Maria Siponen

ORAL LICHEN PLANUS – ETIOPATHOGENESIS AND MANAGEMENT
Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in the Leena Palotie auditorium (101A) of the Faculty of Medicine (Aapistie 5 A), on 28 January 2017, at 12 noon.
Oral lichen planus (OLP) is a chronic immune-mediated mucosal disease with unknown etiology. According to the current view, the pathogenesis of OLP involves activation of T-cell mediated immunity against the epithelial keratinocytes. A proportion of OLP patients are affected by painful symptoms, and the risk of oral cancer is increased in OLP. There is no curative treatment for OLP. Topical corticosteroids are used most commonly in the management of OLP. However, the evidence base for the effectiveness of any therapy is weak.

The objective of this thesis was to study novel aspects of OLP etiopathogenesis and management. An epidemiologic, retrospective case-control study was conducted to determine whether systemic diseases, in particular thyroid diseases, are associated with OLP. In addition, a randomized controlled trial comparing the effectiveness of topical tacrolimus, triamcinolone acetonide and placebo in symptomatic OLP was carried out. Furthermore, immunohistochemical expression of toll-like receptors 4 and 9, hyaluronan and its principal receptor CD44 antigen, hyaluronan synthases 1-3, hyaluronidases 1-2 and cathepsin K was studied in OLP tissue samples and in healthy oral mucosa. The effect of topical tacrolimus on the expression of these molecules in OLP was also studied.

The results of the present study showed that a history of hypothyroidism was associated with an approximately twofold risk of having OLP. Furthermore, both tacrolimus and triamcinolone acetonide were more effective than placebo in reducing the signs and symptoms of OLP. No statistically significant differences were noted in the efficacy between tacrolimus and triamcinolone acetonide. In addition, the expression of the studied molecules was altered in the epithelium or stroma in OLP compared to healthy oral mucosa. Tacrolimus treatment decreased the expression of CD44 antigen in the stroma and the expression of cathepsin K in the epithelium in OLP.

In conclusion, the present study extends our knowledge about systemic associated factors and management of OLP. In addition, the results improve our understanding of molecular level changes that occur in OLP.

**Keywords:** case-control study, cathepsin K, CD44 antigen, hyaluronan, hyaluronan synthase 1, hyaluronan synthase 2, hyaluronan synthase 3, hyaluronidase 1, hyaluronidase 2, hypothyroidism, immunohistochemistry, oral lichen planus, placebo, randomized controlled trial, tacrolimus, thyroid disease, toll-like receptor 4, toll-like receptor 9, triamcinolone acetonide


Yhteenvetona voidaan todeta, että tämä tutkimus lisää tietoa suun punajäkälään liittyvistä systeemistä tekijöistä ja suun punajäkälän hoidosta. Lisäksi löydökset lisäävät ymmärtämystä suun punajäkälässä tapahtuvista molekyylitason muutoksista.

Keskeiset asiasanat: CD44-antigeeni, hyaluronaani, hyaluronaanisyntaasi 1, hyaluronaanisyntaasi 2, hyaluronaanisyntaasi 3, hyaluronidaasi 1, hyaluronidaasi 2, immunohistolokemia, katepsiini K, kilpirauhasen vajaatoiminta, kilpirauhasnaira, lumelääke, satunnaisetut kontrolloitu tutkimus, suun punajäkälä, takrolimuus, tapaus-verrokkitutkimus, tollin kaltaisen reseptori 4, tollin kaltaisen reseptori 9, triamsinoliasetoniti
To my parents
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The work leading to this thesis began in Oulu in 2002 when a novel drug, tacrolimus, had shown promise in the management of oral lichen planus. Randomized controlled trials of topical tacrolimus in oral lichen planus were lacking, and therefore we designed and initiated one. The following process revealed the many different aspects of clinical trials. I would like to thank warmly the patients as well as many colleagues, dental nurses, nurses, pharmacists, laboratory technicians and secretaries at the University of Oulu, Oulu University Hospital and Kuopio University Hospital for their co-operation and effort during the clinical trial.

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Most of all, I am extremely grateful for the support of my dear father, who despite his longstanding grave illness has taken an interest in my wellbeing and supported and encouraged me in my work. Dad, your sophistication and sense of humor inspires me.

Kuopio, November 2016

Maria Siponen
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>atopic dermatitis</td>
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<tr>
<td>AITD</td>
<td>autoimmune thyroid disease</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
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<td>BMZ</td>
<td>basement membrane zone</td>
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<tr>
<td>CTSK</td>
<td>cathepsin K</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C motif chemokine 5 (RANTES)</td>
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<td>CCR1</td>
<td>C-C chemokine receptor type 1</td>
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<tr>
<td>CD1A</td>
<td>T-cell surface glycoprotein CD1a, Langerhans cell marker</td>
</tr>
<tr>
<td>CD4</td>
<td>T-cell surface glycoprotein CD4, helper T cell marker</td>
</tr>
<tr>
<td>CD8A</td>
<td>T-cell surface glycoprotein CD8A alpha chain, cytotoxic T cell marker</td>
</tr>
<tr>
<td>CD44</td>
<td>CD44 antigen, hyaluronan receptor</td>
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<tr>
<td>CD68</td>
<td>macrophilin, macrophage marker</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
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<td>CS</td>
<td>clinical score</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DEFB4A</td>
<td>defensin beta 4A (beta defensin 2)</td>
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<td>e.g.</td>
<td>exempli gratia</td>
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<td>et al</td>
<td>et alia</td>
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<tr>
<td>FFPE</td>
<td>formalin fixed paraffin embedded</td>
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<tr>
<td>HA</td>
<td>hyaluronan</td>
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<td>HAS</td>
<td>hyaluronan synthase</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen, HLA histocompatibility antigen (major histocompatibility complex, MHC)</td>
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<td>HMW</td>
<td>high molecular weight</td>
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<td>HSP</td>
<td>heat shock protein</td>
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<tr>
<td>HYAL</td>
<td>hyaluronidase</td>
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<td>ICC</td>
<td>intracell correlation</td>
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<td>IF</td>
<td>immunofluorescence</td>
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<td>IFNG</td>
<td>interferon gamma</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LP</td>
<td>lichen planus</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Melan-A</td>
<td>melanoma antigen recognized by T-cells 1, melanocyte marker</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>OLP</td>
<td>oral lichen planus</td>
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<td>OLL</td>
<td>oral lichenoid lesion</td>
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<td>OLR</td>
<td>oral lichenoid reaction</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PTPRC</td>
<td>receptor-type tyrosine-protein phosphatase C (CD45 antigen), lymphocyte marker</td>
</tr>
<tr>
<td>RCT</td>
<td>randomized controlled trial</td>
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<tr>
<td>Tg, TG</td>
<td>thyroglobulin</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TPO</td>
<td>thyroid peroxidase</td>
</tr>
<tr>
<td>TPSAB1</td>
<td>tryptase alpha/beta-1 (mast cell tryptase)</td>
</tr>
<tr>
<td>TSHR</td>
<td>thyroid stimulating hormone receptor, thyrotropin receptor</td>
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<tr>
<td>UWS</td>
<td>unstimulated whole saliva</td>
</tr>
<tr>
<td>VAS</td>
<td>visual analogue scale</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


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Introduction

Oral lichen planus (OLP) is an enigmatic disease that has puzzled countless researchers, clinicians and patients alike. While extensive research has been conducted regarding the pathogenetic mechanisms involved, we still do not know the triggering factor(s) of OLP nor much about associated systemic factors (Kurago 2016). The management of symptoms of severe OLP often poses challenges for clinicians, and may leave patients without adequate symptomatic relief thereby lowering their quality of life. Hence, it is clear that more research on the etiopathogenetic mechanisms underlying OLP is needed, with the aim of developing better treatment options.

Several systemic conditions, like hepatitis C and diabetes, and autoimmune diseases such as Sjögren’s syndrome are suggested to be associated with OLP (Likar-Manookin et al. 2013, Lodi et al. 2005, Scully et al. 1998). With the exception of hepatitis C in certain populations, conclusive evidence for these associations is lacking (Eisen 2002, Lodi et al. 2010, Lopez-Jornet et al. 2014). Based on our clinical experience, we postulated that the prevalence of thyroid diseases is increased in OLP patients. To study the possible associated systemic conditions, especially thyroid diseases in OLP, we planned an epidemiologic case-control study.

Symptomatic OLP is typically treated with topical corticosteroids. Since the late 1990s, reports have been published on the use of topical tacrolimus in the management of OLP, mainly in cases not responding to topical corticosteroids (Kaliakatsou et al. 2002, Lener et al. 2001, Morrison et al. 2002, Olivier et al. 2002, Rozycki et al. 2002, Vente et al. 1999). Most of the previous studies of topical tacrolimus in OLP have been open-label, non-randomized or non-controlled. In general, there is only weak evidence for the effectiveness of any specific treatment in OLP (Thongprasom et al. 2011, Thongprasom et al. 2013). Therefore, we designed a randomized controlled trial (RCT) to study the efficacy and side-effects of topical tacrolimus in the management of OLP.

To increase understanding and gain new insights into the molecular factors in OLP pathogenesis, we studied the immunohistochemical expression of certain biomarkers associated with innate immunity, hyaluronan metabolism and protein degradation in archival OLP tissue samples. In addition, the effect of tacrolimus on the immunohistochemical expression of these molecules was investigated in the tissue samples collected in the clinical trial.
1 Review of the literature

1.1 Oral lichen planus

1.1.1 Definition

Lichen planus (LP) is a chronic inflammatory mucocutaneous disease of unknown etiology affecting mainly middle-aged women (Bolognia et al. 2012). The typical oral manifestation of LP is the presence of symmetrical white reticular lesions in the buccal mucosa, although diverse clinical features may be seen. The name lichen means a slow-growing symbiotic organism (of a fungus or fungi and algae or cyanobacteria) which “typically forms a low crust-like, leaf-like, or branching growth on rocks, walls, and trees” (Oxford Dictionaries 2016, Spribille et al. 2016). It derives from Greek leikhēn meaning tree-moss, originally “what eats around itself”, probably from leichein “to lick”, and Latin planus meaning flat, level (Harper 2001–2016, Stedman’s 2006).

LP was initially described by the Austrian dermatologist Ferdinand von Hebra who called the disease “lichen ruber” (Wilson 1866). The first clinical description of LP was reported in 1866 by an English surgeon and dermatologist Erasmus Wilson, who depicted in 4 patients a papular skin eruption that he called “lichen planus” (Wilson 1866). In the same report he also described a 56-year old woman with lesions in the oral mucosa. Later on, Dr. Wilson published a series of 50 patients with LP; three of these patients also had lesions in the mouth: white spots on the tongue, the buccal membrane and the mucous lining of the fauces (Wilson 1869). It was not until 1906 that the microscopic features of OLP were first described by Dubreuilh (Dubreuilh 1906).

Cutaneous lichen planus manifests as small violaceous, flat-topped, polygonal papules, with a shiny or transparent surface and white or grey-white lines (Wickham’s striae) or spots (Bolognia et al. 2012, Sachdeva et al. 2011). The cutaneous lesions most frequently exist on the flexor surfaces of forearms, wrists, dorsal surfaces of hands, anterior side of lower legs, neck and presacral skin (Bolognia et al. 2012). Genital LP occurs in about 50% women and 25% of men with cutaneous LP (Bolognia et al. 2012). Rarely, scalp, nails, esophagus, larynx or conjunctiva may be affected (Eisen 1999, Gorouhi et al. 2014).

LP may appear only on the skin or on the oral mucosa but frequently both lesions co-exist. Approximately 15% of OLP patients have cutaneous, and 20%
genital lesions, although in men the genital lesions are less prevalent (Eisen 1999). On the other hand, oral lesions may occur in 70–77% of cutaneous LP patients (Alrashdan et al. 2016). A form of LP with vulvovaginal and gingival involvement has been termed vulvovaginal-gingival syndrome (Eisen 1994, Pelisse 1989). Similarly, peno-gingival LP in men has been described (Petruzzii et al. 2005, Rogers & Eisen 2003).

### 1.1.2 Epidemiology

About 0.22–1% of the population worldwide is affected by cutaneous LP (Bolognia et al. 2012, Boyd & Neldner 1991). The prevalence of OLP is estimated to be about 0.2–2% (Regezi et al. 2017). Sixty-one to seventy-seven percent of OLP patients are women (Bermejo-Fenoll et al. 2010, Carbone et al. 2009, Eisen 2002, Ingafou et al. 2006, Thorn et al. 1988). The typical age at presentation of OLP is 30–60 years but the disease may start at any age (Regezi et al. 2017). Males may have a younger age at onset of OLP (Ingafou et al. 2006).

### 1.1.3 Clinical features

Clinical manifestations of OLP are variable. Several clinical types, namely papular, reticular, atrophic, plaque-like, erosive and bullous are recognized (Andreasen 1968, Pindborg et al. 1997, Regezi et al. 2017). The various clinical types of OLP may be classified into two major groups: reticular and atrophic-erosive with or without reticular lesions (Bagan-Sebastian et al. 1992, Neville et al. 2016). Often, more than one clinical form manifests on the oral mucosa. OLP lesions are located bilaterally, most often on the buccal mucosa, followed by the tongue and gingiva (Carbone et al. 2009, Eisen et al. 2005).

The most common clinical form of OLP is reticular, which exhibits a lace-like network of white striae, hence the term reticular (Figure 1a). The papular form of OLP presents as small white papules (Figure 1b) and is quite rarely seen, which may reflect the fact that it occurs mainly in the initial phase of the disease (Thorn et al. 1988). Atrophic OLP displays an erythematous appearance of the oral mucosa, often in combination with white lesions (Figure 1d). The atrophic form of OLP affecting the gingivae may be called desquamative gingivitis (Figure 1c). Plaque-like OLP lesions typically affect the dorsal tongue, and may clinically mimic leukoplakia (Figure 1e). It has been reported that the plaque-like OLP is more common in smokers than in non-smokers (Thorn et al. 1988). Erosive OLP
manifests as ulcerative lesions, most commonly in the buccal mucosa (Figure 1f). The rarest clinical form of OLP is the bullous form, with frank blisters (Cheng et al. 2016). Bullae are typically located in the buccal mucosa.

The irritation of oral mucosa from sharp tooth edges, dentures, calculus etc. may trigger OLP lesions, similarly to the isomorphic reaction to trauma in several skin diseases called the Koebner phenomenon (Sagi & Trau 2011).

The majority of patients experience pain and tenderness associated with oral lesions, especially in reaction to spicy, sour or hot food or drink (Carbone et al. 2009, Eisen 2002). Oral hygiene products may cause a burning sensation and especially gingival lesions may impair oral hygiene practices. The oral mucosa may feel rough or rigid. The signs and symptoms of OLP show natural fluctuation with periods of exacerbations and quiescence, but typically persist for several years or decades, in contrast to the self-limiting cutaneous LP (Carbone et al. 2009, Gorouhi et al. 2014). The quality of life in patients with OLP is negatively affected by the disease (Lopez-Jornet & Camacho-Alonso 2010b).
Fig. 1. Clinical forms of oral lichen planus: a) reticular, b) papular, c) atrophic, d) combination of reticular and atrophic forms, e) plaque-like and f) erosive-ulcerative.
1.1.4 Histopathological features

Characteristic, although not specific, histopathological features of OLP include epithelial hyperorthokeratosis or parakeratosis, epithelial atrophy or acanthosis, basal cell layer liquefaction (hydropic) degeneration, basal keratinocyte apoptosis, a subepithelial eosinophilic amorphous band and a dense, band-like lymphocytic inflammatory infiltrate in the superficial lamina propria that migrates to the lower part of epithelium (Kramer et al. 1978, Neville et al. 2016, Pindborg et al. 1997). Epithelial rete ridges are often missing or they may exhibit a “saw-tooth” pattern, but this is more common in cutaneous LP (Cheng et al. 2016, Fernandez-Gonzalez et al. 2011).

Intercellular spaces are widened in basal and spinous epithelial layers (Paul & Shetty 2013, Shklar et al. 1978, Tyldesley & Appleton 1973). Ultrastructural studies have demonstrated basal cells with cytoplasmic processes, which may be longer and narrower in the erosive form of OLP compared to the non-erosive form (Paul & Shetty 2013). Basal cells may also demonstrate acantholytic changes (Shklar et al. 1978), cytoplasmic vacuolization (Paul & Shetty 2013, Tyldesley & Appleton 1973), decrease or loss of desmosomal junctions, nuclear inclusions, and lymphocyte entrapment (Paul & Shetty 2013). The basal and spinous epithelial cells demonstrate thickening of tonofilaments (Hashimoto et al. 1966, Paul & Shetty 2013, Pullon 1969). Reactive epithelial atypia is sometimes seen in OLP, especially when superimposed candidiasis is present or when the biopsy is taken adjacent to an existing ulceration.

The degree of liquefaction degeneration varies from slight to intense and is not directly proportional to the number of apoptotic keratinocytes (Bascones-Ilundain et al. 2007). The apoptotic basal keratinocytes manifest as homogenous eosinophilic globules and are called colloid (hyaline, cytoid, Civatte) bodies (Burgdorf & Plewig 2014).

The basement membrane zone (BMZ) may appear thickened and branching and disruptions in its continuity are seen (Hashimoto et al. 1966, Jungell et al. 1989, Paul & Shetty 2013, Pullon 1969). Disruptions of the anchoring structure of basal keratinocytes including hemidesmosomes, filaments and fibrils have also been demonstrated (Haapalainen et al. 1995). The basement membrane (BM) may show separation from the overlying basal cells (Shklar et al. 1978). The structural changes in the basal cell layer and BMZ make the epithelial-connective tissue interface fragile and may occasionally result in cleft formation (Max-Joseph spaces) that can clinically manifest as blisters (Sugerman et al. 2002).
The characteristic lymphocytic infiltrate in OLP is present immediately subepithelially as a band-like zone. Intraepithelial lymphocytes are detected between basal and spinous epithelial cells (Paul & Shetty 2013). The inflammatory cell infiltrate in OLP consists mainly of T-lymphocytes (Walker 1976). Most T-cells in the epithelium and in the disrupted BM are activated cytotoxic T-cell surface glycoprotein CD8 alpha chain (CD8A)+ lymphocytes, while most T-cells in the lamina propria are helper T-cell surface glycoprotein CD4 (CD4)+ lymphocytes (Hirota et al. 1990, Sugerman et al. 2002). However, Khan et al. (2003) noted that most subepithelial lymphocytes were CD8A+, and CD4+ lymphocytes were located mainly in deeper lamina propria. Although B-cells are not considered to have a major role in OLP pathogenesis, they are present in the inflammatory infiltrate and may, in a proportion of cases be a dominant lymphocyte type (Mattila et al. 2011). Plasma cells are typically not seen in any considerable numbers in OLP inflammatory cell infiltrate, but may be present especially in biopsies from the gingiva where nonspecific chronic inflammation is often present (Cheng et al. 2016). If plasma cells are seen in the infiltrate, a diagnosis of oral lichenoid lesion (OLL) is usually favored (Mravak-Stipetic et al. 2014).

Besides lymphocytes, other leukocytes present in OLP lesions include macrophages, mast cells and dendritic cells (Langerhans cells, LC) (Payeras et al. 2013). Macrophages are seen in close proximity to the basal cell layer in both epithelium and lamina propria (Matthews et al. 1985, Paul & Shetty 2013). Increased numbers of mast cells have been detected in OLP and most of them are degranulated (Jontell et al. 1986, Sharma et al. 2011, Zhao et al. 1997, Zhao et al. 2001). Jontell et al. (1986) observed that most mast cells were located below the subepithelial infiltrate while Zhao et al. (1997) found the greatest density of mast cells in the superficial part of the subepithelial inflammatory infiltrate in OLP. Degranulated mast cells were seen in the basal cell layer and juxtaepithelially by electron microscopy (Paul & Shetty 2013). Langerhans cell density is increased both in the epithelium and stroma in OLP compared to normal oral mucosa (Gueiros et al. 2012, Gustafson et al. 2007, Hasseus et al. 2001, Santoro et al. 2005, Villarroel Dorrego et al. 2002).

Direct immunofluorescence (IF) staining of lesional tissue shows a characteristic linear or shaggy fibrinogen/fibrin deposition in the BMZ in almost all OLP cases, however, this finding is not specific (Buajeeb et al. 2015, Laskaris et al. 1982). Less often, colloid bodies and vessel walls show fibrin deposition (Laskaris et al. 1982). Colloid bodies may also stain positive for immunoglobulin M (IgM), immunoglobulin A (IgA) and complement 3 (C3) (Buajeeb et al. 2015).
Rarely, immunoglobulin G (IgG), IgM, IgA or C3 deposition may be found in the BMZ (Hashimoto et al. 2015, Laskaris et al. 1982, Schiodt et al. 1981).

1.1.5 Diagnosis

The diagnosis of OLP can be made based on the typical clinical features, but may require a histopathologic correlation (Neville et al. 2016). Occasionally, IF studies are needed to rule out other immune-mediated mucosal diseases. Some authorities recommend that both clinical and histopathologic criteria should be fulfilled for the diagnosis of OLP (Cheng et al. 2016). Originally, the World Health Organization (WHO) defined the clinical and histologic diagnostic features of OLP (Kramer et al. 1978, Pindborg et al. 1997). In 2003, a paper proposed a modified set of diagnostic criteria for OLP (van der Meij & van der Waal 2003). Recently, in an American Academy of Oral and Maxillofacial Pathology (AAOMP) opinion paper further amendments were suggested to the diagnostic criteria for OLP (Cheng et al. 2016) (Table 1).

The clinical and histopathologic features of OLP are variable and depend on the site and type of OLP, as well as disease activity at the time of diagnostic assessment (Cheng et al. 2016). In addition, other concurrent oral inflammatory processes may alter the typical features of OLP. Furthermore, many clinical and histopathological features of OLP are shared by a variety of other oral mucosal diseases. It is therefore no wonder that inter- and intraobserver variability has been demonstrated in the clinical and histopathologic assessment of OLP (van der Meij et al. 1999, van der Meij et al. 2002, van der Meij & van der Waal 2003).

Hence, the accurate diagnosis of OLP can be challenging (Cheng et al. 2016). The lack of a clear-cut and reproducible diagnosis is also a factor that may affect the value of studies on OLP.
Table 1. AAOMP position paper diagnostic criteria for OLP (Cheng et al. 2016).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Features</th>
<th>Exclusion criteria</th>
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<tbody>
<tr>
<td>Clinical</td>
<td>Multifocal symmetric distribution</td>
<td>Lesions are not localized exclusively to the site of smokeless tobacco placement</td>
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<tr>
<td></td>
<td>White and red lesions exhibiting one or more of the following forms:</td>
<td>Lesions are not localized exclusively adjacent to and in contact with dental restorations</td>
</tr>
<tr>
<td></td>
<td>Reticular/papular</td>
<td>Lesion onset does not correlate with the start of a medication</td>
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<td></td>
<td>Atrophic</td>
<td>Lesion onset does not correlate with the use of cinnamon-containing products</td>
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<td></td>
<td>Erosive (ulcerative)</td>
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<td></td>
<td>Plaque</td>
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<td></td>
<td>Bullous</td>
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<tr>
<td>Histopathologic</td>
<td>Band-like or patchy, predominantly lymphocytic infiltrate in the lamina propria confined to the epithelium-lamina propria interface</td>
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<td></td>
<td>Basal cell liquefactive (hydropic) degeneration</td>
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<td></td>
<td>Lymphocytic exocytosis</td>
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<td></td>
<td>Absence of epithelial dysplasia</td>
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<td></td>
<td>Absence of verrucous epithelial architectural change</td>
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1.1.6 Differential diagnosis


1.1.7 Oral lichenoid lesions

The terms oral lichenoid lesion (OLL) or oral lichenoid reaction (OLR) are used for lesions that are similar to OLP but lack some of the typical clinical and/or histopathologic features of OLP (Al-Hashimi et al. 2007, Cheng et al. 2016). OLL include oral lichenoid contact lesion, oral lichenoid drug reaction, oral lichenoid lesions of graft-versus-host disease, oral discoid lupus erythematosus, oral lesions...
of systemic lupus erythematosus, erythema multiforme, paraneoplastic pemphigus/paraneoplastic autoimmune multiorgan syndrome, chronic ulcerative stomatitis and lichen planus pemphigoides (Khudhr et al. 2014).

The lichenoid tissue reaction, called “interface dermatitis” in dermatology is a histopathological term originally defined by Pinkus (1973), and encompasses many clinically diverse inflammatory skin diseases (Sontheimer 2009). Lichen planus is the prototypic skin disease with interface dermatitis (Sontheimer 2009). Of note, the chronic inflammation associated with preneoplastic or dysplastic lesions and oral squamous cell carcinoma may sometimes exhibit a lichenoid tissue reaction pattern (Fitzpatrick et al. 2014b).

In contrast to OLP, a causative factor for OLL should be sought and can sometimes be found, most often being drugs or an amalgam filling (Al-Hashimi et al. 2007, Cheng et al. 2016). A wide variety of medications are capable of inducing OLL, including anti-hypertensives, non-steroidal anti-inflammatory drugs, penicillamine and anti-malarials (Al-Hashimi et al. 2007, McCartan & McCreary 1997).

### 1.1.8 Associations with systemic diseases

Although no single systemic factor or disease is consistently associated with OLP, hepatitis C virus (HCV) seropositivity has been demonstrated to be more common in OLP patients (Alaizari et al. 2016, Lodi et al. 2010). This association is especially present in certain geographic areas, being strongest in the Middle-East (Baccaglini et al. 2013). HCV-RNA has been detected in OLP lesions and HCV may attract HCV-specific T-lymphocytes (Kurokawa et al. 2003, Nagao et al. 2000, Pili et al. 2002).

The association of LP and various autoimmune diseases, including alopecia areata and lupus erythematosus, is proposed (Chung et al. 2015, Scully et al. 1998). Regarding OLP, no firm evidence linking it to autoimmune comorbidities exists (Lopez-Jornet et al. 2014).

A few reports suggest that thyroid diseases could be associated with OLP. In a study comparing small groups of HCV-negative and HCV-positive OLP patients, thyroid dysfunction was found in 13% (3/23) of the HCV-negative cases and in 15% (4/26) of the HCV-positive cases (Carrozzo et al. 1999). In addition, serum anti-thyroid antibodies were found in 17% of the HCV-negative and in 4% of the HCV-positive patients. Another study reported anti-thyroglobulin (Tg) antibodies in 21.4%, and anti-thyroid microsomal (TMA) antibodies in 24.4% of OLP patients.
compared to 1.9% and 1.9%, respectively, in the healthy control subjects (Chang et al. 2009). Furthermore, in a relatively large epidemiologic study, patients with LP were found to have a higher prevalence of hypothyroidism than the age and sex matched controls (10% vs. 5.7%) (Dreher et al. 2009). In addition, the use of thyroid therapeutics was reported to be more common in OLP patients than in controls (4% vs. 0.5%), which suggests an association of OLP and hypothyroidism (Kragelund et al. 2003).

Thymomas and Good syndrome (thymoma with hypogammaglobulinemia) are associated with autoimmune conditions such as myasthenia gravis, thyroid diseases, systemic lupus erythematosus, alopecia and lichen planus (Bernard et al. 2016, Motegi et al. 2015, Seneschal et al. 2008). In particular, the erosive form of OLP may be associated with thymoma (Bobbio et al. 2007, Kaku et al. 2011, Motegi et al. 2015, Seneschal et al. 2008).

Stress is often attributed to the onset or exacerbation of OLP, even though no causal relationship has been demonstrated. However, OLP patients are more likely to suffer from anxiety, depression and low self-control than controls (Pippi et al. 2016).

1.1.9 Management

There is no curative treatment for OLP, so therapy is aimed at controlling symptoms (Hodgson & Chaudhry 2010). Elimination of local irritating factors, treatment of possible oral candidiasis and dental and periodontal treatment is important. In fact, patients with gingival OLP typically benefit from calculus removal and rigorous plaque control (Holmstrup et al. 1990, Salgado et al. 2013, Stone et al. 2013, Stone et al. 2015). Regarding medical management, topical corticosteroids are considered the mainstay of therapy of OLP, although the evidence-base for their effectiveness is weak (Thongprasom et al. 2011). There are a wide range of other treatment options available, both topical and systemic, but again with little evidence for their effectiveness in reducing the signs or symptoms of OLP or for any one being better than the others (Cheng et al. 2012, Suresh et al. 2016, Thongprasom et al. 2011). Besides topical corticosteroids, most clinical research in recent years has been done on topical tacrolimus. Conflicting evidence comparing the effectiveness of topical corticosteroids and tacrolimus has been reported (Chamani et al. 2015, Guo et al. 2015, Hettiarachchi et al. 2016, Sivaraman et al. 2016).
**Topical glucocorticoids**

The mechanism of action of corticosteroids is believed to involve the binding of the drug to steroid receptors in the cytoplasm, which results in inhibition of synthesis of proteins responsible for induction of an inflammatory response (Goa 1988). Corticosteroids are known to suppress the production of inflammatory humoral factors, hinder leucocyte migration to sites of inflammation, decrease T-cell proliferation, increase T-cell apoptosis and interfere with the function of endothelial cells, granulocytes, mast cells and fibroblasts (Brazzini & Pimpinelli 2002).

Topical corticosteroid potency is based on their skin vasoconstricting capacity; however, comparable vasoconstricting ability does not denote therapeutic equivalence. Topical corticosteroids are classified from very high potency to low potency (classes I–VII) (Essential Medicines and Health Products Information Portal. A World Health Organization resource 2016).

Several classes and potencies of topical corticosteroids are used in the management of OLP (Thongprasom & Dhanuthai 2008). In Finland the available mid-potency preparations include 0.1% triamcinolone acetonide as a rinse, spray or paste with Orabase® gel, beclomethasone dipropionate or beclomethasone dipropionate monohydrate as a spray, fluticasone propionate spray; potent preparations include 0.1% betamethasone 17-valerate; and high-potency preparations include 0.05% clobetasol propionate. Methylprednisolone is available for intralesional injections.

The most common side effect of topical corticosteroid use in the oral cavity is secondary oral candidiasis (Thongprasom & Dhanuthai 2008). A temporary burning sensation is occasionally reported when topical corticosteroids are applied on the oral mucosa (Laeijendecker et al. 2006, Sonthalia & Singal 2012). Other possible adverse effects of oral topical corticosteroids include taste alteration, xerostomia, nausea and sore throat (Thongprasom & Dhanuthai 2008). Systemic transmucosal absorption of topical corticosteroids may occur (Varoni et al. 2012). This can lead to adrenal suppression clinically manifesting as cushingoid symptoms or Cushing’s syndrome (Decani et al. 2014, Gonzalez-Moles et al. 2002, Lehner & Lyne 1969). However, most patients do not develop systemic side effects, even after prolonged use of topical corticosteroids (Carbone et al. 1999, Plemons et al. 1990).
Topical tacrolimus

Tacrolimus (FK-506) is a macrolide obtained from a soil bacterium, *Streptomyces tsukubaensis* (Kino *et al.* 1987). It is an immunosuppressant that was originally developed for systemic administration to prevent rejection in organ transplants (Fung & Starzl 1995). The mechanism of action of tacrolimus is based on the inhibition of calcineurin, which results in inhibition of the activation and proliferation of T-lymphocytes and suppression of synthesis of pro-inflammatory cytokines (Reynolds & Al-Daraji 2002, Tsuda *et al.* 2012).

A topical formulation of tacrolimus was later developed for treatment of atopic dermatitis (AD) (Ruzicka *et al.* 1999). Since the late 1990s, off-label use of topical tacrolimus with favorable outcomes was reported in OLP not responding to conventional treatment (Hodgson *et al.* 2003, Kaliakatsou *et al.* 2002, Lener *et al.* 2001, Morrison *et al.* 2002, Rozycki *et al.* 2002, Vente *et al.* 1999). In Finland, topical tacrolimus is available as 0.03% and 0.1% ointment preparations.

Topical tacrolimus may cause transient local irritation at the site of oral mucosal application in 16–56% of the patients (Byrd *et al.* 2004, Corrocher *et al.* 2008, Hodgson *et al.* 2003, Kaliakatsou *et al.* 2002, Laeijendecker *et al.* 2006). The irritation is typically felt as a burning or stinging sensation, either when the drug is administered, or is associated with eating or drinking. Other less commonly encountered adverse effects of oral topical tacrolimus use include taste alterations, oral dryness and headache (Hodgson *et al.* 2003, Kaliakatsou *et al.* 2002, Olivier *et al.* 2002, Ribero *et al.* 2015). Systemic absorption of topical tacrolimus through the oral mucosa resulting in detectable blood concentrations of the drug may occur (Kaliakatsou *et al.* 2002). However, hematological renal and liver function tests typically reveal no changes in these cases, and clinically significant adverse effects have not been reported (Hodgson *et al.* 2003, Lopez-Jornet *et al.* 2010). The possibility that topical tacrolimus could contribute to oral cancer development has been suggested in a few case reports (Becker *et al.* 2006, Mattsson *et al.* 2010, Morita *et al.* 2016). At the moment there is no evidence linking topical tacrolimus to malignancies in cutaneous use (Chia & Tey 2015, Cury Martins *et al.* 2015, Siegfried *et al.* 2016), and in mucosal use there is not enough data to make firm conclusions. The long-term safety of topical tacrolimus use in the oral mucosa remains to be established (Lopez-Jornet *et al.* 2010).
**Other therapies**

Besides topical corticosteroids and tacrolimus, numerous therapeutics in various formulations and dosages are reported to have efficacy in OLP (Yang et al. 2016). Many are mentioned in cases refractory to topical corticosteroids.

Topical pimecrolimus, cyclosporine A, aloe vera, hyaluronic acid, chamomile, curcumin, retinoids, tetracyclln, rapamycin, thalidomide, different phototherapies like psoralen and ultraviolet A radiation (PUVA) or low-level or CO2 laser therapy, ozone, surgical excision, cryosurgery, and palatal graft are among the treatment modalities reported (Jajarm et al. 2011, Kazancioglu & Erisen 2015, Kia et al. 2015, Lopez-Jornet & Aznar-Cayuela 2016, Nolan et al. 2009, Pavlic & Vujic-Aleksic 2014, Thongprasom et al. 2013, Yang et al. 2016). However, in Finland these are not commonly in use.

Systemic therapies for OLP reported include corticosteroids, retinoids, thalidomide, mycophenolate mofetil, etanercept, curcumin, glycyrrhizin and extracorporeal photochemotherapy (Yang et al. 2016). In Finland, mainly prednisolone is used for systemic management of OLP.

**1.1.10 Outcome measures in OLP**

Different outcome measures, both investigator-assessed and patient-reported are used in OLP intervention trials (Ni Riordain et al. 2015, Wang & van der Waal 2015). A recent review of the disease scoring systems identified 22 different scales and a lack of universally accepted and validated measure for OLP (Wang & van der Waal 2015). In addition, the measures used frequently lack the necessary sensitivity to detect therapeutic efficacy vs. placebo (Zakrzewska et al. 2005).

**1.1.11 Prognosis**

No studies are available with a decades long follow-up of a large population of OLP patients. However, it is acknowledged that OLP is typically a long-lasting disease, with only a small proportion of patients experiencing recuperation (Carbone et al. 2009, Thorn et al. 1988). The malignant transformation rate of OLP is estimated to be 1.09% (Fitzpatrick et al. 2014a). One study suggested that the malignant transformation is associated with OLL, not OLP (van der Meij et al. 2007). A recent study found that DNA ploidy analysis of a biopsy specimen was able to predict malignant transformation in a proportion of OLP patients (Sperandio et al. 2016).
The time from diagnosis to transformation is on average 51.4 months (Fitzpatrick et al. 2014a).

### 1.1.12 Pathogenesis

The current hypothesis of pathogenic mechanisms in OLP points to a state of immune dysregulation (Kurago 2016, Nogueira et al. 2015, Payeras et al. 2013). Antigen-specific, T-cell-mediated as well as non-specific immune mechanisms are involved in OLP (Sugerman et al. 2002). (Figure 2: Schematic illustration of the hypothetical pathogenesis of OLP).

Although OLP has many features of a true autoimmune disease, like disease chronicity, female predilection, adult onset and autoreactive T-cells, other features like circulating autoantibodies are not found consistently (Ingafou et al. 1997, Sugerman et al. 2002). In addition, the target self-antigen in OLP is not known. Although a lichen planus-specific antibody has not been found in OLP or in oral lichenoid drug eruptions (McCartan & Lamey 2000), some investigators have found basal cell cytoplasmic autoantibodies in OLR (Lamey et al. 1995). In addition, occasional positivity for antiepithelial, and desmoglein 1 and 3 antibodies using indirect IF has been reported (Ingafou et al. 1997, Kinjyo et al. 2015, Lukac et al. 2006). Also autoantibodies against ETS-related transcription factor Elf-3 (ELF3), a transcription factor for keratinocyte differentiation, are present in a proportion of OLP patients (Danielsson et al. 2013).

Although no firm conclusions about the possible genetic basis for OLP can be made at this time, gene polymorphisms of cytokines interferon gamma (IFNG), tumor necrosis factor (TNF), interleukins 4, 10 and 12A (IL4, IL10 and IL12A) may contribute to OLP susceptibility (Jiang et al. 2015, Lu et al. 2015).

### Antigen-specific immune mechanisms

The antigen or antigens triggering the T-cell mediated immune response are suspected to include extrinsic factors like viruses, bacteria, drugs, dental materials or intrinsic factors like self-peptides (Kurago 2016). The virus most commonly linked to OLP is HCV, and in susceptible individuals, the HCV antigens expressed on keratinocyte surfaces could act as targets for cytotoxic T cells (Lodi et al. 2005). An interesting recent finding was the presence of bacteria in keratinocytes and T-cells in OLP tissue (Choi et al. 2016). Altered expression of heat shock proteins (HSP) have been found in OLP so it has been speculated that they could act as

There are two possible mechanisms suggested for the initiation of the T-cell response in OLP. During the initial phase of OLP lesion formation, an antigen may be expressed in association with the human leukocyte antigen (HLA) class I histocompatibility complex on keratinocytes (Sugerman et al. 2002). Cytotoxic CD8A+ T-cells may then, on routine surveillance (“chance encounter”) or via attraction by keratinocyte-derived chemokines (“directed migration”), recognize the antigen, become activated and trigger apoptosis of the basal keratinocytes (Sugerman et al. 2000, Sugerman et al. 2002).

On the other hand, it seems that before CD8A+ T-cell-mediated cytotoxic attack on keratinocytes can occur, CD8A+ T-cells require interaction with activated CD4+ T-cells (Sugerman et al. 2002). Hence, early in the OLP disease process, antigen-presenting cells (APC), including LCs or keratinocytes may present a HLA class II histocompatibility complex associated antigen to CD4+ T-cells, which, with the help of co-stimulatory molecules from keratinocytes and APCs, become activated (Sugerman et al. 2002). The initial encounter of LCs with the triggering antigen typically leads to LC maturation and migration to regional lymph nodes for antigen presentation and subsequent T-cell activation. However, T-cell-LC interactions leading to T-cell activation may possibly occur at a lesion site in OLP (Kurago 2016). The crosstalk between activated CD4+ T-cells and CD8A+T-cells is thought to result in apoptosis of the keratinocytes. The increased number of LCs and CD4+T-cells in lesion sites suggests that they are indeed involved in OLP pathogenesis. Furthermore, early OLP lesions may contain more CD4+ cells with an increase of CD8A+ cells with progression of the disease (Sugerman et al. 2002). Non-specific T-cells are also attracted to the lesion site by secretion of chemokines.

The role of cytokines

The immune dysregulation in OLP is in many ways related to abnormal production of various inflammatory mediators, including cytokines and proteases, which are present both in the lesions and in peripheral blood. These inflammatory mediators orchestrate interactions between many cell types, like keratinocytes, T-cells, and dendritic cells (DCs) in complex signalling networks (Lu et al. 2015). Depending on the effect the mediators have on the cells, the resulting state may be pro-inflammatory or anti-inflammatory.
Among the key molecules that regulate both innate and adaptive immune responses are cytokines, small peptides that are synthesized and secreted by many immune and non-immune cells (Lu et al. 2015). They act by binding to specific cell surface receptors and, depending on the target cell type and the environment, exert different effects on the cell phenotype and function (Lu et al. 2015). The expression of a great range of inflammation-related cytokines in lesional tissue, saliva, peripheral blood mononuclear cells and serum in OLP has been studied. In particular, TNF, IFNG and interleukins have been proteins of interest in OLP (Lu et al. 2015).

Expression of TNF is increased in lesional tissue, saliva and serum in OLP patients compared to controls (Pezelj-Ribaric et al. 2004, Rhodus et al. 2005, Sklavounou et al. 2000, Sklavounou-Andrikopoulou et al. 2004, Younes et al. 1996). Moreover, increased production of TNF has been demonstrated by keratinocytes, mast cells and CD4+ T-cells in OLP (Piccinni et al. 2014, Yamamoto et al. 1994, Yamamoto & Osaki 1995, Zhao et al. 2001). TNF is thought to stimulate adhesion of lymphocytes to the luminal surfaces of blood vessels and their subsequent extravasation into the tissue (Zhao et al. 2002a). In addition, TNF may stimulate the production of other inflammatory mediators, including C-C motif chemokine 5 (CCL5) by T-cells, and matrix metalloproteinase 9 (MMP9) as well as itself (Khan et al. 2003, Zhao et al. 2001, Zhou et al. 2001). One of the mechanisms suggested to induce keratinocyte apoptosis in OLP involves TNF, secreted by CD8A+ T-cells, which may bind to its receptor on the basal and suprabasal keratinocyte surface and eventually activate the caspase cascade leading to keratinocyte apoptosis (Younes et al. 1996).

IFNG positivity and overexpression compared to healthy controls has been detected in OLP subepithelial mononuclear cells, suggesting a Th1 predominant type immune response in OLP (Khan et al. 2003, Wang et al. 2015). IFNG may thus be implicated in the activation of CD8A+ T-cells and subsequent keratinocyte apoptosis (Kurago 2016, Lu et al. 2015). Decreased production of IFNG in peripheral blood mononuclear cells in OLP patients has been observed, while inconsistent results have been reported regarding the expression in saliva and serum of patients (Lu et al. 2015).

Interleukins are produced by numerous cell types and participate broadly in inflammatory and immune-mediated processes (Lu et al. 2015). A wide variety of interleukins have been studied in OLP and implicated in its pathogenesis (reviewed in Lu et al. 2015). It is suggested that, e.g. IL12 and IL18 may stimulate IFNG expression and activate natural killer (NK) cell and T-cell cytotoxicity; in addition
IL18 may stimulate TNF expression (Kurago 2016). IL2 may participate in the signaling between CD4+ and CD8A+ T-cells in OLP (Sugerman et al. 2002).

Among the chemokines implicated in OLP pathogenesis are CCL5 and chemerin that are involved in the recruitment of various inflammatory cells in OLP (Kurago 2016, Sugerman et al. 2002). CCL5 activates the C-C chemokine receptor type 1 (CCR1) in mast cells and stimulates mast cell degranulation with subsequent release of TNF and chymase. Cell surface receptors for CCL5 have been identified in T-cells and mast cells in OLP (Zhao et al. 2002b). Chemerin may be involved in attracting natural killer (NK) cells in OLP, since NK cells were found to express the chemerin receptor in OLP (Parolini 2007).

**The role of proteases**

Matrix metalloproteinases are a family of zinc-containing endopeptidases whose main function is proteolytic degradation of extracellular matrix proteins. MMP1, MMP2, MMP3, MMP7 and MMP9 may play a role in OLP (Ertugrul et al. 2013, Rubaci et al. 2012, Zhou et al. 2001). MMP9 is seen mainly in the inflammatory infiltrate in OLP, and it may be involved in the BM disruption (Sugerman et al. 2002, Zhou et al. 2001). Chymase, secreted by mast cells, may directly cause BM disruption or activate T-cells to secrete MMP9, which causes the disruption (Sugerman et al. 2002). T-lymphocytes may use the BMZ breaks to infiltrate the epithelium (Hashimoto et al. 1966, Zhou et al. 2002).

**The role of adhesion molecules**

The migration of leukocytes to the OLP lesion site is mediated via chemokines and cellular adhesion molecules. Intercellular adhesion molecule 1 (ICAM1) expression is detected in keratinocytes, endothelial cells and leukocytes in OLP (Little et al. 2003). The expression of other vascular cell adhesion molecules, e.g. vascular cell adhesion protein 1 (VCAM1), is also increased in OLP (Regezi et al. 1996, Walton et al. 1994), and probably contributes to lymphocyte extravasation. In addition, components of BM that are overexpressed, laminin, type IV collagen and type VII collagen, in the junctional area may serve as ligands for lymphocyte adhesion molecules in OLP (Eversole et al. 1994).

It is proposed that in OLP, keratinocytes may respond to lymphocyte-mediated damage in the epithelium-connective tissue interface by increased expression of the
interface-associated adhesion molecules, and this could help in resisting epithelial separation from connective tissue (Ramirez-Amador et al. 1996).

Other possible factors contributing to OLP pathogenesis

The specific mechanism that results in keratinocyte apoptosis in OLP is still unclear, but several types of interactions between cytotoxic CD8A+ T cells and basal and suprabasal keratinocytes may occur that activate the caspase cascade. These include mainly TNF-TNF receptor interaction (mentioned above), perforin-1 (PNF1) and granzyme B (GZMB) secreted by cytotoxic T-cells and tumor necrosis factor receptor superfamily member 6–tumor necrosis factor ligand superfamily member 6 (FAS-FASLG) interaction (Sugerman et al. 2002). It has also been suggested that the basal keratinocyte apoptosis may be related to cellular tumor antigen p53 (TP53) or bcl-2-like protein 1 (BCL2L1) proteins (Dekker et al. 1997).

It has been proposed that the BM disruption seen in OLP may be caused by apoptotic keratinocytes that are not able to perform their normal function in the maintenance of BM structure. On the other hand, keratinocytes need a BM-derived cell survival signal to avert the start of apoptosis. Hence, both BM and keratinocytes are dependent on each other for normal function and the breakdown of this equilibrium may lead to a cyclical, chronic disease process (Sugerman et al. 2002).

Keratinocyte differentiation is altered in OLP (Boisnic et al. 1995, Danielsson et al. 2013, Danielsson et al. 2014, Jacques et al. 2009). In fact, several genes involved in epithelial development and differentiation were differentially expressed in OLP epithelium compared to healthy epithelium (Danielsson et al. 2014). The altered keratinocyte differentiation suggests that the normal epithelial barrier function is affected, and may contribute to the pathogenesis of OLP.
Fig. 2. A hypothetical model of pathogenesis of OLP. A. Antigen specific mechanisms. B. Non-specific mechanisms. Please see text. Modified from Sugerman et al. (2002).
1.2 Toll-like receptors 4 and 9 in immune-mediated conditions

Toll-like receptors (TLR) are a family of pattern recognition receptors (PRRs) expressed in various immune and non-immune cells that play a key role in the host defense against infection by sensing invading pathogens (Kumar et al. 2011). The specific recognition of the evolutionarily conserved molecules of pathogens known as pathogen associated molecular patterns (PAMPs) by TLRs is essential in initiating and controlling innate immunity and subsequent activation of the adaptive immune responses (Kawai & Akira 2010). In addition to PAMPs, endogenous metabolites such as nucleic acids, fatty acids and phospholipids, produced by tissue injury or inflammation may act as ligands for TLRs (Miyake & Kaisho 2014).

In humans, 10 different TLRs have been identified (Kawai & Akira 2010, Sasai & Yamamoto 2013). TLRs expressed on cell surfaces (e.g. TLR4) recognize mainly microbial lipopolysaccharides (LPS), lipids and proteins (Kawai & Akira 2010). On the other hand, TLRs that are expressed exclusively intracellularly (e.g. TLR9) mostly sense microbial nucleic acids (Kawai & Akira 2010, Sasai & Yamamoto 2013). The non-microbial, endogenous ligands or self-molecules (constituents of an organism that can be differentiated from foreign substances by the immune system) capable of binding to TLRs include hyaluronan (HA), high mobility group protein B1 (HMGB1), HSP, and defensin beta 4A (DEFB4A) (Lin et al. 2011, Yu et al. 2010). After the stimulation of TLRs by their agonists, they recruit several downstream adaptor molecules (mainly TIR domain-containing adapter molecule (TICAM1) and myeloid differentiation primary response protein MyD88 (MYD88) that mediate further immune signaling to produce inflammatory cytokines, chemokines and co-stimulatory molecules by the immune cells (Gianchecchi & Fierabracci 2015, Li et al. 2009, Lin et al. 2011). TLR activation and specificity are regulated through complex signaling mechanisms (Qian & Cao 2013).

Based on the ability of TLRs to recognize a large range of host-derived molecular patterns and their expression on a wide variety of cell types, it is probable that TLRs participate in the development, progression and resolution of most noninfectious inflammatory and immune diseases (Lin et al. 2011). TLR-mediated immune responses are implicated in chronic epidermal inflammatory diseases such as psoriasis, LP and AD (Ermentcan et al. 2011, Miller & Modlin 2007), as well as mucosal diseases such as chronic rhinosinusitis (Ramanathan et al. 2007), ulcerative colitis, coeliac disease (Ospelt & Gay 2010) and aphthous ulcers (Gallo et al. 2012, Hietanen et al. 2012). Numerous studies have demonstrated the
involvement of various TLRs in initiation and progression of periodontitis, reviewed by Song et al. (2016).

Regarding TLR4 and TLR9 expression in oral diseases, both have been found in the epithelial and connective tissue of healthy gingivae and periodontitis, with a propensity for stronger expression in inflamed tissues (Beklen et al. 2008, Rojo-Botello et al. 2012, Sugawara et al. 2006). In contrast to these findings, Ren et al. did not find expression of TLR4 in healthy periodontal tissue (Ren et al. 2005). TLR4 and TLR9 expression with variable intensities has been reported also in oral chronic hyperplastic candidiasis, leukoplakia and healthy oral sulcular mucosa (Ali et al. 2008).

In previous literature on TLRs and LP, toll-like receptor 9 protein and mRNA expression was shown to be increased in skin lesions compared to healthy skin (Li et al. 2007). In OLP patients, enhanced expression of TLR2 in mucosal lesions as well as in peripheral blood monocytes was reported (Ohno et al. 2011). In contrast to these findings, another research group reported a reduced toll-like receptor 2, but increased toll-like receptor 4 proteins and transcripts expression in lesional epithelium and unstimulated whole saliva (UWS) epithelial cells from OLP patients (Janardhanam et al. 2012). In addition, soluble forms of TLR4 were found to be increased and functional in UWS, but TLR4 mRNA levels of salivary epithelial cells were reduced in OLP patients compared to controls (Zunt et al. 2009).

Two earlier reports have described the effect of tacrolimus on toll-like receptor expression. Kis et al. (2006) reported that TLR4 expression in human keratinocytes was not influenced by tacrolimus and Antiga et al. (2011) showed that topical tacrolimus therapy did not have an impact on the immunohistochemical expression of TLR4 and TLR9 in AD.

1.3 Hyaluronan, its receptor CD44 and its metabolizing enzymes HAS1–3 and HYAL1–2 in immune-mediated conditions

Hyaluronan (HA) is a large polysaccharide composed of alternating N-acetyl-glucosamine and glucuronic acid units that is found ubiquitously in human extracellular matrix tissues (Dicker et al. 2014). HA has diverse biological functions in both normal and pathological processes: it can bind large amounts of water and form viscous gels, function as a filter, and act as a signaling molecule. HA is involved in regulating cell functions, tissue development, embryogenesis, inflammation, wound healing, tumor progression and metastasis (Dicker et al. 2014). It also contributes to the maintenance of tissue homeostasis.
HA is recognized as a significant and active molecule in many aspects of inflammation, participating in the regulation of expression of inflammatory genes, the recruitment of inflammatory cells and the release of inflammatory cytokines (Heldin et al. 2008, Jiang et al. 2011, Petrey & de la Motte 2014). Increased accumulation of HA is noted after tissue injury and during inflammatory conditions (Heldin et al. 2008, Jiang et al. 2011, Monslow et al. 2015, Petrey & de la Motte 2014). Immune cell interactions with HA increase upon an inflammatory response. This may help recruit immune cells to the site of inflammation as well as keep cells at the site and may facilitate their survival and function (Lee-Sayer et al. 2015). During infection and tissue injury, HA becomes fragmented, and the fragments accumulate extracellularly, which provokes an inflammatory response (Lee-Sayer et al. 2015).

HA is synthesized on the plasma membrane by three hyaluronan synthase isoenzymes (HAS1-3), of which HAS2 is the most active and abundant in many cells (Vigetti et al. 2014). An inactive reservoir of HAS is present intracellularly that may be activated upon various stimuli and transported to the plasma membrane for HA synthesis (Rilla et al. 2005). Dysregulation of hyaluronan synthases occurs during tissue injury and inflammation (Siiskonen et al. 2015).

Enzymatic degradation of HA occurs by hyaluronidases (HYAL). Human HYAL1 and HYAL2 are the two major hyaluronidases cleaving HA in somatic tissues (Stern & Jedrzejas 2006). HYAL1 (serum hyaluronidase) is an acid active endo/lysosomal enzyme, while HYAL2 is attached to the plasma membrane via a glycosylphosphatidylinositol anchor (Harada & Takahashi 2007). In addition to HYALs, HA can be degraded by reactive oxygen species created under stress conditions (Dicker et al. 2014). Cleavage of HA increases the permeability of connective tissue and lowers the viscosity of body fluids. Hence, HYALs have been implicated to have a role in bacterial pathogenesis, spread of toxins and progression of cancer, as well as in facilitating direct contact between microbes and host cell surfaces (Girish & Kemparaju 2007).

CD44 is a cell surface adhesion molecule that is widely expressed in a variety of cells, including leukocytes, fibroblasts, epithelial and endothelial cells (Goodison et al. 1999). CD44 may occur as a transmembrane cell surface receptor, in the extracellular matrix as well as in a soluble form, e.g. in saliva (Pure & Cuff 2001). The diverse roles of CD44 include cell–cell and cell–matrix interactions comprising cell proliferation and migration, hematopoiesis and lymphocyte activation, homing and extravasation (Jordan et al. 2015). CD44 is a principal HA-binding receptor (Heldin et al. 2008).
Previous studies have shown that healthy oral epithelium expresses HA both on the cell surface and in the intercellular space in the basal and spinous cell layers (Kosunen et al. 2004, Tammi et al. 1990). The expression of hyaluronan synthases and hyaluronidases in oral mucosal tissue has not been described before, but cultured oral mucosal epithelial cells demonstrated HAS1–3 mRNA (Yamada et al. 2004). CD44 is known to be expressed in normal squamous epithelium, locating on cell membranes of the basal two-thirds of the epithelium (Kosunen et al. 2007). In OLP specimens, Neppelberg et al. (2007) demonstrated that CD44 immunoexpression was comparable to that in healthy control tissue. On the other hand, Chaiyarit et al. (2008) found a decreased expression of the isoform CD44v6 in OLP epithelium compared to healthy oral epithelium.

1.4 Cathepsin K in immune-mediated conditions

Cathepsin K (CTSK) is an acidic cysteine endoproteinase belonging to the papain-like cysteine peptidase family (Novinec & Lenarcic 2013). CTSK was initially identified as an osteoclast-specific lysosomal protease, but has since been found to be expressed in many different cell types and to be involved in various physiological and pathological processes (Novinec & Lenarcic 2013).

Experimental studies have found that CTSK contributes to autoimmune inflammation. CTSK is required for TLR9 signaling by DCs in autoimmune arthritis (Asagiri et al. 2008) and for induction of inflammation and bone erosion, as well as for expression of TLRs 4, 5 and 9 in rheumatoid arthritis (Hao et al. 2015b). In a mouse model of psoriasis, CTSK was involved in the development of psoriasis-like skin lesions through TLR7-dependent Th17 activation (Hirai et al. 2013).

In oral diseases, CTSK was first found to be involved in peri-implantitis and periodontitis (Mogi & Otogoto 2007, Strbac et al. 2006). Further studies have corroborated the important role of CTSK in periodontal disease (Beklen et al. 2015, Chen et al. 2016, Hao et al. 2015b). CTSK is also involved in the bone loss and inflammation associated with periapical disease (Gao et al. 2013, Hao et al. 2015a), and in diffuse sclerosing osteomyelitis (Montonen et al. 2006).
2 Aims of the study

The objectives of this thesis included investigating the prevalence of systemic conditions, especially thyroid diseases, in OLP, and comparing the effectiveness of topical tacrolimus, triamcinolone acetonide and placebo in the management of symptomatic OLP. To increase our understanding of the etiopathogenesis of OLP, the immunohistochemical expression of specific, previously uninvestigated molecules were studied in OLP, as well as the effect of tacrolimus treatment on their expression.

The more specific research questions were:

1. Are thyroid diseases or thyroxin medication more common in OLP and OLL patients?
2. Are topical tacrolimus and triamcinolone acetonide more effective than placebo in the management of symptomatic OLP, and is there a difference in the effectiveness between tacrolimus and triamcinolone acetonide?
3. Are toll-like receptors 4 and 9 (TLR4 and TLR9) associated with OLP pathogenesis?
4. Could hyaluronan (HA), its receptor CD44 antigen (CD44) and its metabolizing enzymes hyaluronan synthases 1–3 (HAS1–3) and hyaluronidases 1-2 (HYAL1–2) be involved in OLP pathogenesis?
5. Is cathepsin K (CTSK) expressed in OLP mucosa, and is its expression associated with TLR4 or TLR9?
6. Does topical tacrolimus have an effect on the expression of 1) TLR4 and TLR9 2) HA, CD44, HAS1–2, and HYAL1–2, and 3) CTSK?
3 Patients and materials

Patients, materials and methods are described in more detail in the original articles (I, III–V) and manuscript (II). The study protocols were approved by The Regional Ethics Committee of the Northern Ostrobothnia Hospital District (I, II, III, IV–V). The Research Ethics Committee of the Northern Savo Hospital District (Kuopio) was notified of the study II. Permission to use archival tissue material (III–V) was given by the National Supervisory Authority for Welfare and Health (VALVIRA). An approval for work II was granted by the Finnish Medicines Agency. The clinical trial (II) was registered at clinical.trials.gov (identifier NCT01544842).

3.1 Patients

The number of cases and controls, and other demographic data of the patients in the studies are shown in table 2. In studies III and IV, the same group of specimens was used.

Table 2. Basic characteristics of the case and control subjects in studies I-V.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>222</td>
<td>27</td>
<td>50</td>
<td>55</td>
<td>25</td>
</tr>
<tr>
<td>Controls</td>
<td>222</td>
<td>n/a</td>
<td>5</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>Female %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>71</td>
<td>85</td>
<td>n/a</td>
<td>n/a</td>
<td>55</td>
</tr>
<tr>
<td>Controls</td>
<td>71</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>71</td>
</tr>
<tr>
<td>Age mean (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>50 (17–77)</td>
<td>56 (37–71)</td>
<td>n/a</td>
<td>n/a</td>
<td>53 (9–88)</td>
</tr>
<tr>
<td>Controls</td>
<td>50 (13–80)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>30 (22–59)</td>
</tr>
<tr>
<td>OLP diagnostic criteria</td>
<td>Cl+Hp</td>
<td>Cl+Hp</td>
<td>Hp</td>
<td>Hp</td>
<td>Hp</td>
</tr>
</tbody>
</table>

n/a not applicable, Cl=clinical, Hp=histopathologic

3.2 Medical records (I)

Patient files of subjects diagnosed with OLP or OLL from 1992 to 2001 at the Institute of Dentistry, University of Oulu and/or the Oral and Maxillofacial Department, Oulu University Hospital were investigated. The control group consisted of age and sex matched persons, who had visited the Institute of Dentistry,
University of Oulu, for general dental care as undergraduate teaching patients during the same period, and their patient files were investigated also.

### 3.3 Medications in the clinical trial (II)

Interventions in the clinical trial included 0.1% tacrolimus ointment (Protopic®, Astellas Pharma, Tokyo, Japan), 0.1% triamcinolone acetonide paste (Kenacort-T®/Kenacort-A® Orabase, Bristol–Myers Squibb, New York, USA/Dermapharm, Grünwald, Germany) and Orabase paste (ConvaTec, Deeside, UK).

### 3.4 Tissue specimens (II–V)

Tissue specimens in the clinical trial (II) were collected from 24 patients before the intervention and after using the study medication for 3–6 weeks. Half of each specimen was fixed in 10% neutral buffered formalin and half was snap frozen in liquid nitrogen and stored at -70°C.

For studies III–V, formalin-fixed, paraffin-embedded tissue specimens (FFPE) diagnosed as OLP were retrieved from the archives of the Department of Pathology, Oulu University Hospital.

As controls in studies III–V, we used a) healthy oral mucosa tissue samples from the archives of the research laboratory of the Institute of Dentistry, University of Oulu, b) healthy buccal mucosal samples obtained from investigators (MS, AK) and from students in Kuopio University Hospital and c) histologically normal looking oral mucosal tissue from oral squamous cell cancer resection specimens (distant from the tumor) from the archives of the Department of Pathology, Kuopio University Hospital.


4 Methods

4.1 Epidemiological study (I)

Study I was conducted as a retrospective case-control study. Age, gender, medical history (medical diagnoses and regular medications), allergies, smoking, oral diseases, main OLP/OLL clinical type and OLP/OLL location were recorded for the cases and relevant corresponding variables also for the controls.

4.2 Clinical trial (II)

The clinical trial (II) was conducted as a double-blind, randomized, placebo-controlled trial.

To measure the outcome of the interventions, a scoring system was developed for the trial, modified from a previously reported assessment method in mucous membrane pemphigoid (Setterfield et al. 1998). This clinical score (CS) was used to measure the clinical signs and subjective symptoms at different time points. The primary outcome variable was the change in CS from baseline to week 3, and the primary research question was whether or not there are differences between the treatment arms.

If a $\geq$ 20% decrease in the CS was noted at week 3, it was considered a response to treatment, while <20% decrease or increase in CS was considered a non-response to treatment.

4.3 Statistical methods (I, II)

I: The clinical features of the patients’ OLP/OLL lesions were presented using frequency and percentage distributions. Cross-tabulation was used to compare patient characteristics between OLP, OLL and control cases. In addition, a multivariable logistic regression method was applied to analyze the associations of selected patient characteristics with the patient groups. Odd ratios (ORs) with 95% confidence intervals (CIs) adjusted by age and gender were reported. Statistical analyses were performed using the R software.

II: The inter-rater reliability of the CS measurements was evaluated with the intraclass correlation coefficient (ICC). The relationships between clinical signs
(1A and 1B of CS) and subjective symptoms (VAS) were analyzed using the Pearson correlation coefficient.

The development of CS and VAS scores in different treatment groups during the study was visualized graphically with line plots. Repeated measures t-test was performed to evaluate the statistical significance of the changes in the CS and VAS values between the different time points.

The main outcome variable was the change in CS values between the baseline and week 3 measurements. To estimate if there were statistically significant differences in CS changes between the treatment groups, analysis of variance (ANOVA) was performed. Tukey's test was applied to identify pairwise differences.

Differences in the treatment responses between the interventions at week 3 were analyzed using cross-tabulation and the statistical significance of the differences was evaluated with a chi-square test.

Missing values (5%) were imputed with the nearest neighbor method. The primary analysis was performed according to the intention-to-treat principle.

The statistical analyses were carried out using IBM SPSS Statistics version 22. G*Power and SamplePower 3.0 software were used for sample size and power calculations. OriginPro software for scientific graphics was used to provide the line plots.

### 4.4 Immunohistochemistry (III-V)

Immunohistochemical staining methods were used in studies III–V. The primary antibodies used are shown in Table 3. Briefly, 5 µm sections were cut from the FFPE tissue blocks, and the sections were deparaffinized and rehydrated. Antigen retrieval, blocking of endogenous peroxidase and non-specific immunoglobulin binding was done when indicated. For the negative controls, either 1) non-immune serum was used, 2) the primary antibody was omitted, 3) the specimens were predigested with Streptomyces hyaluronidase in the presence of protease inhibitors (HA), or 4) the primary antibodies were treated with corresponding peptides (HAS). Positive controls included tissues previously shown to be positive for the studied antibodies. The bound antibodies were visualized with commercial detection systems.

In the double immunoenzymatic staining, localization of the CTSK antibody with various other antibodies (Table 3) was studied using a sequential staining method. Visualization of the primary antibody reactions was done with a peroxidase-based system combined with red and blue-gray chromogens.
In the double IF staining, sequential and simultaneous staining methods were employed. Fluorescein-conjugated secondary antibodies were used to visualize the antibody reactions and the slides were examined with a fluorescence microscope (Zeiss Imager.D2).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone or code</th>
<th>Mouse/rabbit/goat/ mono/polyclonal</th>
<th>Dilution</th>
<th>Incubation time /temperature</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTSK</td>
<td>E-7</td>
<td>Mouse/monoclonal</td>
<td>1:400</td>
<td>60 min/RT</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>CD44</td>
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<td>overnight/+4°C</td>
<td>Dr. Sirpa Jalkanen</td>
</tr>
<tr>
<td>HA</td>
<td>bHABR</td>
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<td>ref. Tammi 1994</td>
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<td>Double ID</td>
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<tr>
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<td>overnight/+4°C</td>
<td>AbD Serotec</td>
</tr>
</tbody>
</table>

4.5 Evaluation of the immunohistochemical staining

The immunohistochemical staining was evaluated using light microscopy. In study III the scoring of the staining was done by three of the investigators (MS, TS, JK), and in studies IV (MS, AK, TS) and V (MS, CCB) by two of the investigators in a consensus manner. In study V, the samples before and after tacrolimus treatment
were assessed individually and blinded to the medication status by two of the investigators (MS, CCB).

III: In both OLP and control samples, the staining intensity of TLR4 and TLR9 in the basal, intermediate and superficial layers of the epithelium as well as subepithelial inflammatory infiltrate was rated using the following categories: absent (0), weak (1), moderate (2) or strong (3). The site of the densest subepithelial inflammatory infiltrate in OLP was selected for the assessment. In each sample, the cell (e.g. mast cell) that showed the strongest staining intensity in the sample was regarded as an intrinsic reference for strong (3) staining.

IV: In OLP and control specimens, the staining intensity was assessed in the basal, intermediate, and superficial layers of the epithelium. A three-level scale (0 = no staining, 1 = some staining, 2 = considerable staining) was used for HA, CD44, and HAS1-2 scoring and a four-level scale (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) was used for HYAL1-2 scoring. HAS3 staining was almost uniformly negative, so it was not scored.

V: Epithelium as a whole was scored by evaluating the number of CTSK-positive cells using grades 1, 2, 3, 4, and 5 corresponding to 0–5%, 6–25%, 26–50%, 51–75%, and 76–100% of cells positive, respectively. Stroma as a whole was scored by assessing the intensity of staining using grades 0 = no staining, 1 = mild, 2 = moderate, 3 = strong, and 4 = very strong.

The specimens before and after tacrolimus treatment were evaluated quantitatively. Three photographs were taken of each slide (left, middle, and right side of the section) with a 200 x magnification and the images were scored utilizing ImageJ software (Rasband). A grid with area per point of 3.00 inches² was placed on the image and the number of cells in the squares was counted using a cell counter: in the epithelial area, the middle right square of the grid was used as a starting point, continuing in a counterclockwise manner until at least 100 epithelial cells were counted. The cells positive for CTSK were counted in the same squares to yield a percentage value of positive cells in the epithelium. The stroma was scored in a similar fashion using the square in the middle right as a starting point and continuing down left diagonally and from there counterclockwise until at least 100 cells in the stroma were counted. The numbers of CTSK-positive cells in the same squares were then counted, yielding a percentage count of positive cells in the stroma. A mean count of the three measurements of each slide was used in the analysis.
4.6 Statistical methods (III–V)

III: The distributions of the TLR4 and TLR9 staining intensity by layers of epithelium were illustrated graphically. A non-parametric Mann-Whitney test was used to evaluate the statistical significance between the control, white lichen and atrophic lichen groups. The Wilcoxon paired samples test was used to evaluate the statistical significances between layers of samples so that layers from the same sample were treated as pairs.

Statistical analyses were done using IBM SPSS Statistics for Mac version 16.0 and Prism 4 for Macintosh (GraphPad, La Jolla, CA, USA).

IV–V: Frequency and percentage distributions of the staining intensity between control and OLP groups were compared using cross-tabulation and presented also graphically. The statistical significance of the differences between distributions was evaluated with a chi-square test.

The agreement between the scores of the investigators among the tacrolimus-treated cases (V) was evaluated using ICC. Repeated-measures t-test was used to evaluate the change in the percentage of CTSK-positive cells before and after treatment with tacrolimus.

The statistical analyses were performed using IBM SPSS Statistics version 21.
5 Results

5.1 Association of thyroid disease and OLP (I)

Fifty-three percent of the cases (both OLP and OLL) and 49% of the controls reported having at least one general medical condition. Regular medications were used by 52% of the cases (51% OLP/53% OLL) and by 49% of the controls.

Thyroid diseases were found to be one of the most common medical conditions in OLP/OLL patients. They were reported by 14% (n=31) of the OLP/OLL patients compared to 8% (n=18) of the control subjects. The odds ratio (OR) for thyroid diseases was 1.95 (95% CI 1.04 to 3.73) when adjusted for age and sex. Looking separately at the OLP/OLL cases, 15% (n=22) of the OLP and 13% (n=9) of the OLL patients had thyroid disease the estimated ORs (with 95% CIs) being 2.12 (1.06 to 4.21) for OLP and 1.57 (0.62 to 3.73) for OLL.

Sixty-eight percent (n=21) of the OLP/OLL cases and 61% (n=11) of the controls with thyroid gland diseases had hypothyroidism. The association of hypothyroidism with OLP seemed stronger than the association with OLL, since the estimated ORs (with 95% CIs) were 2.39 (95% CI 1.05–5.61) for OLP and 1.73 (0.56–4.90) for OLL. However, the sizes of pertinent subgroups of cases and controls were rather small, and the CIs quite wide, so this difference in the OR estimates could be explained by random variation. Thyroxin medication use per se, analyzed separately, was not associated with OLP/OLL (OR 0.94, 95% CI 0.27 to 5.20).

Diabetes was reported by 4% (n=8) of the cases (2 with OLL and 6 with OLP) and 4% (n=8) of the controls.

A history of allergy was reported more frequently by the OLP/OLL patients (n=91) than by the controls (n=69). The overall OR for the association of reported allergy and OLP/OLL was 1.58 (95% CI 1.07 to 2.35).
5.2 Topical tacrolimus and triamcinolone acetonide in the management of symptomatic OLP (II)

5.2.1 The clinical score reliability and validity

The inter-rater reliability of CS was found to be strong (ICC 0.959). On the other hand, the correlation between clinical signs (1A+1B of the CS) and symptoms (VAS) was very weak (Pearson correlation coefficient 0.180).

5.2.2 Patient flow and characteristics

The baseline clinical and demographic data of the study groups are presented in tables in the study II manuscript (table 1, supplementary table 1 and supplementary figure). A total of 27 patients were randomized and allocated to receive either 0.1% tacrolimus ointment (n=11), 0.1% triamcinolone acetonide paste (n=7) or Orabase (placebo) paste (n=9). The patient flow is illustrated in the flow chart (study II manuscript, figure 1). Due to slow participant intake and lack of financial resources and collaborators, the trial was ended before the target sample size was reached. Therefore, the actual power of the study is 0.70.

5.2.3 Primary and secondary outcomes

The results of all the primary and secondary outcome analyses are presented in the study II manuscript. All the participants were analyzed for the primary outcome variable (change in CS from baseline to week 3). CS from baseline to week 3 decreased 37% (mean decrease 10.5, SD 8.6, p=0.002) in the tacrolimus group (n=11) compared to 32% (mean decrease 10.3, SD 6.8, p=0.007) in the triamcinolone group (n=7). In the triamcinolone group that responded to intervention (n=4), and that did not respond to intervention (n=3), the CS decreased 41% (p=0.027) and 19% (p=0.026), respectively. The CS increased slightly (0.8%) in the placebo group (p=0.927). Tacrolimus (p=0.012) and triamcinolone (p=0.031) were more effective than placebo in reducing the CS at week 3. There was no difference in the efficacy between tacrolimus and triamcinolone (p=0.997).

The response to intervention (≥20% decrease in CS from baseline) in the intervention groups was assessed at week 3. Tacrolimus (p=0.005) and triamcinolone (p=0.019) groups responded to treatment more often than the
placebo group, and tacrolimus and triamcinolone groups were equally effective in producing a treatment response (study II manuscript, table 2).

Regarding the effect of interventions on symptoms, tacrolimus and triamcinolone seemed to reduce pain more than placebo at week 3, but the differences were not statistically significant (study II manuscript, figure 2, supplementary tables 2 and 3).

Some secondary outcome analyses could not be completed for all the patients. At week 3, the triamcinolone group was divided into responders and non-responders to treatment, resulting in small sample sizes for further measures. Therefore, although an obvious effect was seen in many of the outcome measures in those intervention groups, a statistical significance was not observed (study II manuscript, figure 2, supplementary tables 2 and 3).

5.2.4 Adverse reactions to the study medications

Altogether 74% of the participants experienced local side effects in association with the use of study medications. During tacrolimus, triamcinolone acetonide or placebo use, 73%, 43% and 33% of the patients, respectively, reported local adverse effects. The most common local adverse effect in tacrolimus users was a burning or smarting sensation either when administering the drug on the oral mucosa or when eating or drinking. This occurred in 57% (n=12) of the patients. Forty-three percent (n=9) of tacrolimus users experienced increased sensitivity to hot, cold or spicy food, and 19% (n=4) reported altered taste sensation. Other less common side effects in tacrolimus users were an unpleasant sensation in the mouth, xerostomia and hoarseness (1 patient each). Triamcinolone acetonide use was associated with a smarting feeling in the mouth (n=1) and tenderness in the gingiva (n=2). Placebo users experienced burning and sensitivity to hot food or drink, tenderness in the gingiva and increased salivary flow after administering the paste (1 patient each).

Before the trial started, oral candidiasis was diagnosed in 37% of the participants (36%, 29% and 44% in the tacrolimus, triamcinolone acetonide and placebo groups). None of the participants who used tacrolimus during the initial trial period of 6–9 weeks were diagnosed with secondary oral candidiasis. On the other hand, 50% (n=2) of those who used triamcinolone acetonide for 6 weeks, 33% (n=1) of those who switched from triamcinolone to tacrolimus at week 3, 25% (n=2) of those who switched from placebo to tacrolimus at week 3 and 1 patient who used placebo for 6 weeks developed oral candidiasis.
Two (18%) patients in the initial tacrolimus group reported systemic adverse effects: nausea (n=2), headache (n=1) and nightmares (n=1).

5.3 Immunohistochemical expression of the studied molecules in OLP and the effect of tacrolimus (III–V)

5.3.1 Toll-like receptors 4 and 9

TLR4 and TLR9 immunoreactivity was observed mainly intracellularly in both normal and OLP mucosa. In normal oral epithelium, TLR4 was expressed only in the basal layer (Figure 3a). The expression was weaker than in the basal layer of OLP epithelium (p<0.001). In OLP, TLR4 staining intensity decreased gradually from the basal to the superficial layer of the epithelium (Figure 3b), and the differences between the basal and intermediate layers and basal and superficial layers in both clinically white and atrophic OLP were substantial (p<0.001). The difference in TLR4 staining intensity between the intermediate and superficial layers of the epithelium in clinically atrophic OLP was considerable (p<0.05), while in the clinically white OLP, the difference did not reach statistical significance. In the subepithelial inflammatory cells, TLR4 staining intensity did not differ markedly between OLP and normal oral mucosa. The subepithelial inflammatory infiltrate showed weaker TLR4 expression compared with the basal layer of the epithelium (P<0.001) in clinically atrophic and white OLP but when compared with other layers of the epithelium, no notable differences were found.

TLR9 immunoreactivity in normal oral mucosa was only observed in the superficial layer of the epithelium and the staining intensity was weaker than in the superficial epithelial layer in OLP (p<0.001). In OLP, the distribution of TLR9 staining exhibited a reversed pattern compared to TLR4, as TLR9 staining intensity increased gradually from basal to the superficial layer of the epithelium. The difference in the staining intensity in clinically white and atrophic OLP was substantial between basal, intermediate and superficial layers (p<0.001). The subepithelial inflammatory infiltrate in OLP demonstrated weaker TLR9 expression compared to the superficial layer of the epithelium (p<0.001) and stronger expression than the basal layer of the epithelium (p=0.001). No appreciable difference in the TLR9 staining intensity was found between the intermediate layer and the subepithelial inflammatory infiltrate (p<0.33).
No change in the TLR4 and TLR9 staining intensities were seen in a patient case where samples before and after treatment with topical 0.1% tacrolimus ointment were examined.

![TLR4 and TLR9 staining](image)

**Fig. 3. Schematic illustration of TLR4 and TLR9 staining in healthy oral mucosa (a, c) and OLP (b, d).**

**5.3.2 Hyaluronan, its receptor CD44, and its metabolizing enzymes HAS1–3 and HYAL1–2**

Hyaluronan and its main receptor CD44 were expressed homogeneously on the plasma membrane mostly in the basal and intermediate layers of the epithelium in both normal (control) and OLP epithelium (Figure 4a–d). However, HA staining intensity was stronger in the basal layer in OLP compared to normal oral epithelium ($p<0.001$). In the intermediate epithelial layer, more control than OLP samples showed considerable staining, fewer control than OLP samples showed some staining, and 17% of the controls showed no staining compared to none of the OLP samples ($p<0.001$). HA expression did not differ markedly in the superficial
epithelial layer in normal and OLP epithelium. In the subepithelial connective tissue, intense HA immunoreactivity was found in both controls and OLP samples.

CD44 expression localization and intensity in the epithelium did not show considerable differences between normal and OLP samples (Figure 4c and d). However, in the subepithelial tissues OLP specimens demonstrated stronger CD44 staining than the control samples, probably due to increased numbers of subepithelial inflammatory cells expressing the receptor in OLP. It is also possible that more mesenchymal cells (e.g. fibroblasts) express CD44 in OLP than in normal mucosa. CD44 staining in some subepithelial connective tissue fibers in control samples presumably represents soluble CD44 shed by epithelial cells.

HAS1 and HAS2 expression of variable intensity was seen in the cytoplasm and occasionally on the plasma membrane in both OLP and control samples (Figure 5a–d). HAS1 was not detected in the basal epithelial layer of healthy controls. HAS3 immunoreactivity was low in both control and OLP mucosa, showing only faint staining in some nuclei of the epithelial and inflammatory cells, and this was regarded as non-specific.

The staining intensity of HAS1 (p=0.001) and HAS2 (p<0.001) was stronger in the basal layer of the epithelium in OLP than in the control samples. In the intermediate epithelial layer, there was no difference in the HAS1 or HAS2 staining intensity between OLP and controls. In the superficial epithelial layer, both HAS1 and HAS2 showed greater staining intensity in control than in OLP samples (p<0.001). Notably, HAS1 and HAS2 showed a strong cytoplasmic granular-like staining pattern in the upper stratum intermedium and stratum superficiale in the normal epithelium that was not detectable in OLP samples nor in HAS3-stained control specimens. Inflammatory cells in the epithelium and in the connective tissue expressed HAS1 and HAS2 in OLP. Fibroblasts in the lamina propria showed HAS1 and HAS2 immunoreactivity in both control and OLP specimens.

HYAL1 and HYAL2 expression was localized mainly intracellularly, and HYAL2 also occasionally faintly on the plasma membrane (Figure 5e–h). HYAL1 and HYAL2 staining intensity were stronger in all layers of the epithelium in OLP than in control samples (p=0.011, p=0.004 and p<0.001 in the basal, intermediate and superficial layers, respectively, for HYAL1 and p=0.001, p=0.175 and p=0.008 in the basal, intermediate and superficial layers, respectively, for HYAL2). HYAL1 and HYAL2 were expressed in the subepithelial inflammatory cells and fibroblasts in OLP while in controls, their expression was detected mainly in stromal fibroblasts and endothelial cells.
The clinically white (reticular or plaque-like) and red (atrophic, erosive) types of OLP did not show marked differences in the immunoexpression of HA, CD44, HAS1–2, and HYAL1–2.

In the patient cases (n=3) treated with topical 0.1% tacrolimus, the samples taken after treatment showed that CD44 staining was decreased in the stroma and HYAL2 staining was slightly increased in the basal layer of the epithelium compared to the samples taken before the treatment. HA, HAS1–2 and HYAL1 did not show a change in immunoexpression before and after tacrolimus treatment.

Fig. 4. Schematic illustration of HA and CD44 staining in healthy oral mucosa (a, c) and OLP (b, d).
Fig. 5. Schematic illustration of HAS1–2 and HYAL1–2 staining in healthy oral mucosa (a, c, e, g) and OLP (b, d, f, h).
5.3.3 Cathepsin K

In general, CTSK was expressed with variable intensity as small granules in the cytoplasm of macrophages, fibroblasts, melanocytes, a few endothelial cells and probably some basal keratinocytes in OLP (Figure 5b), as demonstrated in part by double IHC. In addition, CTSK was noted extracellularly. In the normal oral mucosa, the epithelium was negative for CTSK immunostaining while some stromal fibroblasts expressed CTSK (Figure 5a).

CTSK positive cells were more numerous in OLP epithelium than in normal (control) oral epithelium (p<0.001). In the stroma, CTSK staining intensity was greater in OLP compared to control samples (p<0.001).

Langerhans cells, lymphocytes and mast cells did not show immunoreactivity for CTSK, as indicated by double IHC and IF. CTSK co-localized with TLR4 and TLR9 in some mucosal cells, presumably macrophages and melanocytes.

In the tacrolimus treated OLP patient cases (n=9), the number of CTSK positive cells decreased in the epithelium after treatment with tacrolimus. However, the observed changes were appreciable only as evaluated by one of the two investigators. In the stroma, there was a tendency for the number of CTSK positive cells to increase after treatment with tacrolimus, but the change did not show statistical significance.

![CTSK staining](image)

**Fig. 6. Schematic illustration of CTSK staining in healthy oral mucosa (a) and OLP (b).**
6 Discussion

6.1 Hypothyroidism and OLP

A few previous studies indicated that thyroid disorders may be associated with OLP. In a small sample of HCV-negative and HCV-positive OLP patients, thyroid dysfunction was found in 13% of the HCV-negative patients and in 15% of the HCV-positive patients (Carrozzo et al. 1999). Another study reported increased prevalence of serum anti-thyroid antibodies in OLP patients compared to controls (Chang et al. 2009). Furthermore, an epidemiologic study suggested an association of LP and hypothyroidism (Dreher et al. 2009). In addition, thyroxin medication was found to be more common in OLP patients compared to controls (Kragelund et al. 2003). After our finding of the association of hypothyroidism and OLP was published (study I), several other groups have reported also increased prevalence of thyroid diseases in patients with OLP in Northern and Southern Europe and Brazil (Ebrahimi et al. 2012, Garcia-Pola et al. 2016, Hirota et al. 2011, Lauritano et al. 2016, Lo Muzio et al. 2013, Robledo-Sierra et al. 2013, Robledo-Sierra et al. 2015). Interestingly, studies from Iran, Sicily and Croatia/Australia found no significant association of OLP and hypothyroidism (Compilato et al. 2011, Lavaee & Majd 2016, Vucicevic Boras et al. 2014).

Autoimmune thyroid diseases (AITD) are multifactorial T- and B-cell mediated organ-specific diseases thought to result from immune dysregulation (Lee et al. 2015, Ramos-Levi & Marazuela 2016). Two main AITDs are Graves' disease and Hashimoto's thyroiditis, manifesting as thyrotoxicosis and hypothyroidism, respectively (Antonelli et al. 2015). Both genetic and environmental factors trigger AITDs (Antonelli et al. 2015). AITD are associated with other autoimmune diseases such as rheumatoid arthritis, Sjögren’s syndrome and systemic lupus erythematosus (Antonelli et al. 2015, Lee et al. 2015).

The mechanism behind the association of thyroid disease and OLP is unknown. However, it has been proposed that circulating thyroid antibodies could trigger, even in subclinical asymptomatic chronic autoimmune thyroiditis, an organ-specific autoimmune response also in the oral mucosa (Lo Muzio et al. 2013). This hypothesis is based on a finding that most patients presumably developed OLP after the onset of thyroid dysfunction (Lo Muzio et al. 2013). Also in a study by Robledo-Sierra et al. (2015) the vast majority of OLP patients reported that the diagnosis of thyroid disease and start of thyroxin medication antedated the onset of OLP.
However, the prevalence of elevated serum antithyroid antibodies thyroid peroxidase (TPO) and thyroglobulin (Tg) did not differ between OLP patients without thyroid disease and the general population in a study done by the same group (Robledo-Sierra 2015). Of note however, they found that OLP patients without previously diagnosed thyroid disease had elevated levels of thyroid stimulating hormone (TSH), and low levels of free thyroxine 4 (FT4) more often than the general population, indicating a higher prevalence of hypothyroidism in OLP patients (Robledo-Sierra 2015). Furthermore, the expression of thyrotropin receptor (TSHR) was found in the basal epithelial layer in OLP lesions but not in the healthy oral mucosa, and thyroid stimulating hormone receptor (TSHR) expression had a tendency to be higher in OLP than in healthy oral mucosa, suggesting their possible association with OLP disease mechanism (Robledo-Sierra 2015). Also skin keratinocytes express functional TSHR and TG (Slominski et al. 2002). In our material we could not reliably determine if thyroid dysfunction was preceded by OLP.

The association of thyroid disorders and OLP or OLL raises the question of whether thyroid medication could be the causative factor of the oral disease. However, when only patients with a history of thyroid disease were analyzed, thyroxin medication per se was not associated with OLP or OLL in our data. Of note, lichenoid drug reactions caused by thyroid hormone supplementation are exceedingly rarely reported. In fact, only one case report describes a lichenoid eruption of the skin in association with thyroxin overdose (Kaur et al. 2003). Also the authors of the study where thyroid hormone supplementation use was associated with OLP, conclude that the thyroid disease is probably behind the association rather than the medication (Robledo-Sierra et al. 2013).

Furthermore, the association of thyroid dysfunction and OLP could be explained by impaired immune system function associated with genetic factors. However, only a few of the same genes are implicated in both diseases. Genes linked to susceptibility to AITD include 1) MHC class II, especially human leukocyte antigen (HLA)-DRB3, 2) immune-regulatory genes CD40 molecule (CD40), cytotoxic T-lymphocyte associated protein 4 (CTLA4), protein tyrosine phosphatase, non-receptor type 22 (PTPN22), forkhead box P3 (FOXP3) and interleukin 2 receptor subunit alpha (IL2RA) and 3) thyroid-specific genes TG and TSHR (Hasham & Tomer 2012). In OLP, increased prevalence of several MHC genes has been reported, namely HLA-Bw57 (Porter et al. 1993), HLA-DRw9 (Watanabe et al. 1986), HLA-DRB3 (Jontell et al. 1987) and HLA-
In addition, FOXP3 gene expression levels in OLP patients are higher than in controls (Lei et al. 2014).

6.2 Topical tacrolimus and triamcinolone acetonide in the management of OLP

Topical corticosteroids have long been the drug of choice for treating patients with symptomatic OLP (Bagan et al. 2012). Even though placebo-controlled RCTs, albeit with their limitations, are considered the gold standard in intervention trials (Bothwell et al. 2016), we have not identified any previous studies comparing topical triamcinolone acetonide or tacrolimus with placebo in OLP. A few placebo-controlled trials of topical corticosteroids in the management of OLP do exist, but many of them suffer from methodological issues related to outcome measures and reporting (Cilurzo et al. 2010, Kazancioglu & Erisen 2015, Tyldesley & Harding 1977, Voute et al. 1993) and blinding (Carbone et al. 1999) (Listed in table 4, in comparison with the present study II). The latest Cochrane review of interventions for treating OLP states that no RCTs that compare steroids with placebo in patients with symptomatic OLP were found (Thongprasom et al. 2011). Also relatively few RCTs that compare topical tacrolimus with corticosteroids in OLP are available, some showing no difference in the efficacy, some showing that tacrolimus is more effective, and one reporting that topical corticosteroids are more effective (Azizi & Lawaf 2007, Corrocher et al. 2008, Laeijendecker et al. 2006, Radfar et al. 2008, Revanappa et al. 2012, Sonthalia & Singal 2012, Sivaraman et al. 2016) (Listed in table 5, in comparison with the present study II). Some of these studies also have the above-mentioned methodological issues. In general, evaluation and comparison of the results of the published RCTs in OLP is challenging due to heterogeneous reporting (Lopez-Jornet & Camacho-Alonso 2010a).
<table>
<thead>
<tr>
<th>Author (year)</th>
<th>n</th>
<th>Therapy and its duration</th>
<th>Main findings</th>
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<tbody>
<tr>
<td>Tyldesley &amp; Harding (1977)</td>
<td>23/20*</td>
<td>Active: betamethasone valerate aerosol (n=11) 2 puffs 4 x daily (total daily dose 800µg) 8 weeks, most patients did not take the full dose. Placebo: aerosol containing Arcton-11 and -12 (n=9) 2 puffs 4 x daily 8 weeks</td>
<td>Response to treatment better in the betamethasone valerate group.</td>
</tr>
<tr>
<td>Voute et al. (1993)</td>
<td>40</td>
<td>Active: 0.025% fluocinonide in an adhesive base (n=20) at least 6 x daily 6 weeks. Placebo: adhesive ointment base with 40% hyromellose in paraffin (n=20) at least 6 x daily 6 weeks</td>
<td>Fluocinonide more effective than placebo in reducing signs and symptoms.</td>
</tr>
<tr>
<td>Carbone et al. (1999)</td>
<td>60/50*</td>
<td>Active: 0.05% clobetasol propionate ointment (n=20) 2 x daily 4 months + 1 x daily 2 months or 0.05% fluocinonide ointment (n=20) 3 x daily 2 months + 2 x daily 2 months + 1 x daily 2 months, both in 4% hydroxyethyl cellulose gel + miconazole gel + CHX rinse Placebo: 4% hydroxyethyl cellulose gel (n=10) 2 x daily 6 months</td>
<td>Clobetasol more effective than fluocinonide or placebo in reducing signs and symptoms. No difference between fluocinonide and placebo.</td>
</tr>
<tr>
<td>Cilurzo et al. (2010)</td>
<td>48</td>
<td>Active: 24µg clobetasol-17 propionate in mucoadhesive tablet (n=16) or clobetasol ointment mixed with Orabase 123 µg/application mixed with Orabase&lt;sup&gt;TM&lt;/sup&gt; (n=16) 3 x daily 4 weeks Placebo: polysodium methacrylate, methylmethacrylate + hydroxypropylmethylcellulose (HPMC)+MgCl&lt;sub&gt;2&lt;/sub&gt; mucoadhesive tablets (n=16) 3 x daily 4 weeks</td>
<td>Clobetasol more effective than placebo in reducing signs and symptoms.</td>
</tr>
<tr>
<td>Author (year)</td>
<td>n</td>
<td>Therapy and its duration</td>
<td>Main findings</td>
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</table>
| Kazancioglu & Erisen (2015)   | 120 | *Active*: 0.05mg/ml dexamethasone mouthwash + nystatin solution 4 x daily 1 month (n=30) or low level laser therapy (LLLT) (n=30) or ozone (n=30), both 2 x weekly, max 10 sessions  
*Placebo*: Solution with base ointment (n=30) rinse 4 x daily 1 month                                                                 | Dexamethasone and ozone more effective than LLLT and placebo in reducing signs. Dexamethasone, ozone and LLLT more effective than placebo in reducing symptoms |
| Study II manuscript (2016)    | 27  | *Active*: 0.1% triamcinolone acetonide paste (n=7) or 0.1% tacrolimus ointment (n=11) 3 x daily 6-9 weeks + 6-month follow-up with sporadic use for tacrolimus and intermittent use for topical corticosteroid if needed  
*Placebo*: Orabase paste (n=9) 3 x daily 3-6 weeks                                                                                                                                             | Triamcinolone and tacrolimus equally and more effective than placebo in reducing signs and symptoms |

*completed the study*
<table>
<thead>
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<th>Author (year)</th>
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<th>Therapy and its duration</th>
<th>Main findings</th>
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<tr>
<td>Laeiendecker et al. (2006)</td>
<td>40</td>
<td>TAC: 0.1% ointment (n=20) 4 x daily 6 weeks Control: 0.1% triamcinolone acetonide in hypromellose 20% ointment (n=20) 4 x daily 6 weeks</td>
<td>TAC more effective than triamcinolone in reducing signs</td>
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<td>Azizi &amp; Lawaf (2007)</td>
<td>60</td>
<td>TAC: 0.1% ointment (n=30) 4 x daily 4 weeks Control: 0.1% triamcinolone in orabase (n=30) 4 x daily 4 weeks</td>
<td>Both as effective in reducing signs and symptoms</td>
</tr>
<tr>
<td>Radfar et al. (2008)</td>
<td>30</td>
<td>TAC: 0.1% ointment (n=15) 4 x daily 2 weeks + 3 x daily 2 weeks + 2 x daily 1 week + 1 x daily 1 week + nystatin rinse 1 x daily Control: 0.05% clobetasol ointment (n=15) 4 x daily 2 weeks + 3 x daily 2 weeks + 2 x daily 1 week + 1 x daily 1 week + nystatin rinse 1 x daily</td>
<td>Both as effective in reducing signs and symptoms</td>
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<tr>
<td>Corrocher et al. (2008)</td>
<td>32</td>
<td>TAC: 0.1% ointment prepared (n=16) 2 ml 4 x daily 4 weeks Control: 0.05% clobetasol ointment (n=16) 2 ml 4 x daily 4 weeks</td>
<td>TAC more effective than clobetasol in reducing signs and symptoms</td>
</tr>
<tr>
<td>Sonthalia &amp; Singal (2012)</td>
<td>40</td>
<td>TAC: 0.1% ointment (n=20) 2 x daily 8 weeks + CHX rinse 3 x daily 8 weeks Control: 0.05% clobetasol propionate ointment (n=20) 2 x daily 8 weeks + CHX rinse 3 x daily 8 weeks</td>
<td>Both as effective in reducing signs and symptoms</td>
</tr>
<tr>
<td>Revanappa et al. (2012)</td>
<td>60</td>
<td>TAC: 0.1% powder in orabase (n=30) 3 x daily 2 weeks Control: 0.1% triamcinolone acetonide in orabase (n=30) 3 x daily 2 weeks</td>
<td>TAC more effective than triamcinolone acetonide in reducing signs and symptoms</td>
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<td>- not available in PubMed</td>
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<tr>
<td>Hetiarachchi et al. (2016)</td>
<td>68</td>
<td>TAC: 0.1% cream (n=34) 2 x daily 3 weeks Control: 0.05% clobetasol propionate cream (n=34) 2 x daily 3 weeks</td>
<td>Both effective in reducing signs and symptoms (interventions not compared)</td>
</tr>
<tr>
<td>Author (year)</td>
<td>n</td>
<td>Therapy and its duration</td>
<td>Main findings</td>
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<tr>
<td>Sivaraman et al. (2016)</td>
<td>30</td>
<td>TAC: 0.03% ointment in Orabase (n=10) 4 x daily 6 weeks</td>
<td>Clobetasol and triamcinolone more effective than TAC, and clobetasol more effective than triamcinolone in reducing signs</td>
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<tr>
<td></td>
<td></td>
<td>Controls: 0.1% triamcinolone acetonate ointment (n=10) 4 x daily 6 weeks or 0.05% clobetasol ointment (n=10) 4 x daily 6 weeks</td>
<td></td>
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<tr>
<td>Study II manuscript (2016)</td>
<td>27</td>
<td>TAC: 0.1% ointment (n=11) 3 x daily 6-9 weeks</td>
<td>TAC and triamcinolone equally effective and better than placebo in reducing signs and symptoms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls: 0.1% triamcinolone acetonide paste (n=7) or Orabase paste (placebo) (n=9) 3 x daily 6-9 weeks</td>
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One of the most important aspects of intervention trials is the assessment of outcome. Numerous different OLP scoring systems have been created, but none have gained universal acceptance (Chainani-Wu et al. 2008, Escudier et al. 2007, Lopez-Jornet & Camacho-Alonso 2010a). Especially for future research purposes, a validated, commonly approved outcome measure for OLP would be useful (Wang & van der Waal 2015). Recently, it has been proposed that quality of life-measures could be applied to assess the outcome of intervention in OLP (Wang & van der Waal 2015). Furthermore, the amount of change in the outcome measures regarded as indicative of therapeutic effect seems to vary between the publications. For example, Laeijendecker et al. (2006) graded ≥30% improvement in the extent and the severity of the lesions as improved and Sonthalia & Singal (2012) defined >50% improvement in their net clinical score (CS) as partial improvement. In our novel CS (study II), we set a ≥20% decrease in the score value as an indicator of therapeutic response. This cut-off value, similar to previously published ones, was not based on any further analyses or calculations.

In our CS the inter-rater reliability was strong, but correlation of clinical signs and symptoms reported by the patients was weak. Similarly, a previous study showed that the correlation of symptoms and clinical signs scores in OLP varied from minimal to high (Chainani-Wu et al. 2008). Visual analogue scale (VAS), one part of our CS has been shown to be a valid measure for symptom assessment (Flaherty 1996, Miller & Ferris 1993). Furthermore, the expected decrease in CS values in the active comparator groups compared to placebo comparator group supports the validity of our CS. For this reason, we believe that the present CS is a valid instrument in evaluating treatment response in OLP and could well be used in the future intervention trials.

One of the strengths of the clinical trial (II) is its design, but unfortunately it is underpowered, and is therefore considered a pilot study. However, the results indicated, for the first time, that topical 0.1% triamcinolone acetonide or 0.1% tacrolimus are more effective than placebo in reducing the signs and symptoms of OLP after three weeks of use. Both drugs showed equal efficacy. Interestingly, during the first week of intervention, placebo or triamcinolone acetonide produced a greater reduction in pain/discomfort than tacrolimus, probably reflecting 1) placebo effect and 2) tacrolimus’ known ability to cause burning at the initial phase of use. Although both active drugs demonstrated a similar decrease in CS and VAS from baseline to 6 months, the change was statistically significant only in the tacrolimus group, reflecting the small sample size in the topical corticosteroid
group during the 6-month follow-up. However, the VAS values assessed at home during the 6-month follow-up period showed that tacrolimus had a tendency to provide a better long-term pain reduction than topical corticosteroid.

In conclusion, the findings of the study II corroborate the results of a few previous clinical trials (Azizi & Lawaf 2007, Radfar et al. 2008, Sonthalia & Singal 2012). Topical tacrolimus may be considered an effective alternative for management of symptomatic OLP, especially in cases refractory to topical corticosteroids. Although the adverse effects of topical tacrolimus use in OLP are typically local and benign, the long-term safety of the drug remains to be established.

6.3 The role of toll-like receptors 4 and 9 in OLP

Pathogenetic mechanisms of many chronic inflammatory and autoimmune diseases are believed to associate with inappropriate response of the TLR, due to particular genetic factors, or from inadequate quantity or quality of ligands (Gianchecchi & Fierabracci 2015, Ospelt & Gay 2010).

Literature on the participation of TLRs 4 and 9 in the pathogenesis of LP or OLP is relatively scarce. Earlier, elevated expression of toll-like receptor 4 protein and transcripts was reported in OLP epithelium as well as in UWS keratinocytes compared to controls (Janardhanam et al. 2012). Therefore, our finding of increased immunohistochemical expression of TLR4 in OLP (study III) confirms the results of Janardhanam et al. (2012). In their report, normal epithelium expressed TLR4 mainly in the lower spinous epithelial layer, while in our study it was located only in the basal epithelial layer. Recently, equal TLR4 cytoplasmic immunostaining was observed in the lower aspect of epithelium in OLP and non-specific inflammatory control tissue, but stromal inflammatory infiltrate contained more TLR4 staining cells in OLP than in the non-specifically inflamed controls (Sinon et al. 2016). We observed that while OLP samples had more inflammatory cells in the lamina propria than healthy controls, the TLR4 staining intensity in the inflammatory cells was only slightly greater in OLP sections.

The present study shows, for the first time, increased immunohistochemical expression of TLR9 in OLP compared with healthy oral mucosa. This finding is in accordance with a previous report of elevated toll-like receptor 9 protein and mRNA expression in cutaneous LP compared with controls (Li et al. 2007).

In contrast to our finding that TLR9 staining was seen only in the superficial epithelial layer in healthy controls, an earlier report described that TLR 9
immunoreactive cells were more numerous in the basal epithelial layer in healthy gingival tissue, and gradually decreased toward the superficial layer (Beklen et al. 2008). That study and our results are similar in the weaker expression of TLR4 and TLR9 in healthy oral mucosal connective tissue than in inflamed tissue. Weak immunodetection of TLRs 4 and 9 in the healthy oral mucosa observed in the present study may be due to a protective mechanism to prevent constant activation of the immune system in an environment replete with micro-organisms, as suggested in the case of skin (Ospelt & Gay 2010).

The mechanism behind TLR 4 and TLR9 induction in OLP could be associated with bacteria or viruses. Lipopolysaccharide in the outer wall of Gram-negative bacteria is a known ligand for TLR4 (Molteni et al. 2016). Previously, activation of TLR2 and TLR4 by periodontal Gram-negative bacteria was reported (Kikkert et al. 2007). Interestingly, increased numbers of Gram-negative bacteria have been detected (samples collected by non-invasive methods) in gingival LP lesions compared with non-LP gingival sites in the same person (Bornstein et al. 2008). In addition, bacteria have been reported to be present in OLP lesions in the epithelium and lamina propria, suggesting that in OLP the epithelial barrier is breached enabling the invasion of bacteria into tissue (Choi et al. 2016). In the same study also the bacteria found to be increased in OLP mucosa were periodontitis-associated, often gram-negative bacteria (Choi et al. 2016). Hence, the observed increased expression of TLR4 in OLP could be due to the increase in Gram-negative bacteria in OLP lesion sites.

Bacterial or viral DNA-containing unmethylated (deoxycytidyl-phosphate-deoxyguanosine) CpG motifs are recognized by TLR9 (Ospelt & Gay 2010). Also TLR4 is able to bind viral structural proteins (Kawai & Akira 2010, Molteni et al. 2016). At the moment, the association of viral or bacterial DNA in the oral mucosa and TLR activation in OLP is unexplored. However, HCV-RNA strands have been detected in oral mucosa in patients with chronic HCV-infection both in OLP and non-OLP cases (Arrieta et al. 2000, Kurokawa et al. 2003). As mentioned above, bacteria were demonstrated in epithelial and T-cells in OLP (Choi et al. 2016). Taken together, the role of micro-organisms in the initiation of OLP is still hypothetical and their detection in OLP lesions does not prove a causal relationship, but could be the result of altered mucosal surface or increased permeability of the mucosa in OLP (Choi et al. 2016).

Endogenous ligands that can activate TLR-mediated signaling include molecules that are derived either from the extracellular matrix or from cells, either
actively secreted or passively released (Molteni et al. 2016). Some of these molecules or proteins have been studied in OLP, namely HSPs, DEFB4A and HA. Increased expression of the antimicrobial peptide DEFB4A has been reported in OLP epithelium (Abiko et al. 2002). HSPs are released by cells after cellular damage or under stressful conditions (Calderwood 2007). Many reports have shown altered HSPs immunoeexpression in OLP (Bramanti et al. 1995, Chaiyarit et al. 1999, Chaiyarit et al. 2009, Garcia-Garcia et al. 2013, Sugerman et al. 1995), although contrasting results have also been published (Seoane et al. 2004). Of note, the role of HSP70 as an endogenous TLR4 ligand has been questioned, since many studies describing this ligand receptor interaction used a recombinant protein which may have been contaminated by endotoxin (LPS) (Ospelt & Gay 2010). HA is a large extracellular matrix molecule that is degraded into smaller fragments during tissue injury or inflammation, and these fragments may bind to TLR4 (Lee-Sayer et al. 2015, Termeer et al. 2002). As we observed increased expression of HA in OLP (study IV), it cannot be excluded that HA acts as a TLR ligand in OLP.

Genetic variation in TLRs is associated with susceptibility to infectious and autoimmune diseases (Netea et al. 2012). In OLP, TLR3 gene polymorphism associated with an increased risk of the disease (Stanimirovic et al. 2013). In addition, downregulation of TLR-mediated signaling pathways in OLP was recently reported (Sinon et al. 2016). In cutaneous LP, impaired TLR signaling pathways are also suggested to play a role; agonists of TLR7/TLR8 or TLR9 were shown to overcome impaired IFN-α secretion in LP (Domingues et al. 2015). In the future, in TLR gene variations in OLP may provide more interesting findings.

### 6.4 The role of hyaluronan, its receptor CD44 and its metabolizing enzymes HAS1–3 and HYAL1–2 in OLP

Previously, the expression of HA and its metabolizing enzymes have not been described in LP or OLP. The study IV demonstrates the distribution of HA, its synthases (HAS1, 2 and 3), and degrading enzymes (HYAL1 and 2) in OLP by immunohistochemistry. HA was expressed homogeneously on the plasma membrane mostly in the lower part of epithelium in OLP and the staining intensity of HA was strongest in the basal layer. In the healthy control epithelium, HA immunoreactivity was detected similarly mostly in the basal and intermediate epithelial layers confirming the findings of previous studies (Kosunen et al. 2004, Tammi et al. 1990). In contrast to OLP however, the staining intensity was most
intense in the intermediate epithelial layer in the control samples. Subepithelial connective tissue exhibited strong HA staining in both OLP and healthy controls.

The more intense staining intensity of HA in the basal epithelial layer compared to healthy epithelium may indicate increased synthesis of HA in that location in OLP, as we also observed increased staining of HAS1 and HAS2 in OLP in the same location. However, although the upper intermediate and superficial epithelial layers exhibited stronger immunoreactivity for HAS1 and HAS2 in control samples compared to OLP, a corresponding increase in HA immunoreactivity was not seen in those areas. This discrepancy between HAS and HA expression could possibly be explained by HAS not being enzymatically active (Rilla et al. 2005). Furthermore, HAS1 and HAS2 staining was observed in a granular or vesicular pattern intracellularly in the upper intermediate and superficial layers in healthy epithelium, probably located in intracellular glycogen vesicles. Interestingly, in OLP epithelium these vesicles were not seen.

We observed elevated HYAL immunoreactivity in OLP epithelium compared to healthy epithelium; only for HYAL2 in the intermediate layer of the epithelium, the increase in immunohistochemical expression did not reach statistical significance. Increased HYAL expression could be expected to lead to decreased HA expression, but this was not seen in the present study. However, as both accelerated HA synthesis and degradation with increased HA accumulation is often noted in pathological conditions, such as in the tumor microenvironment (Schmaus et al. 2014), these could similarly occur in OLP.

Although HA expression was increased basally in OLP epithelium, expression of CD44, the main receptor for HA, was not altered in OLP epithelium compared to control tissue, which confirms the results of a previous study (Neppelberg et al. 2007). Contrary to these findings, decreased expression of the isoform CD44v6 (Chaiyarit et al. 2008), and increased expression of CD44 in OLP lesional epithelium compared to healthy controls has been reported (Santarelli et al. 2015). However, in the study IV, stronger CD44 immunoreactivity was noted in the stroma in OLP compared to controls, probably due to an increased number of inflammatory cells and maybe mesenchymal cells expressing CD44 in OLP. The binding avidity of CD44 for HA may increase during inflammation as a consequence of simultaneous elevation of HA synthesis and release of inflammatory mediators (Heldin et al. 2008). For example, activated T-cells are able to bind HA when stimulated by cytokines IL2 and TNF and chemokines such as IL8 and CCL5, and monocytes may be activated to bind HA by TNF, IFNG and IL1A (Lee-Sayer et al. 2014).
Enhanced HA interaction by immune cells during inflammation may assist in recruiting more immune cells to the site of inflammation, keep the cells at the site as well as increase their survival and function (Lee-Sayer et al. 2015).

In tissue homeostasis and resolution of inflammation, HA exists as high molecular weight (HMW)-HA and has protective and anti-inflammatory effects. At sites of inflammation and injury, HA is fragmented to smaller sizes leading to increased amounts of HA and different biological functions compared to HMW-HA (Monslow et al. 2015, Petrey & de la Motte 2014). In general, low molecular weight (LMW)-HA enhances the inflammatory response by promoting angiogenesis, inflammation, maturation of APCs and cell migration (Bollyky et al. 2009, Termeer et al. 2000), mediated via, for example, MMPs and pro-inflammatory cytokines such as TNF and IL12. In addition to purely CD44-mediated effects of HA fragments, they may mediate pro-inflammatory or anti-inflammatory signals via TLR2 and/or TLR4 (Lee-Sayer et al. 2015, Monslow et al. 2015). Furthermore, HMW-HA is capable of promoting immunosuppression via binding to TLR4, leading to enhanced release of the immunosuppressive cytokine IL10 (Asari et al. 2010). In fact, it was demonstrated in a mouse model that oral administration of HA900 modulated Th-1-type autoimmune disease and inflammation via TLR4 in intestinal epithelial cells (Asari et al. 2010). As we and others have reported increased expression of TLR4 in OLP epithelium, HA–TLR interaction may have a role in OLP pathogenesis (Janardhanam et al. 2012). However, in the present study, we did not assess the molecular size of HA nor the possible co-localization of HA and TLR in OLP; these investigations would provide interesting research topics for the future.

Interestingly, the management of OLP with topical hyaluronic acid has shown efficacy in reducing pain and lesion size in two RCTs (Nolan et al. 2009, Shetty et al. 2016). In light of the known HA–TLR4 interaction, it is interesting to speculate that topical HA could have produced immunosuppressive effects and clinical improvement in OLP. The molecular weight of HA defines its function, and the different size fragments of the HA molecule present in the tissue can exert alternate, often contrasting effects (Monslow et al. 2015). Even small changes in the proportion of the different size HA fragments may shift the outcome of cellular functions. It is therefore possible that therapies may change the ratio of the active HA fragments and change the balance of signaling in favor of restoring homeostasis (Monslow et al. 2015).
Overall, the results from the present study indicate that HA metabolism is altered in OLP, as signs of both increased and decreased HA synthesis, and increased HA degradation were seen in the epithelium.

6.5 The role of cathepsin K in OLP

Study V showed that CTSK immunoexpression was significantly increased in OLP compared to healthy oral mucosa. CTSK staining was most intense around the epithelial-stromal interface where some of the characteristic pathologic changes in OLP occur. The cells mainly expressing CTSK were macrophages and epithelial DCs, some of which were identified as melanocytes. Although melanocytic tumors express CTSK, its expression in melanocytes in inflammatory mucocutaneous disease has not been reported before (Rao et al. 2014). We suspected that the CTSK-positive, morphologically dendritic cells in the epithelium would be LCs, although we could not confirm this by double immunostaining. Previously however, bone marrow-derived DCs were shown to express CTSK (Asagiri et al. 2008).

In line with our findings, activated cultured monocyte-derived but not normal resident macrophages, were shown to express CTSK (Punturieri et al. 2000). We observed that only a few basal keratinocytes and endothelial cells stained for CTSK in OLP. In periodontitis however, gingival keratinocytes and endothelial cells were found to express CTSK (Beklen et al. 2015). The same study also showed that more mucosal macrophage-like cells and fibroblast-like cells stained for CTSK in periodontitis compared to normal oral mucosa, and that TNF enhanced the secretion of active CTSK from fibroblasts in culture. Moreover, CTSK was expressed in macrophages and DCs and implicated in inflammation and bone erosion in a mouse model for periodontitis (Hao et al. 2015b). We could not detect CTSK in lymphocytes, and this finding is in line with a study on experimental osteoarthritis where splenic T-cells did not show CTSK expression (Asagiri et al. 2008).

CTSK involvement in the innate immune processes may be associated with its ability to proteolytically process TLRs, so that their structure becomes suitable for antigen recognition (Ewald et al. 2011). CTSK may also participate in the cleavage of proteins that interfere with the association of pathogen-derived unmethylated DNA with TLR9 (Asagiri et al. 2008, Takayanagi 2010). In addition, TLR4 activation may be required for CTSK expression (Abdollahi-Roodsaz et al. 2009, Hirabara et al. 2013). These findings are interesting in relation to earlier reports of
toll-like receptors 4 and 9 induction in OLP (Ge et al. 2012, Janardhanam et al. 2012). Our observation that CTSK was co-located with TLR4 and TLR9 in some oral mucosal cells, likely in macrophages and melanocytes, suggests that these molecules could be interacting in OLP.

Although primarily implicated for lysosomal proteolysis, cysteine cathepsins are known to participate also in the extracellular degradation of proteins (Fonovic & Turk 2014). During physiological as well as pathological conditions, cysteine cathepsins can be secreted into the extracellular space, where their activity and stability are modified by glycosaminoglycans (Fonovic & Turk 2014). We noted that CTSK was located also extracellularly in OLP. This could be due to, for example, macrophages or fibroblasts releasing the enzyme into the extracellular milieu. Since our results suggest that HA metabolism is altered in OLP, it is an intriguing possibility that HA and CTSK could interact in and contribute to the pathogenesis of OLP.

CTSK is a powerful elastinolytic protease. Although CTSK is not required for extracellular elastin degradation (Punturieri et al. 2000), it may have a role in ECM degradation. Expression of ECM proteins, including collagen type 1, are altered in OLP (Becker & Schuppan 1995). In addition, elastin in the connective tissue is required for the non-keratinized phenotype of normal oral epithelium, and aberrant keratinization of the oral mucosa in reticular OLP may be related to elastolysis by elastases (Hsieh et al. 2010, Liao et al. 2012).

Taken together, our results suggest that macrophages, melanocytes and to some extent keratinocytes and DCs could be activated in OLP possibly partly due to the involvement of CTSK in OLP. Based on our findings, CTSK may be implicated in OLP pathogenesis, although we have no proof that CTSK is active and functioning in OLP. The role of CTSK in OLP should be more thoroughly investigated in the future.

6.6 The molecular level effect of tacrolimus on the studied molecules

Topical tacrolimus seemed to decrease the expression of CD44 in the stroma and CTSK expression in the epithelium, and increase slightly the expression of HYAL2 in the basal cell layer and CTSK expression in the stroma. However, these observations must be interpreted with caution because of the small sample size, and ideally should be repeated with a larger sample size. The decrease in CD44 expression after tacrolimus treatment is probably associated with a reduced number of lymphocytes and
macrophages since T lymphocytes, after activation by antigen, and monocytes after stimulation by inflammatory agents, are induced to bind CD44 to HA (Johnson & Ruffell 2009). In addition, experiments using monoclonal antibodies against CD44 in mouse models of chronic inflammatory disease have demonstrated reduced disease severity that is believed to result from decreased leukocyte recruitment (Johnson & Ruffell 2009). In addition, the reduction of soluble CD44 in connective tissue fibers may have accounted for the decrease of CD44 expression, although a previous study did not find reduced CD44 expression in cultured rat cardiac rejection or normal fibroblasts after tacrolimus treatment (Johnsson et al. 2004). The observed slight increase of HYAL2 expression in the basal epithelial layer may suggest that during tacrolimus treatment and subsequent resolution of inflammation in OLP, the degradation of HA is increased. The tendency of CTSK expression to diminish in the epithelium and increase in the stroma after treatment could be explained by the fact that tacrolimus reduces the amount of epithelium infiltrating macrophages that express CTSK, and the number of stromal lymphocytes (negative for CTSK), leading to a relative increase in CTSK-positive cells (fibroblasts, endothelial cells, and macrophages) in the stroma.

6.7 Methodological considerations

6.7.1 Epidemiologic study

Because of the retrospective nature of the case-control epidemiologic study (I), some studied characteristics could not be controlled or reliably measured. In particular, the medical histories in the patient files where the data were collected could have been incomplete due to inaccurate reporting by the patients, doctors or students. However, normally the medical histories are recorded very carefully in the hospital and university clinics where the data were gathered. In addition, it is reasonable to assume that there was no difference in the accuracy of the medical histories between cases and controls.

Diagnosing patients as having OLP or OLL based on the information in the patient files includes a possibility for error. The reporting of clinical features of oral disease is likely to be inaccurate in some cases. The histopathologic diagnosis is subjective and inter- and intra-rater variability is noted (van der Meij et al. 1999). However, both clinical and histopathological diagnoses were combined, and the
final diagnosis was rendered by using the suggested modified WHO criteria (van der Meij & van der Waal 2003).

6.7.2 Clinical trial

In RCTs there are a multitude of patient-, investigator-, intervention- and trial design-related factors that can never be fully controlled. Regarding the treatment groups, the patients in study II were randomly allocated to any one of groups. However, because of the small sample size, the baseline characteristics of the groups were somewhat dissimilar. In this kind of clinical trial, it would be difficult to have totally comparable baseline characteristics for the study groups.

Although a placebo comparator is considered necessary in medication intervention trials, we know that a pure placebo effect is difficult to measure (Finniss et al. 2010). The placebo response includes also the natural healing that may occur. It is also known that if the participant is aware that there is an active comparator, and if he/she believes he/she is receiving it, that probably affects the results. Therefore, it would be more ideal to compare placebo medication to no medication at all (Hrobjartsson & Gotzsche 2010). In a disease like OLP, the symptoms are known to fluctuate naturally. However, in this study we did not have a no treatment group. When comparing the active and placebo treatments, a recent systematic review and meta-analysis observed that active and placebo treatments often have similar effect sizes (Howick et al. 2013). On the other hand, a systematic review of randomized trials with a placebo group versus no intervention group, found that placebo interventions had no clinically important effect in general (Hrobjartsson & Gotzsche 2010). However, in some trials the placebo may have produced some effect for patient-reported outcomes, especially for pain and nausea (Hrobjartsson & Gotzsche 2010). Indeed, in this study (II) we detected an initial effect in the patient-assessed VAS scores for discomfort/pain in the placebo group, but the effect diminished at week 3, whereas the placebo had no effect on the CS.

Giving placebo medicine to a symptomatic patient when a therapy that is likely to relieve his/her symptoms is available, may be considered unethical. In the present trial, we partly resolved this problem by switching the medication to tacrolimus at week 3 if no treatment response was apparent. In addition, the patients were informed that they could withdraw from the trial at any point.

Blinding in clinical trials is important to increase objectivity of assessment of outcomes. In the present trial, for reasons mentioned above, we could hold the design strictly double-blind up to week 3, when those patients who did not respond
to intervention were switched to tacrolimus. The patients were told they were switched to active medicine, but the investigator was aware that it was tacrolimus. The patients that responded to intervention at week 3 continued to use the same medication for another 3 weeks and thus, for those patients the trial design was double-blind up to 6 weeks. This difference in the blinding among the treatment groups could have affected the results.

The medicines used in the trial were prepared by the hospital pharmacists, and even though they were blinded so that the containers were identical and the labels had no indication of the intervention, they could not be made exactly identical in consistency or color. This could have affected the patients’ perceptions of what intervention they were receiving.

We found that patient cooperation in general was good, and we lost contact with only one patient at 6 months. However, the assessment of discomfort/pain by patients at home was quite varied, and shows that detailed patient instruction in the beginning and revision at visits, as well as checking the assessments is extremely important during a clinical trial.

Unfortunately, the sample size of the present trial was below optimal, which lowered the power of the study. The problem with accruing the pre-specified number of patients was mainly due to strict inclusion criteria, in which only patients with relatively severe disease were considered for inclusion. In addition, we had difficulties to engage collaborators from Finland. Of note, for a period, funding for the study was cut off, which also affected the final number of patients included. The aforementioned reasons underline the challenges of investigator-initiated clinical trials of a relatively rare disease, in a small country and in the present research environment. Future RCTs of OLP definitely need multicenter collaboration and funding (Pihlstrom BL & Curran AE 2008).

The primary outcome measure (CS) used was similar to many that were previously employed (Wang & van der Waal 2015). To date, none of the scoring systems of OLP is widely accepted for use. Therefore, for future research purposes and comparison of results of clinical trials, it would be beneficial to have a uniform outcome measure for OLP.

6.7.3 Immunohistochemistry

IHC is a highly valuable tool for direct visualization of biomarker expression in different tissue levels. At best, it confirms the presence and localization of an
antigen in the tissue and gives an indication of the amount of antigen. However, IHC cannot determine if the observed biomarkers are active and functional in the tissue. Moreover, the validity of IHC depends to a great extent on the quality of the immunostaining. Major factors affecting this quality are antibody quality, tissue fixation and processing, antigen retrieval (unmasking of epitope) and sensitivity of the detection method (Werner et al. 2000). Dilution of the antibody also affects the results of IHC, and may greatly influence quantification (Walker 2006). The tissue used in the IHC studies was archival, formalin-fixed, paraffin-embedded, and the oldest blocks were approximately 20 years old. The antigenicity of the tissue may be altered during the long storage, thus possibly affecting the results. However, the risk of degradation of epitopes is mainly associated with stored, precut unstained slides (Bertheau et al. 1998, Grillo et al. 2015). In this study we used freshly cut slides from the blocks, so the possibility of false negative or positive results due to decreased immunoreactivity of the tissue is estimated to be low. However, we do not have records of the exact time interval between cutting of the paraffin slides and their staining. Also pre-staining conditions might affect the results of IHC. The formalin fixation of tissue by itself is able to cause epitope masking or denaturation (Werner et al. 2000). Too short or too long a fixation time, old tissue processing solutions, and excessive paraffin temperature during embedding may affect antigen expressivity (Werner et al. 2000). Although we don’t have exact information about these factors for the specimens used, the biopsies were taken in the hospital or university clinic where the fixation and processing of tissue is done by a fixed routine. The storing conditions (e.g. temperature, humidity) of the specimens are expected to be fairly stable in the hospital pathology department.

The use of positive and negative controls is of paramount importance for the validity of immunohistochemistry (Hewitt et al. 2014, Torlakovic et al. 2015). In the present study, negative and positive controls (tissue previously shown to express the antigen) were employed in order to ensure the specificity of the staining. However, the negative controls were not always ideal, as omission of the primary antibody instead of non-immune IgG was used.

Different antigen retrieval methods were tested for some antibodies until the staining result was considered optimal. Mainly routinely used detection methods were applied for the staining, but some required additional testing.

Double staining is a useful tool for studying two different antigens in a single tissue section. Using this method, we determined which cells express CTSK, and whether it co-localized with TLR4 and TLR9 in OLP tissue. In any double staining, the characteristics of the primary antibodies determine the protocol used, and
should be optimized first (van der Loos). Cross-reactivity of primary antibodies is a potential problem, and may be overcome by different procedures (van der Loos 2008). If a simultaneous staining protocol is used, the primary antibodies should, in general, be from different species. We used a sequential staining method to be able to use two mouse antibodies. The chromogen combination is of utmost importance since optimal contrast is needed between the two color reagents and in the mixed color at sites of co-localization (van der Loos). Normally, sequential double staining is applied for the identification of two different cell types or cell components. It is not recommended for the observation of co-localization by mixed colors (van der Loos 2008). However, we used a red-bluegray-color combination, which yielded a fairly good contrast-mixed color at sites of suspected co-localization. In addition to the selection of chromogens, the order of use of the chromogens requires consideration. We observed that use of the red color first yielded the best results. The use of IF double staining has additional drawbacks compared to the immunoenzymatic method, namely quenching and fading of the signal and autofluorescence due to formaldehyde fixation (van der Loos).

The interpretation of immunohistochemical staining is traditionally done visually by a pathologist and is therefore subjective. In research, it is preferable to have at least two investigators evaluate the staining to increase objectivity (Fedchenko & Reifenrath 2014), as we also did in this thesis. The scoring of staining is often done semi-quantitatively yielding ordinal variable data. Currently, digital image analysis systems are also used to facilitate the scoring process and to yield more accurate data of the scoring intensity with continuous variable data. The parameters that are chosen for scoring should reflect the research question and take into account the expression pattern of the studied biomarker. Therefore, the scoring method and interpretation of staining is usually designed by the investigators for each antibody individually, as was done in this thesis.
7 Conclusions

The present study revealed new findings regarding OLP etiopathogenesis and management. First of all, we found that the prevalence of thyroid diseases, especially hypothyroidism, is significantly higher in OLP patients compared to an age- and sex-matched population without OLP from the same Northern Finland area. Secondly, in a clinical intervention trial comparing topical 0.1% tacrolimus, 0.1% triamcinolone acetonide and placebo, we observed that both active comparators were more effective than placebo in reducing the signs and symptoms associated with OLP at week 3. In fact, the efficacy of topical tacrolimus and triamcinolone acetonide compared to placebo is shown for the first time in OLP. Thirdly, the immunohistochemical expression of TLR9, HA, HAS1–3, HYAL1–2 and CTSK is described for the first time in OLP, and was found to be altered compared to healthy oral mucosa. Fourthly, we observed that treatment with topical tacrolimus affected the expression of CD44 and CTSK in OLP.

In conclusion, our findings provide evidence for a multi-factorial etiologic basis in OLP, reflected by the association with the autoimmune disease hypothyroidism found in some patients. The observed involvement of several previously undescribed molecules related to innate and adaptive immunity and extracellular matrix modulation suggests their role in the pathogenesis of OLP. In the management of symptoms and lesions associated with OLP, topical tacrolimus seems equally effective compared to triamcinolone acetonide, but may provide better long-term pain control.
References


Sivaraman S, Santham K, Nelson A, Laliytha B, Azhalvel P & Deepak JH (2016) A randomized triple-blind clinical trial to compare the effectiveness of topical triamcinolone acetonate (0.1%), clobetasol propionate (0.05%), and tacrolimus orabase (0.03%) in the management of oral lichen planus. J Pharm Bioallied Sci 8(Suppl 1): S86–S89.


**Original publications**


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Original publications are not included in the electronic version of the dissertation.
1387. Ronkainen, Justina (2016) Role of Fto in the gene and microRNA expression of mouse adipose tissues in response to high-fat diet
1388. Ronkainen, Eveliina (2016) Early risk factors influencing lung function in schoolchildren born preterm in the era of new bronchopulmonary dysplasia
1389. Casula, Victor (2016) Quantitative magnetic resonance imaging methods for evaluation of articular cartilage in knee osteoarthritis: free-precession and rotating-frame relaxation studies at 3 Tesla
1390. Alahuhta, Ilkka (2016) The microenvironment is essential for OTSCC progression
1391. Hagnäs, Maria (2016) Health behavior of young adult men and the association with body composition and physical fitness during military service
1394. Erani, Antti (2016) The role of electrocardiographic abnormalities, obesity, and diabetes in risk stratification for sudden cardiac death in the general population
1395. Kubin, Minna (2016) Glucocorticoid receptors in inflammatory skin diseases: the effect of systemic and topical glucocorticoid treatment on the expression of GRα and GRβ
1396. Määttä, Juhani (2016) The heritability and morphology of lumbar Modic changes and their association with pain
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