturk P, Sayar H, Karakas T, Akman Y. Erythema multiforme as a result of Orf disease. *Acta Dermatovenerol Alp Pannonica Adriat*. 2012;21:45-46.
19. Ma JH, Smith S, Gordon LA. Acute HIV infection presenting as erythema multiforme in a 45-year-old heterosexual man. *Med J Aust*. 2015;202:273-275.
20. Cieza-Díaz DE, Campos-Domínguez M, Santos-Sebastián Mdel M, et al. Erythema multiforme in a newborn associated with acute acquired cytomegalovirus infection. *Pediatr Dermatol*. 2013;30:e161-e163.
21. Vitiello M, Echeverria B, Elgart G, Kerdel F. Erythema multiforme major associated with CMV infection in an immunocompetent patient. *J Cutan Med Surg*. 2011;15:115-117.
22. Miranda AM, Antunes SL, Nery JA, Sales AM, Pereira MJ, Sarno EN. Erythema multiforme in leprosy. *Mem Inst Oswaldo Cruz*. 2012;107:34-42.
23. Kasuya A, Sakabe J, Kageyama R, Ikeya S, Fujiyama T, Tokura Y. Successful differentiation of herpes zoster-associated erythema multiforme from generalized extension of herpes by rapid polymerase chain reaction analysis. *J Dermatol*. 2014;41:542-544.
24. Kishore BN, Ankadavar NS, Kamath GH, Martis J. Varicella zoster with erythema multiforme in a young girl: a rare association. *Indian J Dermatol*. 2014;59:299-301.
25. Esnault J, Jeannel J, Oehler E. Erythema multiforme due to varicella-zoster virus. *Presse Med*. 2014;43:1149-1150 [in French].
26. Park IH, Kang JN, Seol JE, Sung HS, Kim H. A case of erythema multiforme followed by herpes zoster. *Infection*. 2014;42:799-800.
27. Schmutz JL, Barbaud A, Trechot P. Vaccination for human Papillomavirus and erythema multiforme. *Ann Dermatol Venereol*. 2011;138:166-167 [in French].
28. Wiwanitkit S, Wiwanitkit V. Erythema multiforme after rabies vaccination. *Pediatr Dermatol*. 2013;30:e299.
29. Verma P. Erythema multiforme possibly triggered by rabies vaccine in a 10-year-old boy. *Pediatr Dermatol*. 2013;30:e297-e298.
30. Storie EB, Perry A. Erythema multiforme following smallpox vaccination. *Mil Med*. 2014;179:e113-e115.
31. Rosenblatt AE, Stein SL. Cutaneous reactions to vaccinations. *Clin Dermatol*. 2015;33:327-332.
32. Kaygusuz I, Gumus II, Sarifakioglu E, Eser A, Bozkurt B, Kafali H. Autoimmune progesterone dermatitis. *Taiwan J Obstet Gynecol*. 2014;53:420-422.
33. Raghunath RS, Venables ZC, Millington GW. The menstrual cycle and the skin. *Clin Exp Dermatol*. 2015;40:111-115.
34. Harr T, French LE. Stevens-Johnson syndrome and toxic epidermal necrolysis. *Chem Immunol Allergy*. 2012;97:149-166.
35. Schofield JK, Tatnall FM, Leigh IM. Recurrent erythema multiforme: clinical features and treatment in a large series of patients. *Br J Dermatol*. 1993;128:542-545.
36. Levin J, Hofstra T. Recurrent erythema multiforme. *JAMA*. 2014;312:426-427.
37. Tatnall FM, Schofield JK, Leigh IM. A double-blind, placebo-controlled trial of continuous acyclovir therapy in recurrent erythema multiforme. *Br J Dermatol*. 1995;132:267-270.
38. Lozada-Nur F, Gorsky M, Silverman S Jr. Oral erythema multiforme: clinical observations and treatment of 95 patients. *Oral Surg Oral Med Oral Pathol*. 1989;67:36-40.
39. Samim F, Auluck A, Zed C, Williams PM. Erythema multiforme: a review of epidemiology, pathogenesis, clinical features, and treatment. *Dent Clin North Am*. 2013;57:583-596.
40. Dilek N, Özkol HU, Akbaş A, et al. Cutaneous drug reactions in children: a multicentric study. *Postepy Dermatol Alergol*. 2014;31:368-371.
41. Brzeziński P. Herpangina and erythema multiforme in a three-year boy. *Przegl Lek*. 2013;70:764-766.
42. Khan MA, Fitzgerald K. Low-dose sertraline-induced erythema multiforme in a child. *J Child Adolesc Psychopharmacol*. 2012;22:179-180.
43. Sanders JE, Tay ET. Baby with a rash. Erythema multiforme. *Ann Emerg Med*. 2014;64:119:136.
44. Ang-Tiu CU, Nicolas ME. Erythema multiforme in a 25-day old neonate. *Pediatr Dermatol*. 2013;30:e118-e120.
45. Cho YJ, Huh SY, Hong JS, Jung JY, Suh DH. Neonatal erythema multiforme: a case report. *Ann Dermatol*. 2011;23:382-385.
46. Huff JC, Weston WL, Tonnesen MG. Erythema multiforme: a critical review of characteristics, diagnostic criteria, and causes. *J Am Acad Dermatol*. 1983;8:763-775.
47. Miller RM. Diagnosis of herpes simplex virus in patients with erythema multiforme. *JAMA*. 2014;312:1060-1061.
48. Shah SN, Chauhan GR, Manjunatha BS, Dagrus K. Drug induced erythema multiforme: two case series with review of literature. *J Clin Diagn Res*. 2014;8:ZH01-ZH04.
49. Ladizinski B, Carter JB, Lee KC, Aaron DM. Diagnosis of herpes simplex virus-induced erythema multiforme confounded by previous infection with *Mycoplasma pneumonia*. *J Drugs Dermatol*. 2013;12:707-709.
50. Tomasini C, Derlino F, Quaglino P, Caproni M, Borroni G. From erythema multiforme to toxic epidermal necrolysis. Same spectrum or different diseases? *G Ital Dermatol Venereol*. 2014;149:243-261.
51. Mii Y, Fukuoka E, Murata K, et al. A case of erythema multiforme induced by regorafenib therapy for metastatic colon cancer. *Gan To Kagaku Ryoho*. 2014;41:1841-1843 [in Japanese].
52. Shioya M, Nishimura T, Nishida A, et al. Two cases of successful sorafenib retreatment with the addition of steroid therapy following sorafenib-induced erythema multiforme in two patients with hepatocellular carcinoma. *Nihon Shokakibyo Gakkai Zasshi*. 2014;111:1424-1432 [in Japanese].
53. Namba M, Tsunemi Y, Kawashima M. Sorafenib-induced erythema multiforme: three cases. *Eur J Dermatol*. 2011;21:1015-1016.
54. Ikeda M, Fujita T, Mii S, et al. Erythema multiforme induced by sorafenib for metastatic renal cell carcinoma. *Jpn J Clin Oncol*. 2012;42:820-824.
55. Sawamura S, Kajihara I, Ichihara A, et al. Crizotinib-associated erythema multiforme in a lung cancer patient. *Drug Discov Ther*. 2015;9:142-143.
56. Edwards D, Boritz E, Cowen EW, Brown RS. Erythema multiforme major following treatment with infliximab. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2013;115:e36-e40.
57. Brănişteanu DE, Voicu CM, Creţu A, Dimitriu A, Luca MC, Sălăvăstru CM. Adverse reactions of biological therapy for psoriasis. *Rev Med Chir Soc Med Nat Iasi*. 2015;119:38-44.
58. Yamany T, Levender M, Silvers DN, Grossman ME. Erythema multiforme-like reaction with mucosal involvement following administration of idelalisib for relapse of chronic lymphocytic leukemia. *Leuk Lymphoma*. 2015;56:1872-1873.
59. Pietrzak A, Chodorowska G, Jazienicka I, et al. Psoriatic erythroderma coexisting with erythema multiforme-like lesions induced by retinoids or retinoids combined with an antibiotic: case report. *Dermatol Ther*. 2011;24:587-590.

60. Bertulyte I, Schwan S, Hallberg P. Identification of risk factors for carbamazepine-induced serious mucocutaneous adverse reactions: a case-control study using data from spontaneous adverse drug reaction reports. *J Pharmacol Pharmacother*. 2014;5:100-138.
61. Upadhyaya SK, Raina RS, Sharma A, Thawani V, Dimari D. Carbamazepine-induced erythema multiforme major in an epileptic patient with bipolar affective disorder. *J Pharmacol Pharmacother*. 2012;3:202-204.
62. Lozada F, Silverman S Jr. Erythema multiforme. Clinical characteristics and natural history in fifty patients. *Oral Surg Oral Med Oral Pathol*. 1978;46:628-636.
63. Chodkiewicz HM, Cohen PR. Radiation port erythema multiforme: erythema multiforme localized to the radiation port in a patient with non-small cell lung cancer. *Skinmed*. 2012;10:390-392.
64. Han JH, Yun SJ, Nam TK, Choi YD, Lee JB, Kim SJ. Erythema multiforme after radiotherapy with 5-fluorouracil chemotherapy in a rectal cancer patient. *Ann Dermatol*. 2012;24:230-232.
65. Vern-Gross TZ, Kowal-Vern A. Erythema multiforme, Stevens Johnson syndrome, and toxic epidermal necrolysis syndrome in patients undergoing radiation therapy: a literature review. *Am J Clin Oncol*. 2014;37:506-513.
66. Léauté-Labrèze C, Lamireau T, Chawki D, Maleville J, Taïeb A. Diagnosis, classification, and management of erythema multiforme and Stevens-Johnson syndrome. *Arch Dis Child*. 2000;83:347-352.
67. Canavan TN, Mathes EF, Frieden I, Shinkai K. Mycoplasma pneumoniae-induced rash and mucositis as a syndrome distinct from Stevens-Johnson syndrome and erythema multiforme: a systematic review. *J Am Acad Dermatol*. 2015;72:239-245.
68. Ayangco L, Rogers RS 3rd. Oral manifestations of erythema multiforme. *Dermatol Clin*. 2003;21:195-205.
69. Creţu A, Dimitriu A, Brănişteanu D, Brinişteanu DE. Erythema multiforme—etiopathogenic, clinical and therapeutic aspects. *Rev Med Chir Soc Med Nat Iasi*. 2015;119:55-61.

Reprint requests:

Antonio Celentano, DDS
Department of Neurosciences
Reproductive and Odontostomatological Sciences
University Federico II of Naples
Via Pansini n. 5, Napoli
80131 Italy
Melbourne Dental School and Oral Health CRC
University of Melbourne
Victoria, Australia.
antony.celentano@gmail.com; antonio.celentano@unina.it; acelentano@unimelb.edu.au

LETTERS TO THE EDITOR

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

To the Editor:

Celentano et al.[1] 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.[1] 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.[1-7]

In 2005, Williams and Conklin[2] summarized the dermatologic diagnostic standards for EM, SJS, and TEN, as reported by Bastuji-Garin et al.,[3] Assier et al.,[4] and Auquier-Dunant et al.,[5] and Cote et al.[6] These standards describe EM, SJS, and TEN as separate diagnostic entities with relatively well-defined diagnostic characteristics. Furthermore, Ayangco and Rogers[7] 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.

Ronald S. Brown, DDS, MS
Professor
Department of Comprehensive Dentistry
Howard University College of Dentistry
Washington
DC, USA
Associate Clinical Professor
Department of Otolaryngology
Georgetown University Medical Center
Washington
DC, USA

REFERENCES

1. Celentano A, Tovaru S, Yap T, Adamo D, Aria M, Mignogna MD. Oral erythema multiforme: trends and clinical findings of a large retrospective: European case series. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2015;120:707-716.
2. Williams PM, Conklin RJ. Erythema mulrtiforme: a review and contrast from Stevens-Johnson syndrome/toxic epidermal necrolysis. *Dent Clin North Am*. 2005;49:67-76.
3. Bastuji-Garin S, Rzany B, Stern RS, Shear NH, Naldi L, Roujeau JC. Clinical classification of cases of toxic epidermal necrolysis, Stevens-Johnson syndrome, and erythema multiforme. *Arch Dermatol*. 1993;129:92-96.
4. Assier H, Bastuji-Garin S, Revus J, Roujeau JC. Erythema multiforme with mucous membrane involvement and Stevens-Johnson syndrome are different disorders with distinct causes. *Arch Dermatol*. 1995;131:539-543.
5. Auquier-Dunant A, Mockenhaupt M, Naldi L, Correia O, Schroder W, Roujeau JC. Correlations between clinical patterns and causes of erythema multiforme major, Stevens-Johnson syndrome, and toxic epidermal necrolysis. Results of an international prospective study. *Arch Dermatol*. 2002;138:1019-1024.
6. Cote B, Wechsler J, Bastuji-Garin S, Assier H, Revuz J, Roujeau J-C. Clinicopathologic correlation in erythema multiforme and Stevens-Johnson syndrome. *Arch Dermatol*. 1995;131:1268-1272.
7. Ayangco L, Rogers RS III. Oral manifestations of erythema multiforme. *Dermatol Clin*. 2003;21:195-205.

http://dx.doi.org/10.1016/j.oooo.2015.12.017

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,[1] which highlighted the applicability of diagnostic criteria proposed by several authors in the last two decades.[2-7]

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.

Antonio Celentano, DDS, PhD
Student at University Federico II of Naples
Department of Neurosciences, Reproductive and Odontostomatological Sciences
Naples, Italy

Melbourne Dental School and Oral Health CRC
The University of Melbourne
Victoria, Australia

Serban Tovaru, MD, PhD
Department of Oral Medicine, Faculty of Dental Medicine
University of Medicine and Pharmacy Carol Davila
Bucharest, Romania

Tami Yap, BDSc, FRACDS, PhD
Student at Melbourne Dental School
The University of Melbourne
Victoria, Australia

Daniela Adamo, DDS
Department of Neurosciences, Reproductive and Odontostomatological Sciences
University Federico II of Naples
Naples, Italy

Massimo Aria, PhD
Department of Mathematics and Statistics
Federico II University of Naples
Monte Sant' Angelo, Naples, Italy

Michele Davide Mignogna, MD, DMD
Department of Neurosciences Reproductive and Odontostomatological Sciences
University Federico II of Naples
Naples, Italy

REFERENCES

1. Celentano A, Tovaru S, Yap T, Adamo D, Aria M, Mignogna MD. Oral erythema multiforme: trends and clinical findings of a large retrospective European case series. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2015;120:707-716.
2. Williams PM, Conklin RJ. Erythema multiforme: a review and contrast from Stevens-Johnson syndrome/toxic epidermal necrolysis. *Dent Clin North Am*. 2005;49:67-76.
3. Bastuji-Garin S, Rzany B, Stern RS, Shear NH, Naldi L, Roujeau JC. Clinical classification of cases of toxic epidermal necrolysis, Stevens-Johnson syndrome, and erythema multiforme. *Arch Dermatol*. 1993;129:92-96.
4. Assier H, Bastuji-Garin S, Revus J, Roujeau JC. Erythema multiforme with mucous membrane involvement and Stevens-Johnson syndrome are different disorders with distinct causes. *Arch Dermatol*. 1995;131:539-543.
5. Auquier-Dunant A, Mockenhaupt M, Naldi L, Correia O, Schroder W, Roujeau JC. Correlations between clinical patterns and causes of erythema multiforme major, Stevens-Johnson syndrome, and toxic epidermal necrolysis. Results of an international prospective study. *Arch Dermatol*. 2002;138:1019-1024.
6. Cote B, Wechsler J, Bastuji-Garin, Assier H, Revuz J, Roujeau J-C. Clinicopathologic correlation in erythema multiforme and Stevens-Johnson syndrome. *Arch Dermatol*. 1995;131:1268-1272.
7. Ayangco L, Rogers RS III. Oral manifestations of erythema multiforme. *Dermatol Clin*. 2003;21:195-205.

http://dx.doi.org/10.1016/j.oooo.2016.02.011

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.

Kalyana Chakravarthy Pentapati, MDS[*]
Associate Professor
Department of Public Health Dentistry
Manipal College of Dental Sciences

International Journal of Dermatology

Report

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

Paolo Nuzzolo[1], DDS, PhD, Antonio Celentano[1,2], DDS, PhD, Paolo Bucci[1], MD, DDS, Daniela Adamo[1], DDS, Elvira Ruoppo[1], DDS, PhD, Stefania Leuci[1], DDS, PhD, and Michele Davide Mignogna[1], MD, DDS

[1]Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Naples, Italy, and [2]Melbourne Dental School, The University of Melbourne, Victoria, Australia

Correspondence
Antonio Celentano, DDS, PhD
Department of Neurosciences
Reproductive and Odontostomatological Sciences
University Federico II of Naples
Naples
Italy
E-mail: antony.celentano@gmail.com

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.[1] It affects 1–2% of the general adult population, with the highest frequency in women over 40.[2]

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.[2,3]

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.[4] These variants can also coexist and change during the course of the disease.[5]

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.[1]

Coincident cutaneous lesions appear in approximately 15% of the patients,[4] 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.[2]

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.[5]

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,[6] 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.

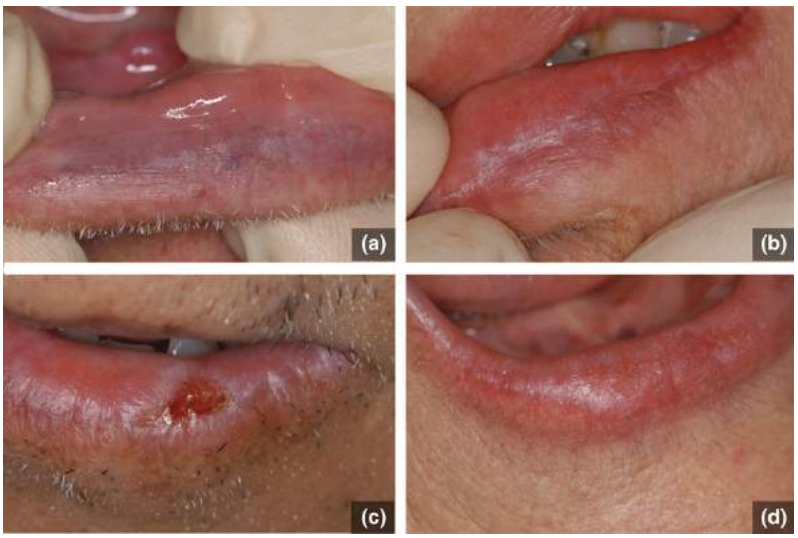


Figure 1 (a) Mild plaque keratosis with multiple micro-erosions of the vermillion. (b) White keratotic striae on labial mucosa with perilesional erythema–exfoliative features of the vermillion with skin inflammation. (c) Ulceration of the vermillion with peripheral keratotic striae. The rest of the lip shows erythema and mild keratosis with exfoliation of the vermillion border that appears undefined. (d) Multiple ulcerations of the mucosal border of the vermillion associated with keratotic isolated papules and striae

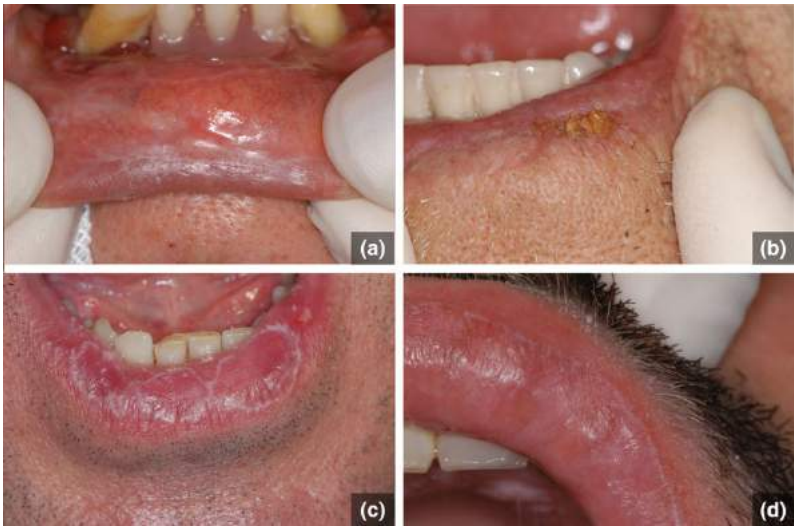


Figure 2 (a) Reticular keratotic lesions of the labial mucosa with keratotic plaques and exfoliation of the vermillion. (b) Squamous cell carcinoma of the lip. (c) Erythema, papules, annular keratotic striae, and erosions of the vermillion interesting also the mucosal side. (d) Linear keratotic lesion with mild plaque. Keratosis of the upper vermillion

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 1 Case series: LP of the lip

Case no.	Year of diagnosis	Patient	Age/ sex	Isolated (oral mucosa)	Symptoms	Clinical form	Skin involvement	Systemic pathologies
1	2002	G G	72/M	Yes (upper)	Burning	Erosive	No	Liver insufficiency
2	2003	C C	80/F	No (lower). Buccal, tongue	Burning	Erosive, keratosic	No	Osteoarthritis
3	2004	S G	78/M	No (lower). Buccal, fold, tongue	No	Atrophic, plaque, reticular	Previously legs and wrists	COPD, former smoker + alcohol, hiatal hernia, ischemic cardiopathy, kidney insufficiency, prostatic hypertrophy
4	2006	B G	77/M	No (lower + upper). Buccal, gingiva, tongue.	No	Erosive, plaque, reticular	No	Former smoker, hypertension, ischemic cardiopathy, prostatic hypertrophy
5	2007	N C	63/M	No (lower). Buccal, gingva	No	Plaque, reticular	Previously lichen ruber	Former smoker + alcohol. celiac disease, rheumatoid arthritis
6	2007	D C	61/F	No (lower). Diffused	Pain, xerostomia	Annular, bullous, LSA	Hands, feet, vagina, nails	Osteoarthritis, osteoporosis
7	2007	S A	77/M	No (lower). Gingiva	Burning	Erosive, keratosic	No	No
8	2007	R A	76/M	No (lower). Buccal, fold, tongue	Burning	Atrophic, erosive, reticular	No	Prostatic hypertrophy
9	2008	P C	78/F	No (lower). Buccal, gingiva, tongue	Burning	Plaque, reticular	Axillas, wrists	Aortic mechanic valve
10	2013	G C	62/M	No (lower). Buccal, tongue	Burning	Erosive, reticular	No	No
11	2014	F A	74/M	Yes (upper + lower)	Burning	Erosive	No	No
12	2014	G V	67/M	No (upper + lower). Gingiva, palate, buccal, tongue	Burning	Reticular, ulcerative	No	Liver insufficiency
13	2014	A A	69/F	No (lower). Gingiva, tongue	No	Keratosic	No	Hypertension

ASA, aminosalicylic acid; COPD, chronic obstructive pulmonary disease; HCV, hepatitis C virus; K lip, OSSC (oral squamous cell carcinoma) of the lip; LP, lichen planus; LSA, lichen sclerosis et atrophicus.

(Table 2). All the cases were presented as true LP, but in three cases, the lesions were diagnosed as concurrent LP and fungal infection,[7] morphea,[8] and systemic lupus erythematosus.[9] A histological specimen was provided in 17 articles.[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,[8,15,19,21–23,25–27] seven from Asia,[7,9–12,16,20] and five from America.[13,14,17,18,24]

Of the 21 case reports and series, 17 described an isolated lip involvement.[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.[11,25]

HCV	Drugs	Biopsy	Diagnosis	Familial	Treatment	Outcome	Notes
Yes	No	Yes	K lip, lichenoid infiltrate	No	Surgical excision	Remission	Died in 2004 of liver insufficiency
Yes	ASA, bisphosphonates	No	LP	No	Cortisone + nystatin	Stable	
No	Antihypertensives, anti-H_2, allopurinol, ASA, beta blockers, statins	Yes	LP	Yes?	Cortisone + nystatin	Stable	Skin involvement appeared and disappeared spontaneously several times
No	Antihypertensives, ASA, beta blockers, statins, nitroglycerin, silodosin	No	LP	No	Nystatin	Regression	
No	No	Yes	LP	No	Nystatin	Stable	Spontaneous remission of skin lesions. Koebner + (buccal lesions)
No	No	Yes	LP	No	Cortisone, nystatin, cyclosporine	Worsening in the mouth, Stable on the lip	Keratosis on the lip only. Skin lesions appeared after oral lesions
Yes	No	Yes	LP	No	Cortisone + nystatin	Remission	LP lasted 10 years, then disappeared
No	Alfuzosin, ASA	No	LP	No	Cortisone + nystatin	Stable	Koebner +
Yes	Allopurinol, bisoprolol, warfarin	No	LP	No	Nystatin	Stable in the mouth, regression on the lips	Skin lesions appeared after oral lesions
No	No	No	LP	No	Cortisone + nystatin	Stable	Lip lesions appeared after oral lesions
No	No	No	LP	No	Cortisone + nystatin	Stable	
Yes	Immunosuppressors	No		No	Cortisone + nystatin	Stable	
Yes	Amiloride + hydrochlorothiazide	No	LP	No	None	Stable	

Discussion

In the literature, the most frequently reported clinical form of LPL is the erosive (10 cases),[7,14,16–20,22,24,25] followed by the reticular/annular (three),[10,11,21] nodular (one),[23] and bullous (one).[15] Accordingly, symptoms such as pain, burning, bleeding, and crusting were reported in 13 cases,[7,14–22,27] while only two cases were completely asymptomatic.[10,11] Only four cases of concurrent skin lesions have been described.[15,20,23,24]

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

Table 2 Review of literature: Case reports/series of LPL

Year	First author/ reference	Country	No. of patients	Age/ sex	Isolated (oral mucosa)	Symptoms	Clinical f eatures	Skin involvement	Systemic pathologies	HCV	Biopsy	Diagnosis	Treatment	Outcome
2012	Domingues[24]	USA	1	44/M	Yes (lower)	Painful, bleeding	Erosive	Yes	Treatment with imiquimod	No	No	LPL	Clobetasol	Remission
2012	Holmukhe[10]	India	1	40/M	Yes (lower)	No	Annular	No	No	No	Yes	LPL	Tacrolimus	NA
2012	Sugashima[11]	Japan	1	32/F	Yes (lower + upper)	No	Annular atrophic	No	Allergy to zinc	NA	Yes	LPL	Tacrolimus	Regression
2011	Gencoglan[12]	Turkey	4	NA	Yes	NA	NA	NA	NA	NA	Yes	LPL	Imiquimod	Recurrence in 1/4
2011	De Moraes[13]	Brazil	1	7/F	No (upper)	NA	NA	NA	NA	NA	Yes	OLP	Corticosteroid	Remission
2008	Johnson[14]	USA	1	42/F	Yes (lower)	Dryness, peeling	Erosive	No	No	No	Yes	LPL	Tacrolimus	Stable
2007	Petruzzi[25]	Italy	10	NA	Yes (lower and/or upper)	Erosions and crusting	Erosive	NA	NA	5/10	NA	LPL	Clobetasol + tocopherol	Remission in 8/10
2007	van Tuyll[15]	Netherlands	1	75/F	No (lower)	Burning, bleeding	Bullous	Yes	No	NA	Yes	OLP	Tretinoin + triamcinolone	Remission
2006	Shichinohe[16]	Japan	2	64, 68/M, M	No (lower), no (lower)	Painful	Erosive	No	NA	NA	Yes	OLP	Tacrolimus	Regression
2005	Donovan[17]	USA	1	51/M	Yes	Painful	Erosive	NA	NA	Yes	Yes	LPL	Tacrolimus	Remission
2003	Yu[18]	USA	1	44/M	Yes (lower)	Burning	Erosive	No	Hypertension	No	Yes	LPL	Clobetasol	Remission
2002	Chiang[7]	Taiwan	1	36/F	Yes (lower)	Painful	Erosive	No	No	NA	Yes	LPL + mycosis	griseofulvin + prednisolone	Remission
2002	Cecchi[19]	Italy	1	45/M	Yes (lower)	Burning, swelling	Erosive	No	No	No	Yes	LPL	Betamethasone	Remission, recurrence on the limbs
2000	Melato[8]	Italy	1	NA	Yes (upper)	NA	NA	NA	Vitiligo	NA	Yes	LPL + morphea	NA	NA
1997	De Argila[26]	Spain	1	51/F	Yes (lower)	NA	NA	No	NA	NA	NA	LPL	Chloroquine phosphate	Regression
1997	Isogai[20]	Japan	1	54/M	Yes	Painful	Erosive	Yes	NA	NA	Yes	LPL	NA	NA
1996	Allan[21]	UK	1	51/M	Yes (lower)	Irritation, scaliness	Reticular	No	No	NA	Yes	LPL	Betamethasone	Remission
1995	Itin[22]	Switzerland	1	44/NA	Yes (lower)	Burning	Erosive	No	NA	NA	Yes	LPL	Acitretin + steroid	Remission
1992	Harland[23]	UK	1	23/M	No (lower)	NA	Nodular	Yes	Former smoker	NA	Yes	OLP	Corticosteroid	Regression, recurrence, K
1978	Piamphongsant[9]	Thailand	2	NA	Yes (lower)	NA	NA	NA	LES	NA	Yes	LPL + LES	NA	NA
1937	Whittle[27]	UK	1	69/M	Yes (lower)	Irritation	Plaque	Anal mucosa	No	NA	No	LPL?	Mercury, arsenic, x-ray	Stable

LES; LPL, lichen planus of the lip; NA, not applicable; OLP, oral lichen planus.

Table 3 Prevalence of lip involvement in oral LP

Year	First author/ reference	Country	No. of patients	Age range	Female/ male	Isolated lip	Lip involvement	Cutaneous involvement (% of patients)
2010	Bajaj[30]	India	95	17–62 (34–36 mean value)	55/40	NA	29.4%. Upper 7.4% (reticular). Lower 22.1% (reticular, erosive)	NA
2009	Carrozzo[34]	Italy	Review	NA	NA	NA	Lower lip 4th most involved site	15
2009	Aminzadeh[2]	Iran	187	46 (mean value)	72%/28%	0.51%	6.3%	1.25
2005	Xue[29]	China	674	10–78 (49–52 mean value)	66%/34%	8.9%	Upper 1.91% (erosive). Lower 32.3% (reticular). Third most common site of involvement	11
2005	Eisen[33]	USA	Review	NA	NA	NA	4th most common site of involvement	15
2002	Eisen[31]	USA	723	13–82 (57–47 mean value)	75%/25%	NA	Upper 2%. Lower 14%	NA
2001	Romero[32]	Spain	62	63 (mean value)	52%/48%	NA	28.6% LP HCV+ vs. 7.3% LP HCV-	NA
1992	Bagan-Sebastian	Spain	205	NA	NA	NA	NA	NA

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.[17,25]

As for the treatment, tacrolimus was used in five cases,[10,11,14,16,17] and the reported outcome was regression in two cases,[11,16] remission in one,[17] and the persistence of the lesion in one[14] (in one there was no reported outcome); corticosteroids, alone or in association with other drugs, were reported to have been used in 10 articles,[7,13,15,18,19,21–25] causing remission of the lesion in most cases.[7,13,15,18,19,21–24] However, in one article, recurrence and malignant transformation was described.[25] 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.[27]

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 32.3% and 6.3%,[28–32] 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.[33,34] 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.*[23] 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,[35] 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,[36] perforin, and granzyme B,[37] and finally caspase 3, Bax, and Bcl-2 associated with apoptosis[38] 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.[37] 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 vermillion.

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.[39] 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

1 Roopashree MR, Gondhalekar RV, Shashikanth MC, et al. Pathogenesis of oral lichen planus – a review. *J Oral Pathol Med* 2010; **39**: 729–734.

2 Sugerman PB, Savage NW, Walsh LJ, et al. The pathogenesis of oral lichen planus. *Crit Rev Oral Biol Med* 2002; **13**: 350–365.

3 van der Meij EH, van der Waal I. Lack of clinicopathologic correlation in the diagnosis of oral lichen planus based on the presently available diagnostic criteria and suggestions for modifications. *J Oral Pathol Med* 2003; **32**: 507–512.

4 Ismail SB1, Kumar SK, Zain RB. Oral lichen planus and lichenoid reactions: etiopathogenesis, diagnosis, management and malignant transformation. *J Oral Sci* 2007; **49**: 89–106.

5 van der Waal I. Oral lichen planus and oral lichenoid lesions; a critical appraisal with emphasis on the diagnostic aspects. *Med Oral Patol Oral Cir Bucal* 2009; **14**: E310–314.

6 Farhi D, Dupin N. Pathophysiology, etiologic factors, and clinical management of oral lichen planus, part I: facts and controversies. *Clin Dermatol* 2010; **28**: 100–108.

7 Chiang CT, Chan HL. Superficial mycosis superimposing on isolated lichen planus of the lip: a case report and review of the literature. *Cutis* 2002; **69**: 305–308.

8 Melato M, Gorji N, Rizzardi C, Maglione M. Associated localization of morphea and lichen planus of the lip in a patient with vitiligo. *Minerva Stomatol* 2000; **49**: 549–554.

9 Piamphongsant T, Sawannapreecha S, Arangson PG, et al. Mixed lichen planus-lupus erythematosus disease. *J Cutan Pathol* 1978; **5**: 209–215.

10 Holmukhe S, Gutte RM, Sirur S. Letter: Isolated annular lichen planus of lower lip. *Dermatol Online J* 2012; **18**: 15.

11 Sugashima Y, Yamamoto T. Letter: Annular atrophic lichen planus of the lip. *Dermatol Online J* 2012; **18**: 14.

12 Gencoglan G, İnanir İ, Sahin O, Gunduz K. Imiquimod 5% cream for isolated lichen planus of the lip. *J Dermatolog Treat* 2011; **22**: 55–59.

13 De Moraes PC, Teixeira RG, Tacchelli DP, et al. Atypical case of oral lichen planus in a pediatric patient: clinical presentation and management. *Pediatr Dent* 2011; **33**: 445–447.

14 Johnson H, Soldano AC, Kovich O, Long W. Oral lichen planus. *Dermatol Online J* 2008; **14**: 20.

15 vanvan Tuyll Serooskerken AM, vanMarion AM, deZwart-Storm E, et al. Lichen planus with bullous manifestation on the lip. *Int J Dermatol* 2007; **46** (Suppl. 3): 25–26.

16 Shichinohe R, Shibaki A, Nishie W, et al. Successful treatment of severe recalcitrant erosive oral lichen planus with topical tacrolimus. *J Eur Acad Dermatol Venereol* 2006; **20**: 66–68.

17 Donovan JC, Hayes RC, Burgess K, et al. Refractory erosive oral lichen planus associated with hepatitis C: response to topical tacrolimus ointment. *J Cutan Med Surg* 2005; **9**: 43–46.

18 Yu TC, Kelly SC, Weinberg JM, Scheinfeld NS. Isolated lichen planus of the lower lip. *Cutis* 2003; **71**: 210–212.

19 Cecchi R, Giomi A. Isolated lichen planus of the lip. *Australas J Dermatol* 2002; **43**: 309–310.

20 Isogai Z, Koashi Y, Sunohara A, Tsuji T. Ulcerative lichen planus: a rare variant of lichen planus. *J Dermatol* 1997; **24**: 270–272.

21 Allan SJ, Buxton PK. Isolated lichen planus of the lip. *Br J Dermatol* 1996; **135**: 145–146.

22 Itin PH, Schiller P, Gilli L, Buechner SA. Isolated lichen planus of the lip. *Br J Dermatol* 1995; **132**: 1000–1002.

23 Harland CC, Phipps AR, Marsden RA, Holden CA. Squamous cell carcinoma complicating lichen planus of the lip. *J R Soc Med* 1992; **85**: 235–236.

24 Domingues E, Chaney KC, Scharf MJ, Wiss K. Imiquimod reactivation of lichen planus. *Cutis* 2012; **89** (276–277): 283.

25 Petruzzi M, De Benedittis M, Pastore L, et al. Isolated lichen planus of the lip. *Int J Immunopathol Pharmacol* 2007; **20**: 631–635.
26 De Argila D, Gonzalo A, Pimentel J, Rovira I. Isolated lichen planus of the lip successfully treated with chloroquine phosphate. *Dermatology* 1997; **195**: 284–285.
27 Whittle CH. Case for diagnosis? Lichen planus of lip. *Proc R Soc Med* 1939; **32**: 1402.
28 Aminzadeh A, Jahanshahi G, Ahmadi M. A retrospective comparative study on clinico-pathologic features of oral lichen planus and oral lichenoid lesions. *Dent Res J (Isfahan)* 2013; **10**: 168–172.
29 Xue JL, Fan MW, Wang SZ, et al. A clinical study of 674 patients with oral lichen planus in China. *J Oral Pathol Med* 2005; **34**: 467–472.
30 Bajaj DR, Khoso NA, Devrajani BR, et al. Oral lichen planus: a clinical study. *J Coll Phys Surg Pak* 2010; **20**: 154–157.
31 Eisen D. The clinical features, malignant potential, and systemic associations of oral lichen planus: a study of 723 patients. *J Am Acad Dermatol* 2002; **46**: 207–214.
32 Romero MA, Seoane J, Varela-Centelles P, et al. Clinical and pathological characteristics of oral lichen planus in hepatitis C-positive and -negative patients. *Clin Otolaryngol Allied Sci* 2002; **27**: 22–26.
33 Eisen D, Carrozzo M. Bagan Sebastian JV, Thongprasom K. *Number V* Oral lichen planus: clinical features and management. *Oral Dis* 2005; **11**: 338–349.
34 Carrozzo M, Thorpe R. Oral lichen planus: a review. *Minerva Stomatol* 2009; **58**: 519–537.
35 Chen J, Feng J, Chen X, et al. Immunoexpression of interleukin-22 and interleukin-23 in oral and cutaneous lichen planus lesions: a preliminary study. *Mediators Inflamm* 2013; **2013**: 801974.
36 Shen Z, Gao X, Ma L, et al. Expression of Foxp3 and interleukin-17 in lichen planus lesions with emphasis on difference in oral and cutaneous variants. *Arch Dermatol Res* 2014; **306**: 441–446.
37 Lage D, Pimentel VN, Soares TC, et al. Perforin and granzyme B expression in oral and cutaneous lichen planus – a comparative study. *Cutan Pathol* 2011; **38**: 973–978.
38 Abdel-Latif AM, Abuel-Ela HA, El-Shourbagy SH. Increased caspase-3 and altered expression of apoptosis-associated proteins, Bcl-2 and Bax in lichen planus. *Clin Exp Dermatol* 2009; **34**: 390–395.
39 Sharma A, Białynicki-Birula R, Schwartz RA, Janniger CK. Lichen planus: an update and review. *Cutis* 2012; **90**: 17–23.

ACTA OTORHINOLARYNGOLOGICA ITALICA 2015;35:449-454; DOI: 10.14639/0392-100X-922

CLINICAL TECHNIQUES AND TECHNOLOGY

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

A. MORO[1,2], C. DE WAURE[3], F. DI NARDO[3], F. SPADARI[4], M.D. MIGNOGNA[5], M. GIULIANI[6], L. CALIFANO[7], A.B. GIANNÌ[8], L. CARDARELLI[1], A. CELENTANO[5], G. BOMBECCARI[4], S. PELO[2]

[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; [3] Institute of Public Health, Università Cattolica del Sacro Cuore, Rome, Italy; [4] Department of Reconstructive and Diagnostic Surgical Sciences, Unit of Oral Pathology and Medicine, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Italy; [5] Department of Integrated Activities Head-Neck, Unit of Oral Medicine, Università di Napoli Federico II. Naples, Italy; [6] Department of Dentistry, Università Cattolica del Sacro Cuore, Rome, Italy; [7] Department of Integrated Activities Head-Neck, Unit of Maxillofacial Surgery, Università di Napoli Federico II. Naples, Italy; [8] Department of Reconstructive and Diagnostic Surgical Sciences, Complex Unit of Maxillofacial Surgery, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, 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 wearing the GOCCLES® spectacles while the light from a dental curing light irradiated the oral mucosa. Lesions were defined as visible potentially malignant lesions and/or fluorescence loss areas. All persisting lesions underwent excisional or incisional biopsy. Sixty-one patients were enrolled. Data from 64 biopsies were analysed. Of the 62 lesions identified by the device, 31 were true positives. The device identified 31 of 32 true positive lesions. One lesion (an invasive carcinoma) was not visible to the naked eye. The device identified all lesions classified as moderate dysplasia to invasive cancer. In 56.7% of cases, true positive lesions showed greater extension when observed through the device. The GOCCLES® medical device allowed the direct visualisation of fluorescence loss in patients suffering from mild to severe dysplasia and in situ to invasive oral cancer. It allowed 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 persistenti 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 tra moderata e severa e ogni carcinoma sono stati correttamente identificati dal dispositivo. Nel Il 56,7% delle lesioni identificate dal dispositivo mostrava 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*

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 [1]. 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 [2]. 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 [2]. The disruption of the extracellular matrix, hyperemia and neo-angiogenesis also contribute to reduce fluorescence emission [2].

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 [3].

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 [4]. 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.

Fig. 1. The GOCCLES (Glasses for Oral Cancer – Curing Light Exposed – Screening) medical device.

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

distance from the oral mucosa and to direct the light perpendicularly to the inspected area. The naked eye inspection and the autofluorescence examination had to be performed by the same operator. All fluorescence loss areas were regarded as potentially malignant lesions of the oral mucosa. The following dental curing lights were used during the study: Elipar S10 3M ESPE (used by the Units of Milan); Led.B Carlo de Giorgi (Units of Rome and Foligno); Optilux 501 Kerr Corporation (Units of Naples). After examinations, all lesions persisting for at least two weeks (detected by at least one between the naked eye inspection and the autofluorescence examination) underwent excisional biopsy. If the excisional biopsy was not feasible, patients underwent incisional biopsy. All biopsies were properly oriented and showed the margins detected by both examinations. If an incisional biopsy including both identified margins was not feasible, two incisional biopsies of the same lesion on different margins were allowed. Different incisional biopsies of the same lesion were considered as a single biopsy. All biopsies underwent histological examination. No blinding on pathologist assessment or data analysts was planned.

For each lesion the following data were recorded: if visible to the naked eye and/or on autofluorescence analysis; which examination showed the greater extension if results were non-overlapping; which margins identified by the two techniques were infiltrated; the histological report. True positive lesions were defined as any dysplasia or cancer of the oral cavity. False positive lesions included negative histological findings and any other disorder of the oral mucosa not related to cancer.

Primary outcomes of the study were: proportion of visible lesions; proportion of infiltrated margins; proportion of true positive, false positive and false negative lesions. Given the study design, it was not possible to assess true negative lesions (no proper follow-up was planned).

The study protocol was approved by the respective ethics committees of the Institutions involved in the study.

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 [4]. 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.

Results

Sixty-one patients were enrolled and all underwent both naked eye inspection and autofluorescence examination of 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 GOCCLES® 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

Table I. Main demographic and clinical characteristics of the patients enrolled in the GOCCLES study, by center

		Rome	Milan	Naples	Foligno	TOTAL
Patients [N]		14	21	11	15	61
Age [mean, (SD)]		67 (14)	66 (16)	66 (13)	63 (11)	66 (14)
Gender [N, (%)]	Female	8 (57.1)	15 (71.4)	8 (72.7)	7 (46.7)	39 (63.9)
Group [N, (%)]	A*	11 (78.6)	16 (76.2)	11 (100)	14 (93.3)	52 (85.2)
	B†	3 (21.4)	5 (23.8)	0 (0)	1 (6.7)	9 (14.8)
Biopsies/patient	1	14 (100)	21 (100)	10 (90.9)	13 (86.6)	58 (95.1)
[N, (%)]	2	0 (0)	0 (0)	1 (9.1)	1 (6.7)	2 (3.3)
	3	0 (0)	0 (0)	0 (0)	1 (6.7)	1 (1.6)

** Patients suffering from suspected dysplastic lesions of the oral mucosa.*

† Patients in follow-up after surgical resection of oral cancer.

Table II. Autofluorescence examination.

Lesion description	Detected by naked eye inspection only	Detected by both techniques	Detected by AF examination only	TOTAL*
False positive† [N (%)]	1 (3.1)	28 (87.5)	3 (9.4)	32 (50.0)
True positive [N (%)]	1 (3.1)	30 (93.8)	1 (3.1)	32 (50.0)
Of which:				
Mild dysplasia [N (%)]	1 (9.1)	10 (90.9)	0 (0)	11 (17.2)
Moderate dysplasia [N (%)]	0 (0)	4 (100)	0 (0)	4 (6.3)
Severe dysplasia [N (%)]	0 (0)	3 (100)	0 (0)	3 (4.7)
Carcinoma in situ [N (%)]	0 (0)	2 (100)	0 (0)	2 (3.1)
Invasive cancer [N (%)]	0 (0)	11 (91.7)	1 (8.3)	12 (18.8)
TOTAL [N (%)]	2 (3.1)	58 (90.6)	4 (6.3)	64 (100)

** 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.*

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

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

	Not visible to AF	AF margins not infiltrated	AF margins infiltrated	TOTAL
Not visible to the NE	-	0 (0%)	1 (3.1%)	1 (3.1%)
NE margins not infiltrated	1 (3.1%)	3 (9.4%)	6 (18.8%)	10 (31.3%)
NE margins infiltrated	0 (0%)	4 (12.5%)	17 (53.1%)	21 (65.6%)
TOTAL	1 (3.1%)	7 (21.9)	24 (75.0%)	32 (100%)

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.

Table IV. Comparison of the performance of the GOCCLES device with different curing lights.

	Auto fluorescence examination results				Lesion margin detection		
	TP lesion	FP lesion	Not detected	p value*	Free margins	Infiltrated margins	p value*
Led.B [N (%)]	15 (46.9)	16 (50.0)	1 (3.1)	0.488	4 (26.7)	11 (73.3)	0.174
Elipar S10 [N (%)]	8 (38.1)	13 (61.9)	0 (0)		0 (0)	8 (100)	
Optilux 501 [N (%)]	8 (72.7)	3 (27.3)	0 (0)		3 (37.5)	5 (62.5)	

*TP: true positive; FP: false positive. * Chi square test.*

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/cm^2; the Elipar S10 LED lamp has a 430-480 nm wavelength and an intensity of 1,000 mW/cm^2; the Optilux 501 is a halogen lamp with a wavelength of 400-505 nm and an intensity of 850-1,000 mW/cm^2. The GOCCLES® device was also previously successfully tested with another halogen lamp with different characteristics (400-500 nm wavelength and 600 mW/cm^2 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 [2 4]. 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 GOCCLES®. Remarkably, however, in one case GOCCLES® 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 [5].

Furthermore, data on its diagnostic performance are lacking because of the small sample size and study de-

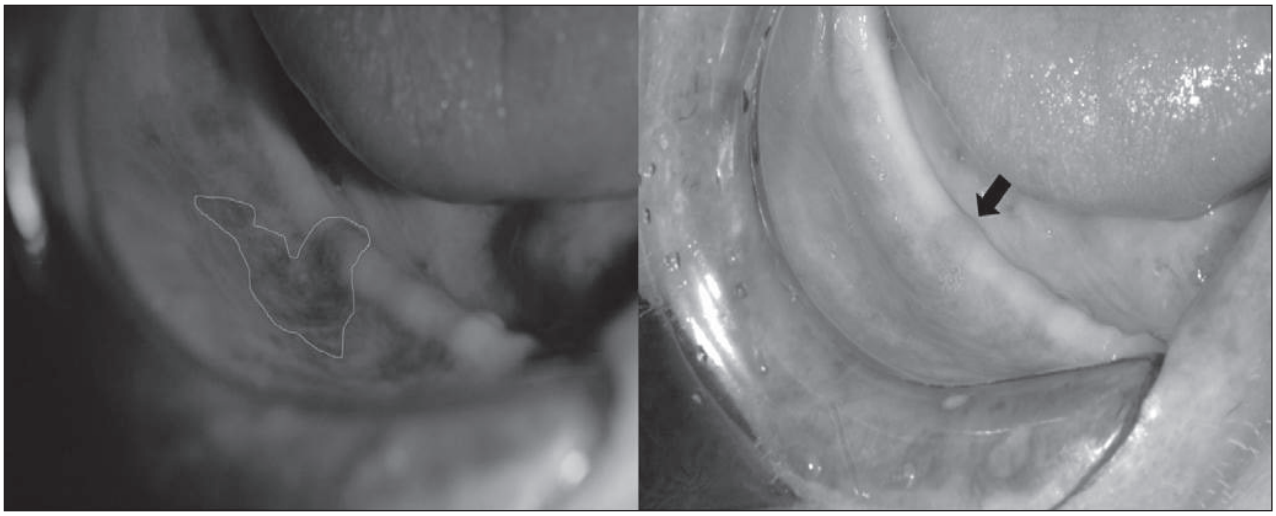


Fig. 2. Oral cancer in an edentulous patient in follow-up after surgical resection of a malignant lesion. Autofluorescence examination (on the left) vs. conventional visual examination (on the right). The lesion is barely visible if the oral examination is performed with superficiality. Loss of fluorescence increased contrast making it easier to see the tumour. Also visible in this figure is a clear difference in the extension of the margins of the lesion: fluorescence loss extended beyond the margins, which were visible to the naked eye. The arrow points to the main lesion. The margins of the lesion (as visible on the autofluorescence examination) are also highlighted.

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

1 Petersen PE. *Oral cancer prevention and control--the approach of the World Health Organization*. Oral Oncol 2009;45:454-60.

2 Monici M. *Cell and tissue autofluorescence research and diagnostic applications.* Biotechnol Annu Rev 2005;11:227-56.

3 Succo G, Garofalo P, Fantini M, et al. *Direct autofluorescence during CO2 laser surgery of the larynx: can it really help the surgeon?* Acta Otorhinolaryngol Ital 2014;34:174-83

4 Moro A, Di Nardo F, Boniello R et al. *Autofluorescence and early detection of mucosal lesions in patients at risk for oral cancer*. J Craniofac Surg 2010;21:1899-903.

5 Sankaranarayanan R, Ramadas K, Thomas G, et al. *Effect of screening on oral cancer mortality in Kerala, India: a cluster-randomised controlled trial*. Lancet 2005;365:1927-33.

Received: September 1, 2015 - Accepted: October 1, 2015

Address for correspondence: Francesco Di Nardo, Istituto di Sanità Pubblica, Sezione di Igiene. Università Cattolica del Sacro Cuore, largo Francesco Vito 1, 00168 Rome, Italy. E-mail: francesco.dinardo2584@gmail.com; francesco_pope84@hotmail.com

Pain Medicine

Pain Medicine 2015; 16: 2171–2179
Wiley Periodicals, Inc.

NEUROPATHIC PAIN SECTION

Original Research Article

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

Daniela Adamo, DDS,* Antonio Celentano, DDS,* Elvira Ruoppo, DDS, PhD,* Claudia Cucciniello, MD,* Giuseppe Pecoraro, MD,* Massimo Aria, MD, PhD,† and Michele D. Mignogna, MD, DMD*

*Department of Neurosciences, Reproductive and Odontostomatological Sciences; †Department of Economics and Statistics, University Federico II of Naples

Reprint requests to: Daniela Adamo, DDS, Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, via Pansini n°5 Naples; 80131 Italy. Tel: +393925253864; +3908119305269; E-mail: daniela.adamo@unina.it.

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 dysgeusia, a bitter/metallic taste, subjective xerostomia, 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 in the absence of hard and soft tissue lesions of any kind; 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, hyposcialia-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 current state. It is a test which is widely used in psychosocial 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, scalding, 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%.

Table 1 Sociodemographic and clinical characteristics of BMS patients

Patient Characteristics	Frequencies (Percentages)	
Gender	Male	19 (25.3)
	Female	56 (74.7)
Education level (in years)	0–4	8 (10.7)
	5–7	27 (36.0)
	8–10	15 (20.0)
	11–13	15 (20.0)
	14–18	10 (13.3)
	Mean ± SD	8.57 ± 4.95
Age (in years)	20–29	1 (1.3)
	30–39	2 (2.7)
	40–49	9 (12.0)
	50–59	22 (29.3)
	60–69	19 (25.3)
	70–79	19 (25.3)
	>79	3 (4.0)
	Mean ± SD	61.17 ± 11.75
Marital status	Single	5 (6.7)
	Married	57 (76.0)
	Divorced	4 (5.3)
	Widowed	9 (12.0)
Job status	Employed	15 (20.0)
	Unemployed	60 (80.0)
Age at disease onset (in years)	20–29	2 (2.7)
	30–39	4 (5.3)
	40–49	13 (17.3)
	50–59	21 (28.0)
	60–69	24 (32.0)
	70–79	11 (14.7)
	Mean ± SD	56.75 ± 12.01
VAS scale	1–4	18(24)
	5–7	29(38.7)
	8–10	28(37.3)

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

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

	Age at Disease Onset	*P* Value
Gender		
Male	60.74 ± 14.06	0.094
Female	55.39 ± 11.05	
Job status		
Employed	46.33 ± 10.66	<0.001**
Unemployed	59.35 ± 10.96	
Marital status		
Single	49.80 ± 23.08	0.193
Married	57.82 ± 10.45	
Divorced	47.25 ± 10.05	
Widowed	58.00 ± 13.45	
Educational level	Spearman ρ −0.314	0.006**

The significance difference between the means was measured by the Anova test procedure.
* Significant $0.01 \leq P \leq 0.05$.
** Significant $P \leq 0.01$.

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).

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

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

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 $0.01 < P \leq 0.05$.
** Significant $P \leq 0.01$.

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 4 Gender, marital status, job status, age, educational level, and age at disease onset differences in relation to symptoms of BMS

Symptoms		Change of Tongue Morphology	Change of Tongue Color	Bitter Taste	Scalding	Burning	Pain	Taste Disturbances	Xerostomia	Scialorrea	Intraoral Foreign Body Sensation
Gender	Male	52.6%	31.6%	42.1%	21.1%	57.9%	42.1%	15.8%	63.2%	5.3%	21.1%
	Female	35.7%	39.3%	41.1%	26.8%	66.1%	42.9%	33.9%	58.9%	0.0%	35.7%
	P value	0.049*	0.377	0.572	0.434	0.521	0.586	0.001**	0.482	0.153	0.045
Marital status	Single	20.0%	40.0%	20.0%	20.0%	40.0%	80.0%	60.0%	60.0%	0.0%	60.0%
	Married	43.9%	35.1%	42.1%	28.1%	70.2%	40.4%	28.1%	57.9%	1.8%	35.1%
	Divorced	0.0%	25.0%	25.0%	0.0%	50.0%	50.0%	25.0%	25.0%	0.0%	0.0%
	Widowed	44.4%	55.6%	55.6%	22.2%	44.4%	33.3%	22.2%	88.9%	0.0%	11.1%
	P value	0.143	0.396	0.350	0.443	0.259	0.308	0.310	0.082	0.907	0.033*
Job status	Unemployed	43.3%	38.3%	43.3%	25.0%	68.3%	41.7%	33.3	60.0%	1.7%	33.3%
	Employed	26.7%	33.3%%	33.3%	26.7%	46.7%	46.7%	13.3	60.0%	0.0%	26.7%
	P value	0.229	0.719	0.482	0.895	0.118	0.727	0.049*	1.000	0.617	0.621
Age (in years)	20–29	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	100.0%
	30–39	0.0%	0.0%	0.0%	50.0%	0.0%	50.0%	0.0%	50.0%	0.0%	0.0%
	40–49	44.4%	44.4%	22.2%	33.3%	66.7%	33.3%	22.2%	33.3%	0.0%	33.3%
	50–59	31.8%	27.3%	40.9%	31.8%	63.6%	40.9%	31.8%	50.0%	0.0%	31.8%
	60–69	47.4%	52.6%	47.4%	21.1%	73.7%	42.1%	26.3%	73.7%	0.0%	36.8%
	70–79	36.8%	47.4%	47.4%	15.8%	63.2%	47.4%	31.6%	68.4%	5.3%	31.6%
	>79	33.3%	33.3%	66.7%	33.3%	66.7%	33.3%	66.7%	100.0%	0.0%	0.0%
	P value	0.523	0.379	0.125	0.320	0.407	0.881	0.637	0.039*	0.810	0.386
Educational level	0–4	25.0%	50.0%	50.0%	12.5%	62.5%	37.5%	37.5%	87.5%	0.0%	25.0%
	5–7	44.4%	55.6%	48.1%	33.3%	77.8%	59.3%	40.7%	59.3%	0.0%	40.7%
	8–10	40.0%	26.7%	66.7%	33.3%	53.3%	33.3%	33.3%	53.3%	0.0%	13.3%
	11–13	33.3%	33.3%	13.3%	20.0%	66.7%	26.7%	6.7%	53.3%	10.0%	40.0%
	14–18	30.0%	20.0%	20.0%	10.0%	40.0%	40.0%	20.0%	60.0%	1.3%	30.0%
	P value	0.825	0.040*	0.022*	0.422	0.238	0.214	0.124	0.476	0.081	0.361
Age at disease onset	20–29	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	50.0%
	30–39	25.0%	50.0%	50.0%	75.0%	25.0%	50.0%	25.0%	75.0%	0.0%	25.0%
	40–49	38.5%	38.5%	30.8%	30.8%	69.2%	30.8%	15.4%	53.8%	0.0%	30.8%
	50–59	28.6%	23.8%	33.3%	23.8%	61.9%	38.1%	33.3%	47.6%	0.0%	38.1%
	60–69	54.2%	54.2%	58.3%	20.8%	70.8%	**50.0%**	29.2%	66.7%	4.2%	33.3%
	70–79	27.3%	45.5%	36.4%	18.2%	72.7%	36.4%	45.5%	81.8%	0.0%	18.2%
	P value	0.291	0.238	0.276	0.246	0.203	0.397	0.516	0.076	0.805	0.884

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

* Significant $0.01 < P \leq 0.05$.

** significant <0.001.

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

Trigger		Undefined	Initiation of Medications	Antecedent Dental Procedures	Antecedent Dental Procedures/Stressful Life Events	Stressful Life Events	*P* Value
Gender	Male	47.4%	5.3%	10.5%	5.3%	31.6%	0.089
	Female	23.2%	3.6%	23.2%	0.0%	50.0%	
Marital status	Single	20.0%	40.0%	0.0%	0.0%	40.0%	0.036*
	Married	31.6%	1.8%	19.3%	1.8%	45.6%	
	Divorced	0.0%	0.0%	50.0%	0.0%	50.0%	
	Widowed	33.3%	0.0%	22.2%	0.0%	44.4%	
Job status	Unemployed	30.0%	3.3%	25.0%	1.7%	40.0%	0.180
	Employed	26.7%	6.7%	0.0%	0.0%	66.7%	
Age (in years)	20–29	100.0%	0.0%	0.0%	0.0%	0.0%	0.137
	30–39	0.0%	50.0%	50.0%	0.0%	0.0%	
	40–49	22.2%	0.0%	22.2%	0.0%	55.6%	
	50–59	22.7%	4.5%	18.2%	0.0%	54.5%	
	60–69	15.8%	0.0%	21.1%	5.3%	57.9%	
	70–79	42.1%	5.3%	21.1%	0.0%	31.6%	
	>79	100.0%	0.0%	0.0%	0.0%	0.0%	
Educational level	0–4	12.5%	0.0%	50.0%	0.0%	37.5%	0.049*
	5–7	29.6%	0.0%	22.2%	0.0%	48.1%	
	8 −10	40.0%	0.0%	13.3%	0.0%	46.7%	
	11–13	13.3%	13.3%	0.0%	6.7%	66.7%	
	14–18	50.0%	10.0%	30.0%	0.0%	10.0%	
Age of disease onset	20–29	50.0%	50.0%	0.0%	0.0%	0.0%	0.383
	30–39	25.0%	0.0%	25.0%	0.0%	50.0%	
	40–49	23.1%	0.0%	30.8%	0.0%	46.2%	
	50–59	19.0%	4.8%	14.3%	0.0%	61.9%	
	60–69	33.3%	4.2%	16.7%	4.2%	41.7%	
	70–79	45.5%	0.0%	27.3%	0.0%	27.3%	

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

* Significant $0.01 < P \leq 0.05$.

** Significant $P \leq 0.01$.

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 catastrophisizing, 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%).

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 (79%); 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 temporomandibolar 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 ($\rho = 0.223$, $P = 0.055$).

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].

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

1 Grushka M, Sessle BJ. Burning mouth syndrome. Dent Clin North Am 1991;35(1):171–84.

2 Bergdahl M, Bergdahl J. Burning mouth syndrome: Prevalence and associated factors. J Oral Pathol Med 1999;28(8):350–4.

3 Headache Classification Subcommittee of the International Headache Society. The International Classification of Headache Disorders: 2nd edition. Cephalalgia 2004;24(Suppl 1):9–160.

4 Lamey PJ, Murray BM, Eddie SA, Freeman RE. The secretion of parotid saliva as stimulated by 10% citric acid is not related to precipitating factors in burning mouth syndrome. J Oral Pathol Med 2001;30(2): 121–4.

5 Klasser GD, Epstein JB, Villines D. Management of burning mouth syndrome. J Can Dent Assoc 2011; 77:b151

6 Charleston L 4th. Burning mouth syndrome: A review of recent literature. Curr Pain Headache Rep 2013;17(6):336.

7 Spanemberg JC, Rodríguez de Rivera Campillo E, Salas EJ, López López J. Burning Mouth Syndrome: Update. Oral Health Dent Manag 2014;13(2):418–24. PubMed PMID: 24984658.

8 Rodríguez-de Rivera-Campillo E, López-López J. Evaluation of the response to treatment and clinical evolution in patients with burning mouth syndrome. Med Oral Patol Oral Cir Bucal 2013;18(3):e403–10. PubMed PMID: 23229252; PubMed Central PMCID: PMC3668864.

9 Rodríguez de Rivera Campillo E, López-López J, Chimenos-Küstner E. Response to topical clonazepam in patients with burning mouth syndrome: A clinical study. Bull Group Int Rech Sci Stomatol Odontol 2010;49(1):19–29. PubMed PMID: 22750263.

10 Soares MS, Chimenos-Küstner E, Subirá-Pifarrè C, Rodríguez de Rivera-Campillo ME, López-López J. Association of burning mouth syndrome with xerostomia and medicines. Med Oral Patol Oral Cir Bucal 2005;10(4):301–8. English, Spanish. PubMed PMID: 16056186.

11 Coculescu E, Tovaru S, Coculescu B. Epidemiological and etiological aspects of burning mouth syndrome. J Med Life 2014;7(3):305–9. Epub 2014 Sep 25. Review. PubMed PMID: 25408745; PubMed Central PMCID: PMC4206710.

12 Aravindhan R, Vidyalakshmi S, Kumar MS, Satheesh C, Balasubramanium AM, Prasad VS. Burning mouth syndrome: A review on its diagnostic and therapeutic approach. J Pharm Bioallied Sci 2014; 6(Suppl 1):S21–5. doi: 10.4103/0975-7406.137255. Review. PubMed PMID: 25210377; PubMed Central PMCID: PMC4157273

13 Grushka M, Ching V, Epstein J. Burning mouth syndrome. Adv Otorhinolaryngol 2006;63:278–87.

14 van der Ploeg HM, van der Wal N, Eijkman MA, van der Waal I. Psychological aspects of patients with burning mouth syndrome. Oral Surg Oral Med Oral Pathol 1987;63:664–8.

15 Forssell H, Jääskeläinen S, Tenovuo O, Hinkka S. Sensory dysfunction in burning mouth syndrome. Pain 2002 Sep;99(1–2):41–7.

16 Speciali JG, Stuginski-Barbosa J. Burning mouth syndrome. Curr Pain Headache Rep 2008;12:279–84.

17 Rodríguez-de Rivera-Campillo E, López-López J. Evaluation of the response to treatment and clinical evolution in patients with burning mouth syndrome. Med Oral Patol Oral Cir Bucal 2013;18(3):e403–10.

18 Loeser JD, Melzack R. Pain: An overview. Lancet 1999;353:1607–9.

19 Kirmayer LG. Culture and the metaphoric mediation of pain. Transcult Psychiatry 2008;45:318–38.

20 le Breton D. Pain and the care relationship. Soins 2010;(749):34–5. French.

21 Wewers ME, Lowe NK. A critical review of visual analogue scales in the measurement of clinical phenomena. Res Nurs Health 1990;13(4):227–36.

22 Italian Istute of Statistics (ISTAT). Available at http://www.istat.it/en/population-and-housing-census/population-and-housing-2011.

23 Schneiderman N, Ironson G, Siegel SD. Stress and health: Psychological, behavioral, and biological determinants. Annu Rev Clin Psychol 2005;1:607–28.

24 Cairns BE. The influence of gender and sex steroids on craniofacial nociception. Headache. 2007;47:319–24.

25 Rohlfs I, Borrell C, do C Fonseca M. Gender inequalities and public health: Awarenesses and ignorances. Gac Sanit 2000;14(Suplemento 3):60–71.

26 Bassols A, Bosch F, Campillo M, Cañellas M, Baños JE. An epidemiological comparison of pain complaints in the general population of Catalonia (Spain). Pain 1999;83(1):9–16.

27 Gorsky M, Silverman S Jr, Chinn H. Clinical characteristics and management outcome in the burning mouth syndrome. An open study of 130 patients. Oral Surg Oral Med Oral Pathol 1991;72(2):192–5.

28 Scala A, Checchi L, Montevecchi M, Marini I, Giamberardino MA. Update on burning mouth syndrome: Overview and patient management. Crit Rev Oral Biol Med 2003;14:275–91.

29 Hagelberg N, Forssell H, Rinnec JO, et al. Striatal dopamine d1 and d2 receptors in burning mouth syndrome. Pain 2003;101:149–54.

30 Leresche L. Defining gender disparities in pain management. Clin Orthop Relat Res 2011;469(7):1871–7.

31 Savage NW. Burning mouth syndrome: Patient management. Aust Dent J 1996;41(6):363–6.

32 Grushka M, Epstein JB, Gorsky M. Burning mouth syndrome. Am Fam Physician 2002;65(4):615–20.

33 Lipton JA, Ship JA, Larach-Robinson D. Estimated prevalence and distribution of reported orofacial pain in the United States. J Am Dent Assoc 1993;124(10):115–121.

34 Mignogna MD, Fedele S, Lo Russo L, Leuci S, Lo Muzio L. The diagnosis of burning mouth syndrome represents a challenge for clinicians. J Orofac Pain 2005 Spring;19(2):168–73.

35 Shima S, Satoh E. Somatoform disorders in the workplace in Japan. Int Rev Psychiatry 2006;18(1):35–40.

36 Grushka M. Clinical features of burning mouth syndrome. Oral Surg Oral Med Oral Pathol 1987;63(1):30–6.

37 López-Jornet P, Camacho-Alonso F, Andujar-Mateos P, Sánchez-Siles M, Gómez-Garcia F. Burning mouth syndrome: An update. Med Oral Patol Oral Cir Bucal 2010;15(4):e562–8.

38 Lamey PJ, Lamb AB. Prospective study of aetiological factors in burning mouth syndrome. Br Med J (Clin Res Ed) 1988;296 (6631):1243–6.

39 Carlson CR, Miller CS, Reid KI. Psychosocial profiles of patients with burning mouth syndrome. J Orofac Pain 2000 Winter;14(1):59–64.

40 Dao T. Sex differences in pain. J Am Dent Assoc 2012;143 (7):764–5.

41 Blanco-Hungría A, Rodríguez-Torronteras A, Blanco-Aguilera A, et al. Influence of sociodemographic factors upon pain intensity in patients with temporomandibular joint disorders seen in the primary care setting. Med Oral Patol Oral Cir Bucal 2012;17:e1034–41.

42 Goulet JP, Lavigne GJ, Lund JP. Jaw pain prevalence among French-speaking Canadians in Québec and related symptoms of temporomandibular disorders. J Dent Res 1995;74 (11):1738–44.

43 Zakrzewska JM. Diagnosis and management of non-dental orofacial pain. Dent Update 2007;34 (3):134–6, 138–9.

44 Eli I, Kleinhauz M, Baht R, Littner M. Antecedents of burning mouth syndrome (glossodynia)–recent life events vs. psychopathologic aspects. J Dent Res 1994;73(2):567–72.

Nodular fasciitis of the tongue

Antonio Celentano, DDS, PhD,[1]* Massimo Mascolo, MD, PhD,[2] Gaetano De Rosa, MD,[2] Michele Davide Mignogna, MD, DMD[1]

[1]Department of Neurosciences, Reproductive, and Odontostomatological Sciences, University of Naples Federico II, Naples, Italy, [2]Department of Advanced Biomedical Sciences, Pathology Section, University of Naples Federico II, Naples, Italy.

Accepted 14 April 2015
Published online 18 July 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/hed.24088

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. © 2015 Wiley Periodicals, Inc. *Head Neck* **38**: E29–E31, 2016

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 Martínez–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.

***Corresponding author:** A. Celentano, Department of Neurosciences, Reproductive, and Odontostomatological Sciences, University of Naples Federico II, Naples, Italy Via Pansini n. 5, Napoli, 80131 Italy.
E-mail: antony.celentano@gmail.com, antonio.celentano@unina.it

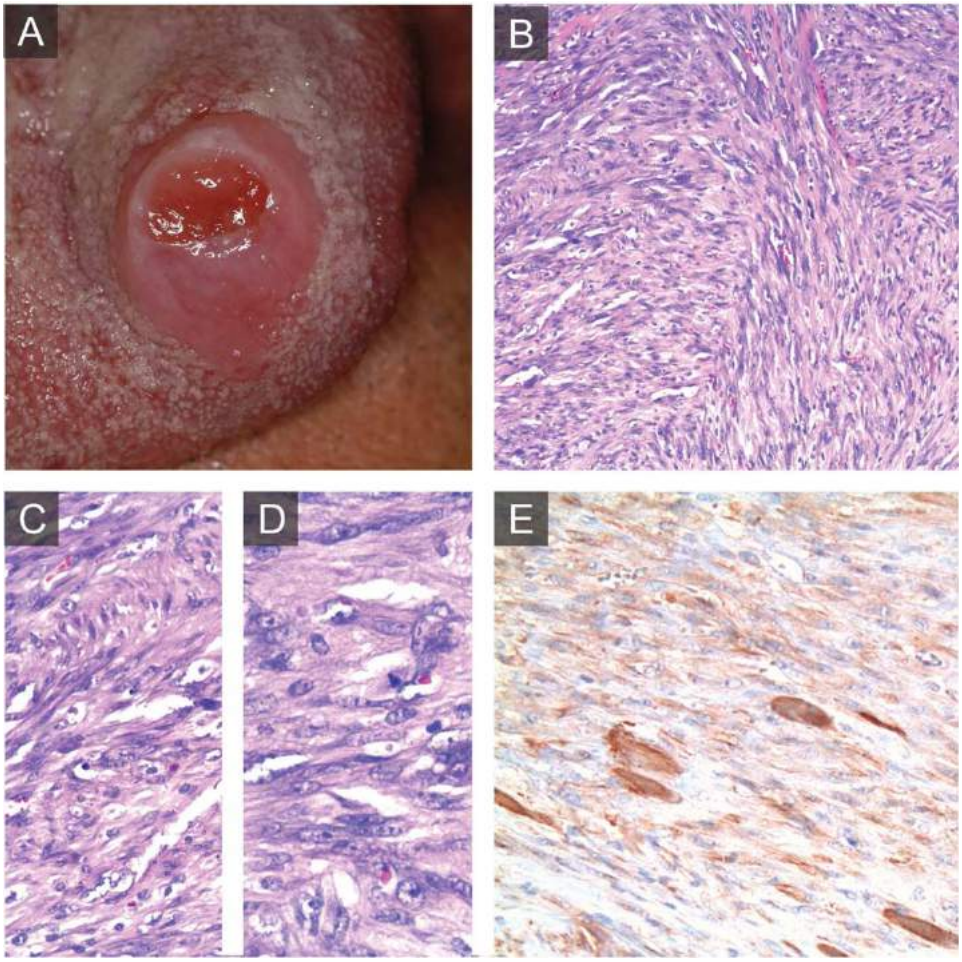


FIGURE 1. (A) A nodular subepithelial mass with ulcerated surface is visible on the tongue tip. (B) A panoramic view of the tumor (hematoxylin-eosin stain, original magnification ×100). The spindle cells showed vesicular nuclei with nucleoli (C) hematoxylin-eosin stain, original magnification ×150 and (D) hematoxylin-eosin stain, original magnification ×200. (E) The tumor cells show a widespread immunoreactivity for smooth muscle actin (SMA; immunoperoxidase stain for SMA, original magnification ×200).

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

1. Konwaler BE, Keasbey L, Kaplan L. Subcutaneous pseudosarcomatous fibromatosis (fasciitis). *Am J Clin Pathol* 1955;25:241–252.
2. Price EB Jr, Silliphant WM, Shuman R. Nodular fasciitis: a clinicopathologic analysis of 65 cases. *Am J Clin Pathol* 1961;35:122–136.
3. Bernstein KE, Lattes R. Nodular (pseudosarcomatous) fasciitis, a nonrecurrent lesion: clinicopathologic study of 134 cases. *Cancer* 1982;49:1668–1678.
4. Takagi M, Ishikawa G. Fibromatosis of the oral cavity. *Bull Tokyo Med Dent Univ* 1982;29:113–122.
5. Martínez–Blanco M, Bagán JV, Alba JR, Basterra J. Maxillofacial nodular fasciitis: a report of 3 cases. *J Oral Maxillofac Surg* 2002;60:1211–1214.
6. Naidu A, Lerman MA. Clinical pathologic conference case 3: nodular fasciitis. Head *Neck Pathol* 2011;5:276–280.
7. Borumandi F, Cascarini L, Mallawaarachchi R, Sandison A. The chameleon in the neck: nodular fasciitis mimicking malignant neck mass of unknown primary. *Int J Surg Case Rep* 2012;3:501–503.
8. Carr MM, Fraser RB, Clarke KD. Nodular fasciitis in the parotid region of a child. *Head Neck* 1998;20:645–648.

Available online at www.sciencedirect.com

SciVerse ScienceDirect

British Journal of Oral and Maxillofacial Surgery 53 (2015) 396–398

BRITISH Journal of Oral and Maxillofacial Surgery
www.bjoms.com

Short communication

Primary oral leishmaniasis mimicking oral cancer: a case report

A. Celentano [a,*], E. Ruoppo [a], G. Mansueto [b], M.D. Mignogna [a]

[a] *Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Italy*
[b] *Department of Advanced Biomedical Sciences, Morphological and Functional Diagnostics, Pathology Section, University Federico II of Naples, Italy*

Accepted 27 January 2015
Available online 18 February 2015

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.

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.

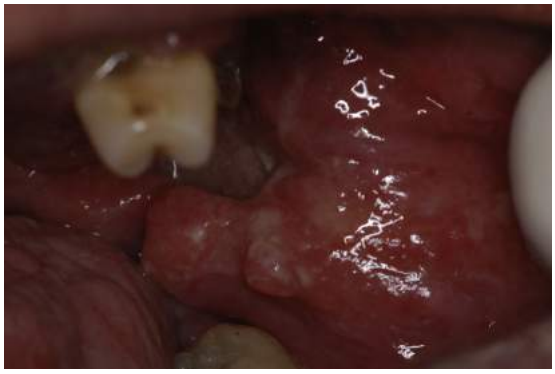


Fig. 1. Clinical features of our case: a widespread exophytic mass of the left buccal mucosa extending to the upper and lower fornix, and the retromolar area. The surface showed erythema, multiple erosions, and mild keratosis that was speckled anteriorly and had a deep ulcer posteriorly.

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

* Corresponding author at: Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Via Pansini n.5, Naples, 80131, Italy. Tel.: +39 0817462181.
E-mail addresses: antony.celentano@gmail.com (A. Celentano), elvira.ruoppo@gmail.com (E. Ruoppo), mansueto.g@inwind.it (G. Mansueto), mignogna@unina.it (M.D. Mignogna).

http://dx.doi.org/10.1016/j.bjoms.2015.01.021
0266-4356/

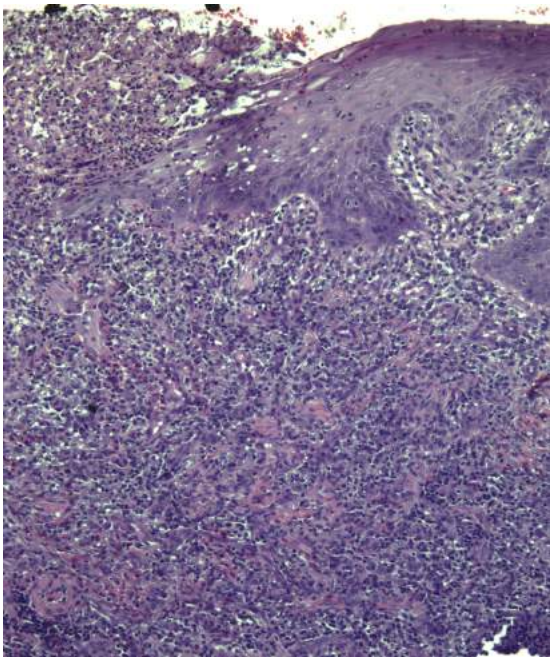


Fig. 2. Histopathological examination of the buccal lesion showing disruption of the epithelial layer, which was partially ulcerated and partially dyskeratotic and hyperplastic (haematoxylin and eosin, original magnification ×10).

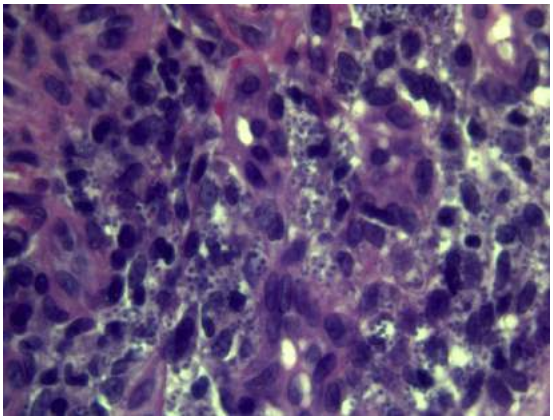


Fig. 3. Further magnification showed a wide number of intracellular and extracellular *Leishmania* spp. amastigotes (haematoxylin and eosin, original magnification ×63).

There was a widespread exophytic mass of the left buccal mucosa that extended to the upper and lower fornix, and to the retromolar area. The lesion was hard, and the surface characterised by erythema, erosions, and keratosis, partly speckled and ulcerated.

Clinical features were consistent with malignancy. Examination of an incisional biopsy specimen showed a large number of *Leishmania* spp. amastigotes (Figs. 2 and 3). Dermatological examination and abdominal computed tomography excluded systemic involvement and confirmed a diagnosis of primary oral leishmaniasis.

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,[3–5] 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 antimonial drugs.[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

1. Ready PD. Epidemiology of visceral leishmaniasis. *Clin Epidemiol* 2014;**6**:147–54.
2. Stockdale L, Newton R. A review of preventative methods against human leishmaniasis infection. *PLoS Negl Trop Dis* 2013;**7**:e2278.
3. Strazzulla A, Cocuzza S, Pinzone MR, et al. Mucosal leishmaniasis: an underestimated presentation of a neglected disease. *Biomed Res Int* 2013;**2013**:805108.
4. World Health Organization. *Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases Technical Report no 949*. Geneva: WHO; 2010.
5. World Health Organization. *World Health Report 2000. Health Systems: improving performance*. Geneva: WHO; 2000.

6. Grimaldi G, Schottelius J. Leishmaniases – their relationships to monoxenous and dixenous trypanosomatids. *Med Microbiol Immunol* 2001;**190**:3–8.
7. Mignogna M, Celentano A, Leuci S, et al. Mucosal leishmaniasis with primary oral involvement: a case series and a review of the literature. *Oral Dis* 2015;**21**:e70–8.
8. Masmoudi A, Hariz W, Marrekchi S, et al. Old World cutaneous leishmaniasis: diagnosis and treatment. *J Dermatol Case Rep* 2013;**7**: 31–41.
9. Warnakulasuriya S. Living with oral cancer: epidemiology with particular reference to prevalence and life-style changes that influence survival. *Oral Oncol* 2010;**46**:407–10.

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

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.

JADA 2014;145(9):950-954.

doi:10.14219/jada.2014.58

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.[1]

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.

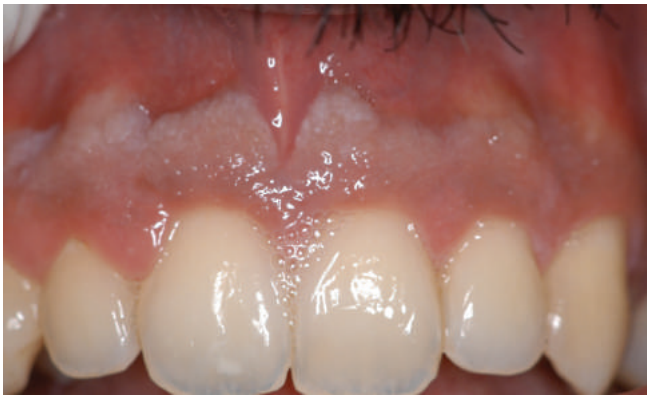


Figure 1. Clinical features of case 1: widespread keratotic lesions and micropapillomatosis of the maxillary gingiva.

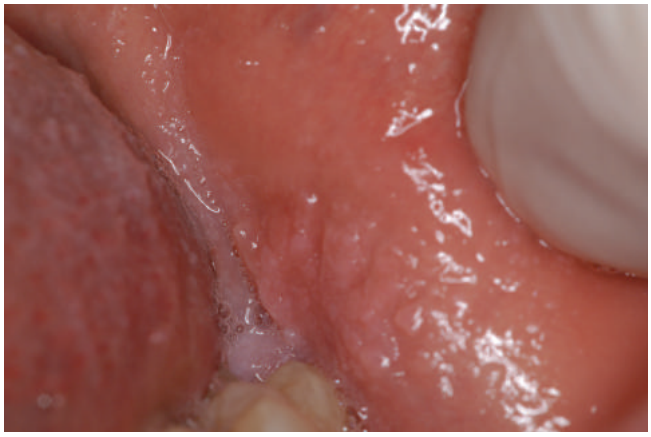


Figure 2. Clinical features of case 2: exophytic lesions of the left buccal mucosa with spongiotic aspect.

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, chlorine, 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 three clinicians (A.C., D.A., M.D.M.) performed an incisional biopsy of the left buccal mucosa, and the histopathological examination revealed multiple fibropapillomas, with well-differentiated cells and parakeratosis, and an absence of inflammatory cells.

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

ABBREVIATION KEY. **BRRS:** Bannayan-Riley-Ruvalcaba syndrome. **CD:** Cowden disease. **LDD:** Lhermitte-Duclos disease. **PHTS:** PTEN hamartoma tumor syndrome. **PTEN:** Phosphate and tensin homolog. **RBC:** Red blood cell.

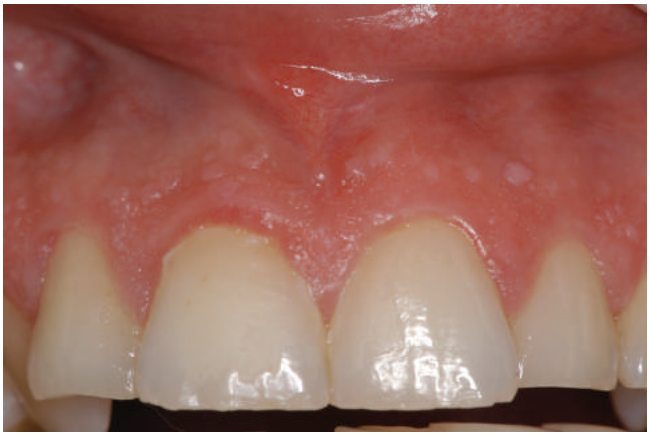


Figure 3. Clinical features of case 2: widespread papillomatous lesions of the maxillary gingiva.

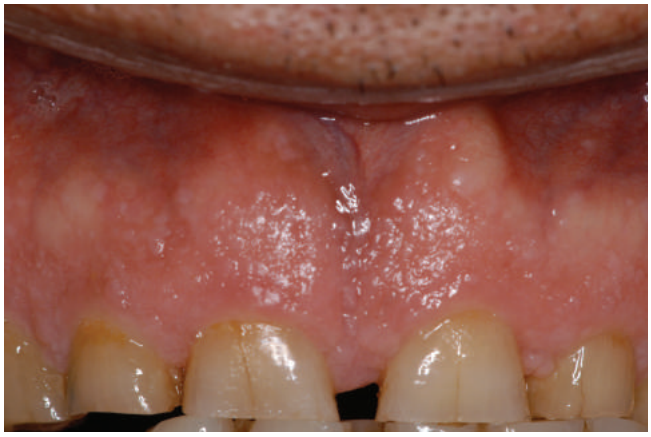


Figure 4. Clinical features of case 3: widespread papillomatosis of the maxillary gingiva.

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,[2] a description then extended by Weary and colleagues[3] 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.[4,5]

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.[6] 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.[7,8] 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[9]).

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.[10] 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.[1]

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.[11]

Thyroid function tests, thyroid ultrasonography, complete blood count, complete urine analysis, Papanicolau (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.[12]

Box 2[10] 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.[1] Starink and colleagues[13] 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, lipoid 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)
Macrocephaly (an occipital-frontal head circumference in the 97th percentile or higher): 58 centimeters for females, 60 cm for males)
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 palmoplantar 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 acanthosis (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]

multiple endocrine adenomatosis, acanthosis nigricans, pseudoepitheliomatous hyperplasia and squamous cell carcinoma.[14]

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 of 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]

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 20s.[15-19]

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.

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. ■

Dr. Celentano is a doctoral student, Department of Neurosciences, Reproductive and Odontostomatological Sciences, Faculty of Medicine and Surgery, University of Naples Federico II, Via Pansini no. 5, Naples 80131, Italy, e-mail antony.celentano@gmail.com.Address correspondence to Dr. Celentano.

Dr. Adamo is an oral surgical specialist, Department of Neurosciences, Reproductive and Odontostomatological Sciences, Faculty of Medicine and Surgery, University of Naples Federico II, Italy.

Dr. Leuci is a clinical researcher, Oral Medicine Unit, and a postdoctoral fellow of oral medicine, Department of Neurosciences, Reproductive and Odontostomatological Sciences, Faculty of Medicine and Surgery,University of Naples Federico II, Italy.

Dr. Mignogna is an associate professor of oral medicine and the head, Oral Medicine Unit, Department of Neurosciences, Reproductive and Odontostomatological Sciences, University of Naples Federico II, Italy.

Disclosure. None of the authors reported any disclosures.

1. Pilarski R, Burt R, Kohlman W, Pho L, Shannon KM, Swisher E. Cowden syndrome and PTEN hamartoma tumor syndrome: systematic review and revised diagnostic criteria. J Natl Cancer Inst 2013;105(21):1607-1616.

2. Lloyd KM II, Dennis M. Cowden's disease: a possible new symptom complex with multiple system involvement. Ann Intern Med 1963;58(1): 136-142.

3. Weary PE, Gorlin RJ, Gentry WC Jr, Comer JE, Greer KE. Multiple hamartoma syndrome (Cowden's disease). Arch Dermatol 1972;106(5): 682-690.

4. Nelen MR, van Staveren WC, Peeters EA, et al. Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. Hum Mol Genet 1997;6(8):1383-1387.

5. Liaw D, Marsh DJ, Li J, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 1997;16(1):64-67.

6. Gorlin RJ, Cohen MM Jr, Condon LM, Burke BA. Bannayan-Riley-Ruvalcaba syndrome. Am J Med Genet 1992;44(3):307-314.

7. Marsh DJ, Kum JB, Lunetta KL, et al. PTEN mutation spectrum and genotype-phenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome. Hum Mol Genet 1999;8(8):1461-1472.

8. Pilarski R, Stephens JA, Noss R, Fisher JL, Prior TW. Predicting PTEN mutations: an evaluation of Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome clinical features. J Med Genet 2011;48(8): 505-512.

9. Hendriks YM, Verhallen JT, van der Smagt JJ, et al. Bannayan-Riley-Ruvalcaba syndrome: further delineation of the phenotype and management of PTEN mutation-positive cases. Fam Cancer 2003;2(2):79-85.

10. National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology. Genetic/Familial High-Risk Assessment: Breast and Ovarian. http://demystifyingmedicine.od.nih.gov/DM10/0413-BreastCancer/NCCN%20br%20genetics_screening.pdf. Accessed July 23, 2014.

11. Hartmann LC, Schaid DJ, Woods JE, et al. Efficacy of bilateral prophylactic mastectomy in women with a family history of breast cancer. N Engl J Med 1999;340(2): 77-84.

12. National Comprehensive Cancer Network. 2009 Cowden syndrome clinical practice guidelines in oncology. www.nccn.org/international/international_adaptations.aspx. Accessed Aug. 5, 2014.

13. Starink TM, van der Veen JP, Arwert F, et al. The Cowden syndrome: a clinical and genetic study in 21 patients. Clin Genet 1986;29(3):222-233.

14. Fistarol SK, Anliker MD, Itin PH. Cowden disease or multiple hamartoma syndrome: cutaneous clue to internal malignancy. Eur J Dermatol 2002;12(5):411-421.

15. Eng C. Will the real Cowden syndrome please stand up? Revised diagnostic criteria. J Med Genet 2000;37(11):828-830.

16. Starink TM, van der Veen JP, Arwert F. The Cowden syndrome: a clinical and genetic study in 21 patients. Clin Genet 1986;29(3):222-233.

17. Li JC, Yen C, Liaw D, et al. PTEN: a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275(5308):1943-1947.

18. Eng C. Role of PTEN, a lipid phosphatase upstream effector of protein kinase B, in epithelial thyroid carcinogenesis. Ann N Y Acad Sci 2002;968:213-221.

19. Allain DC. Genetic counseling and testing for common hereditary breast cancer syndromes: a paper from the 2007 William Beaumont hospital symposium on molecular pathology. J Mol Diagn 2008;10(5):383-395.

ORAL DISEASES

Oral Diseases (2015) 21, e70–e78 doi:10.1111/odi.12268

www.wiley.com

REVIEW ARTICLE

Mucosal leishmaniasis with primary oral involvement: a case series and a review of the literature

MD Mignogna[1], A Celentano[1], S Leuci[1], M Cascone[1], D Adamo[1], E Ruoppo[1], G Favia[2]

[1]*Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Naples;* [2]*Head Oral Surgery Unit, Interdisciplinary Department of Medicine, University of Bari, Bari, Italy*

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 in 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.

Oral Diseases (2015) **21**, e70–e78

Keywords: primary oral leishmaniasis; mucosal; immunocompetent; head–neck leishmaniasis

Correspondence: Mignogna Michele Davide, MD, DMD, Department of Neurosciences, Reproductive and Odontostomatological Sciences, University Federico II of Naples, Naples, Italy. Tel: +39 0817462182, Fax: +39 0817462181, E-mail: mignogna@unina.it
All persons and only persons who meet authorship criteria and participated in the preparation of the manuscript are listed as authors.
Received 11 April 2014; revised 6 June 2014; accepted 9 June 2014

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 (Prabhu *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

Subsequently, we retrospectively reviewed all articles from 1950 to 2013 focused on cases of ML in immunocompetent/otherwise healthy patients, by searching in the PubMed database on each of the following keywords: 'mucosal', 'oral', 'buccal', 'nose', 'nasal', 'pharynx', 'pharyngeal', 'esophagus', 'esophageal', 'larynx', 'laryngeal' in association with 'leishmaniasis', and alternatively with 'healthy' and 'immunocompetent'.

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 leishmaniasis 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.

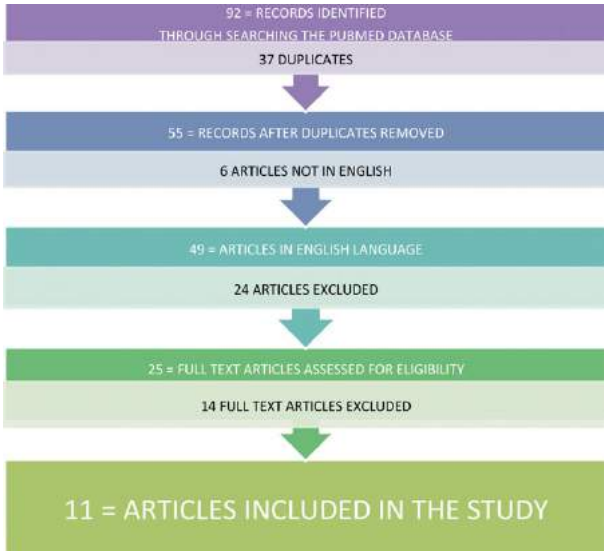


Figure 1 Flow chart

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 fissurations.

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

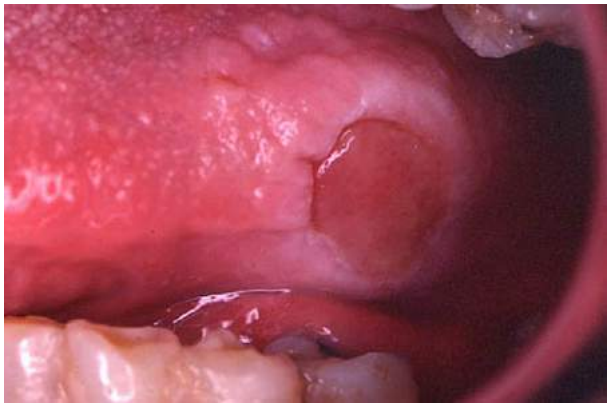


Figure 2 Clinical aspect of the Case n. 1

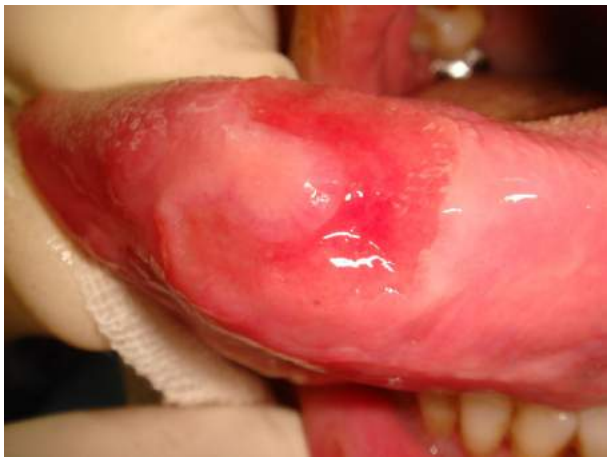


Figure 3 Clinical aspect of the Case n. 2

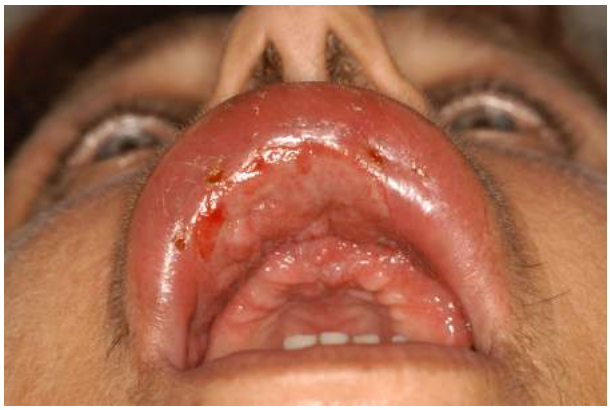


Figure 4 Clinical aspect of the Case n. 5

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.

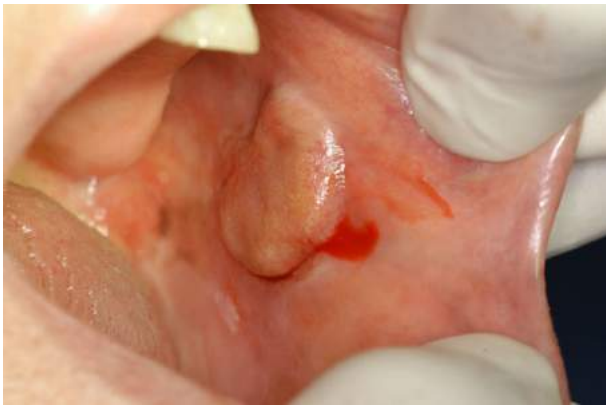


Figure 5 Clinical aspect of the Case n. 7

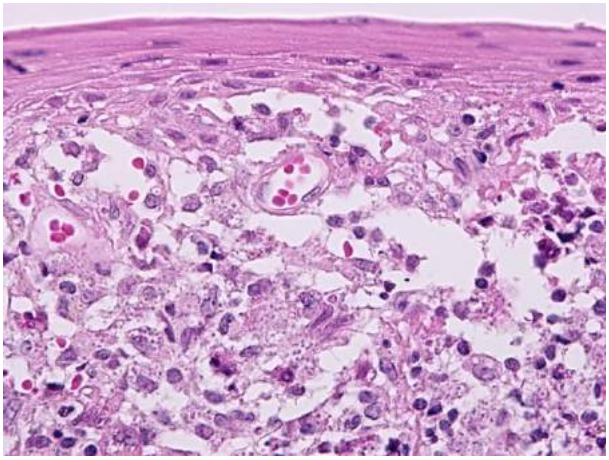


Figure 6 (Case n. 1) Histopathological examination (Hematoxylin-Eosin, X400) of the lingual lesion showed disruption of the basal-intermediate epithelial layers due to inflammatory infiltration (lymphocytes, macrophages and focal plasma cells), intra- and extra-cellular *Leishmania* spp. amastigotes and endothelial damage

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 others 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

Reference	*Country*	*Age*	*Sex*	*Underlying or past diseases*	*Predisposing risk factors*	*Local impairment*
Palmeiro *et al* (2007)	Brazil	75	M	None	Endemic area Farmer	Deficient oral hygiene, poor tooth conservation
Pellicioli *et al* (2012)	Brazil	71	M	Hypertension (atenolol and hydrochlorothiazide treated)	Farmer	–
Cocuzza *et al* (2013)	Italy	64	M	(COPD) since 20 years. Hypersensitivity to non-steroidal treatment Anti-inflammatory	Endemic area	Former smoker (25 cig day^{-1})
Oryan (2013)	Iran	42	M	None	Endemic area	–
Oryan (2013)	Iran	32	M	None	Endemic area	–
Pau *et al* (2009)	Italy	52	M	Hypertension (by ACE inhibitors treated)	Endemic area, Shepherd	None
Pau *et al* (2009)	Italy	71	M	Ischemic cardiopathy (coronary by-pass surgery 2002)	Endemic area, Farmer	None
Habibzadeh *et al* (2005)	Iran	40	M	None	Endemic area	None
Guddo *et al* (2005)	Italy	59	M	COPD, Low level of IgM (28.8 mg 100 ml^{-1})	Endemic area	Smoker
Casolari (2005)	Italy	53	M	None	Endemic area	–
Tiseo *et al* (2008)	Italy	64	M	Well-compensated type 2 diabetes mellitus	Endemic area	Until 8 years previously, he had been a heavy smoker (50 cig day^{-1})
Mathur *et al* (2006)	India	45	M	Hemoglobin = 11.2 G% Leukocyte tot. count: 12 000 mm^{-3}	Endemic area	–
Kassam *et al* (2013)	England	66	M	COPD (diagnosed in 2002) raised ESR (34 mm h^{-1})	–	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

–, Not reported/unknown; ACE, angiotensin converting enzyme; COPD, chronic obstructive pulmonary disease; ESR, erythrocyte sedimentation rate.

2–2.4 million disability-adjusted life years lost (World Health Organization, 2000; Hotez *et al*, 2004).

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 the flagellated, motile stage that reside extracellularly in sand fly vectors of the genera *Phlebotomus* or *Lutzomyia*. Virulent metacyclic promastigotes are inoculated into the dermis of mammalian hosts during sand fly blood meals. Macrophages rapidly internalize the promastigotes forming a parasitophorous vacuole 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. amazonesis* 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 Schottelius, 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

Table 2 Data of 13 patients from pubmed search: Clinical features

Reference	*Involved sites*	*Symptoms*	*Lesion description*
Palmeiro *et al* (2007)	Palatal mucosa (hard and soft palate) Uvula gingiva	Swallowing difficulties	Granular area with presence of coarse granulation Fine granulation
Pellicioli *et al* (2012)	Palatal mucosa (hard and soft palate) oropharynx	Weight loss (approximately 8 kg). Slight pain and no bleeding upon palpation	Largest diameters measuring approximately 5 × 4 cm. Multiple ulcerated nodules, with granulomatous appearance and fibro-elastic consistency
Cocuzza *et al* (2013)	True vocal cords	8 months history of hoarseness and discomfort	Two focal hard and whitish lesions
Oryan (2013)	Laryngeal mucosa	Several months history of dysphonia, dyspnea, hoarseness, and odynophagia	Single lesion
Oryan (2013)	Epiglottis, cricoarytenoid muscle, laryngeal mucosa	Several months history of: dysphonia, dyspnea, hoarseness, odynophagia. Vocal cords paralysis; swelling, erythema and edema of the epiglottis and aryepiglottic folds	Deep mucosal damaging processes
Pau *et al* (2009)	Endonasal mucosa (anterior part of left cartilaginous septum)	Epistaxis	Bright red, 1 cm nodule bled easily, moderately infiltrated, covered by serohematic crusts
Pau *et al* (2009)	Endonasal mucosa (left nasal vestibule)	Epistaxis, rhinorrhea, sense of obstruction, and respiratory difficulty. Moderate labial edema	Hard erythematosus, polypoid, non-ulcerated and painless, 1.5 cm diameter nodule
Habibzadeh *et al* (2005)	Right side of the tongue (against the first molar tooth)	None	Fleshy mass, measuring 5 × 7 × 5 mm
Guddo *et al* (2005)	Larynx (subglottic mucosa)	Cough, exertional dyspnea, mucus production, bronchiectases	3 mm mucosal polypoid-like lesion
Casolari (2005)	Right epiglottis region Infiltrating the pharyngo-laryngeal wall	Dyspnea, Dysphonia Odynophagia	Whitish, fungating, swelling Lesion
Tiseo *et al* (2008)	Left wall of the pharynx, vocal cords	Dysphonia	–
Mathur *et al* (2006)	Hemilarynx (bilaterally) Epiglottis, involving posterior commissure	Hoarseness, cough and a history of fever for 18 months	Fixed ulcerative growth, With edematous surrounding areas
Kassam *et al* (2013)	Tongue (left mid-dorsum)	3-month history of swelling and soreness of the dorsum of the tongue because of painful ulceration, and some difficulty in swallowing	Lymphoid-like tissue swelling

–, Not reported/unknown.

Table 3 Comparative data on age, gender and sites of involvement

	Our cases (n = *7*)	*Review cases* (n = *13*)	*Total* (n = *20*)
Overall age range (years)	31–66	32–75	31–75
Men	6	13	19
Women	1	0	1
Mean (±SD) age	48 (±14) years	56 (±14) years	53 (±14) years
Larynx	0	7	7
Tongue	4	2	6
Pharynx	0	4	4
Palate	1	2	3
Nasal mucosa	0	2	2
Buccal mucosa	1	0	1
Gingiva	0	1	1
Lip	1	0	1

SD, Standard deviation.

a solid organ transplant. In these clinical settings, leishmaniasis shows the most severe signs and symptoms, characterized by fever, chills, hepatosplenomegaly, pancytopenia, gastrointestinal involvement, and/or ascites.

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 (blastomycosis), tertiary syphilis, sarcoidosis, paracoccidioidomycosis, histoplasmosis, tuberculosis, leprosy, lethal midline granuloma, pemphigus vulgaris, pemphigoid, plasmacytic gingivitis, (deficiencies) anemia, leukemia, antineutrophilic 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

Reference	*Diagnostic tests*	*Treatment*	*Follow-up (months)*	*Outcome*
Palmeiro *et al* (2007)	Montenegro skin test Serology (IIF) Histopathological (hematoxylin–eosin, Ziehl-Neelsen, Silver), Culture	Meglumine antimoniate (5 mg kg^{-1} day^{-1}) i.m. for 20 days	30	Healing
Pellicioli *et al* (2012)	Montenegro skin test 4 Biopsies (inconclusive diagnoses) Histopathological (hematoxylin–eosin, Ziehl-Neelsen, Silver, Pas) Immunohistochemistry	Liposomal amphotericin B for 5 weeks	–	Healing with residual fibrosis
Cocuzza *et al* (2013)	*12-2009* Fibroscopy and laryngeal biopsy (inflammation and hyperplasia)	Amoxicillin		Lack of clinical improvement
	07-2010 Second laryngeal biopsy PCR	Liposomal amphotericin B (3 mg kg^{-1} day^{-1}) for 7 days, then (3 mg kg^{-1} per once a week) for 5 weeks		Achieved complete recovery
	05-2011 Third laryngeal biopsy	Liposomal amphotericin B (3 mg kg^{-1} day^{-1}) for 7 days, then (3 mg kg^{-1} per once a week) for 5 weeks	20	Resolution of symptoms and good health
Oryan (2013)	Laryngoscopy Cytologic (Wright and Papanicolaou) Nested PCR	–	–	–
Oryan (2013)	Laryngoscopy Cytologic (Wright and Papanicolaou) Immunocytochemistry, Nested PCR	–	–	–
Pau *et al* (2009)	Endoscopy Facial X-ray Histopathological (Giemsa)	Meglumine antimoniate 300 mg ml^{-1}: intralesional infiltration (1 ml per weekly) for 4–5 weeks	36	Full resolution
Pau *et al* (2009)	Rhinoscopy Cytological and histopathological (Giemsa) Isoenzymatic characterization	Meglumine antimoniate 300 mg ml^{-1}: intrales. infiltr. (1 ml per weekly) for 4 weeks	12	Healing
Habibzadeh *et al* (2005)	Histopathological Indirect immunofluorescence	Surgical	48	Healing
Guddo *et al* (2005)	Bronchoscopy Histopathological (hematoxylin–eosin, Ziehl-Neelsen, Gram, Grocott, Giemsa) PCR, ELISA test on serum Microbiological	Ciprofloxacin (without clinical improvement) then Amphotericin B: (0.5 mg kg^{-1} day^{-1})	-	Clinical improvement
Casolari *et al* (2005)	Laryngoscopy Multiple Histological (Giemsa), Culture Nested PCR	Liposomal amphotericin B 2 courses, 10 days apart, (3 mg kg^{-1} day^{-1}) for 5 days	12	Healing
Tiseo *et al* (2008)	CT scan Direct laryngoscopy Histological (Giemsa) Antibody title (weakly positive)	Liposomal amphotericin B: starting with 0.5 mg kg^{-1} day^{-1}, Overall dosage: 950 mg	2	Healing Lesion decrease
Mathur *et al* (2006)	Direct laryngoscopy, Histopathological (hematoxylin–eosin, Pas, Giemsa), Serology	Stibogluconate (20 mg kg^{-1} day^{-1}) For 2–3 weeks	3	Significant symptom improvement
Kassam *et al* (2013)	Histopathological (hematoxylin–eosin, Giemsa) PCR	Miltefosine: 150 mg BD for 28 days	10	Healing

–, 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; Teemul *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 Ruoppo 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

Aliaga L, Cobo F, Mediavilla JD *et al* (2003). Localized mucosal leishmaniasis due to *Leishmania infantum* clinical and microbiologic findings in 31 patients. *Medicine* **82**: 147–158.

Alvar J, Vélez ID, Bern C *et al* (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* **7**: e35671.

Balasegaram M, Ritmeijer K, Lima MA *et al* (2012). Liposomal amphotericin B as a treatment for human leishmaniasis. *Expert Opin Emerg Drugs* **17**: 493–510.

Benítez MD, Miranda C, Navarro JM, Morillas F, Martín J, de la Rosa M (2001). Thirty-six year old male patient with dysphonia refractory to conventional medical treatment. *Enfermedades Infecciosas y Microbiología Clínica* **19**: 233–234.

Carranza-Tamayo CO, Carvalhomdo S, Bredt A *et al* (2010). Autochthonous visceral leishmaniasis in Brasília, Federal District, Brazil. *Rev Soc Bras Med Trop* **43**: 396–399.

Casolari C, Guaraldi G, Pecorari M *et al* (2005). A rare case of localized mucosal leishmaniasis due to *Leishmania infantum* in an immunocompetent Italian host. *Eur J Epidemiol* **20**: 559–561.

Cocuzza S, Strazzulla A, Pinzone MR *et al* (2013). Isolated laryngeal leishmaniasis in immunocompetent patients: an underdiagnosed disease. *Case Rep Infect Dis* **2013**: Article ID 165409.

Desjeux P (1996). Public health aspects and control. *Clin Dermatol* **14**: 417–423.

Ferlito A, Pesavento G, Visonà A, Recher G, Meli S, Bevilacqua P (1986). *Leishmaniasis donovani* presenting as an isolated lesion in the larynx. *ORL J Otorhinolaryngol Relat Spec* **48**: 243–248.

Gill G, Beeching N (2009). *Visceral Leishmaniasis. Tropical Medicine*, 6th edn. pp. 74–79.

Goto H, Lindoso JA (2010). Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Rev Anti Infect Ther* **8**: 419–433.

Grimaldi G, Schottelius J (2001). Leishmaniases – their relationships to monoxenous and dixenoustrypanosomatids. *Med Microbiol Immunol* **190**: 3–8.

Guddo F, Gallo E, Cillari E *et al* (2005). Detection of *Leishmania infantum* kinetoplast DNA in laryngeal tissue from an immunocompetent patient. *Hum Pathol* **36**: 1140–1142.

Habibzadeh F, Sajedianfard J, Yadollahie M (2005). Isolated lingual leishmaniasis. *J Postgrad Med* **51**: 218–219.

Hotez PJ, Remme JH, Buss P, Alleyne G, Morel C, Breman JG (2004). Combating tropical infectious diseases: report of the Disease Control Priorities in Developing Countries Project. *Clin Infect Dis* **38**: 871–878.

Kassam K, Davidson R, Tadrous PJ, Kumar M (2013). Lingual leishmaniasis presenting to Maxillofacial Surgery in UK with successful treatment with miltefosine. *Case Rep Med* **2013**: 975131.

Kassi M, Afghan AK, Rehman R, Kasi PM (2008). Marring leishmaniasis: the stigmatization and the impact of cutaneous leishmaniasis in Pakistan and Afghanistan. *PLoS Negl Trop Dis* **2**: e259.

Masmoudi A, Hariz W, Marrekchi S, Amouri M, Turki H (2013). Old World cutaneous leishmaniasis: diagnosis and treatment. *J Dermatol Case Rep* **7**: 31–41.

Mathur SR, Ray R, Handa KK (2006). Isolated laryngeal leishmaniasis in an immunocompetent patient in India. *Trop Doct* **36**: 55–56.

Montalvo AM, Fraga J, Monzote L, García M, Fonseca L (2012). Leishmaniasis diagnosis: going from microscopic observation of parasite to DNA detection. *Rev Cubana Med Trop* **64**: 108–131.

Murray AS, Lynn MA, McMaster WR (2010). The *Leishmania mexicana* A600 genes are functionally required for amastigote replication. *Mol Biochem Parasitol* **172**: 80–89.

Nogueira MF, Sotto MN, Cucé LC (2008). American tegumentary leishmaniasis: langerhans cells in Montenegro skin test. *Rev Inst Med Trop São Paulo* **50**: 283–286.

Oryan A, Shirian S, Tabandeh MR, Hatam GR, Kalantari M, Daneshbod Y (2013). Molecular, cytological, and immunocytochemical study and kDNA sequencing of laryngeal *Leishmania infantum* infection. *Parasitol Res* **122**: 1799–1804.

Palmeiro MR, Rosalino CM, Quintella LP *et al* (2007). Gingival leishmaniasis in an HIV-negative patient. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **104**: e12–e16.

Pau M, Atzori L, Aste N (2009). Two cases of primary endonasal leishmaniasis in Sardinia (Italy). *Dermatol Online J* **15**: 5.

Pellicioli AC, Martins MA, Sant'ana Filho M, Rados PV, Martins MD (2012). Leishmaniasis with oral mucosa involvement. *Gerodontology* **29**: e1168–e1171.

Prabhu SR, Wilson DF, Daftary DK, Johnson NW (1992). *Oral diseases in the tropics*. Oxford: Oxford University Press.

Sacks DL, Saraiva EM, Rowton E *et al* (1994). The role of the lipophosphoglycan of Leishmania in vector competence. *Parasitology* **108**(Suppl.): 5–62.

Stockdale L, Newton R (2013). A review of preventative methods against human leishmaniasis infection. *PLoS Negl Trop Dis* **7**: e2278.

Teemul TA, Williams J, Lester SE (2013). Laryngeal leishmaniasis: case report of a rare infection. *Head Neck* **35**: E277–E279.

Tiseo D, Tosone G, Conte MC *et al* (2008). Isolated laryngeal leishmaniasis in an immunocompetent patient: a case report. *Infez Med* **16**: 233–235.

World Health Organization (2000) *World Health Report 2000. Health systems: improving performance*. Geneva: World Health Organization.

World Health Organization (2010). *Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases*. Geneva, Switzerland: Tech. Rep.

Columbia International Publishing
American Journal of Oral Medicine
(2015) Vol. 1 No. 1 pp. 5-7
doi:10.7726/ajom.2015.1002
Image in Oral Medicine

Grooved Tongue and Congenital Muscular Torticollis

Antonio Celentano[1,2*] and Michele Davide Mignogna[1]

Received: 06 April 2015; Returned for revision: 26 April 2015; Received in revised form: 06 May 2015; Accepted: 06 May 2015; Published online 26 May 2015

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 suprahyoid region muscles was insufficient.

*Corresponding e-mail: antony.celentano@gmail.com; antonio.celentano@unina.it
1 Department of Neurosciences, Reproductive and Odontostomatological Sciences, University of Naples Federico II, Naples, Italy
2 Melbourne Dental School & Oral Health CRC, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Melbourne, VIC, Australia

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.

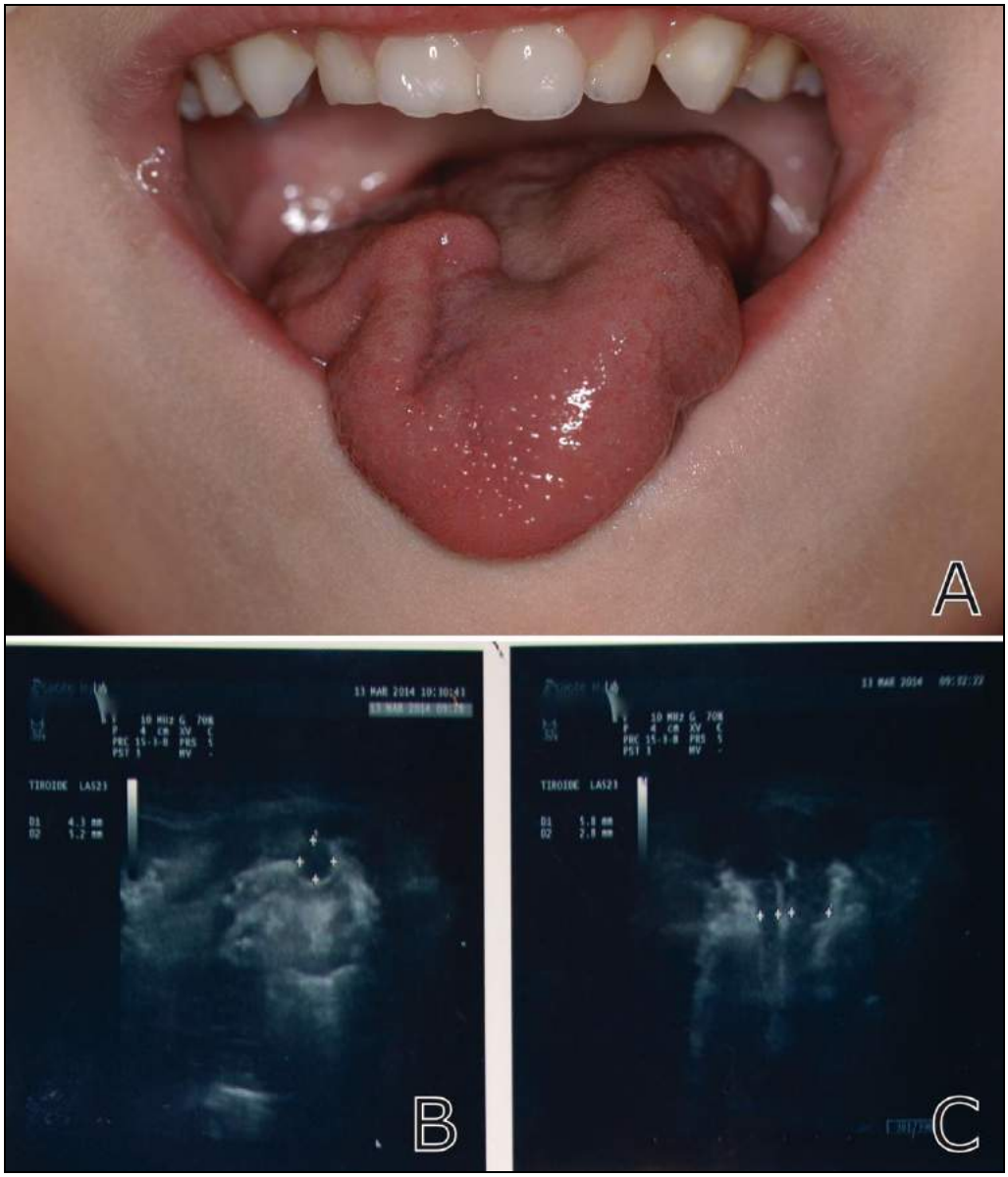


Fig. 1.

Conflict of Interest

None

Funding

None

References

Jung, A.Y., Kang, E.Y., Lee, S.H., Nam, D.H., Cheon, J.H., Kim, H.J. (2015). Factors that affect the rehabilitation duration in patients with congenital muscular torticollis. Ann Rehabil Med, 39(1):18-24. http://dx.doi.org/10.5535/arm.2015.39.1.18

Lee, K., Chung, E., Koh, S., Lee, B.H. (2015). Outcomes of asymmetry in infants with congenital muscular torticollis. J Phys Ther Sci, 27(2):461-4. http://dx.doi.org/10.1589/jpts.27.461

Nilesh, K., Mukherji, S. (2013). Congenital muscular torticollis. Ann Maxillofac Surg, 3(2):198-200. http://dx.doi.org/10.4103/2231-0746.119222