ORAL IMMUNE DEFENSE
against
CHRONIC HYPERPLASTIC CANDIDOSIS

Ahmed S. Ali Musrati

Academic dissertation
Supervised by

1. **Professor Yrjö T. Konttinen**, M.D., Ph.D., Head of Biomaterial and Inflammation Research Center, Institute of Clinical Medicine, Department of Medicine, Biomedicum Helsinki, P.O. Box 700, 00029 HUS, Finland.

2. **Professor Jarkko Hietanen**, M.D., Ph.D., D.D.S., M.Sc., Department of Oral Pathology, Institute of Dentistry, PL 41, 00014 University of Helsinki, Finland.

Reviewed by


2. **Professor Stephen Porter**, BSc MD PhD FDS RCSE FDS RCS Professor of Oral Medicine, Chairman of the Division of Maxillofacial Diagnostic Medical and Surgical Sciences, UCL Eastman Dental Institute 256 Grays Inn Road London WC1X 8LD, UK.

Opponent

- **Docent Aaro Miettinen**, MD, PhD. Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland.

ISBN 978-952-10-4702-2 (PDF)
Helsinki 2008
Yliopistopaino
This thesis is dedicated to.....

... the memory of my late mother, Zakia Mohammed...
(1948-1998)
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ACKNOWLEDGEMENT

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*LIST OF ORIGINAL PUBLICATIONS*

This thesis is based on the following original publications, referred to in the text by their Roman numerals (I-V). The impact factor (IF) was according to rating up to 2006.


* The publishers have granted me their kind permission to use any material in all the papers of this thesis.
LIST OF ABBREVIATIONS

ABC                 avidin-biotin-peroxidase complex
AIDS                acquired immune deficiency syndrome
APC                 antigen presenting cell
ATP                 adenosine triphosphate
BSA                 bovine serum albumin
C. albicans        Candida albicans
CHC                 chronic hyperplastic candidosis
DAB                 diaminobenzidine
DC                  dendritic cell
FBS                 fetal bovine serum
GCP                 granulocyte chemotactic protein
HCl                 hydrochloric acid
HIV                 human immunodeficiency virus
HNP                 human neutrophil peptide
Ig                  immunoglobulin
IL-8                interleukin-8
IL-8 RA             interleukin-8 receptor A
LC                  Langerhans cell
LP                  Leukoplakia
LPS                 lipopolysaccharide
MC_T               mast cell tryptase
MC_Tc              mast cell tryptase and chymase
MDNCF              monocyte-derived neutrophil chemotactic factor
MHC                major histocompatibility complex
NADPH              nicotinamide adenin dinucleotide phosphate
NAF                neutrophil activating factor
NAP                neutrophil activating protein
NCF                neutrophil chemotactic factor
ODF                osteoclast differentiation factor
OPG                osteoprotegrin
OPGL               osteoprotegrin ligand
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PAS</td>
<td>periodic acid Schiff</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PML</td>
<td>polymorphonuclear leukocyte</td>
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<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor κB</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor κB ligand</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency syndrome</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell chemotactic factor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TTC</td>
<td>triphenyltetrazolium chloride</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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</table>
Abstract

*Candida* yeast species are widespread opportunistic microbes, which are usually innocent opportunists unless the systemic or local defense system of the host becomes compromised. When they adhere on a fertile substrate such as moist and warm, protein-rich human mucosal membrane or biomaterial surface, they become activated and start to grow pseudo and real hyphae. Their growth is intricately guided by their ability to detect surface defects (providing secure “hiding”, thigmotropism) and nutrients (source of energy, chemotropism).

The hypothesis of this work was that body mobilizes both non-specific and specific host defense against invading candidal cells and that these interactions involve resident epithelial cells, rapidly responding non-specific protector neutrophils and mast cells as well as the antigen presenting and responding dendritic cell – lymphocyte – plasma cell system. It is supposed that *Candida albicans*, as a result of darwinistic pressure, has developed or is utilizing strategies to evade these host defense reactions by e.g. adhering to biomaterial surfaces and biofilms.

The aim of the study was to assess the host defense by taking such key molecules of the anti-candidal defense into focus, which are also more or less characteristic for the main cellular players in candida-host cell interactions.

As a model for candidal-host interaction, sections of chronic hyperplastic candidosis were used and compared with sections of non-infected leukoplakia and healthy tissue.

In this thesis work, neutrophil-derived anti-candidal α-defensin was found in the epithelium, not only diffusely all over, but as α-defensin-rich front. Once they reach the epithelium, neutrophils, which form the major immigrant host defense cell, organize themselves into microabscess structures (study I). Mast cells, in addition to tumour necrosis factor-α, were found to contain preformed receptor activator of nuclear factor kappa B ligand (study II). This is important for the recruitment and maturation of antigen presenting dendritic cells and T lymphocyte activation (study III). The presence and effects of the chemokine interleukin-8 on the chemotaxis and transmigration of neutrophils was studied (study IV). For the immune system to operate, it has to be invoked first by a set of innate receptors known as Toll-like receptors (TLRs). Only three classes of TLRs seem to be engaged in recognizing *C. albicans*, i.e. TLR2, TLR4 and TLR6. Hypha-rich candidal infection appears to try to elude the host response through stimulating TLR2 rather than TLR4 (study V).
Chronic hyperplastic candidosis provides a system that is very useful to study local and systemic host factors, which under normal circumstances restrain \textit{C. albicans} to a harmless commensal state, but failure of which may lead to chronic infection.
**Anatomical & histological review of the oral cavity:**

The oral cavity can be divided into two parts: the vestibulum oris (vestibule) and the cavum oris proprium (oral cavity proper). The vestibular part is bordered by the lips and cheeks on the outer side and by the teeth and alveolar ridges on its inner side. The oral cavity proper part lies within the dental arches and bones of the jaw, being limited posteriorly toward the pharynx by the anterior pillars of the fauces which is the passage between the back of the mouth and the pharynx (Fig.1, A&B). The oral cavity is mostly lined with mucous membrane and its underlying musculature and connective tissue.

The morphologic structure of the oral mucous membrane varies according to the functional requirements in different areas of the oral cavity and the mechanical influences which affect them. In case of considerable mechanical influences, e.g. around the teeth, on the surface of tongue and on the hard palate where the epithelium comes in contact with the rough surface of masticated food, the mucosa is keratinized and attached densely to the underlying tissue and / or bone. In other areas of the mouth where chewing is not primary concern, the mucosa is nonkeratinized, loose and unsupported by bone e.g. labial and buccal regions. The same applies in those areas which are well-protected with other tissues e.g. the floor of the mouth which is covered with the tongue. Due to its unique function in chewing and tasting, the dorsum of the tongue is covered with a mosaic of keratinized and nonkeratinized epithelium and specialized foliate, fungiform and circumvallate papillae which contain the taste buds (Squier and Kremer 2001).

The oral mucous membrane is composed of two layers, 1) surface epithelium which covers 2) underlying connective tissue. The **lamina propria** is the name used for the connective tissue component of oral mucosa.

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**Figure 1 showing a schematic illustration A, and extraoral photograph B, of the oral cavity (modified from a website)**
**Epithelium**

The oral mucosa, which is covered by stratified squamous epithelium, is traditionally classified into three main types, keratinized, nonkeratinized and specialized. The epithelial covering of the oral mucosa consists of several layers of cells which flatten as they reach the surface. The deepest or the innermost layer which rests on the basement membrane is called basal layer (*stratum basale*) and consists of cuboidal cells. Next to the basal layer is a number of layers of polyhedral cells which form the prickle cell layer (*stratum spinosum*), the name of which was derived from the prickly appearance of the connected cells by their intercellular bridges. The cells of the prickle-cell layer flatten out when they pass into the next two layers; the granular and keratinous. The granular layer (*stratum granulosum*) is called so because its cells contain keratohyaline granules. The surface of oral epithelium is covered with the last keratinous layer which contains dead cells filled with keratin (only in the keratinized areas of the oral cavity). The keratinized layer may take up two morphological forms, orthokeratinized or parakeratinized, with the main difference between the two being the retention of pyknotic nuclei in the cells of the latter.

The epithelium of the oral cavity does not contain blood vessels although some of the nerves actually pass into it. Papillae of underlying connective tissue protrude toward the epithelium.

The epithelium forms reciprocal ridges that protrude toward the lamina propria. These ridges interdigitate with the papillae and are called epithelial ridges or rete pegs. The epithelium is separated from its underlying lamina propria by means of a basement membrane.

**Lamina propria**

The lamina propria of the oral mucosa is a dense connective tissue layer of variable thickness. It lies below and supports the overlying stratified epithelium. Its papillae contain both blood vessels and nerves important also for the epithelium. The papillae are arranged in such a way that the surface area of contact between the lamina propria and epithelium is increased to facilitate exchange of material e.g. nutrients. The lamina propria can be divided into two parts; the papillary superficial part located just beneath the epithelium containing papillae, and the reticular deeper part. Besides blood vessels and nerves, the lamina propria consists of other components of the dense and loose connective tissue e.g. lymphatics, ducts of glands, and sense organs.
The lamina propria is further attached to an underlying connective tissue called submucosa.

**Submucosa**

Submucosa is the connective tissue which lies below the lamina propria and which attaches the oral mucous membrane to the underlying bony or muscular tissues. This tissue contains blood vessels which divide into smaller branches, nerves and adipose tissue. The blood vessel system in this tissue divides into subepithelial capillary network in the papillae, and is accompanied by venous and lymphatic vessels. The sensory nerves, which traverse the submucosa, are myelinated but just before they form their terminal parts lose their myelin sheath and turn unmyelinated. This portion of the oral cavity may contain minor salivary glands.

**Salivary glands & saliva**

The oral mucosa is bathed continually with a clear fluid, saliva, which is mostly secreted by three major paired glands, the parotid, the submandibular and the sublingual. In addition, there are numerous minor salivary glands, perhaps about 500 in number, scattered over most of the oral surfaces with the exception of the gingivae and the anterior third of the hard palate. All salivary glands, which are of merocrine type, empty their secretions into the oral cavity through excretory ducts (e.g. Stensen’s and Wharton’s ducts).

Apart from water, the other major constituents of saliva are mucus, α-amylase, lipase, electrolytes and growth factors (Kagami, Hiramatsu et al. 2000). Besides, saliva, whose electrolyte composition is different from plasma, contains shed epithelial cells, food debris and oral microbiota (Edgar 1992). The basic secretory units of salivary glands are clusters of cells called acinus (plural: acini). The basic morphology of acini differs according to the type of secretion so that the serous acini tend to be circular in shape while those producing mucinous secretions have a rather tubular morphology. These cells secrete a fluid that contains water, electrolytes, mucus and enzymes, all of which flow out of the acinus into the salivary ducts. Ducts of the salivary glands root out from acini as intercalated ducts, which unite to form striated ducts which further coalesce to form the excretory duct of the gland.

Within the ducts the composition of the secretion is altered. The intercalated ducts add bicarbonate to the glandular secretions and reabsorb chloride ion from the primary-produced saliva. The basal parts of striated duct cells are rich in mitochondriae whose alignment together with the basal folds forming the cellular compartments inhabiting
the mitochondria give the ducts a striated appearance (hence its name). The mitochondria of the striated ducts allow pumping of ions across the membrane, thus regulating the ion concentration of saliva, e.g. much of the sodium is actively reabsorbed, and potassium is secreted. These relatively small collecting ducts within salivary glands lead further into a larger terminal (excretory) duct whose main function is to eventually empty the saliva into the oral cavity. Both serous and mucous acini and terminal parts of the secretory intercalated duct system are surrounded by spindle-shaped smooth muscle cells called myoepithelial cells which, by their contraction, help move the saliva from acini to the duct system and thus participate in the secretory process.

Each major salivary gland is characterized by its own type of acini, which corresponds to the type of saliva it produces:

- **Parotid glands** produce a serous, watery secretion
- **Submandibular glands** produce a mixed serous and mucous secretion; however serous cells significantly outnumber the mucous cells.
- **Sublingual glands** are mixed as well but mucous cells predominate.

Secretion of saliva is under control of the autonomic nervous system, which in part controls both the volume and type of saliva secreted. Stimulation of the sympathetic division leads to a rather viscous secretion rich in proteins, while parasympathetic stimulation produces more watery saliva.

**Functions of saliva**

The most important functions of saliva are summarized below:

1. **Lubrication and binding:** the mucin component of saliva lubricates and protects the oral structures by acting as a barrier against irritants. Lubrication aids in speech, mastication and swallowing (Tabak 1990). Lubrication of dry food solubilizes it so that it can be tasted. A salivary protein, gustin, seems to be essential for taste bud growth and development (de Almeida Pdel, Gregio et al. 2008). Salivary mucins are extremely effective in binding masticated food into a slippery bolus that slides easily through the esophagus without inflicting damage to mucosa.

2. **Oral hygiene:** saliva enhances oral health by its almost constant flushing oral tissues, which floats away food debris and microorganisms thereby keeping the mouth relatively clean and hindering microbial colonization. Saliva contains
antibacterial substances e.g. lysozyme, peroxidase, and lactoferrin, which can lyse many bacteria or prevent overgrowth of oral microbiota (Rudney 1995). Saliva participates in the immune response through IgA which it contains and which is known to inhibit bacterial attachment to the oral mucosa by means of clumping the bacterial cells together (Dowd 1999).

3. **Initiation of food digestion:** saliva starts digestion of food. Serous acinar cells secrete an alpha-amylase which digests dietary starch into maltose. However, this function remains minor compared to the pancreatic amylase which breaks down the yet undigested starch in the intestine.

4. **Buffering action:** the salivary content of bicarbonate and urea is of great importance in neutralizing the acidic environment of dental plaques, which protects against dental decay.

5. **Antisolubility:** in the initial stages of dental caries, the hard tissue substance of the tooth (enamel) is dissolved most probably due to the acid produced by acidogenic bacteria. Thus, the mineralized constituents of enamel, calcium and phosphate, are liberated. Instead of losing these essential minerals for good, saliva, already containing considerable calcium and phosphate, when it gets saturated with them partly with the aid of certain salivary proteins, tends to precipitate them again in the enamel (remineralization).
Fungi: Definition, taxonomy and characteristics

Fungi are eukaryotic, plant-like microorganisms which are ubiquitously spread in nature. There are about 80,000 species of fungi which range from the simplest unicellular yeasts to the more complicated multicellular mushrooms and mildews.

There are many ways to classify fungi, but the most common criterion upon which fungi can be classified is according to their manner of reproduction and formation of spores. With respect to reproduction, fungi are classified into five major groups:

- **Ascomycetes** - characterized by production of microscopic spores inside elongated cells or sacs, known as asci.
- **Basidiomycetes** - the spores in this group are produced on the end of specialised cells called basidia.
- **Deuteromycetes** - this class includes all fungi which reproduce only by asexual spores without any known sexual reproduction.
- **Oomycetes** - the life cycle of these fungi include two phases: asexual (through zoospore), and sexual (oospore)
- **Zygomycetes** - the hyphae of these fungi form coenzytic mycelia. They reproduce both asexually (chlamydomyces) and sexually (formation of zygote).

Fungi grow well in dark and moist conditions; hence they thrive most often in soil and aquatic environments. One of the important characteristics of fungi is that they lack chlorophyll and therefore draw their nutrition from decaying organic matter of living or dead plants and animals which they use as sources of energy. For that reason, fungi are considered heterotrophic and said to be saprophytes. Due to their lack of chlorophyll, modern biologists tend to place fungi in their own kingdom rather than in the plant kingdom as used to be previously.

Many fungi play an important role in the natural cycle as they decompose organic matters and return their end products to the soil. Fungi are even used for medical purposes, such as species within the penicillium genus which provide antibiotics, e.g. penicillin.

There are two basic morphological varieties of fungi, **yeasts** and **molds**. Fungi in the yeast phase tend to form moist and shining colonies reproduce asexually and their cell walls are sometimes surrounded with a capsule, e.g. *Cryptococcus neoformans*.

In contrast, fungi in the mold form consist of masses from which filamentous projections, known as hyphae, branch out. The hyphae may be septate (where the
multicellular hyphae are separated by crosswalls) or nonseptate (called coenocytes, multinucleate without partitions). Hyphae usually grow along a surface and branch together forming tufts, collectively called mycelium. Common septate filamentous fungi are Aspergillus, Fusarium, Cephalosporium, Paecilomyces, and Penicillium species. The nonseptate filamentous fungi include the Mucor species.

**Candida**

The genus *Candida* belongs to yeasts. It is also the most common cause of opportunistic mycoses worldwide. It is a frequent colonizer of human skin and mucous membranes. *Candida* is a member of normal flora of skin, mouth, vagina, and bowel. In addition to being a colonizer and a pathogen, it is found in the environment, particularly on leaves, flowers, water and soil. The genus *Candida* includes around 154 species. Among these, six are most frequently isolated in human infections. While *Candida albicans* is the most abundant and significant species, *Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida krusei,* and *Candida lusitaniae* are also isolated as causative agents of Candida infections. Importantly, there has been a recent increase of infections due to non- *albicans Candida* spp., such as *Candida glabrata* and *Candida krusei* (Abi-Said, Anaissie et al. 1997). Patients receiving fluconazole prophylaxis are particularly at risk of developing infections due to fluconazole-resistant *Candida krusei* and *Candida glabrata* strains (Barchiesi, Morbiducci et al. 1993). The diversity of *Candida* spp. that are encountered in infections is expanding and the emergence of species that were rarely described in infections in the past is now likely (Blinkhorn, Adelstein et al. 1989).

**Candida albicans**

*C. albicans* belongs to the Ascomycota class of fungi and is the most commonly studied species because it causes a variety of mycotic infections in humans (Siqueira and Sen 2004).

**Taxonomy**

- Kingdom: fungi
- phylum: ascomycota
- Subphylum: ascomycotina
- Class: ascomycetes
- Order: saccharomycetales
- Family: saccharomycetaceae
- Genus: Candida
- Species: albicans

**Morphology**

The fungal pathogen *C. albicans* can be found in three morphological states as yeasts, hyphae or intermediate forms i.e. pseudohyphae (Sudbery, Gow et al. 2004) (Fig. 2, 3). Some mycologists prefer to consider the pseudohyphal and hyphal forms as one entity; therefore *C. albicans* is usually said to be **dimorphic** in nature as it has two distinct shapes. Yeast cells are unicellular and spherical or oval in shape, and normally form smooth, white dome-shaped colonies. Yeasts multiply by a specific process of mitotic division known as **budding**, in which daughter cells exude from the mother cells. Several classes of fungi, including *C. albicans*, are featured with the ability to form spores i.e. blastospores and chlamydospores (which are spherical, smooth surfaced and highly refractile (Nobile, Bruno et al. 2003), thus more favorable for candidal survival). Pseudohyphae are considered modified yeasts which continued in polarized growth without separation from the mother cell at the end of each cell cycle (Sudbery, Gow et al. 2004). Pseudohyphae are also characterized by unequal width of their cellular projections, being wider at the center than at ends (Merson-Davies and Odds 1989). Hyphae are microscopic tubes which contain compartmentalized cell units separated by septa, these units arise initially from blastospores or also from already existing hyphae (Webb, Thomas et al. 1998). When it takes up the hyphal form, it forms filamentous projections with parallel-sided walls, so keeping the width of their compartments the same throughout the branched portion. Germ tube is a term applied to the projecting hyphae in the first cell cycle just before septation (Calderone, Suzuki et al. 2000).
*C. albicans* is well known for its morphological plasticity, i.e. its ability to transform from one morphological pattern to another known as **switching** (Whiteway and Oberholzer 2004), which is thought to promote the pathogenicity of the organism (Lo, Kohler et al. 1997). There are other minor morphological changes which take place during switching. For example, opaque phase is a variety where the cell becomes oblong instead of the usual oval form of the yeast. Cell signal transduction pathways and various transcriptional effects have been linked to the different array of morphological forms and switching of *C. albicans* (Liu 2002; Dhillon, Sharma et al. 2003).

**Biofilm formation**

Microorganisms can exist either in a floating planktonic state or attached to an external surface. A biofilm is an assembly of surface-coating microbial cells that is attached to the surface and enclosed in a matrix of polysaccharide material such as alginate, Psl and Pel-encoded polysaccharide (Donlan 2002; Ryder, Byrd et al. 2007).

In addition to its association with many hospital acquired nosocomial infections (Potera 1999) biofilm formation is of clinical significance since it confers the associated microorganisms an ability to resist external threats, e.g. antimicrobial drugs (Baillie and Douglas 2000; Mah and O'Toole 2001; Mukherjee, Chandra et al. 2003). A biofilm formed of *C. albicans* renders it a hundred times more resistant to the antifungal fluconazole and 20-30 times more resistant to amphotericin B than planktonic cells (Kumamoto 2002). Both yeast and hyphal forms of *C. albicans* can participate in biofilm formation. Due to biofilm formation, *C. albicans* yeast is considered as one of the most common microorganisms found in the bloodstream in hospitalized patients, in whom it originates from the biofilm composed of yeast cells embedded in a protective matrix of extracellular protein (Chaffin, Lopez-Ribot et al. 1998; Crump and Collignon 2000; Soustre, Rodier et al. 2004). Biofilm of *C. albicans* has been noticed in dentures as well as other biomaterials, e.g. stents, shunts, endotracheal tubes and catheters (Andes, Nett et al. 2004; Bulad, Taylor et al. 2004). In order to form a biofilm *C. albicans* has to adhere first to a medical device, colonize it and then establish a biofilm. Biofilm formation by *C. albicans* depends on many factors, e.g. nature of the device surface, whether host-derived conditioning film is present and liquid flow (Chandra, Kuhn et al. 2001; Kuhn, Chandra et al. 2002). Colonization of different biomaterials by *C. albicans* has been accepted as an important major cause of medical device failure (Jones, McGovern et al. 2001). In the biofilm composed of *C. albicans* a layer of yeast
cells is located lowermost attached to the device, follows a layer above the yeast layer composed of filamentous cells in the hyphal form surrounded by extensive exoplasmic matrix (Baillie and Douglas 1999).

**Biological behavior**

Generally speaking fungi have the ability to adapt themselves to different environmental conditions. Two important biological features seem to contribute to such adaptation, chemotropism and thigmotropism.

**Chemotropism** is the growth of an organism along a concentration gradient toward a particular chemical in its environment. Despite some doubts, it is argued that *C. albicans* hyphal tips might excrete enzymes e.g. SAPs (secreted aspartyl proteinases), phospholipases, which break cellular components of the host, which are then sensed back by receptors located on the hyphal apices (Davies, Stacey et al. 1999). This might be used as a directional guidance for the candidal hyphae so they decrease or even stop growing in the surface plane and extend instead down to the underlying cellular tissue. Chemotropic response has been suggested to play a role when *C. albicans* infects the vascular endothelium from where the candidal hyphae grow down to the underlying cellular tissue due to chemical signals released from the endothelial cells and detected by the newly formed germ tubes (Rotrosen, Edwards et al. 1985).

**Thigmotropism** is the ability of the organism to sense and respond to changes in surface topography upon which it rests. There are two types of thigmotropism. Positive thigmotropism causes an organism to grow toward an object and negative thigmotropism away from it.

Thigmotropism has been demonstrated in hyphae of *C. albicans* (Sherwood, Gow et al. 1992). This contact sensing phenomenon seems of importance in candidaemia where invasion of both epithelium and endothelium ensues. Although invasion of endothelium involves direct penetration of this layer without any requirement of surface exploration, epithelium is grown over by blastospore-derived hyphae prior to penetration (Filler, Swerdloff et al. 1995). This might indicate that thigmotropism can be of importance in both surface growth and invasion (Davies, Stacey et al. 1999). The molecular events involved in the regulation of thigmotropism are not yet known.
**Culture media**

In general, fungi are cultivated in three basic types of culture media, natural, dehydrated and synthetic.

The most frequently used medium for culturing *C. albicans* is SDA (Sabouraud Dextrose Agar) which belongs to the dehydrated media. Basically it contains dextrose and beef extract and is useful in clinical applications since this culture medium allows growth of *Candida* while inhibiting growth of other microorganisms, e.g. bacteria. SDA is seldom used alone since it is estimated that more than one type of *Candida* species occurs in about 10% of oral samples (Williams and Lewis 2000). Examples of media used in combination with SDA include Pagano-Levin agar, or commercially available chromogenic agars (discussed in the next section). In SDA candidal colonies are white to cream colored with smooth contours and glabrous shape (Fig. 4).

**Identification**

There are many species of the genus *Candida* e.g. *C. glabrata*, *C. krusei*, and *C. tropicalis*. To differentiate *C. albicans* from the other species, certain identification tests must be employed. *C. albicans* can be distinguished from other species by means of three main criteria, chromogenic, morphological and physiological criteria. Only the chromogenic and morphological criteria will be clarified.
Table 1. Chromogenic criteria in agar culture to differentiate *C. albicans* from other *Candida* species.

<table>
<thead>
<tr>
<th><strong>Agar medium</strong></th>
<th><strong>Basis of differentiation</strong></th>
<th><strong>Candida sp. identified (color)</strong></th>
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</thead>
<tbody>
<tr>
<td>Pagano-Levin</td>
<td>Reduction of TTC (triphenyltetrazolium chloride)</td>
<td><em>C. albicans</em> (cream), other species (red or pink)</td>
</tr>
<tr>
<td>CHROMagar Candida</td>
<td>Chromogenic substrate for hexosaminidase</td>
<td><em>C. albicans</em> (green) <em>C. tropicalis</em> (blue), <em>C. krusei</em> (pale rose)</td>
</tr>
<tr>
<td>Albicans ID</td>
<td>Chromogenic substrate for hexosaminidase</td>
<td><em>C. albicans</em> (blue), other species (cream)</td>
</tr>
</tbody>
</table>

Table 2. Morphological criteria to differentiate *C. albicans* from other *Candida* species (Williams and Lewis 2000).

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germ tube</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>+</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>some</td>
</tr>
<tr>
<td><em>C. stellatoida</em></td>
<td>+</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>-</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>-</td>
</tr>
<tr>
<td><em>C. guilliermondii</em></td>
<td>-</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>-</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>-</td>
</tr>
</tbody>
</table>
Pathophysiology of *Candida albicans* infection

Relationship between *C. albicans* and the host

- **C. albicans** as a component of the host microflora

Candidal species are found in about 40-70% of healthy individuals, the main species being *C. albicans* (Lo, Kohler et al. 1997; Watts, Very et al. 1998; Liu 2001; McCullough, Jaber et al. 2002). *C. albicans* comprises a component of the benign commensals living in a variety of body locations (Lo, Kohler et al. 1997; Lockhart, Pujol et al. 2002; Soll 2002). It belongs to the normal microbial flora which colonizes mucocutaneous surfaces asymptotically e.g. oral cavity, gastrointestinal tract, genitourinary tract of healthy human host (Elahi, Pang et al. 2001; Newman, Bhugra et al. 2005). In the vagina *C. albicans*, which colonizes the epithelial surfaces of 5 – 25% of healthy women (Sobel 1988; Hauman, Thompson et al. 1993), is the causative agent of vulvovaginal candidosis which affects 50 – 75% according to estimates of those women at least once before their menopause (Kent 1991; Sobel 1992), while 5-10% have a recurrent type of vulvovaginal candidosis (Fidel 2005).

- **When does *C. albicans* cause infection?**

As mentioned above, candidal yeasts can form a part of the normal microflora of the body. Candidal infection ensues when the number of yeasts increases so it exceeds the tolerance of the host tissue and causes inflammation, e.g. mucositis. In other words, it is the immune host defense status of the host which is to be blamed rather than the microorganism itself since as long as the host defense is intact with proper functional integrity, the latter remains innocent. When the local or systemic host defense becomes compromised, the delicate balance between *C. albicans* and the host is broken and the fungus becomes pathogenic in which case it causes an infection called **candidosis** or **candidiasis**. Factors which alter the status of the local and / or general host defense and hence favor the growth of the candidal yeasts in the oral cavity will be discussed shortly below as predisposing factors. Therefore, candidal infections have received the designation “disease of the diseased”. Recently, the incidence of candidosis has increased which is mainly due to the increasing number of immunocompromised patients (McCullough, Jaber et al. 2002; Wellington, Bliss et al. 2003). Candidosis ranges from simple superficial oral and vaginal infections to life-threatening systemic candidosis in severely immunocompromised patients (Naglik, Newport et al. 1999;
Whiteway 2000). Biomaterials, when implanted to a host, form a good habitat for *C. albicans* to organize a biofilm community which in turn can contribute to disease (Kumamoto 2002).

- **Role of *C. albicans* in nosocomial infections & candidaemia**

*C. albicans* has been claimed to be the fourth most common nosocomial infectious agent (Schaberg, Culver et al. 1991; Ashman, Farah et al. 2004). In addition to the role of *C. albicans* in mucosal and mucocutaneous infections, it has an ability to invade also other vital systems in the body. In patients whose immunity is suppressed, these forms of invasive candidal infections, including blood-disseminated candidosis (candidal sepsis) are usually associated with high morbidity and mortality despite the use of appropriate antifungal agents (Fridkin and Jarvis 1996; Andriole 1999; Kao, Brandt et al. 1999). In susceptible hosts *C. albicans* enters the blood compartment and causes deep-seated infections in target organs (Ibrahim, Filler et al. 1998). Once in blood *C. albicans* may disclose its virulence factors and so the condition transits from candidaemia (mere presence of candida in the blood) to lethal septicemia (presence of symptoms caused by candidal toxins) the morbidity of which has been estimated to be around 50% (Edmond, Wallace et al. 1999; Barelle, Bohula et al. 2003).

**Oral candidosis**

- **Definition and epidemiology**

Oral candidosis is an opportunistic infection of the oral cavity caused by the overgrowth of *Candida* species, usually of *C. albicans* (Fotos and Hellstein 1992; Muzyka and Glick 1995). *Candida* species are present as commensal organisms of the oral microbiota in about 20-60% of normal human population (MacFarlane TW 1989). In the mouth, the primary site where *C. albicans* is located is the dorsum of tongue, while other places such as tooth surfaces covered with plaque are less commonly colonized (Arendorf and Walker 1980). There are certain factors which affect the pattern of distribution of *C. albicans* in the mouth (Webb, Thomas et al. 1998). They may include:

1. **Saliva:** One study showed that saliva has the ability to reduce candidal attachment to the acrylic surface of oral biomaterials (Samaranayake, McCourtie et al. 1980), although mannoprotein on the surface of *C. albicans* may also selectively absorb
salivary mucins, which can enhance candidal attachment to acrylic (Edgerton, Scannapieco et al. 1993).

2. **pH**: The exact pH at which *C. albicans* adheres best to oral cells cannot be defined precisely since the effect of pH varies considerably depending on the candidal strain and type of mucosal cell (Mehentee and Hay 1989) but generally speaking, low pH has been suggested to favor the growth and colonization of *C. albicans*.

3. **Adhesion**: The interaction between oral cells and *C. albicans* is thought to be mediated through ligand-receptor interactions. Mannoprotein, for example, represents a ligand on the candidal surface while there are many mammalian cell proteins acting as receptors, e.g. iC3b, fibrinogen and laminin (Calderone and Braun 1991).

4. **Cell surface hydrophobicity**: Candidal cells can be hydrophilic or hydrophobic depending on the composition of cell wall in the protein structure of the cell wall (Hazen, Lay et al. 1990). When candidal cells are hydrophobic, they can bind diffusely to hydrophobic surfaces of host cells without ligand-receptor interactions to areas which are free of macrophages (Hazen, Brawner et al. 1991).

5. **Oral bacteria**: The growth and colonization of *C. albicans* may be augmented by the presence of some bacteria e.g. *Streptococcus sanguis*, *Streptococcus gordonii* (Hsu, Minah et al. 1990).

6. **Hyphae**: *C. albicans* is a biphasic fungus. The hyphal form is associated with more invasive potential than the yeast form (Kimura and Pearsall 1980). This is thought to be due to the release of some proteases during switching of *C. albicans* from the unicellular yeast to the hyphal form (Borg and Ruchel 1988; Cutler 1991).

- **Etiology and predisposing factors**
  Among the 150 *Candida* species known today, the most common etiological microorganism of oral candidosis is *C. albicans* (Cannon, Holmes et al. 1995). This, however, does not preclude the other human pathogenic species from being direct causative agents in some cases of oral candidosis, namely *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. pseudotropicalis*, or even *C. dubliniensis*, which has first more recently been associated with oral candidosis in HIV-infected patients (Odds 1988; Sullivan, Westermeng et al. 1995). There are many factors which predispose to the development of oral candidosis. These can act either by enhancing the growth and colonization of candidal yeast or suppressing the immune system of the host or both.
They are summarized in table 3. Some factors, which are considered common, are detailed further below.

1) **Prostheses**

Dental prosthesis, especially if they are ill-fitting and accompanied with poor oral hygiene, may become a substrate for candidal growth. Constant physical irritation can cause local microscopic breaches in the oral mucosa, which provide an entrance route to the fungus. It has been noticed that salivary yeast counts are much higher in patients wearing full dentures than in dentate subjects (Parvinen 1984). Since dental prostheses may restrict the diffusion of oxygen and flow of saliva to the underlying tissue, a local stagnant environment with a low pH and oxygen content may be produced. Such an environment favors fungal overgrowth and ingrowth into the porous acrylic resin matrix (Shay, Truhrler et al. 1997). *C. albicans* adheres avidly to denture-base materials *in vitro* and this has been attributed to its hydrophobicity (Radford, Challacombe et al. 1999).

2) **Epithelial alterations**

Intact oral mucous membrane provides an effective physical barrier against ingress of fungal or bacterial cells. When the turnover rate of epithelial cells alters, e.g. due to radiation therapy or anticancer medication, the integrity of oral epithelial seal is impaired, which predisposes the mouth to candidal infection (Bunetel and Bonnaure-Mallet 1996).

3) **Endocrine disorders**

Deficiencies of certain hormones predispose to the emergence of oral candidosis e.g. diabetes mellitus, hypothyroidism, hypoparathyroidism, hypoadenalism and Addison’s disease (Fotos and Hellstein 1992). More commonly, the mucocutaneous form of candidosis has been associated with multiple endocrine disorders (Firth, O’Grady et al. 1997) where the triad of chronic mucocutaneous candidosis, hypoparathyroidism and adrenocortical failure comprise a compex condition known as APECED (Autoimmune Polyendocrinopathy-Candidosis-Ectodermal Dystrophy) (Perheentupa 2006). Studies have found that *Candida* species also in asymptomatic patients are more common in the oral cavity of diabetic patients than in healthy people (Dourov and Coremans-Pelseneer 1987). Although it is still obscure, the mechanism of this overgrowth of *Candida* is thought to be xerostomia, elevated glucose levels and impaired neutrophil function (Rossie and Guggenheimer 1997).
4) Infectious and immunologic disorders

The cell-mediated and humoral immunity are of paramount importance in protecting the oral mucosa against candidosis (Hedderwick and Kauffman 1997). Since Candida species are opportunistic pathogens, fungal infections are common in patients whose immune system is compromised, e.g. acquired immune deficiency syndrome (AIDS) in which more than 90% of affected patients at some stage of the disease develop oral candidosis (Ellepola and Samaranayake 2000). Human immune deficiency virus (HIV) alters cell-mediated immunity so that T-cell function becomes impaired. Oral candidosis in HIV +ve patients can take up a variety of expressions ranging from pseudomembranous, atrophic and hyperplastic, all of which resemble clinically the lesions seen in noninfected individuals. HIV-related oral candidosis can also extend beyond the frontiers of the oral cavity to involve other organs such as the esophagus and the trachea, which will cause dysphagia and restrosternal discomfort. Severe combined immunodeficiency syndrome (SCID) is another condition characterized by defects in the cell-mediated and humoral immune reactions. Chronic mucocutaneous candidosis is often noticed in patients with SCID. Patients on immunosuppressive drugs, e.g. following organ transplantation, are also susceptible to oral candidosis.

Table 3. Summary of factors predisposing to oral candidosis

<table>
<thead>
<tr>
<th>Promote the growth of yeasts</th>
<th>Suppress the host defense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrinsic</td>
<td>Extrinsic</td>
</tr>
<tr>
<td>Imbalance of oral microflora</td>
<td>High carbohydrate diet</td>
</tr>
<tr>
<td>Low pH</td>
<td>Dental prostheses</td>
</tr>
<tr>
<td>Poor oral hygiene</td>
<td>Broad spectrum antibiotics</td>
</tr>
<tr>
<td>Already existing lesions</td>
<td>Malnutrition or malabsorption, e.g. iron deficiency</td>
</tr>
<tr>
<td></td>
<td>Radiation therapy</td>
</tr>
<tr>
<td></td>
<td>Heavy smoking</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Local</td>
<td>Systemic</td>
</tr>
<tr>
<td>Hyposalivation</td>
<td>Extremity of age, i.e. infancy/senility</td>
</tr>
<tr>
<td>Epithelial changes</td>
<td>Immunosuppressive infections e.g. AIDS</td>
</tr>
<tr>
<td></td>
<td>Immunosuppressive therapy</td>
</tr>
<tr>
<td></td>
<td>Endocrinopathies e.g. diabetes</td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
</tr>
</tbody>
</table>

Radiation therapy
Heavy smoking
Classification of oral candidosis

**Group 1: Candidosis confined to the oral mucosa**

1. **Acute**
   - Acute pseudomembranous candidosis (thrush)
   - Acute atrophic (erythematous) candidosis

2. **Chronic**
   - Chronic atrophic candidosis (denture stomatitis)
   - Candida-associated angular cheilitis
   - Median rhomboid glossitis
   - Chronic hyperplastic candidosis (candidal leukoplakia)

**Group 2: Oral manifestations of generalized candidosis**
- Chronic mucocutaneous candidosis

Clinical variants of oral candidosis:

1) **Acute pseudomembranous candidosis (thrush)**
   This type is characterized by the presence of extensive white superficial patches. These curd-like patches are nothing but pseudomembranes consisting of desquamated epithelial cells, fibrin and fungal hyphae (Akpan and Morgan 2002). The pseudomembranes can be wiped off leaving an erythematous base. This lesion can be found anywhere in the oral cavity, most often on the dorsum of tongue or on labial and buccal mucosae. Young infants and old people are frequently affected.

2) **Acute atrophic candidosis**
   This form of candidosis is also called acute erythematous candidosis. It is not as frequent as thrush except perhaps after the use of broad spectrum antibiotic, but when it occurs it causes painful burning sensation of the mouth (Lehner 1967). These lesions are characterized by red, atrophic changes occurring most commonly on the palate and tongue. Loss of tongue papillae (depapillation) leads to a bright red appearance and soreness (Palmer, Robinson et al. 1996). This clinical type of candidosis is commonly seen in HIV infections and after use of corticosteroids or broad-spectrum antibiotics.

3) **Chronic atrophic candidosis (denture stomatitis)**
   This particular form is characterized by the presence of chronic erythematous and edematous lesions, which range from small to large. It affects at some stage about half of all complete denture wearers (Budtz-Jorgensen 1990). As the synonym suggests, it is
associated with ill-fitting and/or poorly cleaned dentures. Therefore, it tends to be found exclusively in the denture-wearing areas, especially the palate. Clinically, it manifests as bright red, somewhat velvety to pebbly surface.

4) Candida-associated angular cheilitis
Since angular cheilitis (perleche) is commonly associated with denture stomatitis, it belongs to the same category as chronic atrophic candidosis but has a different location (RA 1966; Budtz-Jorgensen 1974). As the name points, it affects the angles of the oral cavity (commissures) and it starts as fissures which allow pooling of saliva and incubation of candidal cells leading to erythematous lesions which may encrust or erode and hence turn painful. Other predisposing factors include deep nasolabial folds (Shay, Truhlar et al. 1997) and long-term use of dentures (Penhall 1980).

5) Median rhomboid glossitis
Median rhomboid glossitis is a chronic symmetrical depapillated area of the tongue anterior to the circumvallate papillae. The region of papillary atrophy is usually elliptical or rhomboid in shape and situated at the midline of the tongue (Scully, el-Kabir et al. 1994).

6) Chronic hyperplastic candidosis
This clinical manifestation was the pivot of my study and is considered separately below in more detail.

7) Chronic mucocutaneous candidosis
This term, which comprises an array of clinical manifestations, is applied when the candidal infection involves organs other than the mouth e.g. skin, nail beds, vagina etc. Four subtypes have been recognized, associated with familial susceptibility; endocrinopathy; both familial susceptibility and endocrinopathy; and onset later than 10 years of age (Higgs and Wells 1974).
Chronic hyperplastic candidosis (CHC)

Lehner first reported the presence of candidal infection in an oral leukoplakia and called it candidal leukoplakia (Lehner 1964; Lehner 1967). Despite the confusion it may cause, most histopathologists prefer to use the term “chronic hyperplastic candidosis” instead of candidal leukoplakia. CHC is considered the most important clinical form of oral candidosis due to its association with the development of malignancy at the lesion site (Cawson and Lehner 1968; Bartie, Williams et al. 2001). This tendency should prompt the oral diagnostician to consider an already-forming real cancer as a differential diagnosis.

Clinical manifestations

Chronic hyperplastic candidosis appears as discrete, raised lesions. These hyperplastic, sometimes nodular lesions may be speckled or homogenous and they range in size from small, palpable lesions to large dense plaques. They are most often white or cream-colored (Fig. 5) but occasionally red (Fig. 6) (Appleton 2000). The red and speckled lesions have the clinical appearance of numerous white nodules on an erythematous base (Cawson and Lehner 1968). They occur on the buccal mucosa, palate, or dorsum of the tongue (Daniels, Schwartz et al. 1985). These lesions cannot be wiped off.

Histopathology

The clinical presentation of CHC (whether it is homogeneous or speckled) may reflect the histopathological picture of the lesion so the more nodular the lesion is, the more dysplasia the epithelium tends to show. Histopathologically candidal cells, yeasts and hyphae (Fig. 7), are seen on the uppermost tissue surface, and when they invade the epithelium (hyphal and pseudohyphal invasion), they rarely penetrate beyond the spinous layer (Reichart, Philipsen et al. 1995). The lesions show hyperparakeratotic or hyperorthokeratotic epithelium with irregular separation and epithelial hyperplasia. There is a higher mitotic activity, but it is restricted to the basal and suprabasal layers of the epithelium. A
typical feature is the presence of microabscesses, which are collections of polymorphonuclear leukocytes in the epithelium. Lamina propria contains an inflammatory cell infiltrate composed mostly of lymphocytes, macrophages and plasma cells.

**Diagnosis**

To reach a precise diagnosis of oral candidosis, as with any other oral lesion, a thorough medical and dental history has to be obtained from the patient. The oral diagnostician has to conduct a careful clinical examination where they first inspect the lesion to see if its appearance matches any of the known clinical presentations of the disease. Therefore, a wide knowledge of oral medicine is mandatory. The next step in clinical examination is palpation which can indicate an important diagnostic feature i.e. whether the lesion can be rubbed off or not.

If the clinical diagnosis is still provisional or doubtful, other tests are also available such as exfoliative cytology, culture or tissue biopsy (Epstein and Polsky 1998; Sherman, Prusinski et al. 2002). Exfoliative cytology is performed by scraping superficial cells to samples. A swab culture should be taken on the undersurface of a denture when denture stomatitis is suspected. If the candidal infection is thought to have already invaded the tissue, sampling some tissue in form of a biopsy should be considered (Fotos and Hellstein 1992). Hematological screening might be useful since up to some 40% of patients with oral candidosis may have some hematological abnormalities (Challacombe 1986).

**Management**

a) **Elimination of the predisposing factors**

Once a diagnosis of oral candidosis is assured, the first line of treatment should aim to eliminate or alleviate any dental and/or medical factors which contribute to the occurrence of candidal infection in the oral cavity. The oral diagnostician may find it necessary to refer his/her patient, in case there is suspicion of any pertinent medical condition, to their physicians to seek for advice. Patient cooperation is sometimes crucial especially when the predisposing factors are social rather than medical, e.g. smoking. One of the most important factors contributing to chronic hyperplastic candidosis is smoking; therefore, cessation of smoking is mandatory to relieve the disease. Chronic atrophic candidosis is mostly associated with edentulous patients who
wear dentures; therefore patient education and motivation to cleanse their dentures regularly and apply the oral hygiene measures is necessary to restore the infected edentulous mucosa to normalcy. If patients with acute atrophic candidosis are on broad-spectrum antibiotics or corticosteroid, discussion with their physicians about the possibility of withdrawal or substitution of the medicine could resolve the problem.

b) Antifungal treatment

Antifungal therapy has been used successfully in the management of oral candidosis. Prior to prescription of any antifungal agents, advising the patient to gargle with a physiological saline solution helps to decrease the oral fungal counts and thus soothe the associated symptoms (Appleton 2000). Pharmacological treatment of oral candidosis should be tailored to the individual patients according to their current medical status and severity of infection (Sherman, Prusinski et al. 2002). Antifungal agents are available in different forms (i.e. gels, ointment, creams, suspension, lozenges, and tablets) and the dentist should manage to select the proper form upon writing a prescription. There are many drug categories available to treat the different clinical presentations of candidosis, summarized as follows:

- Polyenes: The candidal cell wall is composed of many layers, the innermost of which is the plasma membrane. Polyenes are potent agents which act by binding to the sterol part of the candidal cell membrane and disrupt its osmotic integrity leading to leakage of essential ions e.g. potassium and magnesium. This category includes nystatin and amphotericin B. Nystatin, which is of bacterial origin, is the drug of choice for treatment of oral candidosis. It should be prescribed cautiously in patients with uncontrolled diabetes or xerostomia. Recently, liposomal nystatin (Nyotran) has been designed where nystatin was incorporated into liposomes and it is now in late phase clinical trial (DiDomenico 1999). The preferred topical application of nystatin is oral suspension (100,000 U/ml) or pastilles (100,000 IU) for 7-14 days (Farah, Ashman et al. 2000). Amphotericin B can be used to treat superficial candidosis i.e. Fungizone (oral), or more wide-spread systemic involvement i.e. Ambisome (intravenous).

- Antimetabolites: This group contains only one agent: flucytosine (5-fluorocytosine). It is candidastatic since it inhibits the candidal protein synthesis by replacing uracil with 5-flourouracil in fungal RNA. Its use has declined due to the emergence of candidal resistance (Appleton 2000).
- **Azoles:** This class includes two major categories: imidazole (e.g. clotrimazole, miconazole, econazole, ketoconazole) and triazole (e.g. fluconazole, itraconazole). Although their effect targets the plasma membrane (same as polyene), they are fungistatic rather than fungicidal (Wynn, Jabra-Rizk et al. 1999). Fluconazole is considered as the first line of treatment in many cases of candidosis but its main drawback is the emergence of candidal resistance.

- **Glucan synthesis inhibitors:** these (e.g. caspofungin) act by inhibiting the essential component of the fungal cell wall, glucan.

- **Miscellaneous agents:** there is only one drug available of this class of current use i.e. griseofulvin which exerts its effect through disrupting the mitotic spindle.

![Figure 8 showing a schematic diagram of C. albicans cell constituents as targets of antifungal agents](image-url)
Table 4 showing a summary of the most commonly prescribed antifungal agents for treating oral candidosis

<table>
<thead>
<tr>
<th>Agent name</th>
<th>Administration (route and dose)</th>
<th>Treatment course</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nystatin (Mycostatin®)</td>
<td>Oral- lozenge (200.000 U): 1X4 - suspension (100.000 U/ml): 1tsp (for 2 mins) X4</td>
<td>10-14 days</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Oral- tablet (10 mg): 1X4</td>
<td>4-6 weeks</td>
</tr>
<tr>
<td>(Fungizone®)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clotrimazole (Mycelex®)</td>
<td>Oral-troche (10 mg): 1X5 - cream (10mg/g): X4</td>
<td>Troche: 14 days Cream: 1-8 weeks</td>
</tr>
<tr>
<td>Miconazole (Daktarin®)</td>
<td>Oral- gel (2%): 2.5 mlX4</td>
<td>Treatment continues 1 week after resolution of the infection</td>
</tr>
</tbody>
</table>

**Leukoplakia (LP)**

The old official definition of leukoplakia (LP) was formulated in 1978 by the World Health Organization (WHO) as “A white patch or plaque that cannot be rubbed off or characterized clinically or pathologically as any other disease” (Sciubba 1995). WHO stated in a newer definition in 1997 as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion.

LP can appear at any time in life, but it occurs most common in the elderly people. Clinically LP is a white or gray patch that develops on the tongue, inside of the cheek or anywhere in the mouth. The lesion may have developed slowly over weeks to months and be thick and slightly raised, eventually with a hardened and rough texture. It is usually painless, but may be sensitive to touch, heat, spicy foods, or other irritation.

The transformation liability of LP to oral cancer is well-documented in the literature and is considered to have the most precancerous potential among oral lesions (Warnakulasuriya 2000). The highest malignant potential is seen in the nodular, exophytic as well as speckled forms of LP and erythroplakia (Axell, Pindborg et al. 1996). Hansen et al., in 1985, suggested the term proliferative verrucous leukoplakia (PVL) for leukoplakias which tend to recur and may change to precancerous lesions (Hansen, Olson et al. 1985). Many studies screened the frequency of PVL and its progression to carcinoma in variable samples in the western countries and the results
ranged from 60 to 100% (Fettig, Pogrel et al. 2000; Bagan, Jimenez et al. 2003; Morton, Cabay et al. 2007)

LP is thought to be a reaction of the oral mucosal membrane to chronic irritation of the mucous membranes. Hairy LP of the mouth is an unusual form of LP that is seen mostly in HIV patients (but can be found in some other immunodepressed patients). It appears as fuzzy (hence the name hairy) white patches on the tongue and less frequently elsewhere in the mouth.

**Etiology**

- Irritation from rough teeth, fillings, or crowns, or ill-fitting dentures
- Chronic smoking, pipe smoking, or other tobacco use
- Alcohol abuse
- Sun exposure to the lips

**Diagnosis**

An experienced clinician may suspect LP upon examination; however, a biopsy is usually necessary to rule out other causes, such as oral cancer. During the biopsy, a small piece of tissue from the lesion is removed to be examined histopathologically in a laboratory.

**Treatment**

Treatment, if needed, involves removal of the source of irritation. For example, if LP is caused by a rough tooth or an irregular surface on a denture or filling, the offending tooth should be smoothened and dental appliances repaired. If LP is caused by smoking, quitting of smoking is critical in relieving the lesion.

LP is usually harmless, and lesions usually clear in a few weeks or months after the source of irritation has been removed. If elimination of the source of irritation is ineffective in reducing LP, the lesion may need to be surgically removed.
Host defense against oral candidosis

Under normal conditions, the host tolerates the existence of *C. albicans* and other eventual candidal components of the oral microflora. The host safeguards itself by means of natural defense measures, e.g. the physical integrity of the intact epithelium, mucosal membrane, which serves as a natural barrier against foreign intruders (Marodi 1997). Thereby it acts as a selective gatekeeper allowing the passage of certain substances but prevents ingrowth of *Candida* and other microbes (Dale 2002). This relationship between the host and microflora is maintained and enables commensalism as long as the innate and adaptive immunity of the host remain competent. Once the host defense is compromised, opportunistic species of the microflora take benefit of it breaking the mutual balance by invading the host. The host tries to defend itself against such saprophytic opportunistic microbes by two main ways, utilizing both nonspecific and specific host defense.

**Nonspecific host defense (innate immunity)**

This branch of the host defense system is the first line to become engaged when a host encounters a pathogen. As the name indicates, it does not base its recognition mechanisms on learning (adaptive behavior) and immunological memory; rather, it senses and identifies any foreign substance, which imposes a threat to the host right away. Any host is naturally armed with such a protective system; therefore, it is also called innate immunity. Although it may utilize ligand specific receptors, these receptors are not drawn from a huge number of different specificities but represent rather broad recognition ranges. When a pathogen invades a host, the innate immunity serves two major tasks. It recognizes the invading organism and tries to clear it in a rapid but robust way. Second, it triggers a subsequent cascade whereby a more sophisticated machinery of the immune system (specific or adaptive) receives a danger signal (secondary stimulus) necessary for its activation by specific antigens (primary stimulus). Understandably, the innate immunity is phylogenetically ancient unlike adaptive immunity which is only found in vertebrates (Raj and Dentino 2002). Innate immune defense utilizes many mechanisms involving various molecules. There are some agents which have a shared task and form a bridge between innate and adaptive immune response e.g. defensins. To avoid repetition, the key players of the innate host defense will be considered under only one heading.
1. Toll-like receptors (TLRs)

Toll was first discovered in *Drosophila melanogaster* in 1996 (Lemaitre, Nicolas et al. 1996), its human homologues Toll-like receptors (TLRs) comprise an important family of receptors for the detection of microbial components of a broad variety of pathogens, which triggers an inflammatory host defense response and alert the adaptive arm of the host defense (Netea, Van der Meer et al. 2004). In mammals, Toll has not only been maintained but has developed to comprise 11 identified receptors which belong to the family of TLRs (Takeda and Akira 2005). Structurally, TLRs are composed of an extracellular portion, which harbors leucine-rich repeats and an intracellular domain having a high degree of similarity to interleukin-1 receptor (Takeda and Akira 2004; Takeda and Akira 2005). Zymosan is a generic name for the polysaccharide component of the fungal cell wall (including yeasts). Zymosan stimulates many host cells via TLR2 (Underhill, Ozinsky et al. 1999). The immunostimulatory properties of zymosan are most probably conferred by β-glucan (Kataoka, Muta et al. 2002). In addition, TLR2 and TLR4 can sense *C. albicans* through its cell wall constituents phospholipomannan and mannann, respectively (Roeder, Kirschning et al. 2004). It is still debatable whether the morphological form of *C. albicans* is a determinant factor in excitation of or escapes from the host defense mechanisms through TLR2 and TLR4 which could perhaps explain some apparently conflicting results (Netea, Sutmuller et al. 2004; Netea, Van der Meer et al. 2004; Villamon, Gozalbo et al. 2004; Villamon, Gozalbo et al. 2004). While epidermal keratinocytes have been found to express TLR2 and TLR4 in response to microbial pathogens including *C. albicans* (Pivarcsi, Bodai et al. 2003), very few studies have investigated, so far, the eventual participation of such recently discovered structures in the immunity of the oral mucous membrane against candidosis.

2. Proteins

a) Natural antimicrobial peptides

One of the mechanisms used by the host when it is invaded by a pathogen is production and / or release of substances having antimicrobial effects in response to the microbe-host cell contact (Yang, Biragyn et al. 2002). These substances are called antimicrobial peptides. As the name denotes, these are small molecular-weight peptides, which are engaged in killing or inactivating a peptide typical spectrum of pathogenic invaders, i.e. viruses, bacteria or fungi (Bastian and Schafer 2001). The list of such natural defense
agents is already long but only the best known antifungal peptides will be presented in this thesis.

- **Defensins**

The discovery of defensins goes back to the time when a class of peptides, found to have antimicrobial properties, was isolated from macrophages of rabbit lung (Selsted, Brown et al. 1983). A couple of years later, similar peptides were found in human neutrophils and therein the term *defensin* was coined (Ganz, Selsted et al. 1985). Defensins are cysteine-rich antimicrobial peptides containing three pairs of disulfide bridges (Lehrer and Ganz 1999). They have a molecular-weight of 2000-6000 Da and their amphipathic properties allow hydrophilic, hydrophobic and cationic clustering (Marshall 2004). There are three classes of defensins: alpha (α), beta (β) and theta (θ), of which only the first two exist in humans (Izadpanah and Gallo 2005). Alpha-defensins are composed of 29-35 amino acid residues and they are shorter than β-defensins, whose amino acid number ranges from 38 to 42 (Raj and Dentino 2002). These defensins have different locations of cysteine residues and disulfide motifs. Alpha-defensins are subcategorized into six types designated from 1 to 6. The first four subclasses (1 to 4) are produced by polymorphonuclear leukocytes (therefore, they are referred to as Human Neutrophil Peptide or HNP) while the last two are produced by Paneth cells of the intestine and the female genitourinary tract (Quayle, Porter et al. 1998). The four types of β-defensins are produced by epidermal keratinocytes of the skin and epithelial cells of the mucous membranes of the mouth, gastrointestinal and genitourinary tracts (Raj and Dentino 2002).

Microbial membranes are the main targets of the microbicidal effects of defensins. Because of their positive charge, defensin peptides interact with the negatively charged components or pathogen-associated molecular structures of the microbial membranes. This antimicrobial protein-microbial membrane interaction results in disruption of the latter and leakage of the microbial cell membrane leading to osmotic rupture and death of the microbial cell (Weinberg, Krisanaprakornkit et al. 1998).

Along with their obvious role in innate immunity, α- and β-defensins are also linked to the adaptive immune response. They augment the adaptive immune response of the host through recruitment of inflammatory cells to the site of the microbial invasion. Alpha-defensins act directly as chemotactic agents to recruit CD8 and CD4 T cells as well as immature dendritic cells. They also stimulate release of the chemokine interleukin 8 by
epithelial cells which attracts neutrophils to the site of infection (Van Wetering, Manness-Lazeroms et al. 1997; Zasloff 2002).

**Histatins**

Histatins, which are small histidine-rich cationic peptides, comprise an important group of the antimicrobial peptides of saliva. They are secreted by the parotid and submandibular salivary glands and their molecular size ranges from 7-38 amino acids (Kavanagh and Dowd 2004). The proteolytic degradation of the products of the two histatin genes (HIS1 and HIS2) results in the formation of at least 12 histatin fragments in human saliva, of which histatin 5 is the most active form against *C. albicans* (Xu, Levitz et al. 1991; Ruissen, Groenink et al. 2002). Histatin 5 tends to retard the transition of *C. albicans* from the unicellular to the fungal form, a step necessary for the tissue invasion and penetration (Cannon, Holmes et al. 1995). It has been postulated that histatin 5, as all other cationic antimicrobial peptides, disrupts the osmotic integrity of *C. albicans* by creating permanent pores in its cell membrane. Consequently, the membrane loses its ability to control the passage of ions in and out of the microorganism leading to its demise. Also other antifungal mechanisms have been reported to histatin 5, e.g. binding to the fungal mitochondria which cause release of cellular ATP out of the cell so that the candidal cell becomes depleted of its energy supply (Helmerhorst, Breeuwer et al. 1999), and generation of lethal reactive oxygen species (Helmerhorst, Troxler et al. 2001). The ability of histatin 5 to kill azole-resistant fungi has made it a topic of intensive research as a potential synthetic chemotherapeutic antifungal agent (Tsai and Bobek 1997).

**Protegrins**

Protegrins belong to a larger group of cathelin-containing antimicrobial peptides called cathelicidins (Zanetti, Gennaro et al. 1995). Protegrins are small (2 kDa) as they contain only 16 to 18 amino acids particularly cysteine and arginine. They were first isolated from porcine leukocytes (Fahrner, Dieckmann et al. 1996) and they occur in five sequential types (PG-1 to PG-5).

**b) Chemokines and cytokines**

Chemokines are small secreted proteins whose main function is to attract leukocytes to move in a specific direction, along the gradient of the chemokine concentration. In other words, chemokines are chemotactic cytokines (hence their name, *chemo* for chemotaxis and *kine* for cytokine) (Baggiolini 2001). Cytokines are regulatory proteins, such as the interleukins and lymphokines that are released by cells of the immune
system and act as intercellular mediators in the generation of an immune response. Thus, cytokine is a more general term than chemokine, which also participate in intercellular communications in a well defined and narrow area. Structurally, chemokines form a family of structurally related glycoproteins with potent leukocyte chemotactic and activating properties. The molecular weight of chemokines ranges from 8 to 10 kD and they are 70 to 90 amino acids in length, having 20 to 70% homologies in their amino acid sequences (Luster 1998). Most of them fit into two subfamilies with four cysteine residues. These subfamilies segregate based on whether the two amino terminal cysteine residues are immediately adjacent to each other (cc chemokines) or separated by one amino acid (cxc or alpha chemokines). The CXC group includes molecules such as interleukin-8 and platelet factor 4 most of them being chemoattractants for neutrophils. Examples of the CC group (beta chemokines) include eotaxin and monocyte chemoattractant protein-3, and such chemokines generally attract monocytes, lymphocytes, basophils, and eosinophils (Luster 1998). There are also two other small subgroups. The C group has one member (lymphotactin) which lacks one of the cysteines in the four-cysteine motif, but shares homology at its carboxyl terminus with the C-C chemokines. The C chemokine seems to be specific for lymphocyte. The fourth subgroup is the C-X3-C subgroup. The C-X3-C chemokine (e.g. fractalkine / neurotactin) has three amino acid residues between the first two cysteine amino acids. It is tethered directly to the cell membrane via a long mucin stalk and induces both adhesion and migration of leukocytes.

Chemokines can also be classified to either inflammatory or homing (homeostatic): inflammatory chemokines are widely distributed in most tissues and their expression is triggered by inflammatory stimuli, while homing chemokines are constitutively expressed within lymphoid tissues (Baggiolini 2000). Chemokines exert their effects, as any protein ligand, through receptors. This chemokine-receptor binding may, in the future, be employed to provide a successful chemotherapy of certain drastic diseases, e.g. AIDS. This is exemplified by the finding that the HIV antigen gp120 recognizes overlapping receptors epitopes to which chemokines can bind (Baggiolini 1998). Chemokines act via many seven-transmembrane-domain receptors, two of which, CXCR1 (IL-8RA) and CXCR2 (IL-8RB), are for interleukin-8.

In this thesis, only two cytokines, IL-8 and RANKL, will be discussed
a) **Interleukin-8 (IL-8)**

Interleukin-8 (IL-8) is a chemotactic cytokine which attracts leukocytes and stimulates monocyte adherence (Lane, Lore et al. 2001). The main cell target for the chemotactic effect of IL-8 is neutrophil. The chemotactic effect of IL-8 results in recruitment and transmigration of neutrophils across the microvasculature to the site of inflammation (Schall and Bacon 1994). IL-8 is also a chemoattractant for T lymphocytes (Larsen, Anderson et al. 1989) and basophils. IL-8 was also referred to as neutrophil chemotactic factor (NCF), neutrophil activating factor (NAF) (Walz, Peveri et al. 1987), neutrophil activating protein (NAP), monocyte-derived neutrophil chemotactic factor (MDNCF) (Yoshimura, Matsushima et al. 1987), T cell chemotactic factor (TCF), granulocyte chemotactic protein (GCP) and leukocyte adhesion inhibitor (Yang, Huang et al.). It is released by several cell types including monocytes, macrophages, neutrophils, T-lymphocytes, fibroblasts, endothelial cells, and epithelial cells after an inflammatory stimulus, e.g. IL-1, TNF, LPS, or viruses (Yoshimura, Matsushima et al. 1987; Peveri, Walz et al. 1988; Strieter, Kunkel et al. 1988; Strieter, Phan et al. 1989; Palter, Mulayim et al. 2001). IL-8 belongs to the CXC subfamily of chemokines, is a member of the beta-thromboglobulin superfamily and structurally related to platelet factor 4. IL-8 may play an important role in the progression of many cancerous diseases, e.g. melanoma, breast cancer and lung cancer (Reed and Purohit 1997; Poppenborg, Knupfer et al. 1999; Penson, Kronish et al. 2000). It was found that IL-8 is produced by fibroblasts in the dental pulp when it is stimulated with substance P (Park, Hsiao et al. 2004) or black-pigmented *Bacteroids* (Yang, Huang et al. 2003). IL-8 induces its chemotactic effect via two receptors: CXCR1 and CXCR2, also known as IL-8RA and IL-8RB, respectively (Murphy and Tiffany 1991). Both receptors are found plentifully on neutrophils while they are expressed less on monocytes and myeloid cell lines (Sprenger, Lloyd et al. 1994). Beside its chemotactic effect and recruitment of neutrophils to the site of inflammation, IL-8 has been claimed to trigger the oxidative burst response in those cells (Baldwin, Weber et al. 1991), thereby aiding their microbicidal efficacy.

In the oral mucosa, IL-8 plays a major role in combating pathogens such as *C. albicans* through recruitment of neutrophils and lymphocytes to the site of infection.

b) **RANKL**

The cytokine receptor activator of nuclear factor κB ligand (RANKL) is a membrane-bound ligand that belongs to the tumor necrosis factor family (Hofbauer and Heufelder et al. 2000). RANKL is involved in the regulation of osteoclast differentiation and bone resorption (Trinchieri and Flament 1999; Ott, Gunther et al. 2000). It consists of a membrane-bound form (RANKL) and a soluble form (sRANKL). RANKL binds to the receptor activator of nuclear factor κB (RANK) on osteoclasts and osteoclast precursors, leading to their differentiation and activation (Trinchieri and Flament 1999; Ott, Gunther et al. 2000). RANKL also binds to the receptor engaged by TNF receptor (RETN) on T lymphocytes, leading to the release of TNF-α and other cytokines (Trinchieri and Flament 1999; Ott, Gunther et al. 2000). RANKL plays a crucial role in the maintenance of bone homeostasis and the regulation of immune responses (Trinchieri and Flament 1999; Ott, Gunther et al. 2000).
2001), which bestows RANKL an other synonym: TRANCE (TNF-related activation-induced cytokine). It is a protein consisting of 316 amino acids with a molecular mass of 35 kDa (Yasuda, Shima et al. 1998). RANKL is produced by osteoblastic lineage cells (Gori, Hofbauer et al. 2000), fibroblasts (Mandelin, Li et al. 2003), immune cells (Anderson, Maraskovsky et al. 1997) and some cancer cells (Nagai, Kyakumoto et al. 2000). The main function of RANKL is to orchestrate formation, maturation and activation of osteoclast (Jeziorska, McCollum et al. 1998; Liu, Xu et al. 2003); therefore, RANKL was also known as osteoclast differentiation factor (ODF). The inductive action of RANKL on osteoclast progenitors is mediated through binding to its receptor RANK. Binding of RANKL to RANK is counteracted by a decoy receptor called osteoprotegerin (OPG), which is the reason why RANKL was also called osteoprotegerin ligand (OPGL). The neutralizing effect of OPG on RANKL-RANK interaction was based on the observation that OPG (-/-) knockout mice develop osteoporosis and arterial calcification of aorta and renal arteries (Schoppet, Preissner et al. 2002). RANKL can occur in three forms: either as a cell-bound, truncated or secreted (Hofbauer, Shui et al. 2001). The role of RANKL in bone metabolism is manifested in many pathological conditions, e.g. rheumatic disease (Haynes, Barg et al. 2003), atherosclerosis (Collin-Osdoby 2004), cancers such as multiple myeloma (Farrugia, Atkins et al. 2003) and prostate cancer (Lynch, Hikosaka et al. 2005), periodontitis (Crotti, Smith et al. 2003; Liu, Xu et al. 2003) and aseptic bone loss in total hip replacement (Gehrke, Sers et al. 2003). The expression of RANKL is upregulated by some factors which have been associated with bone resorption, e.g. glucocorticoids, interleukins (IL-1, IL-6, IL-11 and IL-17), TNFα and PTH (Kong and Penninger 2000). Another important role of RANKL, beside its osteoclastogenetic effect, is immunity. The fact that activated CD4+ and CD8+ T lymphocytes display RANKL in lymphoid structures, such as lymph nodes, Peyer’s intestinal patches and thymus (Lacey, Timms et al. 1998), and that RANK is expressed by dendritic cells evoked the concept of RANKL contributing to immunological processes. So far, no analytical study has been conducted to assess the role of RANKL and RANK interaction in an infectious disease, let alone candidosis.

3. Cells
Along with the cytokines and the natural antimicrobial peptides, there are certain cells which belong to the innate immune system and aid in extinguishing the infectivity of pathogens.
Such cells include phagocytic cells (macrophages and neutrophils) that can engulf (phagocytose) foreign substances. Macrophages are thought to mature continuously from circulating monocytes.

Phagocytic cells respond to the chemotactic stimuli by carrying out three main steps which guide their migration towards the chemotactic stimulus. These steps are margination and rolling, adhesion and transmigration, and then the final migration towards the site of inflammation. The journey of the phagocytic cells to their destiny is guided by chemotaxis. Chemotaxis is the process by which phagocytes are attracted to where the microorganisms are located by means of chemotactic stimuli such as microbial products, complement, damaged cells and white blood cell fragments. Chemotaxis is followed by a direct contact of the phagocyte with the microorganisms. This intimate adhesion is enhanced by opsonization, where protein components (called opsonins) coat the surface of the pathogen. This is followed by ingestion, in which the phagocyte extends projections, or pseudopods that engulf the foreign organism. Finally, the pathogenic microbe in the phagosome fuses with a lysosome and is digested by enzymes in the phagolysosome, a process involving also reactive oxygen species. Many important cells are involved in the host immune defense against infection such as neutrophils, dendritic cell and mast cells.

a) Neutrophil

The first phagocyte a pathogen is likely to encounter is a neutrophil. A neutrophil is a type of white blood cell which plays major early role in the cellular innate immune response against foreign invaders. Neutrophils are produced in the bone marrow at a rate of $10^{11}$ cells per day, and then circulate around the blood stream as mature cells for approximately ten hours before migrating into the peripheral tissue pools, where they reside for their 1 to 2 day long living period (Smith 1994). After that neutrophils undergo apoptosis (Savill, Fadok et al. 1993). It is thought that the short life span of neutrophils might be due to their involvement in the maintenance of body homeostasis which renders them susceptible to cellular stress (Smith 1994), to minimize propagation of those pathogens that parasitize phagocytes, and to diminish the damaging effects of the antimicrobial products on the host tissues.

Neutrophils constitute 50 to 60% of the total population of leukocytes (Smith 1994). Like other phagocytes, they are recruited to the tissue site where the noxious substance is located. Once there, they approach the invading stimulus and display a variety of antimicrobial processes, e.g. elaboration of natural antimicrobial peptides, enzymes
(e.g. peroxidase, hydrolytic enzymes), and eventually engulf it. Because of their peculiar nuclear morphology (the nucleus is termed segmented or polymorphonuclear), neutrophils belong to the polymorphonuclear leukocytes (PMLs) (Huizinga, Roos et al. 1990). They contain granules and are also known as granulocytes. These cytoplasmic granules are tiny vesicles containing enzymes that help the cell to efficiently perform its microbicidal function. Neutrophils utilize two major microbicidal mechanisms, namely oxidative and enzymatic processes. In this way, it kills and digests microorganisms that it has already engulfed by phagocytosis. Degranulation process of the cytoplasmic granules of neutrophils also causes an extracellular release of its destructive mediators. Neutrophils are active phagocytes, capable of only one phagocytic event, expending all of their glucose reserves in an extremely vigorous respiratory burst. The respiratory burst involves activation of an NADPH oxidase enzyme, which produces large quantities of superoxide converted to other reactive oxygen species. Beside their role as direct scavengers of toxic substances in the body, neutrophils can also aid in the regulation of the inflammatory process by secreting of humoral mediators such as cytokines, e.g. TNF-α (Lloyd and Oppenheim 1992), IL-1β (Palma, Cassone et al. 1992), IL-6 (Melani, Mattia et al. 1993) and IL-8 (Strieter, Kasahara et al. 1990). Being highly motile, neutrophils quickly congregate at the site of infection, dragged by cytokines expressed by cells at the site of action, e.g. activated endothelium, mast cells and macrophages. Neutrophils are also under normal circumstances produced in huge numbers, but this may increase as much as 10-fold in response to an infection (Cannistra and Griffin 1988).

In candidosis, neutrophils play a major role in combating the pathogen *C. albicans* by expressing either candidastatic and/or candidacidal mechanisms. Neutrophils contain calprotectin in their cytoplasm which inhibits the growth of the fungus without killing it (Sohnle and Collins-Lech 1990). Neutrophils can eliminate the fungal pathogen from the host by producing candidacidal proteins, e.g. HNP 1-4. The hyphal and pseudohyphal forms of the organism can be engulfed and damaged by neutrophils through oxidative mechanisms (Diamond, Clark et al. 1980). The importance of neutrophils in defending the host against *C. albicans* is supported by the fact that candidal infection predominates in patients with chronic granulomatous disease in which leukocytes suffer from NADPH oxidase deficiency (Kim, Rodey et al. 1969).
b) Dendritic cells

Dendritic cells (DCs) belong to a class of cells known as antigen presenting cells (APCs). They are derived from haematopoietic stem cells in the bone marrow and were first identified in 1973 (Steinman and Cohn 1973; Scimone, Lutzky et al. 2005). Possibly excluding the brain, testes and cartilage, DCs patrol almost all other tissues of the body (Cutler, Jotwani et al. 2001). They are found most commonly in places with direct contact with the environment, in skin and mucous membranes where they serve as sentinels against ingress of pathogens. In humans, DCs are classified into three subsets. Two are myeloid cells, namely Langerhans cells and dermal DCs (both of which can be generated in vitro from human cord blood CD34+ hemopoietic progenitors) (de Saint-Vis, Fugier-Vivier et al. 1998) and the third one is lymphoid DCs (Cutler, Jotwani et al. 2001). When they circulate in the blood, DCs are indistinguishable from monocytes but when they reside in peripheral tissues, e.g. skin and mucosa, they develop long spiky projections called dendrites, from which their name is derived (Cutler and Jotwani 2004). DCs are considered to represent an important link between innate and adaptive immune responses (Palucka and Banchereau 1999). In the mucous membranes and epidermis, DCs are present as Langerhans cells (LCs), while in the dermis they are known as dermal DCs. Despite their old discovery in 1868 by Paul Langerhans (after whom LCs are named), LCs were categorized as a class of DCs very recently, in 1979 (Palucka and Banchereau 2006). LCs are found above the basal layer in skin and mucosal surfaces of the mouth, nose, gastrointestinal tract and genitourinary tract (Jotwani and Cutler 2003). The primary function of LCs (and other DCs) is to control the potential pathogen entry sites and hence they are considered as immunosurveillant cells. Having not exposed to an antigen yet, LCs remain in a resting and immature state but characterized by high endocytotic activity. When an antigen is encountered, e.g. C. albicans, LCs capture it and actively internalize it by phagocytosis (Ramachandra, Chu et al. 1999). The manner of phagocytosis depends on the morphological form of the fungal part being engulfed. LCs engulf yeasts through coiling phagocytosis while zipper-like mechanism is the prevalent phagocytosis type for hyphae (Montagnoli, Bacci et al. 2001). In the cytoplasmic vacuolar compartments, LCs process the fungal cell, degrade it into fragments and then associate it with major histocompatibility complex-antigen (MHC I or II, depending on the antigen) (Romagnoli, Nisini et al. 2004). After the LCs have packaged the antigen with MHC, they extrude the whole complex to the cell surface for
recognition by T cells (Adams, O’Neill et al. 2005). If antigen-specific T cells are encountered, a response ensues and LCs transform to the active, mature form, they migrate via blood or lymphatic vessels to the regional lymph nodes where they interact with naïve T cells (Banchereau and Steinman 1998). This interaction is mediated through upregulation of certain cell surface receptors, e.g. CD80 and CD86, which aid in priming and enhancing the T lymphocytes.

c) Mast Cells

Paul Ehrlich, when still a medical student in 1878, was the first to describe mast cells whose large size and metachromatic granular appearance led him to the mistaken belief that they were either well-fed or feeding the surrounding tissues, and therefore gave them the name ‘mastzellen’ which means “feeding cells”. Mast cells develop in the bone marrow from CD34+ pluripotent stem cells. Being still immature, mast cells circulate in the blood and lymphatics to home to tissues where they undergo maturation by a locally-produced growth factor (Prussin and Metcalfe 2003). Throughout the human body mast cells (infrequently known as mastocytes) are found in connective tissues and submucosal areas (Kelley, Chi et al. 2000), preferably around the blood vessels and beneath the epithelium (Church and Levi-Schaffer 1997). The role of mast cells in the pathogenesis of many human diseases e.g. atherosclerosis, has become strongly evident (Kovanen 1995). Although some researchers prefer to classify mast cells according to their location into mucosal and connective tissue mast cells (Gemmell, Carter et al. 2004), the granular content of mast cells remains the prominent criterion for categorizing them into mast cell tryptase (MC\textsubscript{T}) and mast cell tryptase and chymase (MC\textsubscript{TC}). As the name denotes, MC\textsubscript{T} contains tryptase in their granules and are found most abundantly in the respiratory and intestinal mucosa and conjunctiva (Irani, Schechter et al. 1986). On the other hand, MC\textsubscript{TC} contains both tryptase and chymase and predominate in the skin and submucosa of the small intestine and subepithelial area of the conjunctiva (Irani, Bradford et al. 1989). Mast cells are known best for their participation in allergy through their expression of high affinity receptor (Fce\textsubscript{RI}) for immunoglobulin E. The prominent location of mast cells in the vicinity of blood vessels most probably dictates their primary function in the regulation of the vascular muscle tone in the inflammatory process. Beside tryptase and chymase, mast cell granules contain and / or rapidly produce an array of active mediators and cytokines, e.g. histamine, heparin, carboxypeptidase, prostaglandins (PGD\textsubscript{2}, PGE\textsubscript{2}), thromoxanes, IL-4, TNF-\textalpha, RANKL and growth factors (Kelley, Chi et al. 2000). When mast cells are
stimulated, they elaborate their granular contents to the extracellular environment by exocytosis, a process known as degranulation. Mast cells are considered an integral part of the immune system. In tissues, mast cells remain in close proximity to T cells (Mekori and Metcalfe 1999) since the former has the ability to engulf and process antigens, and present them to the latter, thereby aiding in the initiation of an adaptive immune response (Malaviya, Twesten et al. 1996). Release of IL-16 by mast cells is thought to be an important explanation for the presence of CD4+ T cells at sites of mast cell activation (Rumsaeng, Cruikshank et al. 1997), since IL-16 is a preferential chemoattractant for CD4+ T cells (Center, Kornfeld et al. 1996). Although mast cells have been shown to be involved in the immune defense against many bacteria, e.g. *K. pneumoniae*, there is no study, so far, attempting to clarify whether mast cells play a role in the host defense against candidal infection.

**Specific host defense (acquired immunity)**

The second category of the immune system is called specific, acquired or adaptive immunity. As the name suggests, this branch of the host defense is directed against the particular and specific pathogens which have succeeded partially or totally in overwhelming the shields of the innate immune armory system. It takes days for the specific immunity to recruit, activate and expand the antigen-specific lymphocytes, which is much longer than it takes to mount the immediate innate defense response. Therefore, the specific host response is not effective in eliminating infections in their early stages and its major role comes to play in cases of long lasting infections. The specific host defense is divided into two major sections through which it exerts its mechanisms: cell-mediated T cells and humoral (antibodies) immunity.

1. **Cell-mediated immunity**

The cellular arm of the specific immune defense is formed of T lymphocytes, which is abbreviated as T cells. This subclass of white blood cells originates in the bone marrow from pluripotent haematopoietic stem cells, and the newly-formed cells then circulate through the thymus gland (the letter T which represents this class of lymphocytes is derived from thymus) and differentiate to thymocytes (Vallejo, Davila et al. 2004). They are usually divided into two major subsets that are functionally and phenotypically different, cytotoxic T cells (CD8+) and helper T cells (CD4+) (Germain 2003). Cytotoxic T cells (sometimes called killer/suppressor, CD8+) are important in the direct destruction of certain tumor cells, virally infected cells and sometimes parasites. Most CD8+ T cells recognize antigens only when they are bound to the MHC
class I (MHC-I) molecules. The other category of T cells is CD4\(^+\) (helper) T cells which constitute a pertinent coordinator of immune regulation. Their main function is to augment or potentiate the immune responses by secretion of specialized factors or cytokines, which activate other white blood cells to fight the infection. Most CD4\(^+\) T cells recognize antigens associated with MHC II (Siu 2001). There are two subsets of T-helper cells: Th1 cells which, through their secretion of IL-2 and INF-\(\gamma\), enhance cell-mediated immune response and in the same time inhibit the other subset of T-helper cells, the Th2 cells. Th2 cells are engaged in minimizing cell-mediated immune response through secretion of certain cytokines such as IL-4 and IL-10. Both types of T cells can be found throughout the body. They often depend on the secondary lymphoid organs, the lymph nodes and spleen, as sites where activation occurs, but they are also found in other tissues of the body, most conspicuously in the mucosal-associated lymphatic tissue in intestinal and reproductive tracts, but also in liver, lung and blood. T cells are responsible for cell-mediated immunity which is of paramount importance in the defense against viral, bacterial and fungal infections. T cells also aid B lymphocytes to produce antibodies, recognize and reject foreign tissues due to their histocompatible tissue type (or actually MHC molecules). T lymphocytes first have to find their way to the site of infection. Once there, they are exposed to an antigen so that the antigen-specific T cells divide rapidly and produce large numbers of new T cells sensitized to the various epitopes of the pathogen specific set of antigens. T lymphocytes play a central role in control of the acquired immune response and, furthermore, serve as crucial effectors through antigen specific cytotoxic activity. As to candidal infections, cell-mediated immunity is necessary in the successful defense of the host against candidosis, especially on mucosal membranes (Fidel 2002). This is based on the experimental observation that T cell-deficient mice fail to resist gastrointestinal candidosis whereas those mice with deficient phagocytic capability and normal T cell function readily cleared the infection (Balish, Filutowicz et al. 1990). Th1 response in particular is the branch of cell-mediated immunity which provides protection against candidosis, while Th2 pathway would lead to attenuation of the Th1 response (Romani, Mocci et al. 1991).
2. **Humoral Immunity**

Humoral immunity is the second branch of the adaptive immune response. It pertains to antibody production and all the accessory processes involved, e.g. Th2 activation and cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation. The main functions of antibodies are pathogen and toxin neutralization, classical complement activation, opsonin promotion of phagocytosis, and pathogen elimination (Cooper 1985; Edelman 1991). The process of antibody production, which is T-cell dependent, is the responsibility of a class of lymphocytes called B cells (B refers to *Bursa* of Fabricius in birds, necessary for the maturation of B cells), which need two signals to initiate the humoral immune response (Gulbranson-Judge and MacLennan 1996). With a T-dependent antigen, the first signal comes from antigen-mediated cross-linking of B cell receptors and the second signal derives from the Th2 cells. First, cross-linking of the T-dependent antigen on B cell ensues, followed by presentation of that antigen associated with MHC II to Th2 cells, which then provide co-stimulation to trigger B cell proliferation and differentiation into plasma cells (Parker 1993; Baker, Gagnon et al. 2000). Cytokines, predominantly IL-4, IL-5, and IL-6, stimulate the B cells to divide and differentiate into antibody-secreting plasma cells. A few activated B cells differentiate into short-lived plasma cells that migrate directly to the medullary cords and begin secreting IgM and later IgG. Isotype switching to IgG, IgA, and IgE and memory cell generation occur in response to T-dependent antigens and cytokines (Jumper, Splawski et al. 1994). In a primary immune response, IgM is secreted before isotype switching occurs and is the predominant isotype produced. Each antibody isotype has its specific effector functions in humoral immunity.

Plasma cells are antibody-producing cells that no longer divide in response to antigen. They are larger than B cells and have more ribosomes, endoplasmic reticulum, and Golgi complexes. Some plasma cells survive only a few weeks, while others continue to produce antibody for longer periods, providing rapid protection against an eventual re-infection. Other B cells become memory cells with high avidity antigen-specific B-cell receptors, which can be rapidly re-stimulated by antigen and are present in higher frequency than the naïve resting B cells were before the infection and antigen-driven oligoclonal expansion of the antigen-specific B cell clones.
There are five different antibody isotypes, IgG, IgM, IgA, IgE, and IgD. All these isotypes seem to have unique features and different implications in the humoral immune response (Han, Mou et al. 1995). When microbes enter the body through the epithelia of the oral mucosa, for example, they may begin replicating locally or get into the circulation and move throughout the body. The Fc regions of the pentavalent IgM, which is secreted first in a primary immune response, allow them to aggregate antigens present at relatively high concentration and subsequently bind to Fc receptors of professional phagocytes to be phagocytosed in e.g. splenic and liver sinusoids. Because IgM is so large, it cannot enter the tissues very efficiently; but it is effective in controlling pathogens in the circulation. Once isotype switching occurs, IgG with the same antigen specificity starts to predominate in serum and in tissues. IgG both neutralizes pathogens and their toxins and opsonizes them for phagocytosis by neutrophils and macrophages (Robbins and Robbins 1986). Interestingly, only IgG seems to be able to cross the placenta and impart immunity to the developing fetus. IgG can also activate complement on the pathogen surface once concentrations are high enough for two IgG molecules to bind to nearby epitopes. IgA is the predominant antibody that is secreted across epithelial cells of the respiratory, digestive and genital tracts to block pathogen entry into the body (Robbins and Robbins 1986). IgE binds to FceRI on mast cells surrounding the blood vessels throughout the body. When a pathogen binds and cross-links the mast cell surface IgE, the mast cell immediately releases inflammatory mediators that trigger coughing, sneezing or vomiting to expel pathogens from the body.

Antibodies against candidal antigens are found in the sera of most people (Lehner, Buckley et al. 1972), being higher in patients with chronic mucocutaneous candidosis in whom the assessed antibodies seemed to be directed against the cell wall component mannan (Lehner, Wilton et al. 1972). Nevertheless, the role of humoral immunity in the defense process against mucosal or systemic candidosis is still questionable (Fidel 2002). There are reports about children having defective B cell functions, either congenital or acquired, who did not show any susceptibility to candidal infection (Rogers and Balish 1980). It is obvious that antibodies \textit{per se} cannot exert any candidastatic or candidacidal activities; rather they contribute by acting as opsonins for neutrophils and macrophages at the site of infection.
AIMS OF THE STUDY

1. To assess and clarify the presence of $\alpha$-defensin-1, its localization and eventual immunohistopathological signs of its participation in the host response against chronic candidal stimulus in CHC compared with candida-negative leukoplakia lesions.

2. When performing immunohistochemical staining of RANKL in CHC samples, we found accidentally that mast cells form a source of RANKL; therefore we aimed to confirm this pioneering discovery in atherosclerotic samples. We chose atherosclerosis because mast cells have a well-established role in the pathogenesis of the disease.

3. To evaluate the histopathological distribution of CD1a Langerhans cells in CHC compared with leukoplakia and healthy controls, and whether the number of these RANK-positive antigen-specific cells correlates with that of RANKL-producing mast cells.

4. To investigate the contribution of the chemokine IL-8 and its receptor IL-8 RA in the host defense against *C. albicans* and check if there is any immunohistopathological signs of their involvement in CHC compared with healthy controls.

5. To study what TLRs (TLR1-9) plays roles in the immune response of the host against *C. albicans* by comparison of the CHC lesions with leukoplakia and healthy mucosa and by studying the effect of those candidal PAMPs on mucosal epithelial cells which differed between CHC and comparators.
MATERIALS AND METHODS

Criteria for patient selection

a) For patients of CHC & LP
The local Ethical committee approved the study protocol. Ten biopsy samples of oral mucosal lesions were obtained from patients with chronic hyperplastic candidosis (n=10) and the same number from patients with leukoplakia (n=10) undergoing examination of mucosal lesions (Table 5). Histopathology of both disease entities disclosed their corresponding typical features. Periodic Acid Schiff (PAS) staining of the biopsy samples and/or fungal culture of saliva samples on Dentocult CA® culture medium (Orion Diagnostica, Espoo, Finland) were used to confirm or exclude the fungal infection. Five biopsy samples were recruited from healthy individuals undergoing dental procedure to serve as controls.

b) For patients of atherosclerosis
Coronary artery samples were collected from left anterior descending coronary arteries from five unselected patients autopsied for medicolegal reasons. All coronary segments contained a large confluent core of extracellular lipid. The local Ethical committee approved the study protocol.

Biopsies: processing and storage
All biopsies (CHC, LP, healthy controls and atherosclerosis) were fixed in formalin (10% phosphate buffered formalin, pH 7) and stored at 4+°C. After fixation, the tissue biopsies were dehydrated by first using graded ethanol solutions, then graded xylene solutions followed by liquid paraffin. The wax was allowed to solidify, forming blocks into which the tissues were embedded in cassettes. Out of these paraffin blocks, 6 µm and 4 µm (for study II) thick sections were cut and mounted on objective glass slides, which were then stored till immunostaining was performed. The modern paraffin is typically a mixture of paraffin wax and resin and it provides excellent morphological detail and resolution.
Table 5. Clinical and demographic data of the patients with chronic hyperplastic candidosis (CHC), leukoplakia (LP) and healthy controls (HC).

<table>
<thead>
<tr>
<th>Number</th>
<th>Gender</th>
<th>Age</th>
<th>Location of the lesion</th>
<th>Clinical presentation</th>
<th>Additional Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CHC</td>
<td>M</td>
<td>59</td>
<td>Tongue</td>
<td>Diffuse keratinization</td>
</tr>
<tr>
<td>2</td>
<td>CHC</td>
<td>F</td>
<td>45</td>
<td>Tongue</td>
<td>Red/white lesion</td>
</tr>
<tr>
<td>3</td>
<td>CHC</td>
<td>F</td>
<td>59</td>
<td>Tongue</td>
<td>Homogenous</td>
</tr>
<tr>
<td>4</td>
<td>CHC</td>
<td>F</td>
<td>67</td>
<td>Palate</td>
<td>Verrucous</td>
</tr>
<tr>
<td>5</td>
<td>CHC</td>
<td>F</td>
<td>55</td>
<td>Cheek/Commissures</td>
<td>Hyperplastic</td>
</tr>
<tr>
<td>6</td>
<td>CHC</td>
<td>F</td>
<td>53</td>
<td>Tongue</td>
<td>Nodular</td>
</tr>
<tr>
<td>7</td>
<td>CHC</td>
<td>M</td>
<td>44</td>
<td>Tongue</td>
<td>Ulcerative</td>
</tr>
<tr>
<td>8</td>
<td>CHC</td>
<td>M</td>
<td>53</td>
<td>Palate</td>
<td>Verrucous</td>
</tr>
<tr>
<td>9</td>
<td>CHC</td>
<td>F</td>
<td>81</td>
<td>Cheek</td>
<td>Papular leukoplakia</td>
</tr>
<tr>
<td>10</td>
<td>CHC</td>
<td>F</td>
<td>85</td>
<td>Tongue</td>
<td>Hyperplastic</td>
</tr>
<tr>
<td>1</td>
<td>LP</td>
<td>F</td>
<td>51</td>
<td>Alveolar ridge</td>
<td>White patch</td>
</tr>
<tr>
<td>2</td>
<td>LP</td>
<td>F</td>
<td>55</td>
<td>Tongue</td>
<td>White patch</td>
</tr>
<tr>
<td>3</td>
<td>LP</td>
<td>F</td>
<td>52</td>
<td>Cheek</td>
<td>Striated patch</td>
</tr>
<tr>
<td>4</td>
<td>LP</td>
<td>F</td>
<td>73</td>
<td>Floor of the mouth</td>
<td>Exophytic</td>
</tr>
<tr>
<td>5</td>
<td>LP</td>
<td>F</td>
<td>66</td>
<td>Palate</td>
<td>Striated</td>
</tr>
<tr>
<td>6</td>
<td>LP</td>
<td>F</td>
<td>64</td>
<td>Floor of the mouth</td>
<td>Homogenous</td>
</tr>
<tr>
<td>7</td>
<td>LP</td>
<td>F</td>
<td>50</td>
<td>Alveolar ridge</td>
<td>Exophytic</td>
</tr>
<tr>
<td>8</td>
<td>LP</td>
<td>F</td>
<td>48</td>
<td>Cheek</td>
<td>Striated</td>
</tr>
<tr>
<td>9</td>
<td>LP</td>
<td>F</td>
<td>84</td>
<td>Alveolar ridge</td>
<td>Exophytic, pigmented</td>
</tr>
<tr>
<td>10</td>
<td>LP</td>
<td>M</td>
<td>49</td>
<td>Alveolar ridge</td>
<td>White patch</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number</th>
<th>Gender</th>
<th>Age</th>
<th>Location of the sample</th>
<th>Reason presenting patient to the clinic (no inflammation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HC</td>
<td>F</td>
<td>56</td>
<td>Upper left sulcular mucosa</td>
</tr>
<tr>
<td>2</td>
<td>HC</td>
<td>F</td>
<td>57</td>
<td>Upper left sulcular mucosa</td>
</tr>
<tr>
<td>3</td>
<td>HC</td>
<td>F</td>
<td>44</td>
<td>Lower right sulcular mucosa</td>
</tr>
<tr>
<td>4</td>
<td>HC</td>
<td>M</td>
<td>32</td>
<td>Upper right sulcular mucosa</td>
</tr>
<tr>
<td>5</td>
<td>HC</td>
<td>F</td>
<td>25</td>
<td>Upper posterior vestibular mucosa</td>
</tr>
</tbody>
</table>

M = Male, F = Female
Histological Staining

a) Periodic acid Schiff staining
Periodic acid-Schiff (PAS) staining of sections from lesions of both chronic hyperplastic candidosis (CHC) and leukoplakia was used to confirm or exclude the presence of candidal hyphae. It is also used for the demonstration of glycogen and neutral mucins. PAS stain consists of 1% Periodic acid and Schiff's reagent (Basic Fuchsin, Potassium metabisulphite, Hydrochloric acid, Deactivated Charcoal and Distilled water).

Procedure
Following deparaffinization sections were 1) immersed in distilled water, 2) treated with 0.5% periodic acid for 5 minutes at room temperature, 3) washed well in distilled water, 4) treated with Schiff's reagent for 20 minutes, 5) washed in running tap water for 10 minutes, 6) counterstained with Mayer's Haematoxylin for 5 minutes and 7) dehydrated in alcohol, cleared in xylene and mounted in Diatex.

b) Immunohistochemistry
Immunohistochemistry is a method used to detect antigens in tissues using antibodies, and it was the main method used in all projects of this study.

Antigen retrieval methods
Antigen retrieval is a process which helps to retrieve the immunoreactivity of a particular antigen after it has been lost due to tissue fixation in formalin. In this study, two methods were used, heat-induced treatment using microwave (for studies I-V), and pepsin treatment (for study III). A special microwave was used in study V.

a) Heat-induced antigen retrieval
This method depends on application of heat treatment to the tissue being stained using microwave. After deparaffinizing the sections, they were immersed in 10% Antigen Retrieval Buffer (ChemMate Detection Kit) (for study I), in Tris EDTA buffer (10 mM Tris and 1 mM EDTA, pH 9.0) (for study III) and in 10 mM citrate buffer (pH 6.0) (for studies II, IV,V), followed by a period of 10 minutes of heating in a microwave at 600 W, with checking of the plastic box after the first five minutes to ascertain that it had enough fluid to evaporate and to avoid drying-up of the slides. In study V, the microwave used was programmed so that the heating cycle would run for 24 minutes at 94 ºC. Slides were kept 30 minutes at room temperature to cool them down.
b) **Pepsin treatment antigen retrieval**

This technique, which was applied only for study III, is based on the fact that pepsin is a proteolytic enzyme and thus may digest (etch) the masking proteins, thereby exposing the covered (hidden) antigen. In this technique, sections were immersed in a solution containing pepsin and distilled water (1:250) followed by addition of 1-N HCl (1:100, 0.1 ml). All sections were kept in +37 °C incubator for 30 minutes and profusely washed with running water, and finally washed in PBS for 5 minutes.

**Primary antibodies used in the study**

The primary anti-human antibodies which were used in the studies I-V are summarized below in the following table.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Source</th>
<th>Subtype</th>
<th>Concentration μg/ml</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-defensin-1</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>IgG</td>
<td>20</td>
<td>Chemicon, Canada</td>
</tr>
<tr>
<td>RANKL</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>IgG&lt;sub&gt;2b&lt;/sub&gt;</td>
<td>5</td>
<td>R&amp;D, USA</td>
</tr>
<tr>
<td>Tryptase</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1.05</td>
<td>Dako, Denmark</td>
</tr>
<tr>
<td>Chymase</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.01</td>
<td>Neomarkers, USA</td>
</tr>
<tr>
<td>CD1a</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.012</td>
<td>Dako, USA</td>
</tr>
<tr>
<td>IL-8</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>IgG</td>
<td>0.2</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>IL-8 RA</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>2</td>
<td>Novus Biologicals, USA</td>
</tr>
<tr>
<td>TLR1</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>0.8</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR2</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>2.6l</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR3</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>2</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR4</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>1.3</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR5</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>1.3</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR6</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>IgG</td>
<td>1</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR7</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>IgG</td>
<td>0.8</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR8</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>1</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>TLR9</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>IgG</td>
<td>0.5</td>
<td>Santa Cruz, USA</td>
</tr>
</tbody>
</table>
Programmable Tech Mate staining robot
In the first study (study I), tissue sections of both CHC and LP were stained using the ChemMate staining system. In this automatic immunostaining protocol, sections were run through three washes in buffer containing carrier protein, detergent and preservative and contributing to blocking of non-specific tissue binding of the immunological reagents used in the protocol. Then the sections were incubated in 20 µg/ml goat anti-human α-defensin-1 IgG (Chemicon, Temecula, CA) in antibody diluent for 30 minutes at room temperature, 2 µg/ml biotinylated rabbit anti-goat IgG for 25 minutes, endogenous peroxidase block solution for 8 minutes and finally with streptavidin conjugated highly purified horse radish peroxidase and 0.03% H₂O₂ with concentrated diaminobenzidine (DAB) as chromogen (160 µl of DAB) in horse radish peroxidase substrate buffer. Sections were counterstained for 30 seconds with Mayer’s haematoxylin solution before mounting.

ABC staining
Avidin-biotin-peroxidase complex (ABC) staining was applied for studies II-V. In this protocol, the endogenous peroxidase enzyme activity of the tissues was quenched by keeping the sections in a solution containing 1.5 ml of 30 % H₂O₂ diluted with 150 ml methanol, for 30 minutes at room temperature. To avoid nonspecific staining, the sections were incubated with normal serum (the normal serum of the species in which the secondary antibody was produced) (1:50 in 0.1% BSA, Vector Laboratory, Burlingame, UK) for 1 hour at room temperature. The sections were then incubated in the primary antibody (please refer to table 2 for details about their types and concentrations) and diluted according to the requirements of the experiment with 0.1% BSA and incubated overnight at 4ºC. Next day, all sections were incubated in biotinylated secondary antibody (dilution 1: 100, Vector) for 1 hour at room temperature, followed by incubation in avidin-biotin-peroxidase complex (ABC) (dilution 1:100; Vector) for 1 hour at room temperature. Finally, the sites of peroxidase binding were revealed with a combination of 300 µl of 3% H₂O₂ and 0.023% 3, 3′-diaminobenzidine tetra-hydrochloride solution (Sigma Chemical Co., St. Louis, MO, USA). All sections were counterstained with Mayer’s haematoxylin solution for 30 seconds, dehydrated in graded ethanol, cleared in xylene and mounted in Diatex (Becker Industrifärg AB, Märsta, Sweden).
Specificity & sensitivity of the primary antibody

During immunohistochemical staining of all the studies (I-V) the specificity of the immunoreaction was asserted by using some sections for negative staining control experiments. These control sections were stained with control antibodies of the same concentration (and isotype when pertinent) as the primary antibody used in this study (if a monoclonal antibody was used for control staining an antibody directed against *Aspergillus niger* glucose oxidase (Dakopatts, Glostrup, Denmark), an enzyme which is not present or inducible in mammalian tissues, was used).

To assess the specificity of the primary antibody in study I, antigen absorption test was done by incubating the primary polyclonal goat anti-human α-defensin-1 IgG antibody with recombinant human α-defensin-1 peptide (GF 099 Chemicon, Temecula, CA, USA) in phosphate buffered saline containing 1% bovine serum albumin, at 1:10 on a molar basis, for 1 hour at room temperature.

In study IV, to preclude any nonspecific binding of the ABC to biotin of the yeast, new sections of CHC were incubated in avidin-biotin-peroxidase complex (ABC) (dilution 1:100; Vector) for one hour at room temperature. Then all steps were followed as mentioned above. No positive staining could be seen in the control sections.

Western blotting

Western blotting technique was used in study II in which mast cells were investigated to assess if they contain RANKL.

a) Cell lysis

The cell pellet of $10^5$ human umbilical cord blood-derived mast cells sensitized with IgE and activated with anti-IgE IgG was lysed with 25µl Passive Lysis Buffer (Promega Corporation, Madison, WI, USA) with gentle shaking at room temperature for 30 minutes. The cell debris was removed by centrifugation at 13 000 g for 10 min at +4°C and supernatant was stored at -70°C.

b) Gel electrophoresis and immunoblot analysis

Cell lysis and culture media (10µl) were mixed with sodium dodecyl sulphate containing sample buffer (5µl) (New England Biolabs, Beverly, MA, USA). The samples were boiled for 5 minutes, and applied to the gels. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed with a 12% polyacrylamide slab gel. After electrophoresis; the gels were blotted onto polyvinylidene fluoride (Millipore Corporation, Bedford, MA, USA).
After blocking overnight with a 3% bovine serum albumin in Tris buffered saline blocking solution and a ten minute incubation in washing solution (0.1% Tween 20, 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5), blotted membranes were incubated with affinity purified polyclonal rabbit anti-human sRANK ligand antibody (0.4µg/ml dilution with washing buffer containing 2% bovine serum albumin) (PeproTech, Rocky Hill, NJ, USA) for two hours at room temperature. The membranes were then washed with washing buffer for 30 minutes with at least 3 changes of buffer. The membranes were further incubated with alkaline phosphatase conjugated goat anti-rabbit Ig (1:5000 dilution with washing buffer containing 2% bovine serum albumin) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for one hour at room temperature. The membranes were then washed with washing buffer for 30 minutes with at least 3 changes of buffer and a final wash in Tris buffered saline (50mM Tris-HCl, 0.9% NaCl pH7.5). The alkaline phosphatase-binding sites were revealed in ten millilitres of colour development solution (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad Laboratories, Richmond, CA, USA) based on the colorimetric detection of alkaline phosphatase with a mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). After 30 minutes the color reaction was stopped by washing the membranes in distilled water for ten minutes.

**Cell culture**

Cord blood-derived clusters of differentiation (CD) 34+ hematopoietic cells (Cambrex, East Rutherford, NJ) were cultured in StemSpan SF expansion medium (StemCell Technologies, Vancouver, Canada), supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and gentamicin (10 µg/ml; Life Technologies, Carlsbad, CA), in the presence of 10 ng/ml of SCF (R & D Systems Inc. Minneapolis, MN) in an atmosphere containing 5% CO2 and 5% O2 for 10 weeks, after which the cells were cultured in the presence of 100 ng/ml of SCF, 10 ng/ml of IL-4 and 50 ng/ml of IL-6 (R & D Systems Inc.) in an atmosphere of 5% CO2 and atmospheric oxygen for at least 4 weeks. The effect of serum-supplementation was investigated by adding 10% fetal bovine serum (FBS; Life Technologies) under different oxygen tensions to cells cultured for 10 weeks in serum-free and hypoxic conditions, in the presence of cytokines as above. The number of viable cells was counted with Trypan blue (Sigma, St. Louis, MO) and is expressed as mean ±SEM.
**Statistical analysis**

In study V, the differences in expression of the TLRs in the three epithelial layers (upper layer, middle layer and basal layer) for the three groups (CHC, leukoplakia and healthy mucosa biopsy samples) were analyzed separately with Pearson’s chi-squared test in which the levels of the TLR expression of the epithelial cells were coded (0 = no expression at all, 1 = 1-24% expression, 2 = 25-49% expression, 3 = 50-74% expression, 4 = 75-100% expression) using the non-parametric Kruskal-Wallis test. Differences between the two groups (candidosis *versus* control, candidosis *versus* leukoplakia, leukoplakia *versus* control) were assessed by the non-parametric Mann-Whitney U test. All statistical analyses were performed using the SPSS version 14.0. p-values less than 0.05 (two tailed test) was considered to be statistically significant.
RESULTS & DISCUSSION

Routine histopathology (for studies I, III, IV, V)
Candidal hyphae were revealed in CHC confined to the uppermost layers of the epithelia with varying degree of penetration, while in leukoplakia no hyphae were observed although some yeast cells were occasionally found on the surface of the mucosa in some of the samples. In CHC, candidal hyphae were usually growing in a perpendicular direction into the epithelium and were randomly distributed along the epithelial surface colonizing some areas while absent in others (Fig. 9, arrows). Numerical grading was used to estimate the number of yeast cells and hyphae in individual cases (included with the result tables of each study below). Staining of CHC sections with hematoxylin and eosin showed parakeratinized epithelium with clear, broad and bulbous rete ridges. In some areas keratin layer had eroded and the underlying epithelium was exposed. Individual neutrophils and microabscesses were often seen in the epithelia of CHC samples, whereas inflammatory cell infiltrates, composed of lymphocytes and plasma cells, were seen in lamina propria in all sections.

Immunohistochemical staining

1. Study I: α-defensin-1 in CHC
The antimicrobial peptide α-defensin-1 was mainly located in neutrophils. Both CHC and LP showed intravascular α-defensin-1 containing neutrophils, which was intense and uniform. In the lamina propria of CHC α-defensin-1 containing neutrophils were also found. There was no peri- or extracellular α-defensin-1 staining in the lamina propria. In the epithelium, an apparent gradient or stratification of α-defensin-1 positive neutrophils was obvious. This accumulation was particularly intense in heavily-Candida-infected and neutrophil-infiltrated samples, which also contained intraepithelial neutrophil microabscesses (Fig. 10). The cytoplasmic staining of many individual neutrophils in CHC epithelia was relatively weak, but associated with peri- and extracellular staining. This was particularly prominent in areas of microabscesses. In addition, CHC epithelia contained an intense superficial band or rim of α-defensin-1
deposition in the uppermost epithelial cell layers (Fig. 10, 11). However, in CHC the number of candidal hyphae did not correlate with the number of \( \alpha \)-defensin-1 positive neutrophils in connective tissue \( (P=0.58) \) or in the epithelium \( (P=0.35) \) (Table 7).

In contrast, the Candida-negative, control LPs contained few neutrophils and very little immunoreactive \( \alpha \)-defensin-1, mostly in intravascular neutrophils. LP epithelium was not infiltrated by neutrophils and no epithelial microabscesses or extracellular \( \alpha \)-defensin-1 staining was observed.

In this work, we were interested in the eventual protective role of the \( \alpha \)-defensin-1 against the opportunistic pathogen \( \text{C. albicans} \) in CHC, and based on our achieved results we can narrate a possible step-by-step story of both the infectious and the defense scenes, focusing on the role of the main spot of our main study, i.e. \( \alpha \)-defensin-1. The first event which follows invasion of the oral mucosa by \( \text{C. albicans} \) is the activation of the host innate immunity that, subsequently, triggers a local inflammatory reaction. Neutrophils, which are capable of killing and digesting smaller invading microorganisms such as planktonized yeast cells in intracellular phagolysosomes (Sohnle and Collins-Lech 1990), are considered the first line of defense against such intruding pathogens, are alarmed and thus recruited from the intravascular compartment to the site of infection. In the extravascular tissue and with the help of some chemokines, e.g. IL-8, neutrophils traverse through the lamina propria towards the location of the pathogen. When neutrophils get into the epithelium they accumulate and form microabscesses (localized collection of PMNs in the epithelium). Due to their activation, neutrophils degranulate their contents extracellularly, and among those substances released is \( \alpha \)-defensin-1. Although \( \alpha \)-defensin-1 is released by neutrophils (irrespective of their location at the time of \( \alpha \)-defensin-1 release), most of our results show that \( \alpha \)-defensin-1 was concentrated in the upper most part of the epithelium. We
hypothesize an explanation of this observation. Whenever α-defensin-1 is released by
the neutrophils, it may move along with the advancing epithelial cells as a result of the
normal epithelial cell growth, migration and shedding cycle (epithelial flow). This will
inevitably lead to the heavy accumulation of this antimicrobial peptide and thus to the
formation of the obvious immunoreactive rim at the most superficial part of the
epithelium. The presence of this α-defensin-1-rich zone might serve as a shield against
the ingress of *C. albicans* in the mucosa. Formation of this neutrophil-derived and
epithelially-deposited α-defensin-1 barrier may lead to an effective anti-candidal shield
against new ingrowths.

When a neutrophil faces a candidal pseudohyphen, it degranulates letting its granular
content with α-defensins come in contact with the candidal yeast membrane and this
can result in inhibition of the growth or even killing of the microorganism. Although
we conducted our study with the first class of α-defensin-1, we believe that it is more or
less similar to the behavior of the other neutrophil granule-derived α-defensin
subclasses as far as mucosal infections are concerned.

Neutrophils can produce, via activation of the cell surface NADPH oxidase, other
antimicrobial substances, e.g. superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$).
The problem with these “chemical weapons” is that their action is immediate; therefore
they seem not to be effective enough against chronic, long standing infections such as
CHC (Dahlgren and Karlsson 1999). Besides, even if these respiratory burst products
succeed in disrupting the pathogen(s), they will probably damage some “innocent
bystanders”, which can trigger an inflammatory reaction. This, however, does not
refrain neutrophils from using other options, e.g. α-defensins. The cooperation between
the immigrant neutrophils, which release α-defensins, and resident epithelial cells
bestows the host an important support in terms of candidal defense (Sawaki, Mizukawa
et al. 2002). As might seem paradoxical, we did not find a significant correlation
between prevalence of candidal hyphae and number of α-defensin-1 expressing
neutrophils. To justify this correlation discrepancy, we suggest that the effects of
epithelial β-defensins may supplement the neutrophil-derived α-defensin-1. This
synergistic action may account for the relatively low content of α-defensin-1 (produced
by the host) compared to the amount of the existing pathogens in some cases.
Table 7. Alpha-defensin-1 in chronic hyperplastic candidosis (CHC) and leukoplakia (LP).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hyphae</th>
<th>Microabscseses</th>
<th>Neutrophil</th>
<th>Dysplasia</th>
<th>α-defensin-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CHC</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>Dysplasia moderata</td>
<td>E+, CT +/-</td>
</tr>
<tr>
<td>2 CHC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>No dysplasia</td>
<td>E-, CT +/-</td>
</tr>
<tr>
<td>3 CHC</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>No dysplasia</td>
<td>E+, CT +/-</td>
</tr>
<tr>
<td>4 CHC</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>No dysplasia</td>
<td>E+, CT +/-</td>
</tr>
<tr>
<td>5 CHC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>No dysplasia</td>
<td>E+ , CT +/-</td>
</tr>
<tr>
<td>6 CHC</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>No dysplasia</td>
<td>E+, CT-</td>
</tr>
<tr>
<td>7 CHC</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>Dysplasia moderata</td>
<td>E+, CT-</td>
</tr>
<tr>
<td>8 CHC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>No dysplasia</td>
<td>E+, CT-</td>
</tr>
<tr>
<td>9 CHC</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>No dysplasia</td>
<td>E+, CT-</td>
</tr>
<tr>
<td>10 CHC</td>
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</tr>
<tr>
<td>1 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Dysplasia levis</td>
<td>-</td>
</tr>
<tr>
<td>2 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>3 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>4 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>5 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>6 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>7 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Dysplasia levis</td>
<td>-</td>
</tr>
<tr>
<td>8 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>9 LP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>10 LP</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>No dysplasia</td>
<td>-</td>
</tr>
</tbody>
</table>

− = Negative, +/- = only occasional, + = some, ++ = moderate numbers of and +++ = high numbers of hyphae, microabscseses, neutrophil infiltrates and α-defensin-1 positive cells. E = epithelium, CT = connective tissue.
2. **Study II: RANKL in mast cell (in atherosclerosis)**

In sections of atherosclerotic left anterior descending arteries taken from patients with coronary artery disease, we found mast cells in the adventitia layer, which displayed strong granular RANKL staining apparently confined to the cellular cytoplasm, without any visible signs of its release into the extracellular space. In contrast, mast cells in atherosclerotic plaques displayed granular cytoplasmic RANKL staining, which was faint and associated with pericellular RANKL-positive granules, apparently as a result of mast cell activation and partial degranulation.

Immunocytochemistry of sections of mature human mast cells (developed from *in vitro* culture of umbilical cord blood-derived CD34+ haematopoietic stem cells) showed a strong and granular cytoplasmic RANKL staining (Fig. 12). Negative control did not show any immunostaining. In Western blotting, lysed mast cells displayed a band which, by its size and immunoreactivity, was shown to represent the soluble form of RANKL (Fig. 13). In fact, the idea of this study emerged when we were doing a series of immunohistochemical staining of some CHC sections with different cytokines using different antigen retrieval protocols. This screening and use of pepsin pretreatment for antigen retrieval culminated in our discovery that mast cells express RANKL, a finding which had not been reported before. Therefore, we decided to assess this observation in a well-known disease called atherosclerosis. As the name suggests atherosclerosis is a general term applied to any obliterating blood vessel atheroma formation (literally it comes from the Greek words *atheroma*, meaning “porridge”, and *Skleros*, meaning “hard”). We investigated the role of mast cells in terms of RANKL expression and whether it is involved in any mechanism behind the process of coronary calcification. There are many processes that are thought to contribute to the
development of atherosclerosis, e.g. oxidant stress (Schulze and Lee 2005). Since the ossified structure of the atherosclerotic plaque was found to have the same histological features of normal bone including trabeculae, lacunae and marrow like spaces, Maria et al. suggested that this structure be called “osteosome” (Jeziorska, McCollum et al. 1998). By our discovery that mast cells form a source of RANKL, we provided new insight for the quantitative assessment of the cytokine RANKL in the pathogenesis of atherosclerosis. RANKL, which is also called osteoprotegerin ligand (OPGL), is a membrane-bound ligand regulating osteoclast maturation and activation (McClung 2006). The interaction of RANKL with its receptor (RANK) or decoy receptor osteoprotegerin (OPG) comprises an essential step in bone physiology and pathophysiology. RANKL and RANK have recently been found both in the endothelial cells as well as the vascular smooth muscle cells (VSMCs) of tunica media of arteries, suggesting that RANKL-RANK interaction is important for blood vessel (patho)physiology (Collin-Osdoby 2004). Our study seems to expand this field by incorporating RANKL-positive perivascular mast cells as new actor into this system. Accordingly, OPG deficiency leads to osteoporosis and arterial calcification. Due to the uncertainty of the exact role of RANKL in the pathophysiology of atherosclerosis, we cannot form an opinion whether expression of RANKL has beneficial or harmful effects in this particular condition. It might be that RANKL plays a protective role in atherosclerosis by means of stimulating osteoclastic progenitors to mature to osteoclasts which can resorb the already formed bone in the calcified plaques, but this cannot be predicted based on only immunohistochemical experiments. The present observations demonstrate that mast cells form a potent cellular source of soluble RANKL, which may contribute to vascular wall inflammation and plaque calcification. Molecular mechanisms responsible for the role of mast cells in the regulation of pathological, dystrophic calcification in atherosclerotic plaques and bone metabolism are only now emerging. It is noteworthy, that mast cells do respond to mechanical stimulation. In this respect it was interesting to note that mast cells in the adventitia were resting mast cells, whereas mast cells in the atherosclerotic plaque were activated, i.e. partially degranulated. This raises the possibility that the shear stress and the lever effect of the hard and calcified plaque may lead to mechanical mast cell activation and degranulation. Degranulation leads to release of biologically active mediators like RANKL and also to release of mast cell proteinases and collagenase activators, which contribute to plaque rupture.
This is the first study to show that mast cells express RANKL, thereby suggesting another critical role of mast cells in atherosclerosis and adding another cellular source of RANKL to the list of RANKL producing cells already reported in the literature. However, whether this affects osteoclast biology in the vicinity of the calcified lesion or some other aspects of endothelial-smooth muscle cell interactions and vascular pathology remains to be elucidated. Therefore, mast cells in general and RANKL in mast cells in particular, together with its co-actors, may deserve more attention in atherosclerosis and calcified tissue research.

3. Study III: Role of RANKL in CHC
Langerhans cells, which are CD1a positive, were found in the epithelia of CHC, LP and healthy controls. In the epithelium of LP and healthy tissue, these CD1a positive cells were mostly found in the middle layer, while in CHC they formed a network which was seen throughout the whole epithelium: in its upper, middle and lower thirds. In CHC, there was an obvious match between the content of Langerhans cells, whose grading values ranged from high (+++) to low (+), and the estimated quantity of candidal hyphae in each case (Table 8). In the epithelium, the morphology of CD1a positive cells was typical of dendritic cells, whereas in the lamina propria, they had the more regular shape of inflammatory mononuclear cells. In some sections of CHC, Langerhans cells were in a very close proximity to, or even traversing the basement membrane (Fig. 14).

In CHC and LP, the epithelia showed some RANKL immunoreaction particularly at the region of the epithelial ridges with stronger staining of the former than the latter. Epithelium of healthy control sections was completely devoid of any immunoreaction to RANKL. RANKL immunostaining was noticed to be dependent upon the method of pretreatment. In case of pepsin pretreatment, the only RANKL-positive cells in lamina propria were typical mast cells which in CHC displayed only faint granular cytoplasmic staining, with pericellular RANKL positive granules and matrix. In contrast, the number of mast cells was relatively low in LP and healthy control sections and RANKL

Figure 14. CD1a LCs traverse the basement membrane towards the lamina propria
staining was in these cells confined to the cytoplasm without any signs of extracellular release of RANKL.

If instead of pepsin, Tris EDTA heat pre-treatment was used for antigen retrieval, also other cells, e.g. fibroblasts and lymphocytes, were found to be RANKL positive. These other RANKL positive cells were more intensely stained than mast cells and they were distributed mainly in the subepithelial areas. Side by side comparison of pepsin pre-treated and Tris EDTA heat pre-treated sections from the same location demonstrates nicely this difference.

Neither CD1a nor RANKL positive cells were observed in negative staining controls. This third work supports the previous study showing that also mast cells in oral mucosa contain RANKL, which was in the second work described in mast cells residing in connective tissue. In this study, CD1a positive Langerhans cells (LCs) were found in mucosal sections from CHC, LP and healthy control tissues. LCs along with neutrophils comprise a very essential nonspecific armory which plays a pivotal role in extinguishing the infectious microbe \textit{C. albicans} from the host. Besides their direct antimicrobial effects i.e. phagocytosis, dendritic cells (immature and mature) can also produce chemotactic factors that activate and recruit neutrophils (Scimone, Lutzky et al. 2005). They are considered the most potent antigen catching, processing and presenting cells of the immune defense system (Steinman 1991). The preferred place of residence for LCs is mucosa where they act as sentinels against any foreign substances. Our results suggest a role for CD1a LCs in the hindrance of the entry of such pathogens. When an antigen source, e.g. candidal cell, succeeds in invading the epithelial natural barrier and intrudes into epithelium, LCs which have already been waiting for such a situation will stand in the way of the invading yeasts and hyphae and will eventually capture and subsequently engulf them. Following endocytosis, LCs process the engulfed candidal cell and associate its degraded components (epitopes) with major histocompatibility complex molecule (MHC II). Dendritic cells undergo maturation and leave the peripheral tissue to migrate to the regional lymph nodes where they present the antigen to naïve T-cells (Cutler and Jotwani 2004). In the present work, we demonstrated and compared the presence of CD1a positive Langerhans cells along with RANKL positive cells in CHC with LP and healthy controls. We also assessed the difference in the immunological response between CHC and LP since the former is considered as LP superimposed by \textit{C. albicans} infection. In CHC, the number of LCs and their pattern of distribution, however, were variable, probably due to recruitment
and migration. The dendritic cell numbers are debatable in other oral infections and inflammations, e.g. chronic periodontitis. In three separate studies, it was found that the number of dendritic cells increased (Saglie, Pertuiset et al. 1987), decreased (Seguier, Godeau et al. 2000), or did not change (Gemmell, Carter et al. 2002) in chronic periodontitis compared to healthy controls. We found a lot of inter- and intraindividual (intrasample) variation in the number and localization of CD1a positive Langerhans cells in CHC, which probably reflects the dynamic nature of their involvement in local disease mechanisms.

Our results showed a close match between the numerical quantities of LCs in the epithelium of CHC sections with the numbers of candidal yeasts and hyphae. However, we still think that the number of LCs per se in such mucosal infectious lesions does not form a reliable parameter to assess the severity of the infection, since otherwise all CHC sections should have shown higher numbers of LCs than their Candida negative counterparts and comparators, i.e. LP and healthy controls, some of which did contain nearly equal or even higher numbers of LCs than CHC. As has been stated, LCs were usually found above the basal layer of mucosal epithelium in oral, nasal, gastrointestinal and other areas (Girolomoni, Caux et al. 2002), which was the case also in our LP and healthy control sections. But in CHC, however, Langerhans cells were seen in all epithelial regions including the basement membrane area at the epithelial-lamina propria junction. The presence of LCs in the upper part of the epithelium can reflect the role of Langerhans cells in capturing and engulfing C. albicans. Once C. albicans (yeast or hypha) is engulfed, LCs start to migrate away from the epithelium towards the lamina propria where they have to traverse the basement membrane, as shown in the Figure 14. The trafficking capability of LCs offers a convincing explanation for the highly variable location of Langerhans cells throughout the epithelium in CHC. The second theme of this work deals with studying the expression of the cytokine Receptor activator of nuclear factor kappa-B ligand (RANKL) and comparing it in CHC with LP and healthy control. RANKL is a membrane-bound ligand which belongs to tumor necrosis factor superfamily. It is produced by osteoblastic lineage cells, mesenchymal cells, VSMCs, chondrocytes, and various immune cells, such as activated T cells (Hofbauer, Shui et al. 2001).
Table 8. Grading* of CD1a and RANKL staining in chronic hyperplastic candidosis (CHC), leukoplakia (LP) and healthy control (H).

<table>
<thead>
<tr>
<th>Cases</th>
<th>Yeasts and hyphae</th>
<th>Keratin</th>
<th>CD1a positive DCs</th>
<th>RANKL positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DCs in epithelium</td>
<td>DCs in CT</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>CHC-1</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CHC-2</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CHC-3</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>++</td>
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<td>CHC10</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Neg.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LP-1</td>
<td>-</td>
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<td>++</td>
<td>++</td>
</tr>
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<td>LP-2</td>
<td>-</td>
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<td>++</td>
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<td>++</td>
</tr>
<tr>
<td>H-3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

* (0) The structure itself is not present, (-) negative, (±) only occasional, (+) some, (++ moderate and (+++) high numbers of positive cell. DC= dendritic cell. CT= connective tissue.
It binds to its receptor RANK on the surface of monocyte/macrophages, osteoclast progenitors, endothelial cells and antigen presenting cells leading to their differentiation (Matsuzaki, Udagawa et al. 1998; Burgess, Qian et al. 1999). T cells can modulate the function of dendritic cells (Josien, Wong et al. 1999) through release of RANKL. This augments the capacity of dendritic cells to stimulate naïve T-cell proliferation in a mixed lymphocyte reaction (Anderson, Maraskovsky et al. 1997). As we found in the previous study that mast cells produce RANKL, we proceeded with this observation to assess whether it holds true for candidal mucosal infections involving antigen presenting cell-T lymphocyte interactions. The antigen retrieval methods seemed to affect the results of RANKL staining. When we treated the samples, which were formalin-fixed and paraffin embedded, with pepsin digestion, mast cells could be specifically studied as probably all cells containing cell surface bound RANKL become negative. In contrast, when Tris EDTA heat treatment was used for antigen retrieval, other RANKL-producing cells were also visible and stained positively, including lymphocytes and fibroblasts. We suggest that the different location of the antigen (RANKL) in different cells as well as to the mechanism of action of the antigen retrieving agent, may account for the disparity of RANKL staining. Pepsin is a proteolytic enzyme so its use entails the risk of cleaving some molecules exposed on the cell surface. In this way, pepsin appears to destroy most of RANKL proteins which were expressed by cells containing RANKL on their surfaces. In mast cells, however, RANKL seems to be stored in cytoplasmic granules. Since pepsin cannot penetrate plasma membranes (either cellular or in particular granular) composed mostly of lipid, RANKL proteins located in mast cells avoids the splitting action of pepsin. The Tris-EDTA based solution, on the other hand, is designed to break the protein cross-links, without endangering the antigen proper itself. Thus, all RANKL positive cells were seen after this method of antigen retrieval. This type of fixation artefact can be used if in particular the eventual role of mast cell RANKL is in focus of interest.

In CHC, RANKL-positive mast cells had weak staining apparently due to release of their RANKL laden-granules extracellularly, while in LP and healthy controls RANKL staining of the mast cell cytoplasm was stronger and completely intracellular. This observation might signify an active role of mast cell RANKL in CHC, which is not taken into use in noninfectious lesion, e.g. LP, or in normal mucosa. We suggest that this mast cell activation and RANKL release perhaps strengthens and speeds up the
maturation of the RANK positive Langerhans cells, when they communicate with the RANKL positive T-cells. In this respect, our results may add a new aspect of the participation of mast cells in the immunological response against pathogens.

4. Study IV: Role of IL-8 and its receptor in CHC

Immunohistochemical staining revealed IL-8 and IL-8 RA in all CHC samples and to a lesser extent in healthy control sections. Deep microvasculature showed IL-8 positive endothelial cells, which was more intense than in the superficial vasculature. In contrast to IL-8, the intensity of staining of both deep and superficial endothelial cells for IL-8 receptor A was much weaker and more variable. Some areas were IL-8 RA negative, although also strongly staining endothelia were found (Table 8). The microvascular endothelia in the healthy control sections were relatively weak in IL-8 and IL-8 RA staining compared to CHC (Table 9, 10).

The basal (lower) cell layer of the CHC epithelium showed stronger staining of IL-8 and IL-8 RA than the spinous (middle) cell layer, whereas the granular (upper) layer was completely devoid of any immunoreactions except for very scarce staining for IL-8 RA in some of the sections. Staining of the healthy control epithelia was generally less intensive than their counterparts of CHC. In the lamina propria and among inflammatory cells, IL-8 was found in many cell types, including plasma cells, mast cells, macrophages and neutrophils. Heavily infected CHC sections, in which there was a heavy neutrophil infiltration, showed intense IL-8 staining of the inflammatory cells. While polymorphonuclear cells had migrated on to the epithelium where they formed intraepithelial microabscesses containing IL-8 RA positive neutrophils, mononuclear inflammatory cells were retained in the submucosa. Negative staining controls confirmed the specificity of the staining.

Figure 15 showing the positive reaction of the mother cell of *C. albicans* to IL-8

Figure 16. The tip of *C. albicans* hyphae seems to be positive to IL-8RA
IL-8 and IL-8 receptor A in candidal cells in tissue and in agar sections

The germs of *C. albicans*, which were found lying on the uppermost layers of the CHC epithelia, stained positively for IL-8 and IL-8 RA. Candidal cells seemed to be polarized so that the mature blastoconidia (candidal cell bodies) only expressed IL-8 (Table 9, Fig. 15), whereas the hyphal tips were IL-8 RA positive (Table 10, Fig. 16).

A set of agar sections with cultured *C. albicans* cells were similarly stained. These experiments confirmed that only the mother cells of *C. albicans* express IL-8, while IL-8 RA is expressed by the outermost parts or the tips of candidal hyphae. The negative staining controls showed neither IL-8 nor IL-8 RA staining.

Table 9. IL-8 in chronic hyperplastic candidosis (CHC) and healthy controls

<table>
<thead>
<tr>
<th>Cases</th>
<th>Candidal mother cell</th>
<th>Candidal hyphae</th>
<th>Epithelium</th>
<th>Lamina propria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keratin layer</td>
<td>Medium layer</td>
<td>Basal layer</td>
<td>Superficial vascular endothelium</td>
</tr>
<tr>
<td>1 CHC</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>2 CHC</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>3 CHC</td>
<td>+++</td>
<td>++</td>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>4 CHC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>5 CHC</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>6 CHC</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>7 CHC</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>8 CHC</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>9 CHC</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>10 CHC</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>1 Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>2 Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>3 Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Negative control staining</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

0 the structure itself is not present, – negative, ± only occasional, + some, ++ moderate and +++ high numbers of positive cells.
Table 10. IL-8 receptor A staining in chronic hyperplastic candidosis (CHC) and healthy controls

<table>
<thead>
<tr>
<th>Cases</th>
<th>Candidal mother cell</th>
<th>Candidal hyphae</th>
<th>Epithelium</th>
<th>Lamina propria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Keratin layer</td>
<td>Medium layer</td>
</tr>
<tr>
<td>1 CHC</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>2 CHC</td>
<td>0</td>
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<td>++</td>
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<tr>
<td>3 CHC</td>
<td>++</td>
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<td>++</td>
</tr>
<tr>
<td>4 CHC</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>5 CHC</td>
<td>++</td>
<td>+++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6 CHC</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>7 CHC</td>
<td>+</td>
<td>++</td>
<td>±</td>
<td>+++</td>
</tr>
<tr>
<td>8 CHC</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>9 CHC</td>
<td>+</td>
<td>++</td>
<td>–</td>
<td>++</td>
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<tr>
<td>10 CHC</td>
<td>0</td>
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<td>1 Control</td>
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<td>2 Control</td>
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<td>3 Control</td>
<td>0</td>
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<td>+</td>
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<tr>
<td>Negative control staining</td>
<td>–</td>
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</tr>
</tbody>
</table>

0 the structure itself is not present, – negative, ± only occasional, + some, ++ moderate and ++++ high numbers of positive cells.

As we have previously mentioned, the first line of cells which are called to the site of candidal infection are neutrophils. But how neutrophils are called and recruited to the place of tissue insult was the objective of this fourth work in which we were interested in the eventual role of IL-8 (a cytokine known for its chemoattractive ability for neutrophils) and its receptor IL-8 RA in neutrophil-mediated defense in CHC. The role of cytokines, when synthesized, is to augment the defensive role of phagocytes (Marodi 1997). In the present study, we were able to show that especially the vascular endothelium and oral epithelial cells seem to react to candidosis by secretion of IL-8. Interleukin-8 is a chemokine which belongs to the CXC subfamily and has potent chemotactic effects on neutrophils both in vivo and in vitro (Baggiolini, Dewald et al.)
Neutrophils apparently respond to IL-8 by migrating through the vascular wall and lamina propria to the epithelium where the actual candidal hyphae are located. Once they infiltrate the epithelium, neutrophils form microabscesses and release the antimicrobial contents of their granules extracellularly. Probably due to the strong innate defense shield, candidal hyphae (despite their penetrative capacity) seldom reach beyond the spinous cell layer of the epithelium. There has long been a debate, due to conflicting data, whether *C. albicans* stimulates epithelial cells to synthesize IL-8 *in vivo* and *in vitro* (Hebert and Baker 1993). Whatever the mechanism, in this work we demonstrate that the growth of *C. albicans* in CHC is associated with relatively strong IL-8 and IL-8 RA staining. IL-8 is a strong chemotactic stimulus to neutrophils, but it may also play a role for candidal cell biology.

The surprising observation was that mother cells of *C. albicans* express IL-8 (or IL-8 – like protein), while IL-8 RA (or IL-8 RA-like protein) was localized mainly in the hyphal tips. Although we did not find candidal homologues of IL-8 or IL-8 RA when we used BLAST to assess whether *C. albicans* has DNA sequences coding these proteins, we still believe there is a possibility that *C. albicans* encodes such proteins since genomic sequencing of *C. albicans* has not been completed yet. These findings, which were first done using CHC tissue sections, were later confirmed by pure candidal cultures in agar. The peculiar location of the positive staining of IL-8 and IL-8 RA by the mother cell and hyphal tips, respectively, seems to exclude the chance of a haphazard occurrence. Based on our findings we assume that the continuous contact of *C. albicans* with oral epithelium in health and disease (since *C. albicans* comprises the major fungal oral microflora in about 25-75% of population) might have driven the fungus to evolve mechanisms counteracting host immune defenses.

*C. albicans* has been demonstrated to have a property of contact sensing or thigmotropism (Sherwood, Gow et al. 1992). This property is important in candidal biofilm formation on intraoral devices such as acrylic dentures (Nikawa, Nishimura et al. 1998). A rather new concept referred to as chemotropism has emerged in candidal biology. Davies *et al.* argued that Candida hyphal tips could elaborate exoenzymes into the underlying host structure. The hyphal tips, maybe through plasma membrane receptors, would be able to detect if this produces any cellular breakdown products (Davies, Stacey et al. 1999). Based on the present work, it might be that *C. albicans* expresses IL-8 RA on its hyphal tips to be able to sense the presence of IL-8, which might indicate potential danger. We tentatively name this phenomenon chemophobia. It
first seemed somewhat paradoxical that candidal cell body itself produces IL-8 or an analogue. However, if the endogenous candidal IL-8 (or analogue) produced by the mother cell communicates with the IL-8 RA (or analogue) at the tip of the hypha, its repulsive effect might help to guide the growth of the hypha in a centrifugal direction, away from the mother cell. When the tip of the hypha has grown to a distance from the mother cell so that the communication between the cell body and hyphal tip ceases, this very same ability might help to keep the sensitive hyphal tip away from IL-8-rich areas, which might be or become heavily infiltrated by neutrophils. Likewise, if the hyphal tip-located IL-8 receptor does not sense any IL-8, *C. albicans* might advance until it is faced by some defense barrier, danger signal or nutrient. This implies that *C. albicans* might have a strategy to sense the most suitable path for epithelial penetration.

In conclusion, IL-8 and IL-8 RA seem to be at work in host defense and may contribute to the recruitment and migration of neutrophils from the vascular compartment through lamina propria to epithelia, where they accumulate and get engaged in anti-candidal defense. At the same time, the evolutionary pressure may have conferred *C. albicans* the ability to utilize IL-8 system in its own survival strategy. The most straightforward explanation for such an arrangement would be that the candidal cell utilizes this system first for internal communication to direct the growth of the hyphae away from the cell body, followed by its use in external intelligence with an aim to keep the vulnerable hyphal tip away from danger.

5. Study V: Differential expression of TLRs in CHC
The pattern of TLR immunostaining in the three categories of samples (i.e. CHC, LP, and healthy controls) was different in terms of intensity and distribution of positive cells. The epithelium of all sections was divided into the three classical layers: lower, middle and upper. In most of the samples the strongest immunostaining was noticed in the basal layer. In CHC the number of positive epithelial cells ranged from none (-) to high (+++) for different TLRs. In hyphae-rich samples of CHC all the epithelium was uniquely positive to TLR 4 while it showed very faint staining for TLR 2, except for very few cells at the very bottom (Fig. 17). The three layers of the epithelia of the CHC sections revealed varying degrees of immunostaining in such a way that the uppermost layers tended to be devoid of staining while the underlying middle layers varied in staining intensity from always weak (in case of TLR 2) to moderately immunopositive (in case of TLR 4) to heavy staining (rest of TLRs), while the lower layer was always
positive. The picture was different in leukoplakia where TLRs staining of the middle and lower epithelial layers was always positive except, in some sections for TLR 9 to which the tissue showed weak staining or was even negative. In contrast, the epithelial layers of the healthy control tissue were always positive except interestingly a few sections where the lower layers were very weakly stained or negative.

The specificity of the TLRs epithelial staining was confirmed by the lack of immunoreactive cells in the negative staining controls.

From a statistical point of view, there was consistently statistically significant lower expression of the TLR2 (P<0.05) among the three epithelial layers either for candidosis versus control or candidosis versus leukoplakia comparisons. In addition to that, among the upper layer in the candidosis versus normal status there was also significantly lower expression of the TLR3-6 (P<0.05), and in the candidosis versus leukoplakia there was a significantly lower expression of the TLR 6 and 7 (P<0.05). Interestingly, there was a statistically significant higher expression of the TLR 4 and 6 (P< 0.05) in the leukoplakia lower layer versus controls. On the other hand, in the upper and middle layers these two TLRs expression were observed to be significantly lowered for candidosis versus leukoplakia or candidosis versus controls.

Lower or higher expression (P>0.05 but <0.066) was also observed for different TLR among the three different epithelial layers.

In this work, the immunohistochemical expression of nine classes of TLRs (TLR1-9) was assessed in a series of sections from CHC, LP and healthy tissue. In oral candidosis, TLRs serve to recognize *C. albicans* (usually through its cell wall component zymosan), thereby igniting a set of intracellular signaling cascades, which end in production of proinflammatory cytokines and chemokines. In this study, we

**Figure 17** showing the numerous candidal hyphae in chronic hyperplastic candidosis (A), in which TLR2 staining was very faint except few cells at the very basal layer (B) while the same section showed strong staining of TLR4 (C). The same finding was noticed in another hyphae-rich section (D, E).
compared the immunochemical staining of all known TLRs (except TLR10) in sections from CHC, LP and healthy tissues. It has been shown that candidal cell wall components constitute pathogen associated molecular patterns (PAMPs) for certain TLRs, e.g. zymosan is recognized by TLR2/TLR6 heterodimers while mannans is a ligand for TLR4 (Netea, Van Der Graaf et al. 2002; Roeder, Kirschning et al. 2004). For practical analysis, the epithelium was divided into three layers namely; upper, middle and lower. All TLRs, except those which are claimed to recognize *C. albicans*, i.e. TLR2, TLR4 and TLR6, were found to be strongly positive especially by the middle and lower layers. This may indicate that when epithelial TLRs (i.e. TLR1, TLR3, TLR5-9) are not stimulated by a particular PAMP (in this context, zymosan or phospholipomannnan), they tend to be constitutively expressed. This explanation is enhanced further by the staining pattern of the healthy tissue to the TLRs (TLR1-9) and by a previous report which has documented the expression of TLRs by a variety of oral mucosal cells (Mahanonda and Pichyangkul 2007). For TLR2 and TLR4, it was another interesting story. In two samples out of five there were high numbers of candidal hyphae compared to the unicellular yeasts and when stained with TLR antibodies, they showed very little staining of TLR2 while TLR4 was comparably strong, but in the other sections in which hyphae were scarce TLR4 was weaker. If we take the dogma of negative regulation of TLRs into account (Han and Ulevitch 2005), then we can suggest that TLR2 is more actively engaged in recognizing and mediating the effects of candidal ligand (and thus more down-regulated) in the sections which contained high hyphae: yeast ratio. Such findings drive us to suggest that candidal hyphae could direct the immune response towards stimulating TLR2 rather than TLR4 which, otherwise, would also be weakly expressed. It has been intensely debated whether *C. albicans* could stimulate TLR 2 and 4 in a manner which is dependent on the fungus morphological form. Our results seem to accord the previous work of Netea et al. who have shown that the opportunistic pathogen can exploit TLR2 to evade the immune system (Netea, Van der Meer et al. 2004). The same group was the first to work on this idea and demonstrated that TLR2 knockout mice had gained more resistance against systemic candidosis compared with wild type due to the less release of anti-inflammatory cytokines, e.g. IL-4 and IL-10 which are elaborated by TLR2 activation (Netea, Sutmuller et al. 2004).
In this work we present a new aspect of *C. albicans* virulence in mucosal candidosis in the sense that candidal hyphae bring down the anticandidal Th1 cytokines (by evading TLR4), and simultaneously enhances the anti-inflammatory Th2 process (through stimulation of TLR2).

The lower layers of all epithelia of CHC showed wider TLRs staining, comparatively, than the superficial layers and this is likely due to the synthesis of TLRs first in the lower layer and then starts to decrease in quantity as it travels upwards along the epithelial flow for a variety of reasons, e.g. limited half-life and down-regulation.

In leukoplakia, the strong epithelial staining (for all TLRs except TLR9, which was quite weak in some sections) extended to the overlying keratinous layer (Fig. 18). If the classical definition of leukoplakia was revised (please refer to the leukoplakia section in this thesis), an explanation can be stated for the less staining of TLR9 noticed in some sections. The definition of leukoplakia denotes that it is a lesion and as a result; the host defense may be elicited and activated. Activation of the defense system may be manifested by an exaggerated expression of epithelial TLRs in order to protect the already diseased underlying tissue. Another important clue which can be drawn from the definition is that the etiology of leukoplakia is not yet known and its pathogenesis has not so far been attributed to any microorganisms. Therefore, we consider it a logical finding that no particular TLR (except TLR9) was down-regulated and, therefore, probably not involved in any long-term ligand binding. The endosomal location of TLR9 parallels its ability to recognize the unmethylated CpG DNA the origin of which can be bacterial, fungal or mitochondrial (Bellocchio, Moretti et al. 2004; Takeda and Akira 2005). Based on that, we suggest that, in leukoplakia, the mucosal host defense system may degrade the invading microbes, whether bacteria or fungi, by phagocytosis, e.g. by PMNs or direct
lysis (by antimicrobial peptides resulting in leakage of the microbial cell contents including its genetic material), which would keep on stimulating TLR9 leading to its decreased expression. We, however, cannot generalize this assumption since it was found only in some sections but this again could be due to involvement of other factors, e.g. degree of the lesion chronicity and local immune status.

Sections of the healthy tissue, in contrast, showed strong expression of all TLRs especially by the middle and superficial layers. The lower layers of some sections showed faint or even absent TLRs staining which may broaden the phenomenon of negative regulation in healthy tissue with unoccupied TLRs.
SUMMARY & CONCLUSION

Candida, which is normally present on the skin and mucous membranes, such as the mouth, gastrointestinal tract, genitourinary tract, is the most frequently isolated fungal pathogen of humans. Among its many species, Candida albicans is responsible for most human candidal infections affecting immunocompromised patients ranging from premature infants to AIDS (acquired immunodeficiency syndrome) sufferers. The immune system of the host, under normal conditions, can maintain a strict control upon C. albicans should it attempt to express its pathogenic potential. It is not a necessity that patients be suffering from immunocompromising pathological conditions to become infected by such opportunistic pathogens, since age extreme individuals, i.e. infants and senile people, as well as denture wearers also remain suitable targets for C. albicans infection. In mucosal niche, C. albicans can thrive in a fertile ground with a moist and warm, protein-rich human mucosal membrane or biomaterial surface where they become activated and start to grow pseudo and real hyphae resulting in colonization. C. albicans can also travel through the blood stream and affect distant sites, e.g. brain, kidney and heart valves, and cause what is known as systemic candidosis.

Infection of the oral mucosa with the opportunistic candidal infection may result in several clinical and histopathological forms ranging from atrophic, pseudomembranous to hyperplastic candidosis. We have adopted the last category (chronic hyperplastic candidosis) to present a model for studying the oral host defense against candidal invasion.

We supposed that the host has to recognize the invading candidal microbe first through binding of certain components of the candidal cell wall to germ-line encoded receptors (the subject of study V). Once the host identifies the identity of the attacking stimulus, it reacts by playing many tactics, e.g. elaboration of natural antimicrobial peptides (e.g. defensins) and recruitment of immune cells (e.g. phagocytes). The style of α-defensin-1 release and its manner of contribution in the defense process against C. albicans was investigated in study I, while the cellular scene of the host defense and the cytokines involved in its regulation were the focus of the other studies (II, III, and IV). Generally speaking, the aim of this thesis work was to draw a scenario of how the host responds to candidal infection of the oral mucosa in terms of natural antimicrobial peptides, cytokines, and cells and try to combine their tasks into a unified theme.
In the first work, we studied the neutrophil-derived anti-candida α-defensin-1 which was found in the epithelium; not only diffusely all over, but as an α-defensin-1 rich shield, while it was hardly seen in the lamina propria. But since the cellular source of α-defensin-1 is neutrophil, which is recruited from the lamina propria one can ask how this antimicrobial peptide reaches the uppermost part of the epithelium without being expressed in the lamina propria? This question drove us to shape a probable step-by-step process which went in accordance with our results and observations and some basic biological facts. We suggested that the neutrophils transmigrate through the microvasculature in the lamina propria and migrate towards the epithelium. On their way, they would maintain their granules intact and advance further till they reach the epithelium where they-upon contact with candidal germs-start to release their granular contents, including α-defensin-1. While neutrophils gather and organize themselves into microabscesses, α-defensin-1 will continue, maybe as a result of epithelial cell flow, advancing towards the uppermost layer of the epithelium and accumulate there, a process which may be responsible for the α-defensin-1 rich frontier shield.

The second work (study II) does not need much to discuss because its main purpose was to confirm the accidental finding that mast cells can express RANKL. The pattern of mast cell staining was different in calcified plaque compared with RANKL-positive mast cells in the tunica adventitia. It seems that
RANKL has a particular role in the calcification process on the plaque since it was partially released from the mast cells, while it appeared completely intracellular in areas not affected by plaques.

The third work (study III) was done to check if mast cells-releasing-RANKL behave in the same way in CHC as in the previous work of atherosclerosis. Mast cells seem to react to the opportunistic *C. albicans* infection by local mast cell hyperplasia and secretion of RANKL. This may be important for the recruitment and maturation of antigen presenting dendritic cells and lymphocyte activation. Some sections showed strong mast cell staining confined to the cell cytoplasm, without any signs of extracellular release of RANKL. Others displayed granular cytoplasmic RANKL staining, which was faint and associated with pericellular RANKL positive granules, apparently as a result of mast cell activation and partial degranulation. Expression of RANKL augments the ability of dendritic cells to enhance naïve T cell proliferation in a mixed lymphocyte reaction. Dendritic cells and T lymphocytes comprise an important category of immune system against fungal infections. Therefore, mast cells seem to participate in the immunological processes conducted by the host against such a pathological insult.

The fourth work (study IV) was conducted to examine the presence and effects of the neutrophil chemokine IL-8 and its receptor IL-8 RA in CHC. We found that most of the host tissue produces IL-8 at high concentrations, e.g. epithelium, endothelium and inflammatory cells, which would activate neutrophils and lead to their degranulation. The most important and novel observation was that the candidal cell body showed IL-8-like immunoreaction whereas the tips of the candidal hyphae expressed IL-8 RA-like immunoreactivity. This is very interesting as we speculate that endogenous candidal-derived IL-8 at low concentrations could lead to centrifugal growth of the hyphae, away from the candidal cell body, via interactions of IL-8 with its receptors at the hyphal tips. At the same time, hyphal IL-8RA could prevent the tips from intruding into dangerous areas where neutrophils will soon migrate or where they are already located.

In the fifth and last piece of work, a comparative analysis of TLRs (TLR1-9) immunostaining in CHC, LP and healthy control was undertaken. From this experiment, we concluded that the resultant immune response following *C. albicans*-TLR binding depends largely on the morphological form of the fungus. In other words, *C. albicans* seems to take advantage of its projecting hyphal form in eluding the host
defense by means of directing the immune defense towards TLR2 rather than TLR4. When oral epithelium is infected with yeast-dominated candidosis, TLR4 seems to be more involved than TLR2. In leukoplakia, the weak status of the diseased tissue makes it vulnerable to further infection and this may explain the strongly positive TLRs staining (except TLR9 which, in some sections, showed weak staining possibly due to their engagement with the nuclear material of any invading microbe).

To summarize, the above long story can be shortened and sectioned into one complete scene (omitting some unnecessary details): The mouth encompasses many candidal cells which are floating around, some are attached. Some such candidal cells attempt to establish adherence to the oral epithelia and invade the underlying tissue. We have two scenarios here. If the host is healthy, such attempts will be doomed to failure due to the strong physical integrity of the epithelium and the strict host defense. If the immune status of the host is compromised, unpleasant consequences may follow. Once *C. albicans* attaches to the oral epithelium, it will be recognized by TLR2, 4 and 6. Based on literature analysis and the pattern of TLR expression, the most important of these in recognizing *C. albicans* seem to be TLR2 and 4. If the invading cells have more unicellular yeast form, TLR4 responds more extensively and the subsequent immune response against candidosis will be promoted through elaboration of pro-inflammatory cytokines and chemokines. IL-8 is important as it recruits the neutrophils to the uppermost layers where the candidal germs are located. During their upward movement, they release some of their α-defensin-1 extracellularly till they reach the superficial layers where they group themselves into collections known as microabscesses (Fig. 19). When the host senses that the infection is going to persist longer (become chronic), antigen-presenting cells are engaged into the host defense in order to pave the way for the more sophisticated specific immune defense. These epithelial dendritic cells are Langerhans cells, which under such circumstances are recruited to the epithelium where they engulf candidal cells (either by zipping or coiling), leave the mucosa and in the regional lymph node present the antigen to naive T cells. During their journey, they get augmented by RANKL secreted by resident mast cells, so that they become more effective in communicating with T cells. On the other hand, *C. albicans* seems to have developed some means to avoid the host defense. One aspect is that when more hyphae are encountered in the infection, then the receptor stimulation may shift towards TLR2 which results in production of anti-inflammatory
cytokines, thereby propagating the potency of the infection. Another smart technique is the ability of the fungus to produce IL-8 or an analogue by the mother cell and express its receptor IL-8RA at the hyphal tip so that it can stimulate itself in an autocrine manner to drive the projecting hyphae away from the cell body. This apparent chemophobic phenomenon may also serve to save the sensitive and more important part of the candidal body (i.e. the hyphal tip- used in thigmotropism and chemotropism) should it sense any risk for approaching neutrophils.

In the end, although the study of the pathogenesis of the opportunistic pathogen *C. albicans* and the host defense against it has advanced progressively, there are still many interesting puzzles which remain to be resolved. First, why does *C. albicans* thrive only in some people? Second, when the host is healthy, why does not *C. albicans* cause any infection? Is it because it stays merely commensal or it actually expresses some pathogenic features which are immediately abrogated by the strong host defense? In case it exerts its pathogenicity only when the host immunity is compromised, how does it sense the change in the defense status? Third, out of study 5, how do candidal hyphae, but not yeast, elude the host response by directing its stimulation towards TLR2? Fourth, is there any purpose behind *C. albicans* chemophobia other than avoiding the source of danger i.e. neutrophils? Fifth, why is it very rare, if ever, that *C. albicans* could invade the mucosa beyond the spinous layer? Is it because of the strong shield of the epithelium, or are some other factors involved? Sixth, what is the nature of epithelial anti-*Candida* activity? Seventh, I wish if researchers, in the future, could reach a clear and satisfactory definition of LP because the one in use today is a bit confusing and ambiguous. Eighth, regarding atherosclerosis, what is exactly the role of RANKL in the pathogenesis of the disease? Is it a causative factor or an effect?

Therefore, further efforts are needed to reveal other anti-*Candida* host defense mechanisms. This might aid in paving the road towards lowering the incidence of candidosis (whether mucosal or systemic) by improving the current medications and developing new therapeutic modalities.
AKNOWLEDGEMENTS

This study was conducted at the Department of Medicine, Institute of Clinical Medicine, Departments of Oral Pathology and Oral Medicine, Institute of Dentistry and Department of Anatomy, Institute of Biomedicine, all part of the Faculty of Medicine at the University of Helsinki, Helsinki, Finland, during the period 2003-2008 (I joined the university in 2002 but I started this thesis project in 2003).

First of all, I thank my Lord “Allah” for His utmost support, not just for this thesis, but rather for the events in all of my life.

I also wish to express my sincere thanks to my dear supervisor Professor of Medicine (and former Professor of Oral Medicine 1999-2003) Yrjö T. Konttinen, who was of great help and support during the whole period of my study. He has helped me get the keys for many scientific research windows thereby giving me the chance to come face to face with the fascinating world of biomedical science. The efforts of his faithful advice and encouragement were so valuable.

Along with Professor Konttinen, my thanks go to my second supervisor Professor Jarkko Hietanen for his supply of the samples and for his kind help and advice, Professor Malcolm Richardson and Dr. Riina Rautemaa for their helpful contribution to our collaborative work, Professor Stephen Porter and Docent Ilmo Leivo for reviewing my thesis and stating their valuable comments.

Moreover, I cannot forget the aid of Dr. Timo Sorsa who made my coming to Finland possible through his certificate of admission which was mandatory for my visa to be issued.

I would like to say to my old colleague Dr. Jian Ma, who is back in China now, thank you for teaching me the principles of staining and lab techniques. I owe you for your help and patience. I wish you a happy and an enjoyable life.

Among Finns whom I know, I have never seen such a kind person with an always smiling face as Marjatta Kivekäs who, beside her nice character, prepared all of my paraffin-embedded sections when needed without even a bit of hesitation.

My many thanks go to all of TULES group members for their good rapport and friendship.

I present my deepest gratitude to my dear brother Professor Mohammed Elmusrati and his family who, upon my first coming to Finland on the 10th of November 2001, offered
me respectful and generous hospitality. Besides, his help in showing me the principles of computer science was really of great support in making my research easygoing and pleasurable.

Coming to my dearest friend Dr. Nabil Nattah, I thank you so much for your explanation and advice about some rules and regulations which I needed to know during my first study days in Biomedicum. Our companionship when we used to and still go out and eat pizzas will never vanish from my memory.

Although I have been living in a completely new culture with different standards for more than five years, I have never had the feeling of being lonely or desolate. Thanks for this go to my Libyan and Arab friends living in Helsinki, Turku and Tampere. The nice moments that we spent together, which were full of useful discussions and amusements, have inspired me with hope and certainty to drill my way ahead towards accomplishing my thesis, and here it is, so thank you very much.

Finally, I am deeply thankful to my wife Dr. Dareen Fteita for her caring and support and since we share the same specialty, her scientific views and comments were of great aid in finalizing this thesis. Our loving son Salem has bestowed more beauty to the picture of my life by letting me taste the meaning of fatherhood.

My last words are devoted for the two persons who together were the reason for my existence before education: my father, Salem Elmusrati to whom I say: Cheer up dad! The very moment that you have grown me for, since I was a kid, has come. Your efforts did not go with the wind. The time is here to harvest what you have sown.

I say to the other most precious person: my mother, Zakia Mohammed who filled my life with kindness and mercy before her passing away. I say to you: Oh mom! How a model mother you were! You suffered a lot to make me stand on my feet. Even if days have parted us farther and farther, you will remain closer and closer to my heart. I will never forget the most beautiful days when you were with us. Being satisfied with the will of Our Lord, I wish you had attended this day even though I am completely sure that you share my happiness in your grave.

I finalize this acknowledgment with thanking the following institutes and foundations for financing me and my work: Libyan Secretary of Higher Education, Finska Läkaresällskapet, The National Center of Excellence in Biomaterials and Tissue Engineering of the Academy of Finland, the National Graduate School of Biomaterials
BGS, and its successor the National Graduate School of Musculoskeletal Disorders and Biomaterials TBGS (2007-), The Sigrid Jusélius Foundation, The Finnish Funding Agency for Technology and Innovation (Microrobotic diagnostics and Therapy), MNT ERA Net (A new Generation of Titanium Biomaterials), MATERA (BioNanoCoRe), Suomalais-Norjalainen Lääketieteen Säätiö, Avohoidon Tutkimssäätiö, Väinö ja Laina Kiven säätiö, Finnish Dental Society (Apollonia), The Invalid Foundation, Viikki Graduate School (for supporting a practical course in Sweden), The University of Helsinki, FEMS (Federation of European Microbiological Societies), ESCMID (European Society of Clinical Microbiology and Infectious Diseases).

Helsinki, 12/05/2008

Ahmed S. Musrati
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