Section of Oral Pathology and Oral Medicine
Department of Odontology
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FACULTY OF HEALTH AND MEDICAL SCIENCES

Oral lesions and contact allergy

-clinical, histopathological, molecular biological and immunological investigations

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PhD Thesis

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Preface

This thesis is based on studies carried out during my employment as a PhD student at the Section of Oral Pathology and Oral Medicine, Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen. The studies are based on an interdisciplinary collaboration between Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen and National Allergy Research Centre, Department of Dermatology and Allergy, Copenhagen University Hospital Gentofte.

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Kristine Røn Larsen, Ballerup, December 2016

List of abbreviations

OLP	Oral lichen planus
OLL	Oral lichenoid lesion
sIgA	Secretory immunoglobulin A
sIgG	Secretory immunoglobulin G
TNF-α	Tumour necrosis factor alpha
INF-ɣ	Interferon gamma
MHC	Major Histocompatibility Complex
IL	Interleukin
FLG	Filaggrin gene
HEMA	2-hydroxyethyl methacrylate
DMF-t/-s	Decayed-Missing-Filled-teeth/surfaces
PI	Plaque index
GI	Gingival index
MSDS	Material Safety Data Sheet
TSH	Thyroid Stimulating Hormone

Summary

The purpose of this thesis was to identify clinical, histopathological, molecular biological and immunological characteristics of patients with symptomatic oral lichen planus, oral lichenoid lesions and generalised stomatitis in order to enable differentiation of oral contact allergic reactions from other mucosal diseases such as oral lichen planus.

Fifty-two Caucasian patients referred to the Clinic for Oral Medicine, Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen were included in the study. Forty-nine patients completed the examinations. Twenty-nine healthy age- and gender matched healthy control subjects were included as well. All participants underwent an interview regarding general health status and medication intake, scoring of xerostomia, sialometry, a clinical oral examination, and a mucosal biopsy from an area with mucosal changes in the patients and from normal buccal mucosa in the controls. The biopsies were checked for histopathological alterations. Based on the results of the clinical and the histopathological examination a diagnosis was made, either oral lichen planus, oral lichenoid lesion or generalised stomatitis. Afterwards the patients were referred to the Department of Dermatology and Allergy at the University Hospital Copenhagen, Gentofte for a dermatological examination and patch testing.

The diagnosis of oral lichen planus was established in 19 patients and additionally 19 patients were diagnosed with oral lichenoid lesions, the remaining were diagnosed with generalised stomatitis. The patients visited their dentist significantly more often than the healthy control subjects and the healthy control subjects had significantly lower number of decayed-missed-filled-teeth/-surfaces than the patients. This illustrates the fact that patients with oral mucosal disease often need more regular dental check-ups, but on the other hand it also leads to enhanced exposure to both dental materials and oral hygiene products. Nineteen patients and 10 healthy control subjects had positive patch test reactions to tested allergens. The patients with positive patch test reactions did not differ with regard to symptoms, clinical and histopathological characteristics or whether lesions were in close proximity to dental restorations or not. Contact allergy to aroma substances was seen significantly more prevalent in the patient group, especially the patients with oral lichenoid lesions than in the healthy control subjects.

There were no significant differences in the presence of mutations in the filaggrin gene between the patients and the healthy control subjects. The number, the severity of oral symptoms and the extension of oral lesions did not differ between patients with the different types of lesions with and without a concomitant mutation in the filaggrin gene. There was no statistical significant difference between the patients and the healthy control subjects with regard to the presence of active dermatoses when excluding cutaneous lichen planus. Immunohistochemical analysis of the oral mucosal specimens revealed an intense immunoreactivity for filaggrin in hyperortho-keratinised epithelium, and a more scattered immunoreactivity in hyperparakeratinised areas. The

immunoreactivity was significantly more intense in the patients with oral lichen planus and oral lichenoid lesions than in the healthy control subjects. This may be explained by the fact that oral lichen planus and oral lichenoid lesions are hyperkeratotic conditions and filaggrin is essential in keratinisation.

Patients reported xerostomia to a higher and more severe degree than healthy control subjects, and this was not related to low saliva flow rates or contact allergy. The total protein concentration and levels of sIgA in saliva samples from patients were higher than in those from the healthy control subjects, irrespective of presence of contact allergy. These findings may be ascribed to a higher degree of anxiety, depression and sleep disturbances in the patient group. All patients but 2 showed normal ranges of TSH, indicating that thyroid disease is not associated with oral lichen planus or oral lichenoid lesions. Overall, the findings obtained from this study indicate that oral lichen planus, oral lichenoid lesions and generalised stomatitis may be associated with contact allergy to substances in oral hygiene products, changes in the distribution of filaggrin, and increased levels of sIgA and salivary total protein concentration. However, these factors are not sufficiently specific to function as discriminatory markers between potentially allergy-induced oral lesions and lesions related to an oral mucosal disease. Consequently, based on the findings it is not possible to determine in which cases patients with oral lichenoid lesions specifically should be referred for patch testing.

Summary in Danish

Formålet med denne afhandling var at undersøge patienter med symptomgivende oral lichen planus, orale lichenoide læsioner og generaliseret stomatitis med henblik at identificere specifikke kliniske, histopatologiske, molekylærbiologiske og immunologiske karakteristika der muliggør differentiering af orale kontaktallergiske reaktioner fra forandringer ved egentlige mundslimhindesygdomme, fx oral lichen planus.

Der blev inkluderet 52 kaukasiske patienter der var henvist til Klinik for Oral Medicin, Odontologisk Institut, Det Sundhedsvidenskabelige Fakultet, Københavns Universitet. I alt 49 patienter gennemførte undersøgelserne. Der blev desuden inkluderet 29 raske alder- og kønsmatchede kontrolpersoner. Alle deltagere blev interviewet om tilstedeværelse og omfang af orale symptomer, almensygdomme, medicinforbrug, tobaks-, alkohol-, og mundhygiejnevaner, og fik efterfølgende foretaget sialometri, en klinisk oral undersøgelse, og en biopsi af de afficerede områder i mundslimhinden og hos raske af normal kindslimhinde. Patienterne blev diagnosticeret med oral lichen planus, orale lichenoide læsioner eller stomatitis på baggrund af de kliniske og histopatologiske fund. Alle deltagerne fik dernæst foretaget dermatologisk og allergologisk udredning med lappetest på Afdeling for Hudsygdomme og Allergi, Gentofte Hospital.

I alt 19 patienter havde oral lichen planus og andre 19 oral lichenoide læsioner, mens 11 patienter havde generaliseret stomatitis. Patienterne havde signifikant højere DMF-t og -s score og gik hyppigere til tandlægen end de raske kontrolpersoner. Dette kan skyldes, at patienter med mundslimhindeforandringer og -gener har et større behov for at gå til tandbehandling end raske personer, men det betyder også at disse patienter er mere eksponerede for dentalmaterialer og mundhygiejneprodukter og dermed potentielle allergener. I alt 19 patienter og 10 raske kontrolpersoner havde kontaktallergi over for nogle af de allergener, der blev undersøgt ved lappetesten. Patienter med kontaktallergi adskilte sig ikke fra dem uden kontaktallergi med hensyn til symptombillede, kliniske og histopatologiske fund og/eller relation til tandrestaurering. Patienterne og især hos patienter med orale lichenoide læsioner havde signifikant flere kontaktallergier overfor aromasubstanser end raske kontrolpersoner. Der var ingen forskel i forekomsten af filaggrin genmutationer hos patienter og raske kontrolpersoner. Ligeledes var der ingen forskel mellem patienter med oral lichen planus og orale lichenoide læsioner og med og uden filaggrin genmutationer i forhold til sværhedsgraden af de orale symptomer og udbredelsen af mundslimhindeforandringerne. Patienterne havde ikke øget forekomst af aktiv hudsygdom (bortset fra kutan lichen planus). De immunohistokemiske analyser viste markant immunreaktivitet for filaggrin i hyperortokeratiniseret epitel og en mindre og mere diffus immunreaktivitet for filaggrin i hyperparakeratiniseret epitel. Denne immunreaktivitet var mest markant hos patienter med oral lichen planus og orale lichenoide læsioner, hvilket kan tilskrives at såvel oral lichen planus som orale lichenoide læsioner er kendetegnede ved at være

hyperkeratotiske slimhindelidelser og at filaggrin netop spiller en central rolle i forbindelse med keratinisering.

Patienterne klagede oftere over xerostomi og mere alvorlig xerostomi end de raske kontrolpersoner. Der påvistes ingen sammenhæng mellem xerostomi og nedsat spytsekretionshastighed, medicinindtag, forekomsten af sygdomme eller kontaktallergi. Koncentrationen af totalprotein og niveauerne af sIgA var højere i spytprøver fra patienter end hos raske kontrolpersoner, uanset tilstedeværelse af kontaktallergi eller ej. Det er muligt, at en højere forekomst af angst, depression og søvnforstyrrelser hos patienterne kan forklare den høje forekomst af xerostomi og de forhøjede værdier af totalprotein og sIgA. Alle patienter, med undtagelse af 2, havde normale serum TSH-værdier, hvilket indikerer at der ikke er sammenhæng mellem thyroideasygdom og oral lichen planus og orale lichenoide læsioner. Samlet set indikerer resultaterne af dette studie, at oral lichen planus, orale lichenoide reaktioner samt generaliseret stomatitis kan være forbundet med øget forekomst af kontaktallergi over for substanser i mundhygiejneprodukter, en ændret fordeling af filaggrin i mundslimhinden samt forhøjede niveauer af sIgA og totalprotein i spyt. Disse fund er dog ikke tilstrækkeligt specifikke til at kunne anvendes i differentieringen mellem allergi-suspekte orale læsioner og læsioner ved andre mundslimhindesygdomme, og heller ikke til at afgøre hvilke patienter der bør henvises til allergiudredning eller ej.

Introduction

The oral cavity and the lips are exposed to irritants and allergens on a daily basis. These include amongst others substances used in oral hygiene products as well as the materials used in diagnosing and treating oral diseases (later on referred to as dental materials). Oral hygiene products and dental materials may be responsible for sensitization and allergic reactions in the oral mucosa (1-7).

The persistent antigenic stimulation from oral hygiene products and dental materials may lead to acute and chronic oral mucosal changes, including allergic reactions (8). The clinical manifestations of oral contact allergic reactions are diverse comprising localized lichenoid lesions, diffuse widespread erythema and swellings including stomatitis and cheilitis, perioral dermatitis, vesicles and blisters as well as ulcerations (9). Symptoms may include burning, stinging and/or tingling sensations in the oral mucosa, xerostomia, dysgeusia and other sensory disturbances (10). As the oral mucosal allergic reactions may resemble changes seen in other oral mucosal diseases, diagnosing and treating the patients may be challenging (11). In large Scandinavian questionnaire studies, adverse reactions to dental materials were estimated to occur in one of 300 patients in prosthodontic practices, in one of 2.600 patients treated in public dentistry, in one of 100 undergoing orthodontic treatment and at a frequency of one every other year per periodontist (12-15). The prosthodontists, orthodontists and public dentists primarily reported type IV allergic reactions, whereas the periodontists primarily reported type I allergic reactions reflecting the type of materials the patients are exposed to and the type of allergic reaction they elicit (12-15).
Only a limited number of studies has investigated the incidence of contact allergic reactions to both dental materials and oral hygiene products in patients with oral lichen planus (OLP) and oral lichenoid lesions (OLL), respectively (4, 16).

The studies referred to in this thesis were carried out in order to identify clinical, histopathological, molecular biological and immunological characteristics of patients with symptomatic oral lichen planus, oral lichenoid lesions and generalised stomatitis to enable differentiation of oral contact allergic reactions from other mucosal diseases such as oral lichen planus.

Background

The oral cavity

The oral cavity is the first part of the digestion system and it consists of the lips, the cheeks, the palate, the tongue, the mucosa covering the alveolar processes and the teeth. The oral cavity is adapted to large fluctuations in temperature, pH value, environmental factors and mechanical stress. In addition, the oral cavity harbours more than 700 species of bacteria that colonise the hard surfaces of teeth and the soft tissues of the oral mucosa (17). Fungi and viruses can be present as well. Dysbiosis, a condition in which the normal microbiome population structure is disturbed and disease-promoting bacteria dominate, may cause oral diseases like caries, gingivitis and periodontitis. Dysbiosis may occur due to poor oral hygiene, insufficient or unhealthy diet, intake of medication, systemic disease and/or oral mucosal disease (18).

The oral mucosa

The oral cavity is covered by a mucosal membrane that consists of stratified squamous epithelium, a basement membrane and an underlying connective tissue (Fig.1). The epithelium is constantly undergoing cell-renewal and hence desquamation of the superficial epithelial cells. The surface of the oral mucosa has several different appearances defined by the functional requirements in the particular area. The gingiva and the hard palate have masticatory mucosa, the dorsum of the tongue has specialised mucosa while the cheeks, the floor of the mouth, the remaining parts of the tongue and the soft palate have lining mucosa (19).

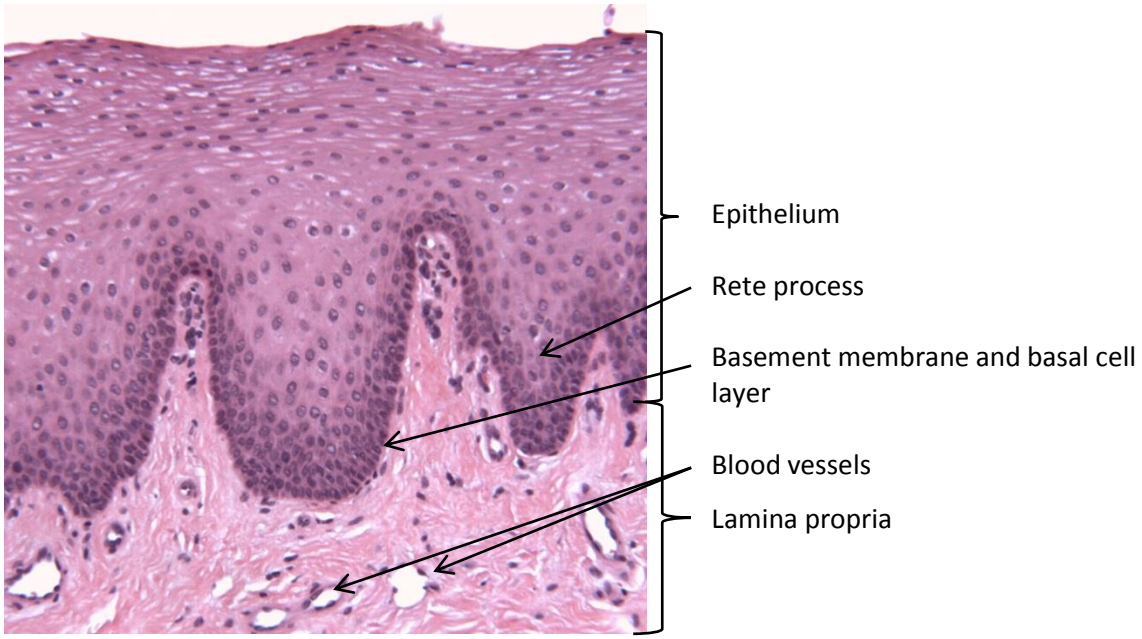


Figure 1: Photomicrograph illustrating the appearance of normal human buccal mucosa (approximate magnification: x100, haematoxylin and eosin staining).

The masticatory epithelium is keratinised and to some extent resembles the structure of the epidermis. Masticatory epithelium is orthokeratinised (the hard palate and parts of the gingiva) or parakeratinised (most of the gingiva). In the masticatory epithelium a stratum granulosum is present and in the hard palate it is well-defined while sparser represented or absent in the gingiva. In stratum granulosum filaggrin granules can be found. The granules are not as well organized as seen in the stratum granulosum of the epidermis. As in the epidermis, filaggrin is formed from profilaggrin in the keratohyalin granules in the stratum granulosum (19). The lining mucosa is non-keratinised. Using immunohistochemistry and molecular-biologically methods, filaggrin and profilaggrin can be detected in these epithelia albeit with variable and significantly lower density (20, 21). In most areas of the oral mucosa the rete processes are well-defined, but in the floor of the mouth they are more sparse and shallow. This reflects the different functions of the surfaces in the oral cavity. Underneath the epithelium lies the lamina propria, a highly vascular collagenous connective tissue. Underneath lamina propria is the submucosa, a loose connective tissue with various amounts of salivary glands and adipose tissue (19).

When evaluating the permeability of the oral mucosa some general rules apply: 1) Molecules penetrate more easily than ions, 2) large molecules penetrate with more difficulty than small molecules and, 3) substances that dissolves in both water and lipid penetrates with most ease. The epidermis provides an extremely good barrier to water, better than any areas of the oral cavity. Within the oral cavity the non-keratinised regions are significantly more permeable to water than the keratinised regions. The integrity of the oral mucosa is maintained by the structural components of the epithelium, including the keratin filaments. The keratin filaments form a coherent skeleton in the epithelium that forms the rigid stratum corneum and filaggrin functions as a matrix in this process (22). The permeability of the epidermis and the keratinised oral epithelium is regulated in the superficial part of the spinous cell layer, where organelles, the membrane-coating granules, excrete small membranous discs into the intercellular space. The discs are then rearranged to form a lamellate sheet in the superficial part of the epithelium that provides a barrier function. The lipid composition of the epidermis and the keratinised epithelium is dominated by acylceramides and ceramides. Non-keratinised oral epithelium shows a different lipid composition, where only small amounts of ceramides can be detected and large amounts of glycosylceramide are present. Simultaneously, the barrier function is provided by small intracellular vesicles that extrude their amorphous content into the intracellular space after fusing with the superficial cell membrane. These differences may account for the greater permeability to water in the non-keratinised epithelium compared to the keratinised. In the non-keratinised oral mucosa, regional differences can be observed with the floor of the mouth being the most permeable area (23).

The oral mucosa as well as the skin reacts to irritants through hyperplastic changes. The hyperplastic changes are characterised by hyperkeratosis and increased thickness of the epithelium. The permeability of such an oral epithelium is, surprisingly, significantly higher maybe

due to the increased cell division and hence the transit time through the tissue. If inflammation is present keratinised epithelium can become non-keratinised and hence the permeability increases. If atrophy and/or necrosis of the oral epithelium are present, which may be seen in oral lichen planus, the permeability barrier is lost. If fibrin is covering the necrotic area it provides a partial barrier. Surfactants, like sodium lauryl sulphate in toothpaste, significantly increase the permeability of the oral mucosa (24).

Saliva

Saliva is a mixed fluid that is in contact with all parts of the mouth. Saliva is essential in the digestive process but also in protecting the teeth and the mucosal membranes through the formation of the acquired tooth pellicle and the mucosal pellicle (25, 26). Salivary gland dysfunction, e.g. changes in the quantity and quality of saliva, may lead to impairment of the oral health. Saliva is primarily produced in the three major paired salivary glands, sublingual, submandibular and parotid salivary glands. These major glands produce approximately 90-95% of the salivary fluid. The remaining part of the fluid is produced by the minor salivary glands, which on the other hand contribute with production of large amount of proteins, especially glycoproteins. Whole saliva contains gingival fluid, food debris, desquamated epithelial cells and microorganisms shed from the oral surfaces (25, 27).

The fluid produced by the parotid salivary glands is watery and rich in alpha-amylase, a digestive enzyme which can split starch into maltose, maltotriose, maltotetrose, and some higher oligosaccharides with an optimum pH about neutrality. The fluid produced by the submandibular salivary glands is more mucin-rich and hence more viscous. The sublingual salivary glands produce an even more mucin-rich and viscous fluid compared to the submandibular glands. The minor salivary glands are located in the labial, buccal, lingual and palatal mucosa. The minor glands in the palate produce a strictly mucous fluid, whereas the lingual glands produce a strictly serous fluid containing lipase. When the salivary glands are stimulated, i.e. by chewing, the saliva produced by the parotid glands poses approximately 50% of the total volume (27). The saliva produced under resting/unstimulated conditions, primarily derives from the submandibular salivary gland (25-27).

Salivary glands are exocrine glands that comprise secretory end pieces (acini) and a connecting duct system. The secretory end pieces consist of acinar cells that can be either mucous or serous. Upon stimulation, the acinar cells produce an isotonic fluid, called primary saliva, which has salt concentrations very similar to those of plasma. As this primary saliva passes through the duct system, energy is expended to reabsorb sodium and chloride, but not water, while secreting potassium. Thus when the saliva reaches the opening of the main excretory duct, the saliva is hypotonic (25).

The final composition of saliva depends on the flow rate. When the salivary glands are stimulated and flow rates are increased, the concentrations of sodium, chloride and bicarbonate increase as less are being reabsorbed and potassium slightly decreases. The saliva flow rates and hence the composition of the final saliva are affected by several factors like the type and size of glands from where the saliva originates, nutritional state of the individual, state of hydration, time of day where saliva is produced, type and duration of stimulus, emotional state and gender. The saliva flow rate is affected by the circadian rhythm. The flow rate rises during the day and peaks in the afternoon; afterwards it then decreases to almost zero during sleep (25).

Xerostomia is the subjective feeling of oral dryness. Xerostomia may occur due to reduced saliva production, hyposalivation and/or compositional changes of saliva. Xerostomia may be indicative of systemic disorders like Sjögren's syndrome and diabetes mellitus. Xerostomia is also a common side effect to a large number of various medications (25, 27, 28).

Besides the water and the mucins in saliva, defence proteins, e.g. lysozyme, lactoferrin and immunoglobulins from the innate and the adaptive immune system can be detected as well (25). The defence proteins are present at low concentrations, but as their effects are additive and/or synergistic, they are efficient in defending the oral cavity against bacteria, virus, fungi and environmental factors. The concentration of the salivary defence proteins is higher in for instance a local area with ulceration, making the concentration efficient locally.

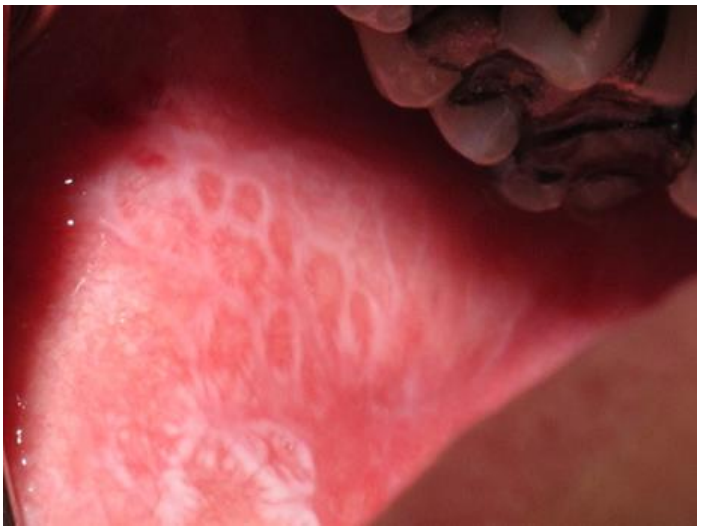
In the human saliva two major classes of antibodies can be detected; secretory immunoglobulin A (sIgA) (90-98%) and IgG (1-10%), but also fractions of IgM, IgD and IgE can be detected mainly deriving from the gingival crevices (29). SIgA is induced in IgA B-lymphocytes in the gut-associated lymphoid tissue. These cells are found in the salivary glands (primarily the parotid glands), where they form an IgA dimer and also produce a joining chain. This dimer and the joining chain are taken up by the poly-IgA receptor on the surface of acinar cells prior to its release into saliva. The secretory component is a small, heavily glycosylated molecule which makes the IgA dimer less susceptible to bacterial proteolytic enzymes than is the single molecule IgA. The functional significance of sIgA is still unclear, as patients with a hereditary lack of IgA do not appear to be more susceptible to oral disease, although they may show an increase in IgM in their saliva (30). Some studies though show that the salivary immunoglobulins inactivate bacteria, viruses and fungi by promoting agglutination hence leading to clearance through the stomach. However, the immunoglobulins may also act via surface immune exclusion, that is promoting the microbial adhesion of (in theory) non-pathogenic bacteria hence excluding disease-promoting microorganisms. SIgA is secreted continuously, also in oral health. The concentration of sIgA in saliva is highly sensitive to general health, mental state, exercise, hydration state, presence of inflammatory disease etc. (31).

Lichen planus, oral lichen planus (OLP) and oral lichenoid lesions (OLL)

Lichen planus (LP) is an inflammatory mucocutaneous disease of unknown aetiology. LP primarily affects the skin and the oral mucosa, but other mucosal membranes and nails may be affected as well. Lichen planus usually debuts in middle-aged adults and women predominate with a female: male ratio of 2:1 (32).

Oral lichen planus (OLP) is one of the most common non-infectious oral mucosal diseases and occurs with an estimated prevalence of 0.5% to 2.2% (33). The clinical manifestations of OLP include reticular, erythematous, erosive/ulcerative, papular, plaque or bullous lesions which may present in combinations. The reticular form of OLP is the most common form. It especially affects the posterior buccal mucosa, but other oral mucosal surfaces can be affected as well. The reticular type of OLP is named from its characteristic clinical appearance, the interlacing white lines, Wickham striae (Fig.2). The Wickham striae can be observed in almost all types of OLP, like for instance in the periphery of an erosive/ulcerative lesion. The reticular lesions rarely cause symptoms, whereas the atrophic and erosive/ulcerative lesions may be associated with significant symptoms such as stinging, burning, itching and stabbing (34).

Figure 2: Patient with the reticular type of oral lichen planus. Note the characteristic Wickham striae in the right buccal mucosa. A few papules are seen in the lower part.



Histopathologically OLP is characterised by a hyperpara- or, more rarely hyperorthokeratinised epithelium. Variation in the thickness of the epithelium is normal and atrophy can often be observed. In the basal cell layer, Civatte bodies (scattered necrotic epithelial cells) are usually seen. They may contain nuclear fragments indicating that they are apoptotic keratinocytes. In the basal cell layer destruction of the cells can be observed referred to as liquefaction degeneration (Fig.3) (35, 36).

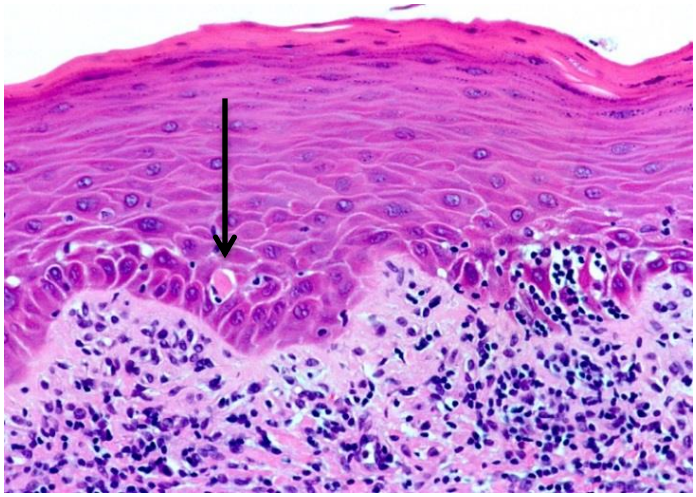


Figure 3. Photomicrograph of an oral lesion in the buccal mucosal showing hyperparakeratosis and a Civatte body (arrow) and basal cell layer liquefaction degeneration (right). Part of the band-like subepithelial lymphocytic infiltrate can also be observed (original magnification x250, haematoxylin and eosin staining).

Lesions that resemble OLP clinically and histopathologically are referred to as lichenoid lesions (OLL). These include oral lichenoid contact lesions, oral lichenoid drug reactions and oral lichenoid lesions of graft-versus-host disease (33).

Presently, and in most countries around the world, the diagnosis of OLP is based on the WHO criteria from 1978 proposed modified by van der Meij and van der Waal in 2003 (Table 1) (35,36).

These criteria were also applied in the present study. Hence, the diagnosis of OLP was established when the lesions met all of the above mentioned criteria. When the clinical and histopathological features of the lesions met most of these criteria, the diagnosis was established as OLL, i.e. patients presenting with typical OLP lesions clinically, but not fulfilling the histopathological criteria or vice versa (36).

Table 1: The criteria used for establishing the diagnosis of OLP and OLL (35, 36)

	WHO criteria	Modified WHO criteria
Clinical criteria	- multiple and often symmetrical distribution of mucosal lesions - white reticular, annular, papular or plaque-type mucosal lesions - white striae radiating from any present papules - atrophic lesions +/- erosion - bullae are rare	-bilateral almost symmetrical distribution of mucosal lesions - a reticular pattern is mandatory - other types of lesions, i.e., erosive or bullous, are only accepted as subtypes if reticular lesions are present as well
Histopathological criteria	- well-defined band-like zone of cellular infiltration consisting primarily of lymphocytes located superficially in the lamina propria - liquefaction degeneration in the basal cell layer - orthokeratosis or parakeratosis - rete ridges have a saw-tooth appearance - epithelial thickness varies - Civatte bodies in the basal layer of the epithelium and/or superficial lamina propria - eosinophilic material in the basement membrane	- well-defined band-like zone of cellular infiltration consisting primarily of lymphocytes located superficially in lamina propria - liquefaction degeneration in the basal cell layer - absence of epithelial dysplasia

Although the exact aetiology for OLP is unknown data suggest that OLP is an immune-mediated disease involving a dysregulation of primarily the T-helper cells. Typically in OLP a CD8+ T-cell epithelial infiltrate bordering apoptotic basal keratinocytes can be observed, that overlies a CD4+ T-cell infiltrate of the lamina propria (37). One study showed that when comparing the reactivity of lesional and non-lesional T-cell clones from patients with LP against lesional and non-lesional autologous keratinocytes, the cytotoxicity of the lesional T-cells were significantly higher than the toxicity of the T-cells from normal skin. The most cytotoxic T-cell clones from the LP lesions were CD8+ and the most non-cytotoxic T-cell clones were CD4+ (39). It has been suggested that CD8+ T-cells from a LP lesion are activated by a keratinocyte antigen leading to keratinocyte apoptosis (39). The triggering antigen, however, has not yet been identified. It has been suggested that Langerhans cells may play a role in presenting antigens to T-cells through major histocompatibility complex class II (MHC II) eliciting a primary immune response (initial sensitivity to the antigen) followed by a secondary immune response leading to clinical signs of mucosal disease (40). The CD4+ cells can differentiate into T-helper 1 cells and T-helper 2 cells depending on the antigen

stimulation (41). The T-helper cells are typically categorized according to their cytokine production. T-helper 1 cells primarily produce interferon-gamma (INF-γ), tumour necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2), that induce cytotoxic CD8+ T-lymphocyte and macrophage activation, hence mediating a local inflammatory immune response in the local cells. T-helper 2 cells secrete mainly IL-4, IL-5, IL-10 and IL-13, all of which are important in the humoral immune response (42). Recently T-helper 17 cells have been isolated and their involvement in the immune system has been studied intensively (43). Maintaining T-helper 17 cells is associated with IL-23 (an IL-12p40/p19 heterodimeric protein) production, a cytokine that is known for its involvement in autoimmune diseases like psoriasis, rheumatoid arthritis and systemic lupus erythematosus (41).

A few studies have shown that the salivary levels of sIgA are elevated in patients with OLP and OLL compared with healthy controls (44-46). In this context it has been suggested that both serum and salivary immunoglobulins, particularly sIgA and IgG, may play an important role in the pathogenesis of OLP, and in 2012 Ghaleyani et al. hypothesised that sIgA and IgG could be of value in the discrimination of OLP from OLL (45). However the findings of these studies suggest that saliva levels of sIgA are not sufficiently sensitive to aid in discriminating between OLP and OLL.

The potential of OLP lesions to undergo malignant transformation is widely discussed. The transformation rates of malignancy range from 0% to 9% depending on the population studied and the criteria used for diagnosis (47-52).

Stomatitis

Inflammation of the oral mucosa is referred to as stomatitis. The diagnosis of stomatitis comprises several subgroups, i.e. recurrent aphthous stomatitis, denture stomatitis and contact stomatitis (5, 53, 54). Histopathologically, contact stomatitis can be characterised by hyperkeratosis, vasculitis, presence of lymphocytic infiltration, and plasma cells (55). In the present study, the diagnosis of stomatitis was based on diffuse mucosal erythema ranging from a barely visible erythema to a bright red, widespread erythema without signs of OLP (Fig. 4). Erosions and hyperkeratosis could also be seen (5).

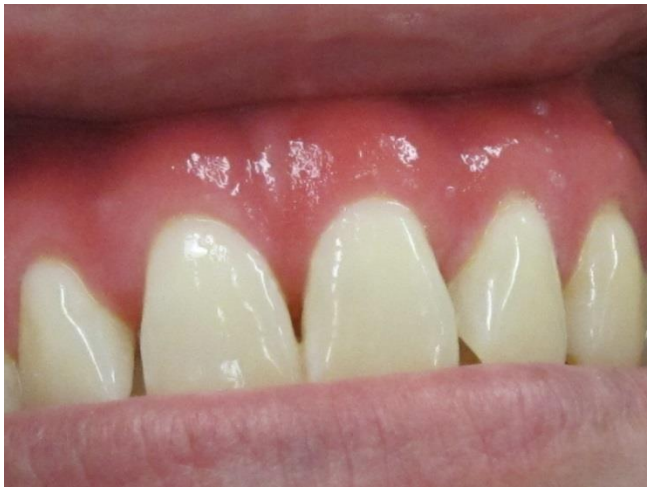


Figure 4. A female patient with stomatitis in terms of bright red erythema on the gingiva

Dental materials

Dental materials are defined as materials used in production of all types of dental restorations. Furthermore, dental materials can be categorized as preventive materials, restorative materials or auxiliary materials. The preventive materials include pit and fissure sealants. The restorative materials are used in replacing lost tooth substance/making a dental restoration, whereas the auxiliary materials are used in manufacturing the dental restorations. Due to its more or less transient nature, materials used in orthodontic treatment can be categorised as both dental materials and auxiliary materials (56).

When a dental material is introduced in the oral cavity it is not inert. Due to the alternating environment of the oral cavity, degradation of all types of dental materials takes place leading to a release of components from the material. There has been immense focus on the release of mercury from amalgam fillings related to general health problems. Mercury is released into the oral cavity as vapour, ions and amalgam fragments. There is an association between the amount of mercury released and the number of tooth surfaces with amalgam (57).

Other metals, most often in the form of an alloy, besides mercury are used in making crowns, bridges and implants. All metals introduced to the oral cavity undergo degradation due to electrochemical corrosion. The degree of corrosion is determined by the type of alloy, the phase and surface structure of the alloy, history of treatment/thermal treatment, combination of alloys in the oral cavity and functioning time in the oral cavity (58).

Acrylates in all its forms is probably the most used material in modern dentistry. Acrylates are present in composite fillings, dental prostheses, temporary crowns/bridges, cements as well as in orthodontic appliances for children and adults. When acrylates are used in dentistry, monomers are trapped in the polymerisation process and the conversion rate is never 100%. Hence different

monomers can leak from the material and into the oral mucosa and the oral cavity. A higher conversion rate leads to a lower amount of leaking monomers (59, 60). All monomers are known sensitisers (3).

Approximately 2.71 mio composite fillings were made in adult Danish patients (18 years and older) in 2015 and approximately 62.000 amalgam fillings. In September 2007 the Danish Health Authority decided that the use of amalgam as a dental filling material should be phased out. Prior to this decision, the numbers of amalgam fillings made annually were about 650.000 and the number of composite fillings 2.1 mio (61).

Acrylates are not only used in dentistry. The exposure to acrylates is increasing among people working in the beauty and nail artist industry, and the popularity of these treatments is still growing (62). A large retrospective study including 455 patients who were patch tested to acrylates (dental, nail, printing and adhesive) in the period 2008-2014, showed that 54 (11.9%) of the patients had clinically relevant positive reactions, and that the annual increase in allergy was significant. 81.2% of the patients were allergic to 2-hydroxyethyl methacrylate (HEMA). The age of the patients ranged from 14 to 80 years and the majority were female (91%). Thirty-seven of the patients had non-occupational allergic contact dermatitis and 30 of these cases were considered related to nail products containing acrylates (63).

Oral hygiene products

Oral hygiene products are used by most people several times every day. They are applied in the oral cavity and usually expectorated after use. Oral hygiene products often contain several known sensitisers like limonene, cinnamon, spearmint and preservatives (4, 6). Despite the fact that oral hygiene products contain known sensitisers, the sensitising potential of this type of product is still debated as oral hygiene products are introduced at a very young age and used throughout a lifetime (1).

Oral hygiene products are subject to shared EU-legislation via the cosmetic directive. This directive states for example that a list of ingredients has to be present and that this list contains all the ingredients/components, arranged in descending order of weight at the time they are added to the product. Perfume and aromatic components are indicated as "perfume" or "aroma". Twenty-six substances have to be declared at all times if present in the product of more than 0.01% for products rinsed off and 0.001% for products which are not rinsed off. Ingredients at a concentration of <1% may be listed in any order after the other ingredients unless they are one of the 26 substances that are on the EU list of substances that must be declared (64).

Latex and chlorhexidine

Latex is widely used in all parts of the health care system. Allergic reactions to latex were a problem of growing concern as the incidence grew rapidly over the 1980´s where latex gloves were introduced in the health care system. It was later discovered that the powder in the gloves that carried the latex molecule and thereby increased the allergen potential. After removal of powder from latex gloves, the incidence has dropped. However, latex still comprises a major problem as it is widely used in both the health care system, in dental practices and in consumer products (65).

Chlorhexidine has been used for many years in all parts of the health care system and in consumer products due to its bacteriostatic, bactericidal, fungistatic, fungicidal and antiviral abilities (66). A recent Danish study showed that chlorhexidine is present in 3.6% of 2251 cosmetic products (67). In dentistry chlorhexidine is primarily used as mouthwash. Allergic contact stomatitis, urticarial and anaphylaxis have been reported after the use of mouthwash (68).

Allergy

Allergic reactions can be classified as an unwanted "side effect" to a functioning immune system, where the immune system overreacts to non-dangerous substances. The allergic reaction can be divided in to 4 different types based on the immune response. The most common types are Type I and Type IV.

Type I

This type of reaction is known as the immediate/acute reaction due to the fact that the reaction appears within seconds to a few hours after exposure to an allergen. This type of reaction is most often caused by substances like pollen, food items and dust mites. In dental practice type I reactions are very rare, but can be triggered by chlorhexidine, penicillin and latex (69).
When the immune system encounters an antigen the first time, the B-cells produces an antibody specifically targeting the antigen. Simultaneously the B-cell produces plasma cells and induces specific T-cells. Now the individual is sensitised to that particular antigen. At the time, the individual encounters the same antigen again the T-cells immediately recognises it and activates the plasma cells and they produce IgE. IgE are then bound to the mast cells circulating in the blood. When the mast cell comes in to contact with an allergen through the mucosal membrane, it is activated and cell mediators are released leading to histamine release. The histamine release causes an increase in the vascular permeability, oedema in the airways, increased mucus secretion and occasionally bronchospasm (Fig. 5). In the oral cavity swelling of the tongue, lips, mucosa and vesicular formation can be seen. The patients usually report of itching in the palate (70).

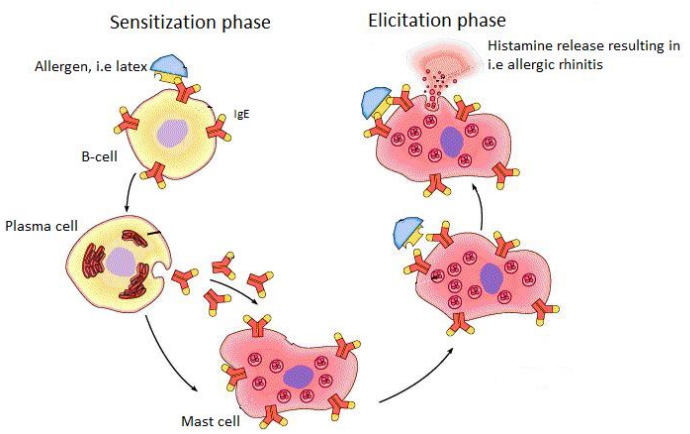


Figure 5. Illustration of the cellular reactions involved in type I allergy.

Modified from Lewis R. Life 3. ed., McGraw-Hill Companies 1998

Type IV

This type of allergic reaction is known as the delayed type of hypersensitivity due to the fact that the reaction appears 48-72 hours after exposure to the allergen. Type IV reactions are most often caused by nickel, perfume and preservatives (71).

This type of allergic reaction occurs due to an immunological response to a reactive chemical with a molecule weight smaller than 500 Da. The reactive molecules become antigenic when bound to a protein, this complex is referred to as a hapten. The hapten penetrate the skin or mucosa and conjugates to dermal and epidermal proteins, including the MHC I+II and Langerhans cells (72). The hapten is then internalized, processed, transported via the afferent lymph system to the nearest draining lymph node and presented to specific naive T-cells that recognize the allergen MHC-complex. The specific naïve T-cells are activated by the MHC I+II in a matter of days and develop into allergen-specific T-cells, CD8+ and CD4+ respectively. When the naïve T-cells get activated, they secrete IL-2 that is a T-cell growth factor. Parallel to the priming of the naïve T-cells, regulatory T-cells are induced. The regulatory T-cells seem to fail to prevent the allergic contact dermatitis in case of sufficiently strong allergens, whereas they may prevent contact allergies to weak sensitizers. In the lymph node the activated Langerhans cells produce large amounts of IL-12, turning off the IL-4 production, hereby activating the differentiation of T-helper 1 cells. Differentiation of T-helper 1 cells leads to INF-γ production by neighbouring cells like natural killer cells and dendritic cells. This creates a positive feedback system; the INF-γ (released from T-helper 1 cells) promotes the release of IL-12 from Langerhans cells. CD4+ T-cells have different cytokine profiles and this profile determines if they are associated with helper/effector or regulatory/suppressive functions. CD8+ T-cells contribute to formation of allergic contact dermatitis. This phase is known as the induction phase (Fig 6).

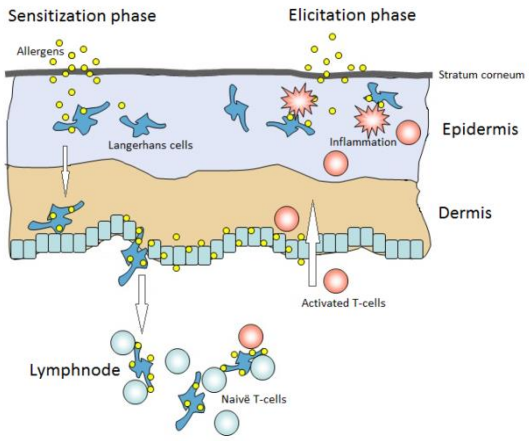


Figure 6. Illustration of the immunological response in a type IV allergic reaction.

Modified from biologicalexceotions.blogspot.dk

Upon re-exposure to the relevant allergen, allergen-specific T-cells accumulate and encounter allergen presenting cells. This sparks production of proinflammatory cytokines and the clinically visible eczema appears. This phase is known as the elicitation phase (Fig. 6) (73-75). If the allergens are structurally related cross-reactivity can be seen. This can be observed with for instance nickel and palladium.

In the oral mucosa the production of proinflammatory cytokines can cause a variety of different clinical manifestations, e.g. cheilitis, stomatitis and lichenoid lesions (76). As mentioned previously the lichenoid lesion resembles the OLP lesions both clinically and histopathologically. Cheilitis may also be seen and manifest as dryness of lips, and fissuring and cracking of the vermillion border (7). Stomatitis is a more diffuse reaction displaying various degrees of diffuse mucosal erythema. Erosions and hyperkeratosis can also be seen. Stomatitis is often accompanied by a burning and itching sensation and tenderness of the involved area (5).

The oral mucosal membrane does not react to contact allergens as readily as does the skin. This can probably be ascribed to the various factors related to the oral environment including abundant vascularisation, saliva and the immunological response. Potential allergens are more rapidly absorbed because of the abundant vascularisation hence a shorter exposure time. Saliva is diluting the potential allergens and also contains factors like sIgA, salivary chaperokine, lysozyme and amylase that inhibit the immunological response (31). The immunological response to allergen exposure in the oral mucosa is slightly different in that of the skin. In the oral mucosa dendritic cells function as antigen presenting cells. The oral dendritic cells resemble the skin Langerhans cells but differentiate by an increased expression of MHC I+II and co-stimulatory molecules and by their stimulatory effect (77, 78).

Filaggrin

The human skin functions as a barrier to external influences like for instance pathogens and chemical compounds. The skin-barrier also prevents loss of water from the body. The outermost layer of the skin (epidermis) is essential to the barrier function, particularly the stratum corneum and its lipids. Filaggrin is observed in between the terminally differentiated keratinocytes in the stratum corneum whereas profilaggrin is present in the stratum granulosum. Translation of the FLG results in the making of profilaggrin. Profilaggrin is then cleaved numerous times and ends up as filaggrin in the stratum corneum (79). In a population with European ancestry, 7-10% of the persons have a mutation in the gene encoding filaggrin. Mutations vary in individual populations but in Europe R501X and 2292del4 are the most common mutations and represent about 80% of the mutations. R2247X is a more uncommon mutation and it represents approximately 3% of all known mutations (80-82). The mutations are deletions, out-of-frame insertions or nonsense mutations that cause loss of performance (81, 83). The importance of filaggrin in maintaining normal barrier function of the epithelium has been elucidated by studies of mutations in the gene encoding filaggrin. Mutations can lead to a variety of skin diseases including atopic dermatitis, due to the loss of performance and the reduced amount of/lack of filaggrin in the epithelium (84). Cross-sectional studies have shown that a defect in the profilaggrin gene is associated with fissured skin on the hands as well as an increased risk and persistence of hand dermatitis (84, 85). Another study has shown a strong relation between perceived dry skin and mutations the profilaggrin gene in the general population (86).

Filaggrin gene mutations might play a role in the development of oral lesions and oral contact allergy. As described earlier the integrity of the oral mucosa is maintained by the structural components of the epithelium, including the keratin filaments. The stratum corneum forms a coherent skeleton of keratin filaments by interacting with filaggrin that functions as a matrix in this process (22).

As in the epidermis, filaggrin is formed from profilaggrin in keratohyalin granula in the stratum granulosum of orthokeratinised oral mucosa, in particular in the hard palate and in parts of the gingiva. Gingiva is mainly parakeratinised and other areas of the oral mucosa, like the buccal mucosa, are non-keratinized. Filaggrin/profilaggrin can, however, still be detected by using immunohistochemical and molecular-biological methods. The density in these epithelia, though variable, is significantly less (Fig. 7A and 7B) (20, 21).

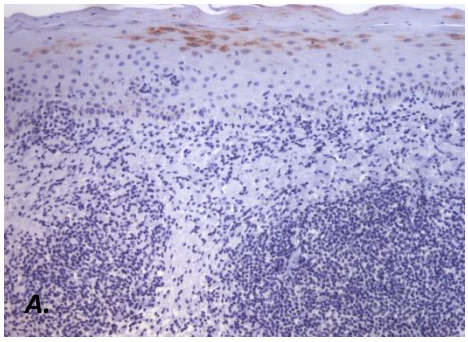


Figure 7A. Photomicrograph of an oral lichenoid lesion in buccal mucosa showing scattered coherent immunostaining for filaggrin in a discontinuous granular cell layer (original magnification x180).

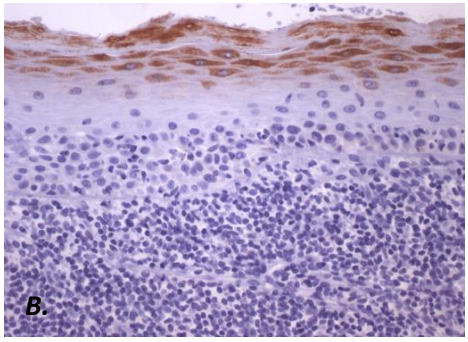


Figure 7B. Photomicrograph of an oral lichen planus lesion in buccal mucosa showing coherent staining in a continuous granular cell layer (original magnification x200).

The permeability of the epidermis and keratinised oral epithelium (palate and gingiva) is also regulated by lipids derived from the lamellar bodies, released from the cells in to the intercellular spaces of the superficial epithelial layer. A different lipid composition is seen in non-keratinised oral epithelium, which may account for the greater water permeability in this type of epithelium as well as the impermeability to larger molecules such as toxins and enzymes (23). Filaggrin binds nickel (87). It is possible that filaggrin also binds other metals and therefore may affect the penetration and accumulation of mercury from amalgam, influencing the risk of oral contact allergic reactions to mercury (88).

Objectives

The overall objective of this thesis is to examine patients with symptomatic oral lichen planus, oral lichenoid lesions and generalised stomatitis to identify specific clinical, histopathological, molecular biological and immunological characteristics that can enable differentiation between oral contact allergic reactions and mucosal changes seen in oral mucosal diseases such as oral lichen planus.

The specific aims are to study:

- the extent to which patch testing of patients with potentially allergy-induced oral lesions identify actual contact allergies to dental materials and oral hygiene products
- oral and cutaneous symptomatology in patients with OLP, OLL and stomatitis
- the total protein concentration and the sIgA levels in saliva from patients with potentially allergy-induced oral lesions, patients with OLP and healthy controls
- the cytokine profile in serum samples from patients with OLP, OLL and stomatitis
- whether potentially allergy-induced oral lesions are associated with mutations in the FLG
- the distribution of filaggrin in the oral epithelium in patients with OLP, OLL and stomatitis, and healthy controls

Hypotheses

Patients with symptomatic OLP, OLL and stomatitis in comparison to healthy controls have:

- more contact allergic reactions
- more contact allergic reactions to dental materials and oral hygiene products
- more active dermatoses, apart from cutaneous LP
- a higher prevalence of filaggrin-gene mutations
- altered expression of filaggrin in the oral mucosa
- more complaints of xerostomia
- lower unstimulated, chewing-stimulated whole saliva and citric-acid stimulated parotid saliva flow rates
- higher serum cytokine levels, salivary levels of sIgA and total protein concentrations.

Furthermore, patients with symptomatic OLP, OLL and stomatitis and a defect in the FLG have:

- more widespread oral lesions and report more symptoms than OLP and OLL patients without a concomitant defect in the FLG.

Additionally, patients with symptomatic OLP and OLL and a concomitant contact allergy:

- report xerostomia more often than patients OLP and OLL without a contact allergy
- have lower unstimulated, chewing-stimulated whole saliva and stimulated parotid saliva flow rates when compared to patients with OLP and OLL without concomitant contact allergy
- higher serum cytokine levels, salivary levels of sIgA and total protein concentrations than patients with OLP and OLL and no contact allergy.

Materials and Methods

Study participants

The participants investigated in the present cross-sectional study were recruited among the patients referred to the Clinic for Oral Medicine, Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen. A total number of 134 patients (112 females and 22 males) were screened for participation in the study during the period of April 2013 and June 2015. A total of 52 Caucasian patients were eligible for inclusion and 49 patients (42 females and 7 males) completed all examinations. All patients had oral symptoms and signs of OLP, OLL or stomatitis. Furthermore, a total number of 29 healthy control subjects, 4 men and 25 women, were included. The selection was based on the requirements that they had no present or past medical history of systemic disease, did not take any medication and matched the patients with regard to age and gender.

Inclusion and exclusion criteria

The participants had to be above the age of 18 years and maximum 75 years and be Caucasian. The age limit of 75 years was set due to the fact that the number of medical diseases and the intake of medications increase with age complicating the interpretation of results. Furthermore, recruitment of healthy and non-medicated subjects becomes difficult with increasing age. We only included Caucasians as in Caucasians two FLG mutations (2282del4 and R501X) account for 80% of the mutations, and three other mutations account for yet 16%. Inclusion of patients irrespective of ethnicity would require inclusion of a significantly larger number of patients and control subjects and investigation of a wider array of mutation types. Moreover, it was a requirement that the patients should have oral symptoms and oral mucosal lesions that clinically could be classified as OLP, OLL or generalised stomatitis (please see previous description of definitions).

The exclusion criteria included pregnancy and lactation, inability to understand the information for participants, alcohol and drug abuse, intake of medication, burning mouth syndrome, on-going infection (including oral candidiasis) or other acute illness. A further exclusion criterion was suspicion of medication-induced lichenoid lesions.

The inclusion criteria for the healthy control subjects was based on the requirements that they had no present or past medical history of systemic disease, did not take any medication, did not have any known allergies and matched the patients with regard to age and gender. Contraception, vitamin and mineral supplements were accepted.

The study was approved by the Regional Ethics Committee, Copenhagen, Denmark (Protocol no. H-3-2013-033, approved March 26th 2013).

All participants underwent the same examinations in the order as listed below. The methods used in the study are described in details in manuscripts I, II, III and IV. In brief, the odontological part of the study included

- An interview based on standardised questions regarding the onset and degree of oral symptoms (including xerostomia, dysgeusia, oral pain or discomfort), comorbidity, current intake of medication, smoking habits and alcohol consumption, and oral and dental hygiene habits (paper I and III)
- The interview was followed by sialometry including measurements of unstimulated and paraffin chewing-stimulated whole saliva flow rates, citric acid stimulated parotid flow rates and collection of saliva samples for analysis (paper III)
- A clinical oral examination with registration of mucosal changes, dental status, periodontal status and also a detailed registration of the dental restorations with regard to materials used (paper I)
- Peripheral venous blood was collected for analyses of serum cytokines (IL-6, IL-10, IL-12p40, IL-12p70, TNF-α and INF-γ (paper IV)
- Finally, an incisional biopsy was taken under aseptic conditions from an affected area of the oral mucosa, mainly the buccal mucosa, and from normal buccal mucosa from the control subjects (paper I and II).

Dermatological examination

The dermatological examination and the allergy testing were carried out at the Department of Dermatology and Allergy, University Hospital Gentofte. A thorough dermatological examination was performed on all participants by the same experienced dermatologist (paper II).

Allergy testing

The patients and the healthy controls were patch tested with the European baseline series, supplementary standard series, declared perfume substances, dental materials and oral hygiene products; the latter two especially developed for this project. There are overlaps of some of the tested allergens in the different series (paper I, appendix).

Skin prick test to latex and chlorhexidine were performed and some patients were skin prick tested to pollen and appropriate food, if they had a history of reactions in relation to either. The allergens for the skin prick testing were provided by ALK Abello (paper I).

The patch testing was performed according to the recommendations of the European Society of Contact Dermatitis (ESCD) (Fig. 8 and Fig. 9). The method is standardised and the risk of actively sensitising the patients is extremely small (72).

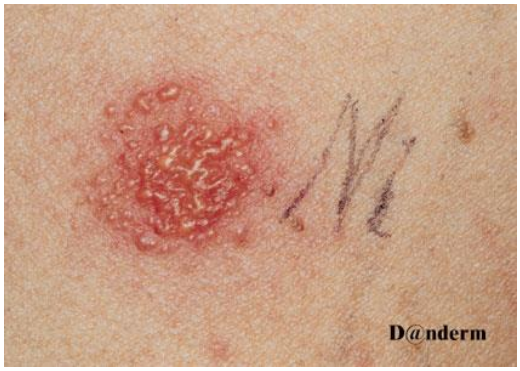


Figure 8. Positive patch test reaction to nickel

Picture printed with permission from Danderm/Niels Veien

Filaggrin genotyping and immunohistochemical analyses

Peripheral blood samples or cotton swabbing from the buccal mucosa were used in analysing genetic loss-of-function variants (R2447X, R501X, 2282del4) in the FLG. Details on the two methods can be seen in paper II.

Immunohistochemical analyses to detect filaggrin were performed on paraffin sections from the biopsy mentioned on page 31 using a rabbit polyclonal antibody to filaggrin (FLGpoly, Sigma #HPA030189) and a mouse monoclonal antibody to filaggrin (FLG01, Abcam #ab3137). Details on the two antibodies and the staining methods can be seen in paper III.

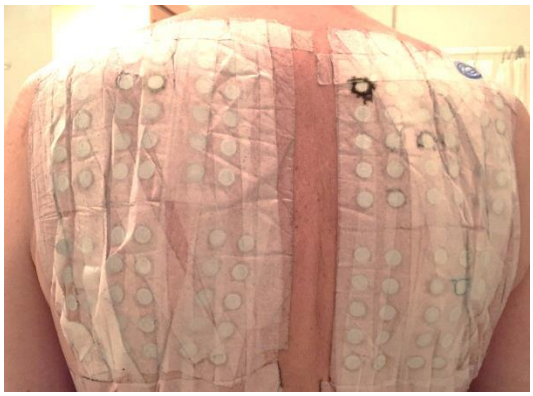


Figure 9. Male participant with patches on the top part of the back

Cytokine analyses

Analyses of the serum cytokines IL-6, IL-10, IL-12p40, IL-12p70, TNF-α and INF-γ were performed on peripheral blood samples. Elisa Duosets® R&D systems was used for detection of human TNF-α, INF-γ, IL-6, IL-10, IL-12p40 and Human IL-12p70 (for further details see paper IV).

Key results

The following section summarises the key results obtained in the study and presented in the four different papers.

Paper I

- The diagnosis of OLP and OLL was established in 38 patients.
- The diagnosis of stomatitis was made in 11 patients.
- The majority of patients reported more than one oral symptom. There was no difference in the symptoms reported by the patients with OLP, OLL and stomatitis.
- The patients with OLP, OLL and stomatitis visited their dentist significantly more often than the healthy control subjects (p=0.0005).
- In the healthy control group, the number of decayed-missed-filled-teeth (DMF-T) was 17% lower and the number of decayed-missing filled-surfaces (DMF-S) 50% lower than in the patient group (*p*=0.033 and *p*=0.010, respectively).
- The plaque index score was lower in the patient group than in the healthy control group, whereas the opposite was the case with regard to the gingival index score being higher in the patient group (*p*=0.01 and *p*=0.0001 respectively).
- Nineteen patients (38.8%) and 10 healthy controls (34.5%) displayed positive patch test reactions to tested allergens.
- The patients with positive patch test reactions did no differ with regard to symptoms, clinical and histopathological characteristics or relation to dental restorations.
- Of the 19 patients with a positive patch test reaction, 12 (63.2%) displayed more than one positive patch test reaction. In the healthy control group, 3 of the 10 (30%) subjects with a positive patch test reaction displayed more than one positive test.
- There was no statistically significant difference in the incidence of contact allergy towards materials used in dentistry, as a whole, between the patients and the healthy control subjects.
- Contact allergy to aroma substances was significantly more frequent in the patient group, especially the OLL group, than in the healthy control group (*p*=0.023).
- In 17 of the 31 (54.8%) patients with doubtful positive patch test reactions, the reactions were interpreted as relevant to their oral symptoms, whereas this was not the case in the healthy controls as they did not have oral symptoms.
- Positive patch test reactions to acrylates were only seen in 3 OLP/OLL patients and in none of the healthy controls.
- Doubtful positive patch test reactions to acrylates used in dentistry were very sparse and the difference between the patients and the healthy control group was not significant.
- None of the study participants had positive skin prick test reactions to chlorhexidine or latex.

Paper II

- The diagnosis of OLP was established in 19 patients and additionally 19 patients were diagnosed with OLL.
- Clinically the patients diagnosed with OLP were characterized by bilateral, reticular symmetrical lesions with or without erosive/ulcerative, atrophic or plaque-like areas. Histopathologically the patients diagnosed with OLP were characterized by a well-defined band-like zone of lymphocytes localized in the superficial part of the connective tissue and liquefaction degeneration and apoptosis in the basal cell layer. Patients with lesions that resembled OLP clinically and/or histopathologically but did not meet the above mentioned criteria were diagnosed with OLL.
- The diagnosis of stomatitis was made in 11 patients.
- Stinging/stabbing or burning sensations were the most common symptoms and the oral mucosal lesions were most often localized on the buccal mucosa and gingiva.
- Neither the number and severity of oral symptoms nor the extension of oral lesions differed between OLP patients with and without a concomitant defect in the FLG.
- There was no statistical significant difference between the patients and the healthy control subjects with regard to the presence of active dermatoses when excluding cutaneous lichen planus.
- There were no significant differences in the presence of FLG mutations between the patients with OLP/OLL and the healthy control subjects.
- The specimens from the oral mucosa of patients stained with the polyclonal antibody showed a positive, intense immunoreactivity for filaggrin in the hyperorthokeratinised layer of the epithelium, and a more scattered immunoreactivity for filaggrin in the areas of hyperparakeratinisation. The immunoreactivity was significantly more intense in the hyperkeratinised layer of the oral mucosa in the patients with OLP and OLL than in the oral mucosa of the healthy control subjects ($p=0.000025$).

Paper III

- Thirty-two patients and 8 healthy control subjects reported daily intake of medication, most often antihypertensives (18 and 4 persons respectively) (ATC code C03-C10).
- Two patients reported hypothyroidism and one reported hyperthyroidism.
- Patients reported xerostomia to a higher and a more severe degree than the healthy control subjects ($p=0.00006$), whereas there was no difference between patients with OLP/OLL with and without a concomitant contact allergy.

- There were no differences in either UWS or SWS flow rates between the patients and the healthy control subjects. The same holds true for patients with OLP/OLL with and without a concomitant contact allergy.
- The total salivary protein concentration in both UWS and SWS samples from patients were higher than in those from the healthy control subjects (p=0.016 for SWS). There was no difference in the total salivary protein concentration between patients with OLP/OLL with and without a concomitant contact allergy.
- The levels of salivary sIgA in both UWS and SWS samples from patients were higher than in samples from healthy control subjects (p=0.008 for SWS). There was no difference in the salivary sIgA levels between patients with OLP/OLL with and without a concomitant contact allergy.
- All patients but 2 had normal serum levels of TSH.

Paper IV

- Serum levels of IL-6 were higher in patients with OLP, OLL and stomatitis when compared to healthy controls.
- There were no differences between patients with symptomatic OLP and OLL with or without a concomitant contact allergy regarding serum levels of IL-6, IL-10, IL-12, TNF-α and INF-γ.

Discussion

The following section includes a discussion of the different aspects of the study presented in the four different papers.

Selection of the study participants and demographic considerations

One hundred and thirty-four consecutive patients referred to the Clinic for Oral Medicine, Department of Odontology, University of Copenhagen, in the period 2013-2015 for investigation of oral mucosal symptoms and lesions, were screened. Of these 52 patients (39%) were eligible for inclusion, and 49 (94.2%) completed the study. There was a large variety of reasons for exclusion of patients, but it was mainly due to non-Caucasian background, suspicion of medication-induced OLL, vitamin- and mineral deficiencies, aphthous stomatitis, oral candidiasis, oral allergy syndrome and pemphigoid. All patients fulfilling the inclusion criteria were asked to participate in the study. Three of them declined (2 females and 1 male). One hundred and twelve of the patients screened for inclusion, were women. Consequently, the female: male ratio being 6:1 was also higher than anticipated in the included patient group. The most commonly reported female: male ratio in OLP is 2:1 (32). The higher female: male ratio in this study may reflect the fact that women seek healthcare for their symptoms and health issues more often than men but it also reflects the fact that gender hormones may play a role in the development and exacerbation of various diseases, including autoimmune diseases and various allergies (89). Although the literature in the area is sparse, the typical age of onset of OLP may also indicate that gender hormones is involved in the pathogenesis via endocrinological changes affecting the oral mucosa and the immunological response hence making the mucosa more susceptible to oral diseases like OLP and allergic reactions (90).

Twenty-nine age- and gender-matched healthy control subjects were also included in this study. The exclusion criteria for this group were current or past medical history of systemic disease, oral mucosal diseases or symptoms, and intake of medication. As it proved difficult to recruit healthy non-medicated control subjects at the age above 60, 4 persons taking antihypertensives on a daily basis were matched to the patients with regard to gender, age and type of medication. These control subjects were otherwise healthy.

The diagnostic process

Caucasian patients with oral symptoms and mucosal changes that were compatible with OLP, OLL or stomatitis could be included in the study. The diagnosis of OLP was established in 19 patients based on a clinicopathological evaluation based on the criteria established by WHO and modified by van der Meij and van der Waal (35, 36). The diagnosis of OLL was established in 19 patients when all of the diagnostic criteria could not be met, i.e. lesions that clinically resembled OLP but

did not meet the histopathological criteria. The criteria used for distinction between OLP and OLL are still a matter of debate and need further validation. Along this line, a set of revised criteria have recently been proposed, comprising additional oral conditions suggested to be excluded in the diagnosis of OLP (91). In the present study, we were not able to identify additional characteristics that would be helpful in the clinical practice and diagnostic process. Moreover, patients with stomatitis did not differ from the patients with OLP and OLL with regard to systemic diseases, medication used, smoking habits and alcohol consumption. It would be obvious to include newer technologies including genomics, metagenomics and proteomics in future studies in the search for factors that can work as discriminatory factors and biomarkers in the future diagnostics as well as in the understanding of the pathogenesis of OLP and OLL.

Dental status

The patients with symptomatic OLP, OLL and stomatitis had higher DMF-T score, DMF -S score and gingival index score, but a lower plaque index score than the healthy controls. The patients visited their dentist significantly more often than did the healthy controls showing that the patients have increased need of regular dental follow-up due to their oral mucosal conditions including problems with gingival inflammation despite good oral hygiene. The patients have an increased need of dental treatment as well. In this respect, they are more exposed to substances in oral hygiene products and dental materials which could lead to sensitisation and development of contact allergies.

Symptoms and oral mucosal lesions

The majority of patients (83.7%) reported stinging, stabbing and/or burning sensation in the oral mucosa and often more than one symptom, which is in line with observations from previous studies (32, 33). There was no difference in the type and severity of symptoms reported by the patients with OLP, OLL or stomatitis. The erosive/ulcerative type of OLP was the most common observed in 78.9% of the patients at the time of the examination. It is well established that the erosive/ulcerative OLP lesions are associated with oral symptoms (32). The lesions were most often localised to the buccal mucosa (77.6%), but 69.4% of the patients also had gingival lesions. There were no associations between the symptoms, the extent of the mucosal lesions and the presence of contact allergy. Accordingly, concomitant allergy does not seem to aggravate the condition. However, in the patients with a positive or a doubtful positive reaction to aroma substances, avoidance of exposure led to disappearance of their oral symptoms within 3 months without further treatment and the improvement persisted for a minimum of 1 year (own unpublished data).

The role of contact allergy

In the present study, the incidences of contact allergy in patients with symptomatic OLP, OLL and stomatitis and in healthy persons with no prior history of allergy were almost identical. A review of data from 1966 to 2007 reports an estimated prevalence of contact allergy to at least one allergen

of 21.2% (range 12.5-40.6%) (71), which are in line with findings in this study. Furthermore, patients with OLP and OLL had a larger number of contact allergies to aroma substances in oral hygiene products than the healthy control subjects. This may be explained by thorough oral hygiene procedures and hence an enhanced exposure to aroma substances as well as a possibly disturbed mucosal barrier and inflammation. The most common aroma substances to which contact allergy were found were spearmint and carvone. Previous studies on adverse reactions to materials used in dentistry have focused on either dental materials or aroma substances. Studies focusing on the metals used in dentistry all found higher numbers of positive patch test reactions in their patients than was the case in the present study (88, 92, 93). This is most likely due to the fact that patients with an already suspected contact allergy were included. They did find contact allergy to metals used in dentistry more prevalent in women than in men, supporting the evidence that gender hormones might be involved in the process as well (90, 93, 95). Previous studies focusing on the oral hygiene products also found higher number of positive patch test reactions in their patients than we did in this study (4, 96, 97). However, again different inclusion criteria were used and patients with an already suspected contact allergy were included. Ahlgren et al. investigated the relationship between OLL and contact allergy to both dental materials and oral hygiene products. They found more positive patch test reactions to carvone and to mercury in patients diagnosed with OLL than in patients with dermatitis (16).

The rate of positive patch test varies tremendously from one study to the other. In general, the rate is often very high in study populations already suspected to have contact allergy to dental materials and oral hygiene products.

The role of filaggrin

In this study, there were no differences in defects in the FLG between patients with symptomatic OLP, OLL and stomatitis and healthy controls, which can be ascribed to the limited number of participants included. We did not analyse for the S3247X filaggrin defect though, which comprises 10% of the known filaggrin mutations in patients with Caucasian ancestry (81). Neither the number and severity of oral symptoms nor the extension of oral lesions differed between patients with OLP or OLL with and without a concomitant defect in the FLG.

The immunohistochemical staining with the polyclonal antibody showed a statistically significant altered distribution of filaggrin in the oral mucosa of patients with symptomatic OLP, OLL and stomatitis compared to that of healthy controls. The distribution was more coherent in patients with OLP, OLL and stomatitis when compared to healthy control subjects. This can possibly be explained by the fact that most lesions were hyperkeratinised whereas control biopsies originated from unkeratinised, normal oral mucosa. A similar change in the filaggrin expression has been shown in patients with leukoplakia and in patients with OLP (98, 99). This alteration in the filaggrin distribution was not associated with defects in the gene encoding filaggrin.

The role of saliva

Xerostomia was reported by significantly more patients than healthy controls in this study. 46.9% of the patients reported daily and extensive xerostomia affecting oral functions. The high prevalence of xerostomia can be ascribed to a high number of women in the study and an average age of 61 years. As the intake of certain medications is associated with xerostomia and reports of xerostomia increases with the number of medication taken on a daily basis, medication-induced xerostomia cannot be ruled out in this study (27, 28, 100, 101). Even though most of our healthy control subjects did not have a daily intake of medications some of them reported xerostomia. However, the sensation of dry mouth was moderate and related to snoring (mouth breathing). Previous studies on xerostomia in OLP have shown that OLP and OLL are associated with an increased prevalence of xerostomia (25, 100, 102, 103). Xerostomia is often associated with salivary gland hypofunction. However, in this study, the presence of xerostomia was not associated with low saliva flow rates. Moreover, the UWS, SWS and stimulated parotid saliva flow rates did not differ between the patients and the healthy controls.

The concentration of total protein was higher in both the UWS and SWS samples from patients than in the healthy control subjects and in the SWS samples this difference was significant. The difference in the salivary protein concentration was independent of the presence of contact allergy.

The levels of salivary sIgA were higher in both UWS and SWS samples from patients than in those from healthy control subjects and in the SWS samples this difference was significant. These findings are in concordance with previous observations of Ghaleyani et al. and Lopez-Jornet et al. (45, 46), but not with those of Gandara et al. (104).

When to allergy test, what to test and what to do in case of contact allergy

Allergy testing patients is an elaborate task that requires many resources, both human and financial. The findings of this study suggest that symptoms, proximity to a dental restoration, xerostomia and histopathological evaluation cannot be used to determine if a patient should be referred for allergy testing or not. In some cases the patient history can contribute to determine if allergy testing is indicated, i.e. temporal correlation between the making of a dental restoration and the onset of oral symptoms.

It may be challenging to identify the specific components of a dental material or an oral hygiene product. In dental materials a material safety data sheet (MSDS) containing details on the composition of the dental material must be provided by the manufacturer. A large Danish study showed that 18.6% (137/738) of MSDSs were inadequate of which 63.1% concerned the R43/H317 section which relates to materials ability to cause sensitisation by skin contact (105). These shortcomings might lead to inadequate allergy testing. Oral hygiene products are, as mentioned earlier, subject to shared EU-legislation via the cosmetic directive. This directive states for example

that a list of ingredients has to be present and that this list contains all the ingredients/ components, arranged in descending order of weight at the time they are added to the product. Perfume and aromatic components are indicated as "perfume" or "aroma". However, it is not required by law to disclose exactly the ingredients of the perfume or the aroma substances, which is used (64). If additional information is to be obtained it requires benevolence from the manufactures.

If allergy testing reveals a contact allergy to a substance in an oral hygiene product, the patient should be guided to avoid exposure prospectively. Toothpaste and mouthwash should be free from aroma substances. However, the effect of this has not yet been established by any studies, but in our study population avoidance of exposure to aroma substances led to disappearance of the oral symptoms without further treatment and the improvement persisted. If a contact allergy towards one or more components in a dental material is present the approach is more complicated. It is expensive and associated with a risk of dental complications, i.e. pulp infections and tooth loss replacing a specific material. However several studies have shown that replacing for example amalgam fillings after having a positive patch test reaction to mercury will result in an improvement or occasional complete remission of the oral mucosal lesions (94, 106, 107). In each case of contact allergy towards a dental material the relevance of the contact allergy and the implications of removing the material have to be evaluated thoroughly.

Strengths and limitations of the present study
The primary strength of this study is that the patient group is not selected on the basis of an already suspected allergy as they were referred to the Clinic for Oral Medicine for examination of oral symptoms and lesions. If contact allergy is already suspected, a higher incidence of positive patch test reactions can be expected. Therefore our study probably reflects the incidence of contact allergy in patients with symptomatic OLP, OLL and stomatitis more accurately. An additional strength is that the participants included are very well characterised.

The limitation of our study is the number of patients and healthy controls included.
As many MSDSs and product labels are inadequate regarding the exact composition of a given material, it may be difficult to determine exactly what potential allergens the patient has been exposed to. Consequently, the patch testing may be insufficient as relevant allergens may be missed leading to failure in diagnosing potential contact allergies, and allergen exposure of the patient continues. However, this is a challenge that other studies in this research field must face and need to take into consideration.

Conclusion

The patients included in this study population were primarily women at the age of 60 and above. The patients had higher DMF-t and DMF-s values and they visited there dentist significantly more often than the healthy controls. The oral lesions were all associated with a variety of oral symptoms, primarily stinging, stabbing and/or burning sensations, which may be ascribed to the presence of erosive/ulcerative lesions at the time of the examination in the majority of patients. Xerostomia including severe xerostomia was also a common symptom in the patients with OLP, OLL and stomatitis. In this study, xerostomia was not associated with the presence of systemic diseases, intake of medication or low saliva flow rates. In fact, there were no differences in unstimulated and stimulated whole saliva and stimulated parotid saliva flow rates between patients and healthy controls. It is likely that the inflammatory changes in the oral mucosa lead to sensory disturbances including sensation of oral dryness as well as dysgeusia, which also was prevalently reported. Overall, the levels of selected serum cytokines were low and some could not be detected. Only serum IL-6 was higher in the patient group than in the healthy control group, and there was no difference in the cytokine profile between groups. Apparently the high number of detected contact allergies did not influence the cytokine profile. Furthermore, there were no differences between the patient groups and the patients and the healthy controls with regard mutations in the gene encoding filaggrin. On the other hand, the immunohistochemical analysis revealed aberrations in the distribution of filaggrin in the oral mucosa of patients, which can be explained by the hyperkeratotic nature of the lesions in OLP and OLL. Finally, the findings of this study do not provide evidence for contact allergy being more prevalent in patients with symptomatic OLP, OLL and stomatitis than in healthy controls, except for reactions to substances in oral hygiene products. Accordingly, this study does not support routine allergy testing in these patients, but the need must be evaluated thoroughly in each case. The various clinical, histopathological and immunological characteristics tested in this study were inadequate to specifically discriminate between patients with OLP, OLL and stomatitis with or without a concomitant contact allergy.

Perspectives

The findings presented in this thesis indicate the need for a future study including a larger number of patients and control subjects. It could be an interdisciplinary multicentre study focusing on the incidence of contact allergies and the potential role of filaggrin gene mutations. As the incidence of contact allergic reactions to acrylates is an increasing problem due to an increasing use of acrylate-containing beauty products and artificial nails, it is anticipated that the problem with allergic reactions in the oral mucosa due to acrylate-containing composite fillings, will increase as well. As the consumers of beauty products and artificial nails are young people, they may potentially face a lifelong problem when undergoing dental treatment. For this reason it would be

interesting to study the incidence of oral mucosal changes in patients already allergic to acrylates due to artificial nails and nail polish, as it can be expected that the incidence of contact allergic reactions in the oral mucosa will rise as a secondary effect. Furthermore, in-depth studies aiming at identifying immunological markers to discriminate between OLP and OLL and understanding the underlying pathogenic mechanisms are also warranted. Accordingly this study will be followed up by studies using genomic, metagenomics and proteomics in the approach to identify more specific disease markers.

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