Oral Lichen Planus
Studies of factors involved in differentiation, epithelial mesenchymal transition

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In memory of my mother
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Chemokines
Autoimmunity
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Transforming growth factor-β and Smad proteins
MicroRNA
miR-21
miR-26b
miR-125b
miR-203
p53 and p63

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Abstract

**Background:** Lichen planus is a chronic inflammation of skin and mucosa with unknown cause. Oral Lichen Planus, OLP, affects around 2% of the population. OLP has been suggested to be an autoimmune disease as OLP has autoimmune features such as female predominance, cyclic nature and cytotoxic T-cell infiltrate. It has been suggested that the intense inflammatory response seen in OLP is caused by factors on the keratinocyte surface triggering the immune system. Chronic inflammation is one of the hallmarks of oral lichen planus and chronic inflammation is connected to increased risk of tumor development. WHO classifies OLP as a potentially malignant condition with increased risk of developing Squamous cell carcinoma of head and neck, SCCHN, but malignant transformation of OLP is a matter of controversy. The aim of these studies was to further elucidate the autoimmune and premalignant character of OLP. Factors involved in differentiation, malignant transformation, autoimmunity and inflammation were analyzed in normal oral mucosa, OLP and SCCHN. Factors studied were the signal transducers of Transforming growth factor-β the Smad proteins, microRNAs, COX-2, the receptor CXCR-3 and its ligands CXCL-10 and -11 and ELF-3.

**Material and methods:** In the study on Smad protein expression formalin fixed and paraffin embedded biopsies from normal oral mucosa, OLP and SCCHN was used. For the remaining studies fresh frozen biopsies from OLP and normal controls was used. All of the fresh frozen OLP samples and their controls were micro dissected to be able to analyze the epithelial part only as well as sections of the whole biopsy. Methods used are immunohistochemistry, qRT-PCR and Western blot.

**Results:** Analyses of smad proteins expression showed a clear increase of smad3 and smad7 in OLP compared to normal oral mucosa. The expressions of smad proteins in the tumors were more heterogeneous. Some of the SCCHN samples showed a similar expression as OLP while others did not. Micro RNA analyzes showed that miR-21 and miR-203 was significantly increased in OLP epithelium compared to normal oral epithelium while the expression of miR-125b and their potential targets p53 and p63 was decreased in OLP. The presence of COX-2 was significantly higher in OLP than normal controls. At the same time the expression of miR-26b, a
suggested repressor of COX-2 was decreased in OLP compared to normal mucosa. The receptor CXCR-3 and its ligands CXCL-10 and -11 were increased in OLP. Expressions of the differentiation involved factor ELF-3 mRNA as well as protein were decreased in OLP.

**Conclusion:** The factors studied are involved in differentiation, malignant transformation and inflammation. Some of the results in these studies indicate a similar expression pattern for OLP and SCCHN. Several of the factors studied are involved in differentiation and their deregulation suggests a disturbed differentiation pattern and this could indicate a premalignant character of OLP but malignant transformation of OLP lesions are relative rare. A lot of these factors are also involved in inflammatory processes and connected to autoimmune diseases and their deregulation in OLP could also support an autoimmune phenotype of the disease. Based on our studies a suggestion is that the disturbed differentiation pattern triggers the intense immune response directed against the epithelial cells seen in OLP.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<td>C/EBPα</td>
<td>CCAAT-enhancer binding protein-α</td>
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<td>Co-smad</td>
<td>Common mediator smad</td>
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<td>COX-2</td>
<td>Cyclooxygenase-2</td>
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<td>CTL</td>
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<td>CXCL-10</td>
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<td>EGFR</td>
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<td>ELF-3</td>
<td>E74-like transcription factor</td>
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<td>ELISA</td>
<td>Enzyme linked immune absorbent assay</td>
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<td>GvHD</td>
<td>Growth factor independent 1 transcription repressor</td>
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<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<td>GFi1</td>
<td>Growth factor independent 1</td>
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<tr>
<td>NOS-2</td>
<td>Nitric oxide synthase-2</td>
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<td>NSAID</td>
<td>Non-steroid anti-inflammatory drug</td>
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<td>OD</td>
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<td>Primary microRNA</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>R-smad</td>
<td>Receptor activated smad</td>
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<td>SCCHN</td>
<td>Squamous cell carcinoma of head and neck</td>
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<td>Zeb-1,-2</td>
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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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Original research articles

This thesis is based on the following paper, which will be referred to by their Roman numerals


IV. Danielsson K, Boldrup L, Rentoft M, Coates PJ, Ebrahimi M, Nylander E, Wahlin YB, Nylander K:. Autoantibodies and decreased expression of the transcription factor ELF-3 together with increased chemokine pathways support an autoimmune phenotype and altered differentiation in lichen planus located in oral mucosa. *Manuscript*

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INTRODUCTION

In this thesis expression of factors involved in different physiological processes such as differentiation, inflammation and epithelial mesenchymal transition are studied in normal oral mucosa, oral lichen planus and squamous cell carcinoma of head and neck. Under normal circumstances these processes are physiological but we also know that they can be deregulated and play a part in pathological processes and disease development.

Lichen planus

Lichen planus (LP) is a mucocutaneous inflammatory disease affecting skin and mucosa. It was first described in 1869 by Wilson 1. LP lesions in the skin are commonly located on the extremities where it manifests with polygonal, flat topped, violaceous papules and plaques with overlaying white reticular scales, and itching lesions 2. LP can affect several kinds of mucosa. The most common sites are the oral and genital mucosa but other mucosal sites such as conjunctiva, nasal mucosa, larynx, oesophagus, urethra and anal mucosa can also be affected as well as scalp and nails. The oral subtype, oral lichen planus (OLP) is a chronic inflammation of the oral mucosa and it is a quite common disorder affecting up to 2 % of the population. The disease occurs more often in women than men 3. The typical OLP patient is a middle aged woman. It is not uncommon that OLP patients have LP lesions elsewhere on skin or mucosa; in a recent study by Ebrahimi et al 50 % of the patients had both oral and genital involvement and 29 % had both oral and skin involvement 4.

Clinical features

OLP lesions usually have characteristic and distinct clinical features and distribution. According to the World Health Organization (WHO) modified diagnostic criteria, OLP lesions are always bilateral with the presence of a white reticular pattern 5 (Table 1). Six clinical subtypes have been described and commonly the subtypes coexist. The white forms include reticular, papular and plaque like forms while the red
forms include atrophic (erythematous), erosive (ulcerative) and bullous.

- The *reticular subtype* presents as a lacy network of white lines and tiny papules, so called Wickham’s striae.

- The *plaque subtype* of OLP appears as white homogenous lesions resembling leukoplakia. The lesion has a smooth to slightly irregular surface and an asymmetric configuration.

- *Papular* OLP is rarely seen and consists of small raised papules.

- In the *atrophic subtype* diffuse red lesions as a result of an atrophic epithelium are seen. A white reticular pattern is present in the margins of the lesion.

- *Erosive* LP is an irregular erosion or ulceration. Red borders and a yellowish pseudo-membrane in the central part are present in the mature lesions. The lesion is surrounded by reticular keratotic striae.

- The *bullous type* is the least common type of OLP and bullae that rupture easily may be seen in the erosive form.

The atrophic (erythematous) and erosive (ulcerative) lesions are often associated with symptoms and discomfort. Reticular OLP can occur as the only sub type whereas the atrophic and erosive forms coexist with other subtypes in the same lesion. The buccal mucosa is the most commonly affected location followed by the lateral borders of the tongue and the gingiva. OLP lesions are characterized by periods of exacerbations and remission.
**INTRODUCTION**

There are several oral lesions that are very similar or even indistinguishable from OLP but have a distinct aetiology and therefore the diagnosis of OLP should be based on both clinical and histological criteria (Table 1). Some of the histopathological features, such as a dense band of inflammatory cells beneath the epithelium, signs of liquefaction degeneration in the basal cell layer and absence of dysplasia, are considered essential for the diagnosis of OLP. The inflammatory infiltrate in the well defined band like zone mainly consists of activated cytotoxic T-lymphocytes. The changes seen in the basal cell layer are caused by liquefactive degenerated basal keratinocytes that can form structures known as Civatte or colloid bodies. Other features that can be seen are atrophic epithelium, parakeratosis and saw-toothed pattern of rete ridges.

**Histopathological characteristics**

There are several oral lesions that are very similar or even indistinguishable from OLP but have a distinct aetiology and therefore the diagnosis of OLP should be based on both clinical and histological criteria (Table 1). Some of the histopathological features, such as a dense band of inflammatory cells beneath the epithelium, signs of liquefaction degeneration in the basal cell layer and absence of dysplasia, are considered essential for the diagnosis of OLP. The inflammatory infiltrate in the well defined band like zone mainly consists of activated cytotoxic T-lymphocytes. The changes seen in the basal cell layer are caused by liquefactive degenerated basal keratinocytes that can form structures known as Civatte or colloid bodies. Other features that can be seen are atrophic epithelium, parakeratosis and saw-toothed pattern of rete ridges.

**Etiology**

OLP is an immune mediated disease with cytotoxic T-lymphocytes, however, of unknown cause. Several factors such as genetic background, infectious agents, immunodeficiency, stress and
trauma have been suggested as possible causes of OLP. It is suggested that LP is an autoimmune disease. T-cells cytotoxic against keratinocytes have been seen in cell lines derived from LP lesions and it has been proposed that changes on the keratinocyte surface cause the intense inflammatory infiltrate seen in OLP. OLP also has some autoimmune features such as a female predominance, a cyclic nature, presence of cytotoxic T-cells, chronicity, adult onset and association with other autoimmune diseases. So far no LP antigen has been discovered but autoantibodies against p63 have been reported in some OLP patients. Several factors involved in inflammatory processes such as TNF-α, IL-1β, IL-2, IL-6 and INF-γ are increased in OLP.

**Malignant transformation**

One of the hallmarks of OLP is the intense inflammation seen in the underlying connective tissue. An association between chronic inflammation and an increased risk of malignant development is known. The World health organization (WHO) classifies OLP as a potentially malignant disorder with increased risk of developing into squamous cell carcinoma of head and neck (SCCHN). The malignant properties of OLP lesions are somewhat controversial and there are different opinions about the premalignant character. There are studies that show an increased risk of developing squamous cell carcinoma in OLP lesions compared to the general population. The reported percentage of tumour development varies between 0.07 - 5.3 percent. There is also reported no malignant transformation of OLP lesions. The lack of accepted specific diagnostic criteria of OLP might contribute to the variation in frequency of reported malignant transformation of OLP. The location most often affected by tumours in OLP patients seems to be the tongue and the buccal mucosa. Both Bombeccari and Gandolfo reported a higher risk for malignant transformation in women with OLP than men with OLP.
Clinical criteria

- Presence of bilateral, more or less symmetrical lesions
- Presence of a lace-like network of slightly raised gray-white lines (reticular pattern)
- Erosive, atrophic, bulbous and plaque-type lesions are only accepted as a subtype in the presence of reticular lesions elsewhere in the oral mucosa

In all other lesions that resemble OLP but do not complete the aforementioned criteria, the term ‘clinically compatible with’ should be used.

Histopathologic criteria

- Presence of a well-defined band-like zone of cellular infiltration that is confined to the superficial part of the connective tissue, consisting mainly of lymphocytes
- Signs of ‘liquefaction degeneration’ in the basal cell layer
- Absence of epithelial dysplasia

When the histopathologic features are less obvious, the term ‘histopathologically compatible with’ should be used.

Final diagnosis OLP or OLL

To achieve final diagnosis clinical as well as histopathologic criteria should be included:

- OLP  A diagnosis of OLP requires fulfilment of both clinical and histopathologic criteria
- OLL  The term OLL will be used under the following conditions:
  1. Clinically typical of OLP but histopathologically only ‘compatible with’ OLP
  2. Histopathologically typical of OLP but clinically only ‘compatible with’ OLP
  3. Clinically ‘compatible with’ OLP and histopathologically ‘compatible with’ OLP

Table 1. Modified diagnostic criteria adopted from van der Meij 5, 28

Treatment

So far no cure for lichen planus is available and the treatment only reduces symptoms but does not stop progression of the disease. Today no treatment is recomended if the lesions are asymptomatic. Individuals with OLP should be recommended to stop any tobacco habits or excessive alcohol use. It is also important that they are informed and instructed in keeping a good oral hygiene 29. In recent
Cochrane reviews concerning treatment for mucosal lichen planus it is concluded that there is no strong evidence supporting any specific treatment as being superior.\textsuperscript{30, 31} Oral lesions may respond to potent topical corticosteroid treatment such as clobetazol, beclomethasone, or budesonide. The lesions are often infected with candida and antifungals are also needed. If no improvement is reached with topical treatment systemic treatment with steroids might be needed. Topical treatment with other immunosuppressors such as tacrolimus and cyclosporine can also be used.\textsuperscript{29} Successful treatment with methotrexate in patients with severe erosive lichen has been reported\textsuperscript{32}.

**Differential diagnoses**

There are several oral lesions that resemble or even are indistinguishable from OLP but have a distinct aetiology. Differential diagnoses include oral lichenoid reactions (OLR), graft versus host disease (GvHD) and leukoplakia.

**Oral lichenoid reaction**

The group of oral lichenoid reactions (OLR) comprises different subgroups with different aetiology. Oral lichenoid contact lesions (OLCL) are a delayed immune mediated hypersensitivity, an allergic contact stomatitis. These lesions are caused by dental restorations and lesions are in close contact with the restorations. In oral lichenoid drug reactions (OLDR) the cause is different kinds of drugs such as non steroid anti inflammatory drugs (NSAID), β- blockers, diuretics and others.\textsuperscript{33}
**Graft versus host disease (GvHD)**

A complication after allogenic haematopoietic stem cell transplantation is GvHD. It is a common complication and is characterized by chronic inflammation affecting skin, mucosa, liver and the gastrointestinal tract. Clinical and histopathological features of GvHD are very similar to OLP but the aetiology is completely different 34, 35.

**Leukoplakia**

Leukoplakia is a white lesion that cannot be characterized as any other definable lesion. It is a clinical term without histological features. Leukoplakia is a potentially malignant disorder. Most of the affected patients are tobacco or betel users or consume alcohol. Other but less common causes are infections such as candidosis, HPV and syphilis. The lesions can occur as single or multiple lesions and in some cases as a diffuse widespread lesion. Most of the leukoplakias are homogeneous white lesions but they can also appear as mixed white and red lesions or warty lesions. A wide range of histopathological features can be seen in leukoplakia from hyperkeratosis without dysplasia to dysplasia, verrucus carcinoma and squamous cell carcinoma, to more lichenoid like features with a band like inflammatory infiltrate 2, 29.

**Squamous cell carcinoma of head and neck**

Head and neck cancers are a group of cancers that involve the oral cavity, larynx and pharynx. Of these cancers the majority are squamous cell carcinomas of head and neck (SCCHN). The WHO estimates that there are around 600,000 new cases of head and neck cancer each year and around 300,000 deaths worldwide. The most common site is the oral cavity with approximately 400,000 cases a year 36. In some parts of the world an increasing trend is reported.
The major risk factors are tobacco and alcohol. Approximately 70% of all head and neck cancer can be explained by these risk factors. Human papilloma virus (HPV) is also a recognized factor especially for oropharyngeal cancer. SCCHN has a relatively poor prognosis and the 5-year survival in Europe is around 50%. One reason for this could be the fact that they are often diagnosed at an advanced stage.

One of the major differences between SCCHN and normal oral epithelium is the increase in immature and less differentiated epithelial cells in SCCHN.

**Oral epithelium**

Stratified squamous epithelium is found in the skin, oral mucosa, genital mucosa, conjunctiva, oesophagus, the gastro-intestinal canal and as a lining around internal organs. It functions as a barrier protecting us from different threats like mechanical insult, bacteria and virus and protects against dehydration.

The oral mucosa consists of two main tissues a stratified squamous epithelium, the oral epithelium and an underlying connective tissue, the lamina propria. The epithelium in the oral cavity consists of cells tightly attached to each other forming a stratified multilayered sheet organized in a number of distinct layers. In the oral mucosa the epithelium shows adaption to different mechanical demands. In the masticatory mucosa, e.g. hard palate and gingiva, which is a subject of mechanical forces from mastication, the stratified squamous epithelium is keratinized and tightly attached to the underlying tissue. The epithelium covering the lining mucosa, which requires a higher flexibility and is covering the floor of the mouth and buccal regions, is nonkeratinized. On the tongue there is a specialized epithelium with a mixture of keratinized and nonkeratinized epithelium. Around 90% of the cells in the oral mucosa are keratinocytes but there are also melanocytes, Langerhans cells, Merkel cells and inflammatory cells such as lymphocytes.
Figure 2: Keratinized and non-keratinized stratified squamous epithelium and the different layers

The oral epithelium maintains its structure by a process of continuous cell renewal. The cells in basal cell layer are mitotic and proliferate. A small portion of the basal cells are epithelial stem cells, providing daughter stem cells and transit amplifying cells. The basal keratinocytes maintain and induce genes required for proliferation. When the cells are moving to the supra basal layers they gradually start to differentiate and are terminally differentiated when they reach the surface. This maturation process is tightly regulated and a lot of factors are involved in maintaining the balance between proliferation and differentiation.

**Differentiation**

In the oral cavity there are keratinized and non-keratinized epithelia representing different patterns of differentiation. In the keratinized epithelium the differentiation leads to production of the stratum corneum, with flattened cells filled with cytokeratin filaments.
Depending on differentiation pattern different keratins are expressed. Cytokeratins keratin 5 and 14 are found in undifferentiated basal keratinocytes in all oral mucosa. In oral keratinized epithelium keratins 1 and 10 are expressed in supra-basal layers while keratins 4 and 13 are expressed in supra-basal layers of non-keratinized epithelium. The accumulation of cytokeratins in the keratinocytes in non-keratinized epithelia is less evident. The terminally differentiated cells in non-keratinized epithelium are large and flat and do not have bundles of filaments. A changed pattern of keratin expression is reported in OLP and dysplasia. Epithelial homeostasis requires that there is a balance between the proliferation in the basal cell layers and the loss of cells from the surface. One of the factors important in epithelial homeostasis is p63, where p63 regulates proliferation and differentiation of keratinocytes. A previous study has reported a decreased expression of p63 in OLP, a down-regulation that could lead to impairment in keratinocyte differentiation. Epidermal growth factor receptor (EGFR) and E-cadherin are other factors reported to play a role in differentiation and also reported to be down-regulated in OLP.

**E74-like transcription factor**

In this thesis, expression of E74-like transcription factor (ELF-3), a factor connected to differentiation of epithelial cells, was studied.

ELF-3 is a member of the ets gene family. In humans this family consists of 27 different members. All family members are characterized by a highly conserved DNA binding domain known as the ETS domain. ELF-3 belongs to a subgroup of the ets family which is a group of epithelial specific transcription factors. ELF-3 has been shown to play a role in the terminal differentiation of epidermal cells and is expressed in the most differentiated cell layers in epidermis. The expression of ELF-3 is induced during terminal differentiation of keratinocytes and ELF-3 is able to transactivate genes involved in keratinocyte terminal differentiation and suppress expression of early differentiation genes such as keratin 4.
There are several studies reporting ELF-3 to be involved in pathological processes, both in tumours and in inflammation. Increased as well as decreased expression of ELF-3 has been observed in primary breast tumours as well as in some breast cancer cell lines. ELF-3 has also been identified as over expressed in lung cancer cell lines, primary lung adenocarcinomas and large cell lung carcinomas. In oral squamous cell carcinoma ELF-3 inhibits tumour cell invasion by suppression of MMP-9, a matrix metalloproteinase implicated in tumour cell invasion. Under normal conditions ELF-3 is exclusively expressed in epithelial cells but studies have shown expression of ELF-3 in other types of cells in rheumatoid arthritis, in a mouse model with vascular inflammation and in airway inflammation. The proinflammatory cytokines IL-1β and TNF-α are able to induce expression of ELF-3 in cells that normally do not express ELF-3. It has also been shown that absence of ELF-3 enhances the infiltration of T-cells and macrophages in response to inflammatory mediators. Both nitric oxide synthase 2 (NOS-2) and cyclooxygenase-2 (COX-2), involved in inflammatory processes, are target genes for ELF-3.

Inflammation

Inflammation is a physiologic response to stimuli such as infection and injury. Acute inflammation is rapidly induced and lasts for a shorter time. Chronic inflammation develops when there is a persisting antigen or, as the case in autoimmune disease, self antigens continuously activate T-cells. An association between chronic inflammation and cancer is known. During an inflammatory response different kinds of inflammatory mediators, such as chemokines and cytokines, are released from cells of both the innate and acquired immune system. These mediators can also be released from epithelial cells such as keratinocytes. A number of cytokines such as IL-1, IL-6, IL-12, IFN-γ and TNF-α are involved in development of acute and chronic inflammation. It has been shown that both IFN-γ and TNF-α play important roles in development of chronic inflammation. IFN-γ is released by T helper-1 cells (Th-1
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cells), Natural killer cells (NK cells) and cytotoxic T lymphocytes (CTL), and TNF-α is secreted by macrophages. In OLP it is known that there are increased expression of several cytokines such as IL-1, INF-γ and TNF-α \(^{13}\).

**Cyclooxygenase-2**

A protein which is directly connected to inflammation and also suggested to be involved in cancer development is cyclooxygenase-2 (COX-2). Cyclooxygenases, also known as prostaglandin G/H synthetases, are enzymes that convert arachidonic acid to prostaglandins. There are three isoforms of COX identified; COX-1, COX-2 and COX-3, where COX-3 is a splice variant of COX-1. COX-1 is a constitutively expressed enzyme found in most tissues. COX-1 synthesizes low levels of prostaglandins and is assumed to function in maintaining physiological functions. COX-2 is an inducible enzyme and is absent in most normal tissues except in some areas of the brain and kidney \(^{64}\). COX-2 is induced in response to growth factors, hormones and cytokines \(^{65}\). COX-2 transforms arachidonic acid to five different primary prostanoids. The prostanoids have many biological functions such as regulation of immune function, modulation of platelet aggregation, vascular homeostasis, body temperature and regulation of inflammation \(^{66}\) (Figure 3).

**Figure 3**: Some of the many diverse activities of prostaglandins
COX-2 expression is implicated in the pathogenesis of many diseases and is also over-expressed in many tumours \textsuperscript{66}. In oral lichen planus, oral dysplasia and SCCHN, an up-regulation of COX-2 expression has been found \textsuperscript{67-69}. It has been reported that the levels of COX-2 are higher in premalignant lesions than in tumours in a variety of organs such as colon, oesophagus and head and neck area. Higher expression of COX-2 in well-differentiated tumours than in poorly-differentiated tumours has also been reported \textsuperscript{70}. COX-2 has been implicated to increase cell proliferation and in some cell lines it has an anti-apoptotic effect \textsuperscript{71}. COX-2 and COX-2 derived prostanoids have a function both in the early stages of inflammation as well as in the resolution phase and the COX-2 pathway has a pro-inflammatory as well as a protective role in the process of inflammation. Defects in inflammatory pathways may predispose for development of chronic and autoimmune disorders \textsuperscript{64}. Both p53 \textsuperscript{65, 72} and p63 has been identified as some of many factors able to regulate expression of COX-2. All three of the ΔNp63 isoforms were able to induce COX-2 expression \textsuperscript{73}. Another factor able to activate COX-2 expression in monocytes is ELF-3 \textsuperscript{62}.

**Chemokines**

Chemokines are small polypeptides that are major regulators of leukocyte traffic. They are important regulators of the homeostatic and inflammatory leukocyte action such as locomotion, degranulation, gene transcription, mitogenic and apoptotic effects. The chemokines are divided in two main families - CXC chemokines and CC chemokines - depending on absence or presence of an amino acid residue between two neighbouring Cys residues. Cells expressing chemokine receptors are often from hematopoietic lineages, but other cells also express chemokine receptors e.g. epithelial cells, neurons, smooth muscle cells, stromal cells and endothelial cells \textsuperscript{74}.

The CXCL-10 and CXCL-11 chemokines have a constitutive expression pattern \textsuperscript{74} and the receptor CXCR-3 is primarily expressed by type 1 T-cells \textsuperscript{75}. CXCL-10 and CXCL-11 can both be induced by
IFN-γ, a factor increased in OLP. Keratinocytes can produce a prominent amount of CXCL-10 and CXCL-11. The receptor CXCR-3 and the ligands CXCL-10 and CXCL-11 are up-regulated in autoimmune disorders and represent a Th1 response, supporting an autoimmune phenotype.

**Autoimmunity**

In autoimmunity the mechanisms of self tolerance that normally protect an individual from self-reactive lymphocytes have an inappropriate behaviour. Healthy persons have mature, recirculating, self-reactive lymphocytes which do not result in autoimmune reactions. In healthy individuals these self-reactive lymphocytes are regulated by clonal anergy or clonal suppression. Anergy is a state of unresponsiveness to antigen; this non-responsiveness is important in peripheral tolerance in preventing the activation of self-reactive clones. Disturbances in this regulation can lead to activation of self-reactive T or B cells which generate a humoral or cell-mediated immune response against self antigens. These reactions can lead to serious damage of organs or cells. The damage to organs or cells can be caused by antibodies or can be mediated by T-cells, as in many autoimmune disorders. The autoimmune diseases can be broadly divided into two categories: organ specific and systemic autoimmune diseases. In systemic autoimmune disorders, immune response is directed against a number of different target antigens and affects a number of organs and tissues, for example as in SLE. Tissue damage is caused by cell-mediated immune responses, autoantibodies, or accumulation of immune complexes. When the immune response is directed to an antigen specific for an organ or a gland, it is an organ specific autoimmune disease. The damage to the cells of the target organ may be caused by humoral or cell-mediated actions, or the antibodies may block or over-stimulate the normal function of the organ. The damaged tissues in the organ are gradually replaced by connective tissue and the function of the organ declines.
OLP displays some autoimmune features such as female predominance, cytotoxic T-cell infiltrate and cyclic nature and OLP is suggested to be an autoimmune disease, but so far no OLP antigen has been discovered even though autoantibodies against p63, desmogleins and an increased expression of serum antibodies has been detected in OLP patients.

**Epithelial mesenchymal transition**

Epithelial mesenchymal transition (EMT) is a process involved in both physiological and pathophysiological events. In embryonic development, EMT generates morphologically and functionally different cell types. In adult tissues, EMT also occurs in wound healing, tissue regeneration, fibrosis and cancer development and metastasis. It has been suggested that EMT should be classified in three different subtypes since it is found in three distinct biological processes. During EMT, epithelial cells switch from an epithelial polarized phenotype to a mesenchymal motile phenotype. The EMT process involves loss of cell-cell adhesion, loss of apical-basal polarity, rearrangement of the cytoskeleton and acquisition of motility. Loss of E-cadherin expression, as well as down regulation of other epithelial markers such as occludin and cytokeratins, are hallmarks of EMT. At the same time, there is up-regulation of mesenchymal markers such as vimentin, α-smooth muscle actin (α-SMA) and fibronectin.

**Transforming growth factor-β and Smad proteins**

Transforming growth factor-β (TGF-β) is a member of the TGF-β superfamily of structurally related proteins. TGF-β signalling regulates tissue homeostasis by modulating cell growth, differentiation, apoptosis, migration, inflammation and angiogenesis. TGF-β is involved in regulating the immune response and is considered as an immunosuppressor under normal conditions.
malignant transformation TGF-β has both a tumour-suppressor effect as well as acts as a tumour promoter.\(^{85}\)

TGF-β is a potent inducer of EMT. Several factors are involved in regulation of EMT and TGF-β is able to control many of these factors.\(^{83}\) Activated TGF-β ligands signal through TGF-β type I (TβRI) and type II (TβRII) receptors and intracellular Smad proteins. Smad proteins are divided into three different subgroups; receptor activated smad (R-smad), common mediator smad (co-smad) and inhibitory smad (I-smad). The activated and phosphorylated R-smads (Smad2 and Smad3) form a complex with the co-smad, Smad4.\(^{86}\) This complex of R-smads and Smad4 then enters the nucleus where it interacts with transcription factors to regulate gene expression. Inhibitory Smad7 is activated by TGF-β and exerts a negative feedback by blocking phosphorylation of R-smads (Figure 4). Smads have low affinity to DNA and interact with co-factors. Some of the identified co-factors are Snail, Zeb1, Zeb2 and AP-1.\(^{87}\)

**Figure 4:** A simplified draft of TGF-β/ Smad signalling. SBE= Smad binding element on target genes.
It has been shown that the Smad-dependent signalling pathway is critical for EMT. Studies have shown that Smad3 is required for the EMT process and both Smad2 and Smad3 are suggested to play a role in promoting an invasive phenotype of SCC. Smad3 is implicated in mediating TGF-β mediated skin inflammation and has been suggested to be important in apoptosis even if results so far are contradictory. Inflammation can drive Smad7 expression since Smad7 can be induced by IFN-γ, IL-1β and TNF-α and inhibition of Smad7 is able to suppress autoimmune encephalomyelitis. Smad7 is connected to apoptosis and can induce apoptosis in epithelial cells. TGF-β plays a part in differentiation and TGFβ/Smad signalling is suggested to be indispensable for epidermal differentiation. Increased expression of Smad7 has been shown to impair terminal epidermal differentiation by negative regulation of KLF-4, an important factor in differentiation of epidermis. Smad proteins also play a role in biogenesis of miRNA through interactions with a subunit of the Drosha complex.

Apart from this canonical Smad signalling pathway there are also non-smad signalling pathways. One factor cross-talking with TGF-β is the tumour suppressor p53. P53 and TGF-β cooperate in cell fate decisions and regulation of cellular homeostatic functions, and mutant p53 has been found to be partly responsible for lost TGF-β sensitivity. Factors shown to be involved in TGF-β induced EMT are COX-2 and p63. Micro-RNA-21, miR-21, is also considered to play a critical role in the TGF-β pathway and it is suggested that miR-21 regulates the ability of epithelial cells to respond to TGF-β, with potential effects on epithelium homeostasis, wound healing and tumourigenesis.

MicroRNA

MicroRNAs (miRNA) are short non-coding RNAs approximately 22nt long, encoded in both protein coding and non-coding areas of the genome. RNA polymerase II transcribes the majority of the miRNAs. After transcription by RNA polymerase II, the primary
miRNA (pri-miRNA) is processed by the Drosha complex into a hairpin structure called precursor miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm and processed once more by a Dicer complex. Processing by Dicer produces a double stranded ~22nt long product comprising the mature miRNA and the miRNA passenger strand. The mature miRNA strand is loaded on to the RISC complex and the passenger strand is degraded. Degradation or repressed translation of the target mRNA is then mediated by the miRNA/RISC complex. Some studies have shown that miRNAs may also act as activators of gene expression in quiescent cells and repressors of gene expression in proliferating cells. MiRNAs are involved in many biological processes such as development, differentiation and proliferation, and are also important players in different diseases. It is estimated that one single miRNA may target several dozen or even hundreds of mRNAs and that the expression of a specific protein may be regulated by several different miRNAs, and a recent study suggested that around 60% of human genes are regulated by miRNAs. The expression of miRNAs is regulated at both transcriptional and posttranscriptional levels and there are also epigenetic events affecting expression of miRNAs.

In this thesis, four selected miRNAs were chosen for analysis in normal oral mucosa and in OLP. All four of the miRNAs are implicated in one or more of the processes of differentiation, EMT, or inflammation and some are known repressors of p53 and p63, factors deregulated in OLP.

**miR-21**

Studies have shown that miR-21 plays important roles in development and morphogenesis. miR-21 is a frequently studied miRNA and is over expressed in many kinds of tumours. It was classified as an oncomiR, a microRNA with a role in cancer, but its’ expression is increased also in other diseases. miR-21 is considered an anti-apoptotic factor. In cell lines miR-21 is induced during differentiation and over expression enhances proliferation. These findings suggest that low levels are required for differentiation and that over expression leads to increased cell proliferation. miR-21 is also implicated in TGF-β induced EMT. Expression of miR-21...
can be activated both at a transcriptional level by for example AP-1, STAT3, p53 and post-transcriptionally by TGF-β/SMADs. Hypoxia is also able to induce miR-21 expression. Several suppressors for miR-21 have also been reported, for example NFI, C/EBPα and Gfi1. miR-21 targets some important tumour suppressor genes such as PTEN, PDCD4 and maspin.

**miR-26b**

miR-26b is reported to act as a tumour suppressor able to induce apoptosis and inhibit cell proliferation. Down-regulation has been reported in several cancers such as breast cancer, squamous cell lung cancer, hepatocellular carcinoma and SCCHN while over-expression has been seen in gastric cancers. COX-2 is an important factor in the inflammatory process and is down regulated by miR-26b. The proinflammatory cytokine IL-1β, which is over expressed in OLP, negatively regulates miR-26b expression.

**miR-125b**

Reports suggest that miR-125b acts as tumour suppressor in some cancers but acts as an oncomir in other types. Down-regulation of miR-125b is seen in SCCHN. mir-125b seems to play different or opposite roles in different tissues/cells. Expression profiling has shown that miR-125b is expressed in most human organs and tissues. The expression of miR-125b in normal skin is seen mainly in fibroblasts, keratinocytes and melanocytes, and levels are decreased in psoriasis, an inflammatory skin disease. In cell lines of human primary keratinocytes miR-125b represses proliferation and promotes differentiation. miR-125b is identified as a negative regulator of p53 and acts as an oncomir suppressing p53 induced apoptosis. Other genes in the p53 network are also targeted by miR-125b. The tumour suppressor p53 has previously been shown to be deregulated in OLP. TNF-α, a factor up-regulated in OLP and
involved in inflammatory processes and in several steps of
tumorigenesis \cite{128}, is also negatively regulated by miR-125b \cite{129}.

**miR-203**

miR-203 is a keratinocyte specific miRNA abundantly expressed in
skin is found in the supra basal layers of the epithelium \cite{117}. miR-203 is
suggested to act as a molecular switch between proliferating basal
cells and terminally differentiated cells, inducing cell cycle exit and
inhibiting cell proliferation \cite{130,131}. In psoriasis, miR-203 levels were
increased \cite{117} while decreased levels have been found in SSCHN \cite{132}.
p63, a factor in the p53 family and important in maintaining the
homeostasis in epithelium, is negatively regulated by miR-203. p63 is
down regulated by miR-203 upon genotoxic damage \cite{133}.

**p53 and p63**

The p53 family may be regarded as a unique signalling network
controlling cell proliferation, differentiation and death. The family
consists of three members; p53, p63 and p73. All three p53-family
proteins have a similar domain organization and are expressed in a
similar set of isoforms. They are also subject to similar post-
translational modifications but with important differences in their
biological role \cite{134}. p53 is a tumour suppressor protein and functions
as a transcription factor involved in induction of cell cycle arrest or
apoptosis allowing DNA repair to occur or apoptotic cell death if DNA
cannot be repaired. Besides being a tumour suppressor, p53 also
regulates other developmental and cellular processes such as
embryonic implantation, inhibition of angiogenesis, innate immunity
and metabolism. A wide variety of stress signals such as genotoxic
damage, loss of normal cell contacts and hypoxia activate p53 \cite{135}.
Nine different isoforms of p53 have been discovered \cite{136}. Inactivation
or mutation of \textit{TP53} often occurs in human tumours and confers
susceptibility to cancer. Previous studies have revealed over-
expression of p53 protein in OLP lesions \cite{44,137,138}. 
The p53 homologue, p63, plays a critical role in development of epithelial structures such as oral mucosa, skin, teeth, salivary glands, hair follicles, mammary glands and prostate. The TP63 gene encodes six different isoforms by alternative splicing and by usage of two different promoters. p63 is also an important player in maintaining homeostasis in stratified squamous epithelia, regulating proliferation and differentiation of keratinocytes. The different isoforms are reported to be deregulated in SCCHN and a decreased expression of p63 is seen in OLP.

There is a crosstalk between p53 and TGF-β pathways, were p53/p63/p73 is reported to assist and be necessary for TGF-β mediation of antiproliferative and proapoptotic factors. Other factors studied are also connected to p53 and p63 (Figure 5).

**Figure 5**
The figure shows interactions found in cell lines and mice between p53, p63 and the factors studied. The numbers beside the lines are reference numbers. The dotted line between p63 and Elf-3 indicates a possible connection. The green line between p53 and TGF-β indicates crosstalk between these two pathways.
AIMS

The overall aim was to analyze expression of factors involved in differentiation, inflammatory, autoimmune processes and epithelial mesenchymal transition, EMT, in an attempt to better understand the pathobiology of OLP and the potentially premalignant character of the disease.

Aims of the studies

- To map and compare expression of TGF-β signalling transducers, the Smad proteins, in normal oral mucosa, OLP mucosa, premalignant oral mucosa and SCCHN in search for signs of EMT related changes.

- To study if expression of three selected microRNAs, miR-21, miR125b and miR203 are deregulated in OLP and if there are signs of correlation with their potential targets p53 and p63.

- To map expression of COX-2 and miR-26b in OLP epithelium and normal oral epithelium to if there was any correlation between COX-2 and its regulator miR-26b in OLP.

- To investigate the potential for an autoimmune aetiology for OLP by mapping expression of CXCR-3, CXCL-10 and CXCL-11 in lesions and control tissue.

- To further evaluate differentiation status in OLP by examining expression of ELF-3 and presence of autoantibodies against this factor in sera from patients with OLP.
MATERIAL AND METHODS

Tissue

*Paper I.* Formalin fixed paraffin embedded (FFPE) biopsies from 22 patients clinically and histologically diagnosed with OLP, (Patient data are shown in Table 2) were retrieved from Clinical Pathology, Umeå University. FFPE biopsies were also collected from 11 patients with sensitive oral mucosa; four men with a mean age of 48 years and seven women with a mean age of 53 years. All of these had developed SCCHN after shorter or longer observation. From six of these patients two to six biopsies were available with diagnosis from benign hyperkeratosis to epithelial dysplasia and SCCHN. From the five remaining patients only one biopsy was available, three showing dysplasia and two SCCHN. Tissue from these patients taken during the observation time was considered premalignant since they all developed SCCHN. Ten normal oral mucosa biopsies were also included, six men and four women. Ethical permission was approved by the Regional Ethical Review Board at Umeå University Dnr 01-210; 03-201; 05-010.

*Paper II, II and IV.* Four mm punch biopsies were collected from twenty patients clinically and histologically diagnosed with OLP (Patient data shown in table 2). Biopsies were also collected from the buccal mucosa of twenty age- and sex-matched controls; 14 women with a mean age of 66 years, range 42-82, and 6 men with a mean age of 59 years, range 43-81. There was no significant difference in age between the groups (p > 0.8). The controls did not suffer from any autoimmune disorder or were receiving any immunosuppressive treatment.

Biopsies were divided, embedded in Tissue TEC OCT and snap frozen in liquid nitrogen. Samples were stored at -80°C until use.

*Paper IV.* In addition to biopsies, sera were also collected from 19 OLP patients and 20 controls (patient data shown in table 2). In the control group, 13 were women with a mean age of 56 years and 7 men with a mean age of 64 years. Ethical permission was approved by the Regional Ethical Review Board at Umeå University Dnr 05-010M and 01-057.
Table 2: Clinical data on OLP patients

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**Immunohistochemistry**

*Paper I.* Antibodies against Smad2, Smad3, Smad4 and Smad7 were used. Sections stained for Smad2, Smad3 and Smad7 were pre-treated by boiling in citrate buffer, while sections stained with Smad4 were boiled in EDTA. Staining was performed using a Ventana staining machine and reagents according to the suppliers’ recommendation. The evaluation of the stained slides was performed independently by two investigators, KD and KN. Results were compared between the two investigators and differences discussed until agreement was reached.

*Paper III.* From the biopsy 5µm thick sections were cut and staining performed with a polyclonal antibody directed against COX-2 (ab15191) Abcam, (Cambridge, UK) at 1:100 dilution, using a BenchMark ULTRA, Ventana Medical system Inc. (Tucson, AZ, USA) staining machine according to the supplier’s recommendation.

**Laser micro-dissection**

*Paper II, III and IV.* Before laser micro-dissection (LCM), biopsies were sectioned in 10µm thick sections and placed on membrane coated glass slides and stained with Histogene staining solution. Laser micro dissection was performed with PALM® micro laser system (PALM GmbH, Germany). Epithelium was collected and placed in tubes with 850 µl TRIzol Reagent (Invitrogen, Sweden). After incubation at room temperature for 30 minutes, tubes were mixed for 5 minutes and centrifuged. Samples were then stored at –80°C until RNA extraction.

**Quantitative RT-PCR and RNA extraction**

*Paper II, III and IV.* Total RNA was extracted with Trizol (Invitrogen, Sweden) extraction method and Qiagen RNeasy micro kit (Qiagen, Germany) was used for purification of total RNA including small RNAs. For microRNA analysis the miRCURY LNA™
Universal RT microRNA kit (Exiqon, Denmark) was used, consisting of first strand cDNA synthesis and real time PCR amplification. Specific primers for miR-21, miR-125b and miR-203 were used and U48 was used as endogenous control. For mRNA analysis, 200 ng of RNA was used for cDNA synthesis with Revert Aid H Minus First Strand cDNA Synthesis kit (Fermentas, Germany). The qRT-PCR was performed using an IQ5 multi color real time PCR detection system with IQ SYBR green supermix (Bio-Rad laboratories Inc, Hercules, CA, USA).

Western blot and protein extraction

*Paper III and IV.* The tissue was pulverized using a Micro-dismembrator (B. Braun Biotech International, Germany) and a lysis buffer added to the samples. Protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific Inc, USA). Thirty µg protein extract was run on 10% Clear PAGE, SDS-polyacrylamide mini-gels (VWR Internationals, Stockholm, Sweden), transferred to a PVDF membrane and stained with Ponceau red for evaluation of transfer efficiency and loading. Primary antibodies used were COX-2 (Cayman Chemicals, USA), ELF-3 and actin (Chemicon International, USA). Peroxidase labelled rabbit anti-mouse (Dako, Denmark) was secondary antibody. For chemiluminescence detection, Chemidoc XRS (Bio-Rad laboratories Inc) was used in combination with ECL advance (Amersham Biosciences, Sweden).

Enzyme linked immune-absorbent assay

*Paper IV.* Wells on a 96 well plate were coated with ELF-3 protein (2 µg/ml) (OriGene Technologies, MD, USA) in coating buffer, pH 9.6 and incubated overnight at room temperature. After incubation the plate was washed 3 times in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). Blocking of non-specific binding was performed with 0.1% non-fat milk at room temperature for 2h. Plates were washed with PBS-T and incubated with sera
diluted 1:200 from patients and controls. After incubation for 2h and washing, 100µl biotinylated anti-human Ig antibody (Amersham) diluted 1:500 was added to each well. Plates were then incubated for 1h at room temperature. One hundred µl Streptavidin-HRP (R & D) diluted 1:200 was after washing added to each well and incubated for 20 min at room temperature. The reaction was stopped with 50µl of 2% H₂SO₄. Optical density, OD, was then measured spectrophotometrically using an EL311 micro plate auto reader (BioTec instruments, USA). Sera were analyzed in duplicate and a mean OD was calculated for each sample.

Statistical analysis

Non parametric analysis was chosen when results did not show a normal distribution, and sample sizes were relatively small. Mann-Whitney U test was performed for calculation of statistical significances between different groups and Spearman’s rank correlation was used for correlation analysis. In paper IV for ELISA analysis the cut-off value for abnormal optical density was the mean of the controls + 2 S.D. Calculations were performed using PASW statistics 18 (SPSS, Chicago, IL, USA). The significance level was set at p < 0.05.
RESULTS AND DISCUSSION

*Increased expression of Smad proteins in OLP, dysplasia and SCCHN (Paper I)*

In paper I, expression of smad proteins was analyzed. We wanted to further explore the issue of OLP as a premalignant condition and thus chose to study if there were any signs of EMT-related changes in OLP lesions compared to normal and sensitive oral mucosa. The latter group of patients had developed SCCHN after periods of diffuse symptoms and observation, and their tissue could thus be considered pre-malignant.

Increased expression of Smad proteins was detected in OLP compared to the normal oral tissue. In normal oral mucosa, Smad2 expressing cells were seen throughout the whole epithelium in 75% of the cells. An increased number of Smad2 expressing cells was seen in OLP lesions as well in the tumour and dysplasia group except in 2 of the 29 dysplasia/SCCHN biopsies (Table 2, *paper I*). The staining was nuclear.

The most obvious increase in OLP compared to normal mucosa was seen for Smad3. All OLP samples except two, which showed similar expression as controls, showed increased Smad3 expression in the whole epithelium and in 75-100% of the cells. An increased expression of Smad3 was detected in most dysplasia and SCCHN samples, but the expression was heterogeneous as three of the 29 samples showed less or no expression (Table 2, *paper I*). The staining was nuclear.

Smad4 expression was detected in occasional basal cells in four of the 10 normal samples. The expression was very weak. In OLP, 50 % of the samples showed Smad4 expression in a few basal cells. 50 % of the dysplasias also expressed Smad4, but at varying degrees and not only in the basal layer but also parabasally (Table 2, *paper I*). The staining for Smad4 was cytoplasmic.

A clear increase in OLP, dysplasia and SCCHN was also seen for Smad7. The expression was seen in most cells throughout the whole epithelium (Table 2, *paper I*). For Smad7, nuclear staining was seen.
Our results for Smad2, Smad3 and Smad4 were not in accordance with a previous study made on erythematous oral lichen lesions (ERY OL), where they saw decreased expression of Smad2/3 and Smad4. The differences could be explained by the fact that they included both OLP and lichenoid lesions, diseases with known differences in aetiology and that they also used a different antibody recognizing both Smad2 and 3.

Smad proteins are signal transducers of TGF-β, a factor known to be a potent inducer of EMT. Apart from being an EMT inducer, TGF-β is also involved in several activities such as apoptosis, differentiation, inflammation and proliferation. The smad proteins are also implicated in different processes.

Smad3 is involved in TGF-β induced EMT. Loss of Smad3 can reduce tumour development and malignant transformation in a chemical skin carcinogenesis model. Smad3 and Smad4 form a transcriptional repressor complex together with SNAIL and repress expression of E-cadherin and occludin in TGF-β induced EMT. The increased expression of Smad3 in OLP together with the previously reported decreased expression of E-cadherin in OLP might indicate an EMT related process in OLP but Smad3 has also been suggested to play a part in apoptosis, a process increased in OLP. However, the role for Smad3 in apoptosis is not clear as some have shown that Smad3 promotes apoptosis while others have shown increased apoptosis in Smad3 knockouts. Inflammation is one of the hallmarks of OLP and Smad3 is suggested as a mediator of inflammation. In studies on Smad3 knockout mice it has been shown that they show a reduced skin inflammation and studies have shown that TGF-β-mediated chemotaxis requires Smad3 in a variety of cells, including neutrophils, macrophages, keratinocytes, and fibroblasts. Loss of Smad3 selectively interferes with the recognition of signals from TGF-β.

Both Smad2 and Smad3 were expressed in the nucleus indicating that they are in an active state. Smad4 could only be detected in the cytoplasm suggesting that the antibody used only detects Smad4 when it is not bound to either Smad2 or Smad3. One explanation may
be that the epitope recognised by the Smad4 antibody is hidden when bound to any R-smad.

Apart from acting as a negative regulator of Smad activation, Smad7 promotes cell cycle arrest and apoptosis regulated by TGF-β\(^ {101, 157, 158}\). The increased expression of Smad7 could play a part in the increased apoptosis seen in OLP. Increased expression of Smad7 could also indicate less differentiated cells in OLP. Increased expression of Smad7 has been shown to impair terminal epidermal differentiation by negative regulation of KLF-4, an important factor in differentiation of epidermis\(^ {102}\).

**Expression of miR-21, miR-125b and miR-203 in oral lichen planus (Paper II)**

The aetiology of oral lichen planus is so far unknown, even if studies have shown deregulation of important proteins like p53 and p63 in OLP lesions\(^ {44}\). Specific miRNAs have been indicated as regulators of these proteins and/or connecting pathways. We therefore mapped expression of three specific miRNAs, miR-21, miR-125b and miR-203, and their potential targets p53 and p63 in OLP lesions and matched healthy controls.

A significantly increased expression of miR-21 (p < 0.01), miR-203 (p < 0.001) (Figure 1, *paper II*) and a significant decrease in p53 (p < 0.001) and ΔNp63 (p < 0.01) mRNA levels in OLP lesions was detected (Figure 2, *paper II*). The decreased expression of miR-125b in OLP compared to normal oral epithelium was not significant (p > 0.05)(Figure 1, *paper II*).

Previous studies have shown up-regulation of miR-21 in other chronic inflammatory skin diseases such as psoriasis and atopic eczema\(^ {117}\) and also in oral squamous cell carcinoma and leukoplakia\(^ {159}\). miR-21 is known to negatively regulate TAp63 but analysis of any potential correlation between them was not possible due to low levels of TAp63 mRNA in our material. In our study, lower levels of p53 mRNA in OLP lesions was seen and previous studies have reported increased
expression of p53 protein in OLP \(^{137, 138, 160-164}\). This discrepancy could be explained by the fact that p53 is regulated mainly at the post-translational level \(^{163, 165, 166}\). One of the hallmarks of OLP, inflammation, is also able to induce p53 \(^{167}\). The increased levels of miR-21 and the decreased p53 mRNA levels could indicate that the known inhibitory effect of miR-21 on p53 activators \(^{168}\) cause lower p53 mRNA levels in OLP. Up regulation of miR-21 has previously been shown to be caused by TGF-\(\beta\) \(^{103}\) and high expression of miR-21 in breast cancer correlates with high levels of TGF-\(\beta\) \(^{169}\). TGF-\(\beta\) and Smad3 have also been shown to be involved in and able to enhance the biogenesis of miR-21. Our previous data showing increased levels of smad proteins in OLP lesions indicative of a higher TGF-\(\beta\) activity could imply a connection between TGF-\(\beta\) and miR-21 also in OLP lesions.

Decreased expression of miR-125b has previously been shown in SCCHN \(^{170, 171}\) as well as in psoriasis and atopic eczema \(^{117}\) and a recent microarray analysis showed a 2-fold down regulation of miR-125b in OLP \(^{172}\). In our material down-regulation in OLP lesions was also seen but the decreased expression of miR-125b was not significant.

miR-203 is abundantly expressed in keratinocytes compared to other cell types \(^{117}\) and in these cells \(\Delta Np63\) and miR-203 are inversely expressed \(^{130}\). In skin, miR-203 represses p63 \(^{131}\) and through this restriction of cell proliferation miR-203 can act as a switch between keratinocyte proliferation and differentiation in adult epidermis and it is suggested that miR-203 regulates \(\Delta Np63\) expression upon genotoxic damage \(^{133}\).

Here we saw increased levels of miR-203 and, in accordance with other studies, decreased levels of \(\Delta Np63\) mRNA in OLP \(^{165}\). Decreased p63 has also been shown at the protein level in OLP compared to normal oral mucosa \(^{44}\). This finding could indicate less proliferative keratinocytes in OLP. However, OLP lesions have a higher proliferative activity than normal oral mucosa \(^{164, 173}\) which could be a secondary effect caused by the intense inflammation \(^{174}\) or by miR-21 which has been connected to increased cell proliferation \(^{175, 176}\). A recent study showed that miR-125b functions as an inhibitor of keratinocyte proliferation and a promoter of terminal differentiation.
and the down-regulation of miR-125b seen in OLP might also contribute to the increased proliferation and disturbed differentiation. A histopathological finding in OLP is atrophic epithelium and in a previous study mice over expressing miR-203 had a thinner atrophic skin\(^{131}\). In theory, this over-expression of miR-203 in OLP could in part be responsible for the atrophic epithelium seen in OLP.

**Increased levels of COX-2 in oral lichen planus (Paper III)**

A protein which is directly connected to inflammation and also suggested to be involved in cancer development is COX-2\(^{66}\). Levels of COX-2 and its potential inhibitor miR-26b were studied in laser micro-dissected epithelium from OLP and normal oral mucosa.

There was a significantly increased expression of COX-2 mRNA in OLP epithelium compared to normal oral epithelium (p < 0.001) and the levels of miR-26 were significantly decreased (p < 0.02) (Figure 1, *paper III*). By the use of immunohistochemistry increased expression of COX-2 protein was also seen in OLP compared to controls which were negative for COX-2 protein expression. Previous studies have also reported an increased COX-2 expression in OLP\(^{68, 69}\) and increased levels of COX-2 have been seen not only in a variety of cancers including SCCHN,\(^{67}\) but also in autoimmune diseases\(^{177-179}\).

The over-expression of COX-2 in OLP could theoretically play a role in the cases of malignant development seen in OLP. Malignant transformation of OLP lesions is however quite rare, 0.07-5.3\%\(^{18-25}\). COX-2 is also over-expressed in autoimmune diseases. A suggestion is that the increased expression in OLP could support an autoimmune phenotype rather than indicating a premalignant character of OLP. This suggestion is supported by the fact that Neppelberg et al\(^{68}\) were not able to connect increased COX-2 expression to malignant progression of OLP lesions. Our findings of lower levels of miR-26b levels in OLP epithelium in concert with higher levels of COX-2 support a previous study suggesting miR-26b as a negative regulator of COX-2\(^{122}\). In OLP there is increased proliferation and miR-26b has
been shown to be an inhibitor of cell proliferation\textsuperscript{120, 122} and low levels of miR-26b could enable the increased cell proliferation in OLP as well as the increased COX-2 levels.

**Increased levels of the receptor CXCR-3 and its ligands CXCL-10 and CXCL-11 (Paper IV)**

OLP has been suggested as an autoimmune disease. A recent study of vulvar lichen sclerosus and vulvar LP showed an autoimmune phenotype in these diseases characterized by an increased Th1 response and strong up-regulation of the expression of proinflammatory chemokines such as CXCR-3 and its ligands CXCL-10 and CXCL-11\textsuperscript{78}.

We identified a significant up-regulation of CXCL-10 (p ≤ 0.021) and CXCL-11 (p ≤ 0.034) in micro-dissected epithelium as well as in the samples containing epithelium and connective tissue (Figure 1, *paper IV*). Significantly increased expression of the receptor CXCR-3 (p ≤ 0.021) was seen in samples containing both epithelium and connective tissue, while no significant difference was seen between normal epithelium and OLP epithelium (Figure 1, *paper IV*). These findings are in accordance with previous findings in OLP, vulvar LP and skin LP\textsuperscript{77, 78, 180}. These increased levels of CXCR-3, CXCL-10 and CXCL-11 support previous suggestions about a Th1 immune response indicating an autoimmune phenotype of the disease.

**Decreased expression of ELF-3 and autoantibodies against ELF-3 in OLP (Paper IV)**

The levels of ELF-3 mRNA were significantly decreased in OLP samples containing both epithelium and connective tissue (whole samples)(p < 0.001) (figure 6B) as well as in micro-dissected OLP epithelium (p < 0.001)\textsuperscript{8}Figure 6 A). Using Western blotting, ELF-3 protein levels were decreased in OLP compared to controls.
RESULTS AND DISCUSSION

(Figure 6 C). In comparison to whole normal oral mucosa, whole OLP samples had significantly decreased expression of ELF-3 (p = 0.48). Autoantibodies against ELF-3 were detected in sera from three OLP patients (Figure 6 D). These were all older women, mean age 77, with a long history of LP lesions. The patient with the highest OD reading had oral lesions only, one patient had both oral and genital lesions and the third patient with the lowest OD had oral, genital and skin lesions. A previous analysis has also shown the presence of autoantibodies against p63 in one of these patients.

Figure 6:

Expression of ELF-3 in controls and OLP. A) ELF-3 mRNA levles in control epithelium and OLP epithelium. B) ELF-3 mRNA levels in whole (epithelium and connective tissue) control and whole OLP samples. C) ELF-3 protein levels in control and OLP. D) Optical density measurements from ELISA analysis of serum from normal controls and patients with OLP. The line representing the cut off value, 0.87 (mean control + 2S.D). *Patient with autoantibodies against p63.
To our knowledge no data on ELF-3 in OLP is available as of today whereas increased ELF-3 expression in some other inflammatory diseases has been reported, suggesting that inflammatory factors such as IL-1β and TNF-α could induce ELF-3 expression in synovial fibroblasts, chondrocytes, macrophages and endothelial cells. However, this does not seem to be the case in our material where decreased levels and expression of ELF-3 were seen in both OLP lesions and separated OLP epithelium. Even though there are elevated levels of inflammatory factors such as of TNF-α and IL-1β in OLP they did not stimulate cells in the connective tissue or epithelium in OLP to produce ELF-3.

Studies on cell lines indicate that ELF-3 inhibits invasion of oral squamous cell carcinoma (OSCC) cells through suppression of MMP-9 and that ELF-3 is down-regulated in Snail induced epithelial mesenchymal transition (EMT) in SCCHN. We previously showed increased expression of Smad3 in OLP and other studies have shown that Snail is a coactivator in Smad3 induced EMT. The decreased ELF-3 expression in OLP raises the question whether lower ELF-3 expression would support an EMT phenotype and premalignant character of OLP.

The decreased levels of ELF-3 seen in OLP indicate more immature and less terminally differentiated cells in this disease. Previous studies in OLP of factors involved in differentiation indicate a disturbed differentiation pattern. Altered expression of keratins has been shown in OLP indicating a disturbed differentiation pattern. We could see a trend of decreased levels of miR-125b in OLP in accordance with a recent study that also showed lower levels of miR-125b in OLP and low levels of miR-125b impair terminal differentiation in keratinocytes. A study by Ebrahimi et al showed levels of p63 to be decreased in OLP, a down-regulation that could lead to impairment in keratinocytes to undergo terminal differentiation. There is a down regulation of E-cadherin and EGFR in OLP and these factors have been shown to be required for normal differentiation of keratinocytes. TGF-β is an inhibitor of ELF-3 and our previous finding of increased expression of Smad3 and 7 in OLP, indicative of increased TGF-β activity, could help explain the decrease of ELF-3 we saw in OLP. Increased expression of
Smad7 has been shown to impair terminal epidermal differentiation by negative regulation of KLF-4, an important factor in differentiation of epidermis. The increased expression of Smad7 that we previously found in OLP could also be an indication of impaired differentiation. Autoantibodies against p63 in patients with OLP has been reported, suggesting a reaction against the process of differentiation in OLP and in this study we were also able to detect autoantibodies against a protein, ELF-3, involved in differentiation. Taken together, there are several findings showing decreased differentiation of keratinocytes in OLP, a process in which the decreased expression of ELF-3 could be involved.

Decreased expression of ELF-3 in OLP indicates a disturbed differentiation pattern of keratinocytes. As low levels of ELF-3 can promote invasiveness in an OSCC cell line, the down-regulation of ELF-3 could promote invasiveness. Accordingly this could indicate a premalignant character of OLP, but malignant development of OLP is rare and ELF-3 is also deregulated in autoimmune and inflammatory diseases. Therefore, the data could support an autoimmune phenotype of the disease, and autoimmune diseases are associated with development of malignancies. We saw an up-regulation of CXCR-3, CXCL-10 and CXCL-11 and autoantibodies against ELF-3 were also found in three patients, which supports an autoimmune phenotype in OLP. A suggestion is that the disturbed differentiation pattern in OLP might induce the inflammatory response directed against the keratinocytes in the epithelium in OLP.
GENERAL DISCUSSION

Even if there are numerous studies concerning LP and OLP the exact cause and pathogenesis of the disease is still not known. Autoimmunity is suggested to play a part in the pathobiology of OLP. It is an immune mediated disease in which there are auto cytotoxic T-cells. Autoantibodies have been found in some patients but no “OLP antigen” has been detected so far. One of the hallmarks of OLP is the sub-epithelial chronic inflammation, and chronic inflammation is known to be connected to increased risk of malignant transformation. WHO classifies OLP as a potentially malignant disorder with an increased risk of developing SCCHN. Malignant transformation of OLP has, however, been a question of some controversy. One aim of this thesis was therefore to shed some light over the potential premalignant character of OLP. It has been suggested that there might be changes on the surface of the epithelial cells acting as a trigger of the immune response. Therefore it was important to analyse the epithelial part separately and we therefore chose to laser micro-dissect the epithelium for separate analysis. The deregulated expression of several of the factors studied indicates that these could play a part in the pathogenesis of OLP. The factors studied are involved in processes such as differentiation, EMT and inflammation and some factors are involved in more than one of these processes (Figure7).

Figure 7 The factors studied and their known involvement in different processes.
The increased expression of smad proteins and especially Smad3 and increased expression of miR-21 indicates possible EMT related changes in OLP. Together with the decreased levels of the differentiation related factor ELF-3 this could indicate a premalignant character of OLP. miR-21 and Smads are, however, also involved in processes like inflammation, apoptosis and cell proliferation, processes known to occur in OLP. The over-expression of COX-2 in OLP could theoretically play a role in the cases of malignant development seen in OLP, however Neppelberg et al. were not able to connect increased COX-2 expression to malignant progression of OLP lesions. The altered expression of miR-21, miR-125b and miR-203 seen in OLP indicates a role for these microRNAs in this specific disease. The increased expression of miR-21 and miR-203 and the decreased expression of their potential targets p53mRNA and p63mRNA could indicate a negative correlation between them in OLP but one must remember that protein expression of p63 and p53 was not analyzed in this study. Previous studies have shown that there is an increased expression of p53 protein in OLP while levels of p63 proteins are reported to be decreased. miR-203 is suggested to regulate ΔNp63 expression upon genotoxic damage, and miR-21 and mir-125 are both suggested to play a part in regulation of inflammatory responses. miR-203 is a player in maintaining the balance between proliferation and differentiation and overexpression can lead to atrophic skin. miR-125b is a factor able to inhibit proliferation and promote terminal differentiation and the lower levels of miR-125 in OLP might contribute to increased cell proliferation and impaired differentiation of the keratinocytes.

Our findings of de-regulated expression of miR-203, miR-125b, p63 and ELF-3 together with previous findings thus point at a disturbed differentiation process in OLP which could support a premalignant character. Malignant transformation of OLP lesions is, however, quite rare and several of the factors studied are de-regulated in autoimmune diseases as well. Therefore, one possibility is that the de-regulated expression seen in OLP supports an autoimmune phenotype as well as indicates a premalignant character. This is further supported by the fact that we saw an up-regulation of CXCR-3 and CXCL-10 and CXCL-11, supportive of an autoimmune phenotype. The fact that we detected autoantibodies against a factor involved in differentiation of keratinocytes together
with previous findings of autoantibodies against p63\textsuperscript{11, 12}, another factor important for normal differentiation, suggests that altered differentiation plays a part in the pathogenesis of OLP. This disturbed differentiation pattern might in turn induce the inflammatory response directed against the keratinocytes in the epithelium in OLP, which could be part of an autoimmune process.
CONCLUSIONS

- Increased expression of Smad proteins and Smad3 in particular was seen. This could indicate an EMT process in OLP. On the other hand Smad3 can also be involved in both apoptosis and inflammation, processes that both are part of OLP. Increased expression of Smad7 also can induce apoptosis.

- Expression of miR-21 and miR-203 was increased in OLP and levels of their potential targets p53 and p63 decreased indicating that miR-21 and miR-203 could play a role in regulating their expression in OLP. Expression of miR-125b was decreased in OLP but not at a significant level. These miRNAs can also be involved in processes such as proliferation, apoptosis and differentiation, which could support a premalignant character of OLP.

- In accordance with previous studies, levels of COX-2 were increased in OLP. The increased COX-2 levels together with decreased levels of miR-26b suggests miR-26b as a negative regulator of COX-2.

- The increased levels of CXCR-3, CXCL-10 and CXCL-11 in OLP support an autoimmune phenotype.

- The decreased levels of ELF-3 in OLP indicate less differentiated cells pointing at disturbed differentiation of keratinocytes. Autoantibodies against ELF-3 were also seen in sera form some patients. Disturbed differentiation could be part of the induction of the inflammatory response seen in OLP.
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