CANDIDA PROTEINASES IN THE DEGRADATION OF ORAL MUCOSAL TISSUE COMPONENTS ASSOCIATED WITH *CANDIDA* INVASION

Pirjo Pärnänen

Institute of Dentistry
University of Helsinki, Finland

and

Department of Oral and Maxillofacial Diseases
Helsinki University Central Hospital (HUCH)
University of Helsinki, Finland

and

Institute of Biomedicine/Anatomy
University of Helsinki, Finland

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki,
for
public criticism in Lecture Hall 2, Biomedicum, Haartmaninkatu 8, Helsinki,
on April 16 th, 2010, at 12 noon.

HELSINKI 2010

Supervised by:

Professor Jukka H. Meurman
Department of Oral and Maxillofacial Diseases
Helsinki University Central Hospital (HUCH), and
Institute of Dentistry
University of Helsinki, Finland

and

Professor Ismo Virtanen
Institute of Biomedicine/Anatomy
University of Helsinki, Finland

Reviewed by:

Professor Erik Dabelsteen
Institute of Dentistry
University of Copenhagen, Denmark

and

Professor Malcolm Richardson
Regional Mycology Laboratory
Wythenshawe Hospital
University of Manchester, UK

Opponent:

Professor Peter Holbrook
Faculty of Odontology
University of Iceland
Reykjavik, Iceland

Cover: *C. tropicalis* ATCC 750; SEM by Kari Lounatmaa, 2008
ISBN 978-952-92-7038-5 (paperback)
ISBN 978-952-10-6147-9 (PDF)
http://ethesis.helsinki.fi
Helsinki University Print
Helsinki 2010

"Nature is the realization of the simplest conceivable mathematical ideas",

Albert Einstein, Oxford lecture, 1933

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following studies, which are referred to in the text by their Roman numerals (I-IV):

I. Pärnänen P, Kari K, Virtanen I, Sorsa T and Meurman JH. Human laminin-332 degradation by *Candida* proteinases. Journal of Oral Pathology and Medicine 2008; 37: 329-335.

II. Pärnänen P, Meurman JH and Virtanen I. Laminin-511 and fibronectin degradation with *Candida* yeasts. Journal of Oral Pathology and Medicine 2009; 38: 768-772.

III. Pärnänen P, Meurman JH, Samaranayake LP and Virtanen I. Human oral keratinocyte E-cadherin degradation by *Candida albicans* and *Candida glabrata*. Journal of Oral Pathology and Medicine 2010; 39: 275-278.

IV. Pärnänen P, Meurman JH and Sorsa T. The effect of *Candida* proteinases on human proMMP-9, and human recombinant TIMP-1, and TIMP-2. Mycoses 2010, accepted for publication.

2. ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacterium actinomycetemcomitans*
AIDS	acquired immunodeficiency syndrome
AJ	adherens junction
APMA	aminophenylmercuric acetate
BL	basal lamina
BM	basement membrane
BMZ	basement membrane zone
C. albicans	*Candida albicans*
cAMP	cyclic adenosine monophosphate
CDH1	CD324, cluster of differentiation 324
CRP	C-reactive protein
DMSO	dimethyl sulfoxide
E-Cad	E-cadherin, epithelial cadherin
ECL	enhanced chemoluminescence
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
Fn	fibronectin
F. nucleatum	*Fusobacterium nucleatum*
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
IL-1	interleukin-1
IL-1Ra	interleukin-1 receptor antagonist
JE	junctional epithelium
Lm	laminin
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MDPF	2-methoxy-2,4-dephenyl-3(2H)-furanone
MHC	major histocompatibility complex
MMP-9	matrix metalloproteinase-9
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
NCAC	non-*Candida albicans Candida*
N-Cad	N-cadherin, neural cadherin
NF- κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
PBS	phosphate-buffered saline
P. gingivalis	*Porphyromonas gingivalis*
P. intermedia	*Prevotella intermedia*
PKA	protein kinase A
PMN	polymorphonuclear leukocyte
RHE	reconstituted human epithelium
RTME	rabbit tongue mucosal explant
RT-PCR	reverse-transcriptase polymerase chain reaction
Sap	secreted aspartic proteinase
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis

SEM	scanning electron microscopy
sIgA	secretory immunoglobulin A
SM	*Streptococcus mutans*
TGF-α	tumour growth factor alpha
TIMP	tissue inhibitor of metalloproteinase
TJ	tight junction
TLR-2	toll-like receptor-2
VE-Cad	vascular endothelial cadherin
WBC	white cell blood count
YPG	yeast peptone glucose

3. ABSTRACT

Oral yeast infections caused by *Candida* are becoming an increasing problem. Most infections caused by *Candida* are superficial, but as the infection persists it tends to invade deeper into the epithelium. Particularly in immunocompromised patients, the invasion by *Candida* may cause systemic infection. Candidosis is the third most common infection causing mortality in hospitalized patients.

The exact invasion mechanisms of *Candida* yeasts through the mucosal epithelial layer remain unknown. The integrity of the epithelium depends on intact and functional structural components.*Candida* use several virulence factors in gaining nutrients from the environment and invading tissue, one of these being proteinases. The aim of this thesis was to compare the degradation of human oral epithelial proteins by proteinases of different *Candida* yeast species. We focused on proteins in the cell-to-cell junction, the basement membrane zone, the extracellular matrix, and local tissue inflammatory regulators. Another main objective was to evaluate the effect of the yeast/hyphal transition and pH on the degradative capability of *Candida*.

To identify the specific *Candida*-mucosal protein interactions without the effect of the modifying host tissue response on *Candida*, we made the study setting simple by using direct degradation assays. The enzymatic activity of the *Candida* proteinases was verified by gelatin zymography. Laminins-332 (Lm-322) and -511(Lm-511) produced by human oral keratinocytes were gathered from the growth media, and E-cadherin (E-Cad) was isolated from the cell membrane of the keratinocytes by immunoprecipitation. The proteins were incubated with *Candida* cells and cell-free fractions, and degradation was detected by fluorography. Fibronectin degradation was visualised by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Matrix metalloproteinase-9 (MMP-9) activation and tissue inhibitor of metalloproteinase-1 (TIMP-1) fragmentation was detected by using the Western blot and enhanced chemoluminescence (ECL) techniques. Residual activity of TIMP-1 was evaluated by a casein degradation assay. A fluorimetric assay was used to detect and compare *Candida* proteinase activities with MMP-9, typical oral bacterial and control enzyme activities.

These studies showed that the ability of the different *Candida* yeast species to degrade human Lm-332, fibronectin, and E-Cad vary from strain to strain and that this degradation is pH-dependent. This indicates that local acidic pH in tissue may play a role in tissue destruction by activating *Candida* proteinases and aid invasion of *Candida* into deeper tissue. A potential correlation exists between the morphological form of the yeasts and the degradative ability; the *C. albicans* yeast form seems to be related to superficial infections, and hyphal forms can apparently invade deeper tissues between the epithelial cells by degradation of E-Cad. Basement membrane degradation is possible, especially in the junctional epithelium, which contains only Lm-332 as a structural component. *Candida glabrata* did not cause E-Cad degradation. Local tissue host inflammatory mediators, such as MMP-9, were activated, and TIMP-1 was degraded by certain *Candida* species, thus indicating the possibility of a weakened host tissue defence mechanism *in vivo*. The inhibition of *Candida* proteinases is a potential field in the development of anticandidal agents. In enzymatic activity studies, we unexpectedly noticed fluorescence produced by *Candida* proteinase interaction with a synthetic substrate. These kinds of fluorigenic substrates of a certain molecule structure seem promising in the development of a rapid *Candida* diagnostic method.

4. INTRODUCTION

The oral mucosa is one of the first barriers against microbial dissemination. To avoid invasion and combat microbes, the structural integrity and normal defence mechanisms of the mucosal epithelial layer are important. Micro-organisms, such as bacteria and yeasts, form a complex biolayer covering the mucosal membranes of the body. Each compartment of the mucosa has a unique local microenvironment with differences in pH, gland secretions and epithelial structure. The balance of the microbial flora in the oral cavity can be disturbed by different factors, such as the use of broad spectrum antibiotics or immunocompromising diseases, and the result can be the overgrowth of yeasts.

The virulence factors of *Candida* yeasts are the focus of many study groups, and the unravelling of the invasion mechanisms is crucial in understanding candidosis. Studies of invasion mechanisms are mostly based on several indirect methods, such as histologically visualizing the loss of components or physical growth of *Candida* in the epithelial layer or interpreting gene expression profiles from infected host tissue material.

Investigating the molecular mechanisms of invasion is nonetheless a daunting task because of the complex structural interactions between the host and *Candida.* By using direct degradation assays in which only one protein is degraded at a time, we could identify and isolate possible individual proteinase-protein interactions and protein modifications that may have a role in the invasion process eventually *in vitro*. The basic question we wanted to answer was whether *Candida* is able to degrade human-derived basement membrane zone (BMZ) proteins, thus making the structure of the epithelial layer more vulnerable to *Candida* invasion. Because most human-derived BMZ and extracellular matrix (ECM) proteins are not commercially available, we isolated two such proteins from a culture medium of oral epithelial cells, one from the cell membranes of oral epithelial cells and one from donor blood.

This thesis focused on the degradation of human oral mucosal proteins by several *Candida* species, particularly those found in the BMZ, namely laminin-332 (Lm-332), laminin-511(Lm-511), fibronectin and E-cadherin (E-Cad). These proteins play an important role in epithelial structural and functional integrity. We compared this degradative ability of the most common *Candida* species.

We also aimed to elucidate the unclear role of hyphae in the invasion capability of yeasts by using hyphal and secreted aspartic proteinase mutants of a known invasive clinical strain of *C. albicans* SC5314. Comparative studies were made with *C. glabrata*, the *Candida* strain that often acquires resistance to the commonly used antifungal group azoles. *C. glabrata* does not form true hyphae, but can still cause infection.

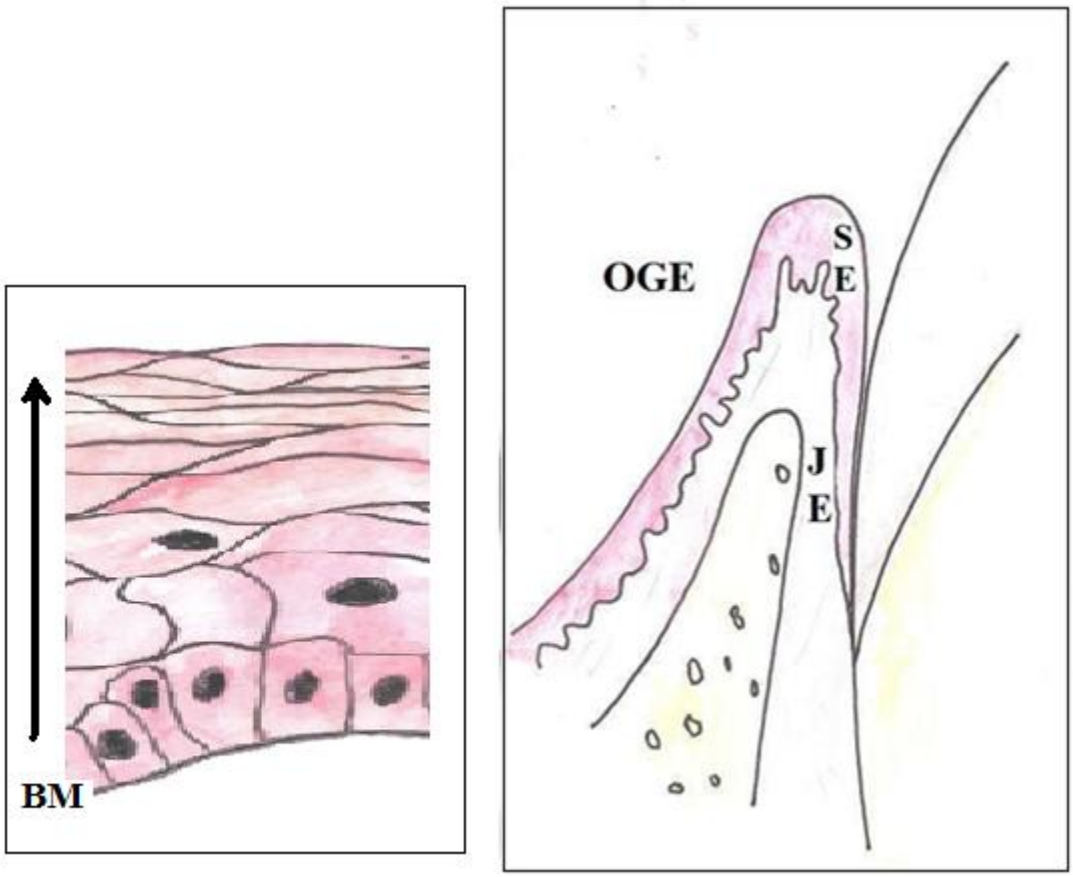
The effect of *Candida* proteinases on host inflammatory molecules, such as matrix metalloproteinase-9 (MMP-9) and tissue inhibitors of metalloproteinase-1 and -2 (TIMP-1 and -2), was evaluated to elucidate the possible impact on host responses. *C. albicans* is known to have a significant role in tissue structure through basement membrane (BM) protein and MMP modulation (Claveau *et al*., 2004).

5. REVIEW OF THE LITERATURE

5.1. Oral mucosa

5.1.1. Anatomy and histology

The oral epithelium is a stratified squamous epithelium comprising the palate, lip, gingival, buccal, tongue and floor of the mouth epithelium. The epithelium of the palate, the dorsum of the tongue and the parts of the gingivae attached to the teeth are keratinized. The keratin layer acts as a mechanical barrier that makes these parts of the epithelium more resistent to wear and injury. The oral gingival epithelium is stratified squamous cell epithelium and can be classified into three distinct entities: 1) keratinized gingival epithelium, 2) non-keratinized sulcular epithelium and 3) junctional epithelium. Epithelial cells shed continuously from the mucoid surface, forming a protective shield against pathogens. The epithelial cells attach to the underlying tissue by hemidesmosomes. Oral stratified squamous epithelium is illustrated in Figure 1A and gingival structure in Figure 1B.



A. **B.**

Figure 1 A. Stratified squamous epithelium; the constant movement of gradually keratinizing epithelial cells moving towards the mucosal surface is indicated with an arrow. BM= basement membrane. **B.** Healthy gingiva. OGE= oral gingival epithelium, SE= sulcular epithelium, JE= junctional epithelium**.**

The gingival tissue around the teeth is called the junctional epithelium (JE) (Bosshardt and Lang, 2005). Laminins -332 (Lm-332, former laminin-5, Lm-5), -311(former laminin-6, Lm-6), -321 (former laminin-7, Lm-7), -511 (former laminin-10, Lm-10) and- 521 (former laminin-11, Lm-11) are glycoproteins, all found in the basement membrane of the oral mucosal epithelium. The gingival epithelium, which has tight junctions, acts as a barrier. JE is more vulnerable, contains no E-Cad and has widened intercellular spaces (Hatakeyama *et al*., 2006). JE cells have fewer tonofilaments and desmosomes than the gingival epithelium (Nanci and Bosshardt, 2006). JE contains only Lm-332 (Oksanen *et al*., 2001). This makes it more vulnerable to breakdown by pathogens. Cells of the defensive system, such as polymorphonuclear leukocytes and T-lymphocytes, move to the gingival pocket through the JE. The gingival connective tissue is composed of collagens, osteonectin, tenascin and elastin. Type I and III collagens are the most abundant and are located immediately under the epithelium, along with the proteoglycans decorin and biglycan. Types IV and VI collagen are found in the BMZ. The periodontal ligament is mainly composed of types I and III collagen and other molecules, including fibromodulin, perlecan, tenascin, fibronectin and vitronectin (Bartold and Narayanan, 2006).

Epithelial junctions

The epithelium has four different adhesive complexes that facilitate adhesion of epithelial cells to each other on lateral sides and to the underlying connective tissue (Meyer *et al*., 1984) (Figure 2).

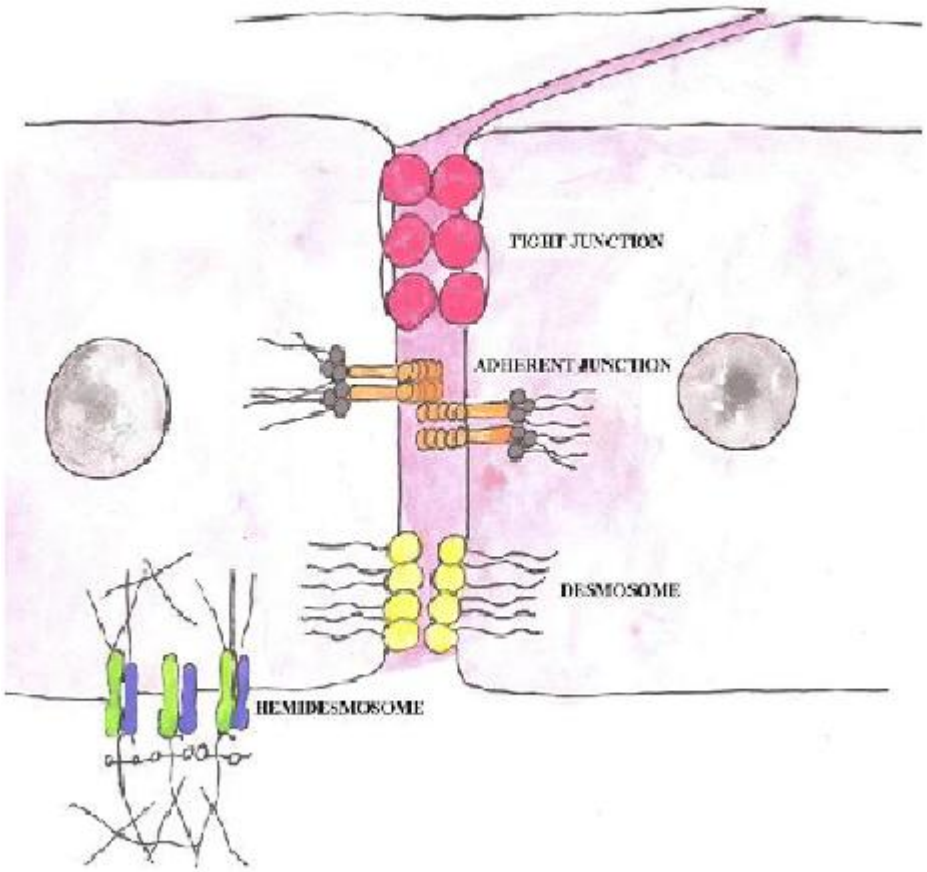


Figure 2. Epithelial adhesive junctions.

Adherens junctions (Figure 3) are Ca^{2+}-sensitive, are located between epithelial cells, are situated more basally than tight junctions and contain such adhesive proteins as E-Cad which binds to β- and γ-(plakoglobin) catenins; these in turn bind to α-catenin. These are anchored to actin filaments (Chitaev and Troyanovsky, 1998).

Hemidesmosomes connect epithelial cells to the basement membrane (Figure 4). Hemidesmosomal α6β4 integrin forms a complex with keratin filament, mediated by plectin and Lm-332, and together they play both a structural and cell signalling role in the epithelium. Other interactive proteins here are cytoplasmic BP230 and transmembrane BP180 (collagen XVII) (Litjens *et al*., 2006). Lm-332 induces hemidesmosome assembly (Jones *et al*., 1998).

Desmosomes, *macula adherentes*, are adhesion plaques attaching two epithelial cells together. The adhesive proteins found in desmosomes are desmogleins 1-3, desmocollin, plakoglobin (γ-catenin) and desmoplakin. Intracellular keratin filaments bind to desmoplakin. The length and homogeneity of the desmosome can be used for diagnostic and prognostic purposes in oral squamous cell carcinoma (Oliveira Crema *et al*., 2005).

Tight junctions, *zonula occludens*, form a barrier connecting two epithelial cells together on the apical side of the epithelial layer. The main proteins present are small transmembrane claudins (Furuse *et al*., 1998), occludins (Furuse *et al*., 1994) and zonulin (Wang *et al*., 2000; Sapone *et al*., 2006). Tight junctions control the movement of fluid and molecules in and out of cells, and, interestingly, the upregulation of zonulin has been speculated to play a pivotal role in development of autoimmune diseases, such as diabetes type 1, by increasing the permeability of the intestine and exposure to environmental antigens (Sapone *et al*., 2006).

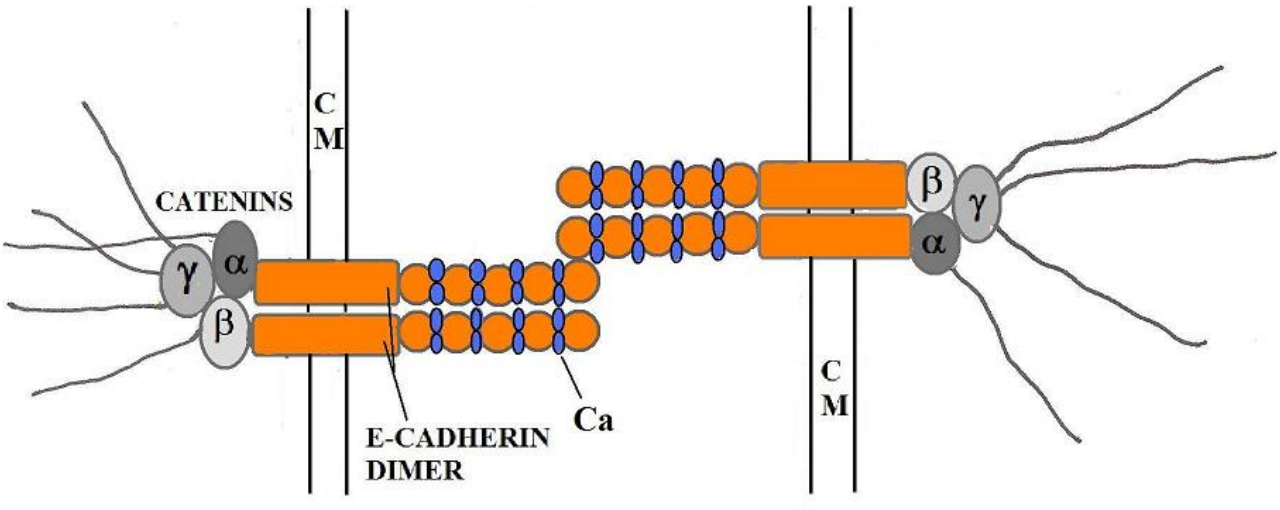


Figure 3. E-cadherin dimer assembly in the adherens junction. CM= cell membrane, Ca= calcium.

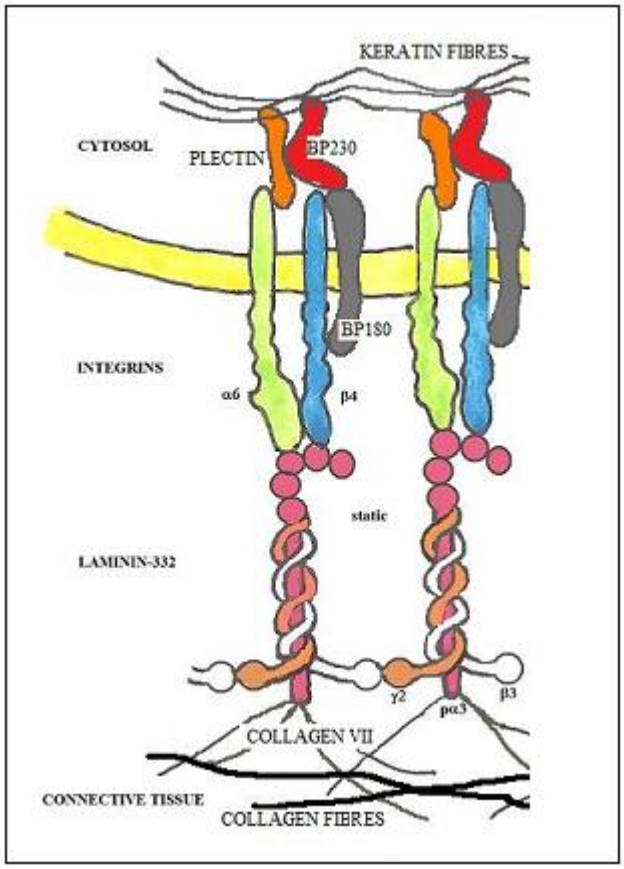


Figure 4. Hemidesmosome structure.

5.1.2. Tissue components

5.1.2.1. Basement membrane

The epithelial cells attach to the underlying tissue by the basement membrane. It is a thin layer of specialized extracellular matrix and can be visualized by electron microscopy. It has classically been considered to be composed of four layers: *lamina lucida*/*lamina rara interna*, *lamina densa*, *lamina lucida*/*lamina rara externa* and *lamina reticularis*. The *lamina rara interna* is an electron-lucid layer, and it contains the glycoproteins laminins, integrins, entactins and dystroglycans. The electron-dense *lamina densa* contains type IV collagen and perlecan (a heparan sulphate). *Lamina rara externa* has a similar composition as the internal lucid layer. The components of the three layers are secreted mainly by epithelial cells. *Lamina reticularis* is secreted by the cells in the connective tissue and contains mainly fibronectin.The

basal lamina (BL) is distinguished from the basement membrane (BM); it is seen only by electron microscopy and lacks the lamina reticularis.

The BMZ is composed of laminins, type IV collagen and other glycoproteins such as nidogen and proteoglycans (Colognato *et al.*, 2000). The BMZ forms a network between epithelial cells and the underlying tissue and has both structural and signalling functions.

Laminins are the most abundant non-collagenous molecules in the BMZ. The first laminin (laminin-1) was discovered by Timpl *et al.* (1979), and since then the family of laminins has been shown to contain at least 15 different laminin isoforms (Colognato and Yurchenko, 2000; Aumailley *et al*., 2005). The role of laminins in the BM has been reviewed by Aumailley and Smyth (1998) and in mammals by Miner (2008). Laminins are heterotrimers composed of α-, β- and γ-chains; each laminin isoform has a distinct chain composition, which affects its function. Five different α-chains, three different β-chains and three different γ-chains have been identified. Laminin chain self-polymerization is crucial for the formation of BMs and naturally occurring networks of laminin depend on isoform constituents (Odenthal *et al*., 2004). The structure of the different laminins can be viewed in the article by Miner (2008).

Integrins α2β1, α3β1, α6β1 and α6β4 are found in the human gingival epithelium. Integrin α6β4 particularly in the junctional epithelium (Hormia *et al*., 1990, 1992, 2001; Thorup *et al*., 1997). Integrins α3β1 and α6β4 are specific for Lm-332 and Lm-511 and integrin α6β1 for Lm-332, Lm-511 and Lm-111 (Nishiuchi *et al*., 2005). Integrin α3β1 is associated with epithelial cell-derived cancer migration (Kreidberg, 2000), and integrin α6β4 has a stabilizing role as an adhesion and signalling molecule in hemidesmosomes attaching to Lm-332 (Borradori and Sonnenberg, 1999).

Extracellular matrix

Extracellular matrix (ECM) is a term for the interstitial matrix between cells in the connective tissue and the basement membrane. The ECM is composed of a network of fibrous collagenous proteins and glycosaminoglycans such as heparan sulphate, chondroitin sulphate, keratan sulphate and the polysaccharide hyaluronic acid. In addition, ECM contains elastin fibres and fibronectin as non-fibrillar proteins. *C. albicans* possesses cell surface integrin-like receptors that bind arginine-glycine-aspartic acid-containing peptides in numerous human proteins (Klotz, 1992). Yan *et al.* (1997) have identified a common haemoglobin-inducible *C. albicans* receptor for laminin, fibronectin and fibrinogen. A cell wall-associated form of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) is able to bind to laminin and fibronectin and may aid *C. albicans* adhesion and invasion (Gozalbo *et al*., 1998). Bouchara *et al.* (1990) have located laminin receptors on *Candida albicans* germ tube tips.

Several studies have shown the ability of different non-*Candida* yeasts (Tronchin et al., 1993; Puccia et al., 1998; Rodrigues *et al*., 2003; Matsuo *et al*., 2005; Moon *et al*., 2006) and *Candida* yeasts (Morschhäuser *et al*., 1997; Rodier *et al*., 1999; dos Santos et al., 2005) to degrade ECM proteins. The interaction of pathogenic fungi with host cells has been reviewed by Mendes-Giannini *et al*. (2005). The structural changes in these proteins may affect epithelial cell motility, signal transduction and cancer metastasis (Patarroyo *et al*., 2002; Katayama and Sekiguchi, 2004; Marinkovich, 2007).

5.1.2.1.1. Laminin- 332

Laminin-322 (formerly known as laminin-5) is found in tissues specialized in protecting and secreting functions (Ghosh and Stack, 2000). It is a glycoprotein of 460 kDa (Tsubota *et al.*, 2000). It is synthesized and secreted in preform, which is proteolytically modified (Miyazaki, 2005). The molecule is composed of α3-, β3- and γ2-chains. The different domains of these chains have specific functions in adhesion to integrins, collagens and other BMZ glycoproteins and also act as part of the signalling cascade between the epithelium and underlying tissue. The localisation of the different laminin chains in the gingival tissue has been described by Pakkala *et al*. (2002).

The proteolytic modification of Lm-332 has been demonstrated to be related to BM dysintegration, altered epithelial cell motility (Pirilä *et al*., 2003; Hintermann and Quaranta, 2004) and epithelial tumour invasion (Katayama *et al*., 2003; Koshikawa *et al*., 2005). Lm-332 γ2-chain levels are elevated in gingival crevicular fluid in chronic periodontitis (Emingil *et al*., 2004). Processing of the human laminin γ2-chain, found in Lm-332, by MT1-MMP may lead to tumour cell scattering and migration (Koshikawa *et al*., 2005). Lm-332 is degraded to c-terminal fragments γ2′ (100kDa), γ2x (85 kDa) and smaller fragments (27 and 27 kDa) from γ2 domain III. The degradation of Lm-332 is outlined in Figure 7.

5.1.2.1.2. Laminin- 511

Laminin- 511 (formerly known as laminin-10) is composed of α5-, β1- and γ1-chains. Lm-10 is found in most tissues, except adult skeletal muscular tissue (Miner *et al.*, 1995). Lm-10 acts in cell adhesion (Tani *et al*., 1999; Ferletta, 2002) and plays an important role in the maturation of most epithelial tissues (Miner 2008). Matrix metalloproteinase-2 (MMP-2; Gu *et al*., 2002) and membrane type 1 metalloproteinase (MT1-MMP; Bair *et al*., 2005) are known to cleave Lm-10 and promote cancer cell migration. It is of interest that Lm-511 is a major neuronal laminin in rat hippocampus, and it is degraded by a tissue plasminogen activator/plasmin protease cascade during excitotoxic injury leading to neuron death (Indyk *et al*., 2003).

5.1.2.1.3. Fibronectin

Fibronectin (Fn) is an adhesive molecule found in most tissues. It is a large glycoprotein and can be found in dimeric (440 kDa) form in plasma, composed of two disulphide-linked monomers (Vartio *et al*., 1987). Plasma Fn is produced by hepatocytes, and it functions in wound healing, blood clotting and phagocytosis (Tamkun and Hynes, 1983). Fn is also produced by epithelial cells and fibroblasts. Fn is in fibrillar form on cell surfaces and in extracellular space. In the periodontal ligament, Fn is found on collagens fibrils (Bartold and Narayanan, 2006). A small amount of escaped cellular Fn can be detected from plasma (Vartio *et al*., 1987).

Studies indicate an integrin-like protein in *C. albicans* (Hostetter, 1999). Beta 1 integrin in *C. tropicalis* has been found to bind Fn (DeMuri and Hostetter, 1996). Haemoglobin-induced Fn binding of *C. albicans* (Yan *et al*., 1998), binding of entactin, Lm and Fn by *C. albicans* (Lopez-Ribot and Chaffin, 1994) and Fn and vitronectin binding by *C. albicans* (Jakab *et al*., 1993) have been detected. Klotz (1994) postulates that binding of *C. albicans* to Fn in blood vessel fibrin-platelet aggregates may help the yeast to avoid normal host phagocytotic removal and take a foothold in the host, similarly as in cancer metastasis.

There is a difference in the binding of soluble vs. immobilized Fn to *C. albicans* surface adhesin. Soluble Fn binds more avidly to *Candida* due to the differentially exposed domain structure (Penn and Klotz, 1994). Klotz and Smith (1995) show inhibition of *C. albicans* adherence to type I collagen, type IV collagen, Fn, and Lm by fragments of gelatin (denatured type I collagen). Threonine-rich repeats in *C. albicans* adhesin Als5p increase the binding to Fn (Rauceo *et al*., 2006). Efg1p mutant of *C. albicans* has downregulated proteome and defective adhesion to ECM components compared with the parental strain (Saville *et al*., 2006).

5.1.2.1.4. E- cadherin

Epithelial cadherin (E-cadherin, E- Cad) is one of the main adhesive proteins that attach epithelial cells to one another in the adherens junction of the epithelial layer in the digestive tract. It is 120 kDa and belongs to the calcium-dependent adhesion molecule superfamily. It is encoded by the CDH1 gene (CD324, cluster of differentiation 324). Two E- Cad molecules bind together in a Ca^{2+} -dependent homophilic way (between identical molecule types; van Roy and Berx, 2008)(Figure 3).

It has been shown by Phan *et al.* (2007) that the *Candida* adhesion protein Als3 has structural similarity to human cadherins and can bind to oral epithelial E-Cad and form a pseudo-homophilic adhesion complex. By this mechanism, *Candida* is endocytosed and E-Cad eventually degraded by fungal and host proteinases. Als3 belongs to the Als adhesive protein family. It binds to neural cadherin (N-cadherin, N-Cad) located in the endothelium or E-Cad in the oral epithelium.

Interestingly, the periodontal pathogen *Porphyromonas gingvalis* has been found to degrade E-Cad *in vitro* (Katz *et al*., 2000, 2002). The loss of E-Cad function or expression leads to decreased cellular attachment, increased cellular motility and even potential cancer invasion into surrounding tissues. McNulty *et al*. (2005) have noted a decrease in motility of CD8 T-cells and reduced E-Cad in oropharyngeal candidosis. Increased human keratinocyte cell motility and loss of E-Cad by *C. albicans* have been observed by Rollenhagen *et al.* (2009) in an *in vitro* study.

The ability of *C. albicans* Sap 5 to degrade E-Cad has been observed (Villar *et al*., 2007). In intestinal adenocarcinoma monolayer Caco2 cells, an extracellular 89-kDa fragment of E-Cad was detected after incubation with the hyphal form of *C. albicans* (Frank and Hostetter, 2007).

5.1.2.2. Matrix metalloproteinases

The matrix metalloproteinase family consists of at least 24 proteinases. They are subdivided depending on their substrate specificity and molecular structure into collagenases, gelatinases, stromelysins, membrane-type matrix metalloproteinases and others (Uitto *et al*., 2003). In cells, they are synthesized in an inactive form and secreted and activated by specific mediators in tissue. MMP-1, -2, -3, -8, -9 and -13 have been identified in the inflamed periodontal tissue (Sorsa *et al*., 2004, 2006).

Matrix metalloproteinase function is regulated by growth factors, cytokines such as interleukin-1 (IL-1), tumour growth factor beta (TGF-β), serum inhibitor alpha macroglobulin and tissue inhibitors of metalloproteinases (Bartold and Narayanan, 2003). The binding of

Candida albicans to rabbit corneal epithelial membrane has been studied by Dong *et al.* (2005). Destruction of the membrane was caused by hyphal forms of *Candida albicans* and the inflammation correlated with an MMP-9 level. MMP-9 has been found to bind to ECM components such as mouse Lm-1, Lm-5 (from 804G cells), collagens types I and IV and serum fibronectin (Mäkelä *et al*., 1998). Several MMPs can modify Lm-332 structure and enhance epithelial cell motility (Pirilä *et al*., 2003). MT1-MMP causes cleavage of Lm-332 and has been suggested to have a role in cancer invasion (Koshikawa *et al*., 2000).

5.1.2.3. Tissue inhibitors of metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) are the main inhibitors of matrix metalloproteinase function. TIMP-1, -2, -3 and -4 are expressed in vertebrates (Brew *et al.*, 2000).

5.2. Oral microbiota

5.2.1. Oral biofilms

There are hundreds of microbial species on the oral mucosal epithelial surface. The majority of the species are still unidentified. Continuously competing with each other for nutrients and space, the microbes form biofilms on the epithelium. The biofilm is composed of yeasts and bacteria.

The most commonly found bacteria are *Aggregatibacter* (*A. actinomycetemcomitans*), *Lactobacilli*, *Streptococcus mutans* (*SM*), *Porphyromonas gingivalis* (*P. gingivalis*), *Prevotella intermedia* (*P. intermedia*) and *Fusobacterium nucleatum* (*F. nucleatum*) (Huovinen 2003). Bacteria use quorum sensing in biofilms: responses to environmental changes in local microniches are transferred from bacteria to bacteria by exchanging genetic material. Antibiotic resistance is also transferred in this way. Yeasts are known to use quorum sensing (Ramage *et al*., 2009).

Biofilms occupy all surfaces in the oral cavity and the growth of microbes on intravenous catheters are one major pathway of microbes leading to systemic infection. The search for materials that prevent biofilm formation is ongoing. Biofilms are found also on foreign objects in the oral cavity such as dental prostheses.

Each individual has a unique microbial balance, called the normal flora. Disturbance of this normal flora by use of broad-spectrum antibiotics or immune-compromising diseases may cause overgrowth of *Candida* yeasts, which normally live as commensals on the mucosal membrane without causing disease. Biofilm formation of *Candida* species has been reviewed by Ramage *et al*. (2009). Comparison of *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. pseudotropicalis* strains revealed that the species that formed the most biofilm was *C. albicans.* Less biofilm formation was seen by the other species, except *C. krusei*, which had an intermediate ability to form biofilm. The *C. parapsilosis* biofilm formation was strain dependant. Biofilm formation depended also on the growth medium glucose/galactose concentration. At least *C. parapsilosis* has been shown to have phenotypic switching, which affects growth speed and biofilm formation (Laffey and Butler, 2005).

5.2.2. *Candida* species

Candida species are prevalent in the oral cavity, in particular among elderly patients with reduced salivary flow and/or dental prostheses and in immunosuppressed patients. The most frequently encountered species is *Candida albicans*. Non-*Candida albicans Candida* (NCAC) strains, however, are isolated in increasing numbers in medically compromised patients. These strains may cause systemic infections and are often resistant to commonly used antifungal agents such as fluconazole. *Candida* species may be capable of metabolizing ethanol to carcinogenic acetaldehyde and can thus induce progression of oral and upper gastrointestinal tract cancer (Uittamo *et al*., 2008). Consequently, more focus should be placed on the diagnosis and treatment of oral *Candida* infections, also on *Candida* species other than *C. albicans*.

C. albicans is the most common species (over 50% of the cases) isolated from the oral cavity of both healthy and diseased individuals. Other species responsible for oral infections have also been identified from the bloodstream in infections (Pfaller *et al*., 1998).These include *C. parapsilosis* (15%), *C. glabrata* (15%), *C. guilliermondii* (6%), *C. tropicalis* (2%) and *C. krusei* (1%). *C. dubliniensis* is often found in immunocompromised patients such as the elderly and HIV patients (Sullivan *et al*., 1997).

Oral candidosis is often detected in the elderly, in patients wearing dentures, in patients positive for the human immunodeficiency virus (HIV), and in acquired immunodeficiency syndrome (AIDS) patients.
All of the *Candida* species cause the same kind of mucositis, but there are significant differences exist in invasiveness and antifungal susceptibility. Oral yeast carriage does not mean infection. For infection, mucositis with clinical symptoms must be present.

Clinical forms of oral candidosis include erythematous candidosis, pseudomembranous candidosis, median rhomboid glossitis, angular cheilitis and candidal leukoplakia. The main local predisposing factors are decreased saliva flow, smoking, mucosal lesions and decreased blood circulation in the mucosa due to, for instance, radiation therapy. Examples of systemic predisposing factors are diabetes mellitus, acquired or inborn immunodeficiency, oral cancer (Menzin *et al*., 2009), malignancies (Laine *et al*., 1993) and malnutrition (Leslie *et al*., 2005).

The ability of *Candida* to adhere to the mucosa and dentures plays an important role in the pathogenesis of oral yeast infections. Adherence is achieved by specific and non-specific mechanisms, as discussed in the previous section. However, the mechanisms remain incompletely understood.

Infections caused by NCAC produce 35-65% of all *Candida* infections (Krcmery and Barnes, 2002). *Candida* species cause 4-9% of bloodstream infections, and multisite colonization among immune-deficient patients confers a high risk for acquiring a systemic infection (Blot *et al*., 2008).

Mortality due to bloodstream infections related to NCAC species is similar to *C. albicans*: 15-35%. *C. parapsilosis* produces the lowest mortality, and *C. tropicalis* and *C. glabrata* cause the highest mortality: 40-70% (Krcmery and Barnes, 2002). Patients with prostheses have a higher carriage percentage of *Candida*, and they tend to have multiple species (Wang *et al*., 2006).

Polymicrobial candidemia has indeed been recorded by several study groups. It is defined as the detection of two or more *Candida* species simultaneously from a patient (Pulimood *et al*., 2002). The main species detected was *C. albicans*, and the other common species were *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei* and *C. kefyr.*

The following *Candida* species described in more detail below are included in the studies of this thesis work. They are the most commonly found species in the human oral cavity.

5.2.2.1. *Candida albicans*

C. albicans is the most commonly (72.1%) found *Candida* yeast in the oral cavity. It exists mainly in two morphologic forms: budding yeast (blastospore) and hyphal form. *Candida* also show phenotypic switching, such as white/opaque change and several colony morphology variants (star, stipple, *etc*.), in response to environmental signals (Slutsky *et al*., 1985, 1987).

5.2.2.2. Candida dubliniensis

C. dubliniensis was long considered the same species as *C. albicans*, but analysis of the genome revealed *C. dubliniensis* to be a separate species. It is the second most common *Candida* found in the oral cavity (Sullivan *et al*., 1995). *C. dubliniensis* is particularly often detected in HIV-infected patients (Sullivan *et al*., 1997).

C. dubliniensis is also able to exist in both blastospore and hyphal forms. It adheres more avidly to human buccal epithelial cells than *C. albicans* when grown in glucose. *C. dubliniensis* is sensitive to azoles and amphotericin B. Seven Sap homologues have been found from *C. dubliniensis* by Southern blot analysis (Gilfillan *et al*., 1998).

5.2.2.3. *Candida parapsilosis*

C. parapsilosis is found as a commensal, but it often causes systemic infections. *C. parapsilosis* infection is related to foreign body insertion, neonates and hyperalimentation (Krcmery and Barnes, 2002). High frequency of colonization with *C. parapsilosis* with reduced susceptibility to fluconazole has been observed on the hands of healthy hosts (hospital workers and non-hospital workers); this may be an infection risk in immunocompromised patients (Bonassoli *et al*., 2005). In a retrospective cohort study of 1995-2003 by Pasqualotto *et al.* (2006), *C. parapsilosis* caused 30% of the NCAC infections. It does not form true hyphae, but is able to form pseudohyphae (Laffey and Butler, 2005).

5.2.2.4. *Candida glabrata*

C. glabrata has recently been found to cause more *Candida* infections than earlier (Li *et al*. 2006). *C. glabrata* is related to *Saccharomyces cerevisiae* (Barnes *et al*., 1991) and is haploid (Doi *et al*., 1992), as opposed to *C. albicans*, which is diploid (Soll, 2000). Lachke *et al.* (2002) noticed switches into four different phenotypes by *C. glabrata* on $CuSO_4$-containing agar. They also observed pseudohyphae and tube structure formation, but no true hyphae. *C. glabrata* and *C. parapsilosis* have been proposed to be the second most common *albicans* species to cause infection in man (Nguyen *et al*., 1996). Azole resistance has been found more frequently in *C. glabrata* infections (35% resistance), and predisposing factors to infection by this species are azole prophylaxis, surgery and catheters. *C. glabrata* causes 5-40% of NCAC infections (Krcmery and Barnes, 2002). The roles of phenotypic switching and pseudohyphal

formation in pathogenesis of *C. glabrata* have not yet been clarified (Li *et al*., 2007 b). For a review of *C. glabrata* and its interaction with the host, see Kaur *et al*. (2005).

5.2.2.5. *Candida krusei*

C. krusei causes 10-35% of NCAC infections. The risk factors for infection are azole prophylaxis, neutropenia and bone marrow transplantation. Of *C. krusei* strains 75% are resistant to fluconazole and 10-15% to amphotericin B (Krcmery and Barnes, 2002). *C. krusei* is able to form hyphae.

5.2.2.6. *Candida tropicalis*

C. tropicalis is found in 10-30% of NCAC infections, and it is also detected in patients with neutropenia and bone marrow transplantation. It is the second most pathogenic *Candida* species (Zaugg *et al*., 2001). Of *C. tropicalis* strains 10-25% are resistant to fluconazole (Krcmery and Barnes, 2002). *C. tropicalis* forms true hyphae.

5.2.2.7. Virulence factors

Candida uses several virulence factors in the infection process. Adhesion molecules (Yang, 2003) are the first molecules that interact with the epithelial surface and attach yeast to the epithelium. *Candida* has several cell surface-bound and secreted enzymes, such as phospholipase (Calderone *et al*., 2001; Yang, 2003) and proteinases (Monod *et al*., 2002; Hube *et al*., 1994; Hube and Naglik, 2001; Schaller *et al*., 2005), that degrade the epithelium. Other virulence factors of *Candida* are haemolytic factor, blastospore/hyphal transition (Gow *et al*., 2002) and phenotypic switching. Phenotypic switching may weaken azole susceptibility (Cetinkaya and Kiraz, 2005). *C. albicans* white phenotype is associated with disseminated candidosis, and this phenotype is less susceptible to host defence than the mating opaque phenotype (Pendrak *et al*., 2004). Altered signal transduction and cytoskeletal organization in host-fungus interaction can be used by yeasts in aiding their virulence in the host (Mendes-Giannini *et al*. 2005). The pH of the infection site regulates the expression of genes in *Candida* essential for survival (de Bernardis *et al*., 1998). Calcineurin is one of the virulence factors crucial for *Candida* survival. It responds to stress such as temperature, pH and cations (Blankenship *et al*., 2003).

5.2.2.7.1. *Candida* proteinases

Candida proteinases are classified depending on the catalytic mechanism they use to cleave peptide bonds as metalloproteinases, serine proteinases and secreted aspartic proteinases (Saps). These proteinases can be bound to the yeast cell wall or to be extracellularly secreted proteinases.

Cell surface-associated proteinases

Sap 9 and Sap10 are secreted aspartic proteinases identified on the cell surface of *C. albicans* (Albrecht *et al*., 2006). Structurally, Sap 9 and Sap10 are anchored by glycosylphosphatidylinositol (GPI) and are located in the cell membrane or the cell wall. Sap 9 and Sap10 seem to be independent of environmental conditions and are constitutionally expressed (Schaller *et al*., 2005).

Secreted aspartic proteinases

Several studies have been conducted on Saps (Schaller *et al*., 1999 a; 1999 b; Hube *et al*. 1994; 1998; Hube and Naglik, 2001; Naglik *et al*., 2008). Saps are important in the invasion process. For *C. albicans*, eight Saps are secreted into the extracellular space (Saps 1-8).

Seven Sap homologues have been found in *C. dubliniensis* (Gilfillan *et al*., 1998). Four Saps have been identified in *C. tropicalis* (Zaugg *et al*., 2001). A Sap multigene family has been observed in *C. guilliermondii* (Monod *et al*., 2002) and one cysteine proteinase and one metalloproteinase in *C. glabrata*. Rüchel *et al.* (1986) identified a 33 kDa secretory proteinase, which was inhibited by pepstatin A. Weig *et al.* (2004) found nine aspartic proteases in *C. glabrata* that have similarity with the aspartic proteinases of *S. cerevisiae*. Three of these seem to be GPI-anchored to the cell wall.

C. albicans Sap 1-3 activity levels depend on the pH of the growth medium (White and Agabian, 1995). The different proteinases have specific optimal activity pH values. Saps 1-3 have an optimal activity at pH 2-5, Saps 4-6 at pH 3-7, as seen in Figure 5 (Hube and Naglik, 2001). Sap isoenzymes may act in acidic microniches (Schaller *et al*., 2001).

A triple deletion of Saps 1-3 causes attenuated virulence (Hube *et al*., 1997). A triple deletion of Sap 4-6 genes has also been noted to cause attenuated virulence in animals (Sanglard *et al*., 1997). Sap 2 is able to degrade collagen, Lm, Fn, α-macroglobulin and all immunoglobulins (Schaller *et al*., 2005).

Saps 1-3 are associated with superficial infection with the blastospore form of *Candida*. Saps 4-6 are found in later infection stages and are related to hyphal forms. Sap 2 may be needed for invasion through the endothelial cell layer barrier, and the SAP2 gene has obsereved to be the most important one expressed in invasive candidosis studied by the reconstituted human epithelial (RHE) model *in vitro* (Lermann and Morschhäuser, 2008; Naglik *et al*., 2008). The action of aspartic proteinases has been investigated by several groups: Saps 4-6 action in murine macrophages (Zepelin *et al*., 1998), Sap 1-3 null mutants in an *in vitro* study by Hube *et al*. (1997), and triple mutant Saps 4-6 *in vitro* (Sanglard *et al*., 1997), revealing the role of these proteinases in the invasion process.

The effect of transcription factor mutants on dissemination has been evaluated *in vivo* with mice and *C. albicans* (Felk *et al*., 2002). The study revealed that hyphal-deficient mutants lacking transcription factor Efg1were unable to invade parenchymal organs such as the liver or pancreas. The result also emphasizes the importance of Sap 4-6 in invasion capability.

C. albicans has been shown by SEM to invade rabbit tongue mucosal explants (RTME) and reconstituted human oral epithelium (RHOE) intercellularly by thigmotropism (Jayatilake *et al*., 2008). The NCAC strains cause intercellular oedema. All of these effects are thought to be caused by enzymes such as Saps and phospholipases.

However, differences still exist between studies on the importance or activity of the different Sap enzymes, which may be due to minor variations in the study protocols or *Candida* strains used (Lermann and Morschhäuser, 2008). Salivary pH affects proteinase activity (Germaine and Tellefson, 1981) and areas of low pH, such as under dental prostheses, may thus enhance proteolytic activity. Stress proteins have higher expression levels at lower pH in *C. glabrata* as opposed to *C. albicans* (Schmidt *et al*., 2008).

5.2.2.7.2. Blastospore/hyphal transition

Blastospore and hyphal forms of *Candida* secrete specific proteinases; *e.g.* genes encoding secreted aspartic proteinases 1-3 (Saps 1-3) are expressed in both yeast and hyphal forms and Saps 4-6 principally in hyphal forms (Schaller *et al*., 2005). Environmental factors, such as temperature and pH, affect the morphology of *Candida*; high pH (over 6.5) and temperature (37°C) favour hyphal formation (Buffo *et al*., 1984). Contact with blood serum also induces hyphal formation. Hyphal forms are more frequently found in chronic infections. Not all *Candida* species form true hyphae despite of their ability to cause systemic infections. This is a subject of major controversy.

Microenvironments in tissue have been suggested to evoke distinct signalling pathways in *C. albicans*, leading to adherence, release of secreted hydrolytic enzymes, hyphal formation and eventually thigmotropic invasion. Regulatory networks controlling *C. albicans* morphogenesis have been described, showing signal pathways, responses to stimuli and the effect of transcription factors Efg1 and Cph1 on hyphal growth (Brown and Gow, 1999).

It is still unclear whether it is the blastospore or hyphal form that is crucial in the invasion process through the epithelium. Non-filamentous *C. albicans* mutants are non-virulent (Lo, 1997). It has been argued that for example, *C. albicans* is able to exist in more than the two morphological forms, and evidence has been shown of the existence of also pseudohyphal and chlamydospore forms; thus *C. albicans* could be called pleomorphic rather than dimorphic. Spore outfits (appareil sporifers) on very long hyphae (verticillia) have been detected by SEM, and these may have some unknown function (Jayatilake *et al*., 2008).

Hyphal formation-inducing transcription factors, such as Efg1p (Stoldt *et al*., 1997), Cph1p (Lo, 1997) and Czf1p (Brown and Gow, 1999), can be activated by different environmental stimuli. The schematic presentation of pathways leading to hyphal development has been described by Saville *et al.* (2003). *CPH1* gene deletion mutant of *C. albicans* has shown suppressed hyphal formation (Liu *et al*., 1994). The pioneer study of constructing isogenic *C. albicans* strains was conducted by Fonzi and Irwin (1993). The *EFG1* gene-associated pathway is a parallel, but independent pathway to *CPH1* (Calderone and Fonzi, 2000).

Efg1 regulator acts downstream of the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway. Cph1 acts in the mitogen-activated kinase (MAPK) pathway (Lane *et al*., 2001). The *efg1* and double mutants *efg1/cph1* do not form hyphae (Korting *et al*., 2003).

Hyphal elements have been found in invasive or chronic candidosis. As all *Candida* species are not able to form true hyphae, the precise function of the hyphal elements remains unclear. *Candida* adapts to environmental stimuli, such as elevated pH or temperature, by forming hyphal structures. Germ tube production has been shown to be glucose-dependent (Vidotto *et al*., 1996). Hyphal-associated proteinases are suitable to act more efficiently in these altered environmental circumstances, and this is crucial to *Candida* survival. The ability to change morphology is thus a powerful virulence factor.

5.3. Mucosal infection

5.3.1. Aetiology

Candida yeasts are in most humans a part of the 10^{14} population of the normal flora of micro-organisms in the gastro-intestinal tract. Approximately 70% of the population has *Candida* in their mucosal flora, mainly as commensals without causing disease. Superficial candidosis can be acute or chronic and atrophic or pseudomembraneous. If the immune defenses are compromised, the initially superficial candidosis can lead to invasive disease.

Candida is the fourth most common cause of infection in hospital patients, but this group has the highest mortality rate (Mavor *et al*., 2005). Keeping in mind the rapidly increasing number of immunocompromised patients (diabetes, neonates, cancer patients, *etc*.) and other predisposing factors, such as side effects of medications (corticosteroids, broad-spectrum antibiotics, antihypertensive drugs, antidepressants), the number of oral candidosis cases is becoming a growing problem. These predisposing factors cause initially superficial candidosis to turn into chronic mucocutaneous candidosis, and the tendency to invade the epithelium increases. Also numbers of *Candida* strains resistant to commonly administered azoles increasing produces pressures to understand the candidosis infection process.

Initial stages of the defence mechanisms

Mucosal infection begins from the early attachment of *Candida* cells to the epithelial cell surface by adhesive proteins to progressive invasion deeper into the epithelial layer if the defence mechanisms of the host fail to eliminate the invader. The mucosal membrane has several defence mechanisms against yeast dissemination: epithelial renewal, mucus secretion with antimicrobial molecules such as lysozyme, lactoferrin, α- and β-defensins, acid pH and the immune system. Hyphal forms of *C. albicans* inhibit β-defensin expression and weaken the innate immune cell chemoattractant role of these antimicrobial peptides (Lu *et al*., 2006).

The mucosal immune system (mucosa-associated lymphoid tissue, MALT) is the first barrier against pathogen invasion. The MALT system differs from the other three immune compartments in the human body (peripheral lymph nodes, peritoneum, skin) in that there are also γ:δ T-cell receptors with different antigen-recognition properties than conventional α:β T-cell receptors. γ:δ T-cells may be classified as an interface between innate and adaptive immunity, responding for instance to cellular stress and injury, promoting repair and recovery of the damaged mucosa. Secretory IgA (sIgA) is the main antibody produced by the mucosa (Janeway *et al*., 2001).

5.3.2. Inflammatory mediators

5.3.2.1. Inflammatory cells

The immune system can be divided into innate and adaptive components. Macrophages and polymorphonuclear leukocytes (PMNs) are cells of the innate immune system responsible for complement-activated pathogen phagocytosis. Macrophages secrete cytokines upon contact with pathogens. The cytokines and chemokines then activate T-cell production and migration to the infection site. Dendritic cells of the adaptive immune system ingest pathogens and present antigens of the pathogens to T-lymphocytes. Cytotoxic T-cells express co-receptor CD8 and bind to major histocompatibility complex (MHC) class I molecules, which then bind

to viral-derived fragments. CD8 T-cells were shown to play a role in oropharyngeal candidosis in a study of HIV patients (McNulty *et al*., 2005). MHC class II molecules present fragments from phagocytosis of bacteria, which are recognized by CD4Th1 cells (helper T-cells). B-lymphocytes are activated by peptides attached to MHC class II molecules, and they start to secrete antibodies.

The immune response varies depending on the morphology of *C. albicans*; hyphal forms activate an early immune response by neutrophils (Wozniak *et al*., 2008). The lymphocyte surface structure Mac-1(CD11b/CD18) is responsible for adhesion of lymphocytes to hyphal forms of *C. albicans* (Forsyth and Mathews, 2002).

As discussed earlier, *Candida* can modify host immunological responses by degrading components of the defence system. Many proteins of the immune system, such as leukocyte enzymes, cathepsin, immunoglobulins, sIgA, complement factors and alpha-macroglobulin, are degraded by Sap 2 (Kaminishi *et al*., 1990; Kaminishi *et al*., 1994, 1995; Naglik *et al*., 2004).

5.3.2.2. Cytokines

Interleukins are a group of inflammatory mediator molecule secreted by macrophages upon microbe contact. They have distinct local and systemic functions in immune response, *e.g.* immune cell recruitment and vascular effects.

Interleukin-12 (IL-12) and tumour necrosis factor alpha (TNF-α) have been found to be important mediators of the inflammatory response to *C. albicans* infection. The innate immune response is responsible for clearance of *Candida,* and the adaptive immune system may limit tissue damage (Ashman *et al*., 2004). Mavor *et al*. (2005) have described host cell cytokine responses in blood vessels upon fungal contact.

Candida hyphal elements have been shown to inhibit the expression of β-defensins (Lu *et al*., 2006). After adhering to the epithelium with special surface proteins, the host is able to recognize fungal 1,3-beta-D-glucan with epithelial Dectin-1, which is a C-lectin-type receptor. This triggers the host innate immune response and activates transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells) through cooperation with toll-like receptor-2 (TLR2) -mediated signalling (Yoshiyuki, 2007). *C. albicans* is also able to induce a TLR4-mediated PMN antifungal defence mechanism (Weindl *et al*., 2007). *C. glabrata* triggers a greater granulocyte macrophage colony-stimulating factor response than *C. albicans,* and insignificant IL-8 or IL-lα secretion (Li *et al*., 2007 b). *C. albicans* SC5314 has been shown to induce IL-1α and interleukin-1 receptor antagonist (IL-1Ra) expression (Jayatilake *et al*., 2007).

5.4. Experimental model systems in oral yeast infection studies

Several different models have been used to investigate the *Candida* invasion process. *In vivo* studies with mice are controversial on ethical grounds, and alternative models have been constructed. Investigations of *Candida* invasion were started with *in vitro* studies by using bovine aortic endothelial cell monolayers on porous support material (Zink *et al*., 1996) and transmigration studies through reconstituted basement membrane (Crowe *et al*., 2003). The *ex vivo* endothelial model of invasion uses the whole liver organ, and is functional up to 12 h and mimics most closely the *in vivo* invasion process (Thewes *et al*., 2007).

Candida dissemination has been studied *in vivo* in mouse models (Rüchel *et al*., 1986; Fallon *et al*., 1997; Chen *et al*., 2006; Yang *et al*., 2009). *In vitro* studies have revealed degradation of endothelial cells of the extracellular matrix and possible penetration of *C. albicans* into circulation and deep organs (Morshhäuser *et al*., 1997). The *Candida* infection mechanism has been studied *in vivo* by injecting *Candida* intravenously into mice (Hube *et al*., 1997; Sanglard *et al*., 1997). The epidemiology, infection process and virulence attributes of systemic fungal infections caused by *Candida* species have been reviewed by Mavor *et al*.(2005), who concluded that more studies are warranted to elucidate the exact virulence factors enabling *Candida* to cause infections.

Epithelial models have been introduced to investigate the invasion process of *Candida* through the epithelium. Different epithelial cell lines have been used in the constructs depending on the model. An *in vitro* model using ventral tongue carcinoma cells grown on polystyrene as monolayers revealed mucosal E-cadherin degradation by *C. albicans* (Villar *et al*., 2007). Reconstituted human skin epidermis cells (Dieterich *et al*., 2002) and intestinal carcinoma cells (CaCO2- cells) (Frank and Hostetter, 2007) have been grown on a biomatrix of collagen I to form multilayers resembling epithelium (Dieterich *et al*., 2002).

A more recent reconstituted human epithelial (RHE) model uses commercially produced oral keratinocytes (squamous cell carcinoma cell line TR146 of the buccal mucosa or human normal gingival keratinocytes) grown on a polycarbonate filter to form a stratified epithelial multilayer in five days (Schaller *et al*., 2006). Another 3D model uses immortalized human oral keratinocytes to form a multilayer, and this model has an additional submucosal component, thus better resembling the normal mucosa and submucosa (Donagari-Bagtzoglou and Kashleva, 2006). RHE models have been used to quantitatively evaluate yeast/hyphal invasion into the epithelium (Jayatilake *et al*., 2006). One limitation of the model is the absence of host immune response elements, but rabbit tongue mucosal explants have been successfully used to circumvent this problem (Jayatilake *et al*., 2008). This model consists of glossal epithelium harvested from rabbits by biopsy and grown on a polycarbonate membrane. This study setting showed the defending capacity of the subepithelial layer seen as less deep penetration of *Candida* hyphae into the epithelium compared with the RHE model, which lacks the submucosal components. More recently, the addition of cells of the immune system (lymphocytes, polymorphonuclear leukocytes, mast cells and dendritic cells) into the RHE model has become possible (Schaller *et al*., 2008).

In addition, many other methods have been used to study and visualize the *Candida* infection process:
Histological sections of infected artificial tissue can be used to detect tissue damage (Schaller *et al*., 1999b) or localization of enzymatic activity (Korting *et al*., 2003; Albrecht *et al*., 2006). **Confocal laser scanning microscopy** (Malic *et al*., 2007), **transmission electron microscopy** and **scanning electron microscopy** have been used to visualize the contact situation of yeasts to epithelial cells (Schaller *et al*., 1999). ***In vivo*** messenger ribonucleic acid (mRNA) extraction and reverse-transcriptase polymerase chain reaction (RT-PCR) enable **genome-wide transcription screening,** in fact the *ex vivo* method gives better results because of more tissue volume and also shows the genes associated with the process. *In vivo* transcription profiling of *C. albicans* has revealed genes essential for interepithelial hyphal dissemination (Zakikhany *et al*., 2007). *In vitro* studies have uncovered the importance of Sap 4-6 in invasion using **confocal laser scanning microscopy** (Malic *et al*., 2007). An *in vitro* study including **immunoelectron microscopy** has revealed localization of Sap 1-3 and Sap 4-

6 and their relation to hyphal, or yeast-formed cells in acidic microniches (Schaller *et al*., 1999).

Proposed mechanisms of invasion

The molecular mechanism by which *Candida* invades the mucosa is still fairly unknown despite the dramatic increase in *Candida* infections. *Candidosis* is most often commensal without causing infection, but can become a persistant chronic superficial mucosal infection. Predisposing factors may further favour systemic spreading and lead to *Candida* abscesses in the inner organs. Invasion takes place in hospitalized patients mainly by trauma caused by catheters. Invasion may also occur by natural routes through the epithelial and endothelial layers, but little is known of these routes.

Many studies have been conducted using a simple model with vascular endothelial cells. The endothelium is a monolayer of cells and is thus different from the mucosal epithelium, which is a multilayer of cells. Despite this anatomical difference, the same invasion mechanisms may be partially applicable to multilayered systems such as the oral epithelium. Frank and Hostetter (2007) demonstrated that the invasion of *C. albicans* occurs between endothelial cells.

Endocytosis

C. albicans is unique among *Candida* species in that it induces endocytosis of the yeast by endothelial cells. Neither *C. glabrata* nor *C. tropicalis* induce endocytosis by endothelial cells (Filler *et al*., 1995). This route may be possible also in the epithelium, combined endocytosis of *Candida* and the action of *Candida* proteinases making a pathway deeper into the epithelium by degrading structural proteins. Hyphal tips are predicted to exert a force from ten to a few hundred μN, acting like a drill pushing through a point of weakest resistance such as degraded structural protein areas. This could lead to invasion of the yeasts sideways and downwards despite the epithelium being in a constant renewal process upwards to the surface opposing the invasion of microbes.

Transmigration between cells

Grubb *et al.* (2008) propose five different mechanisms in the endothelial transmigration by *C. albicans*: 1) endocytosis, 2) endothelial cell destruction by hyphae, 3) translocation between endothelial cells by cyclic switching of adhesion proteins, 4) phagocytosis and trans-endothelial carriage by leukocytes, 5) transmigration through fenestrations in the endothelium.

Filler and Sheppard (2006) proposed two possible mechanisms for *Candida* invasion of human epithelial cells: 1) lytic aspartic proteinases degrading the cell surface, thus providing a route inwards and 2) endocytosis of yeast by epithelial cells. By comparison, the invasion through the endothelium (blood vessels) could occur by 1) endocytosis of yeasts by endothelial cells 2) endocytosis by leukocytes or 3) yeast invasion between endothelial cells.

6. HYPOTHESIS AND AIMS OF THE STUDY

The invasion process of *Candida* yeasts through the oral mucosa has been the focus of many studies, but the precise mechanisms involved remain obscure. Immunohistochemical studies have shown that *Candida* invade tissue and that invasion is more likely to occur after prolonged infection of the mucosa. In chronic candidosis, most *Candida* yeasts form hyphal structures believed to be elementary structures in the invasion. On the other hand, there are *Candida* species that do not form hyphae and they are nevertheless able to cause invasive candidosis. Most studies focus on evaluating the invasion indirectly by visualization. Only a few studies have revealed the mechanisms on a molecular level.

The present series of studies were aimed at clarifying the invasion process on a molecular level with direct proteolytic assays using human-derived oral tissue components. The hypothesis was that *Candida* possesses proteinases capable of degrading oral mucosal components, thus enabling invasion into deeper structures of the oral epithelium. The main questions to be answered were as follows:

1. Is *Candida* able to degrade mucosal epithelial proteins?
2. Do differences exist between *Candida* species in this ability?
3. Are hyphal forms of *Candida* essential in the degradation process?
4. Does *Candida* invade between epithelial cells?
5. Can *Candida* affect the local inflammatory response by degrading host proteinases/ inhibitors?

Based on the different invasion models, hypothetical routes of *Candida* invasion through the oral epithelium are proposed and depicted in Figures 5, 6 and 7.

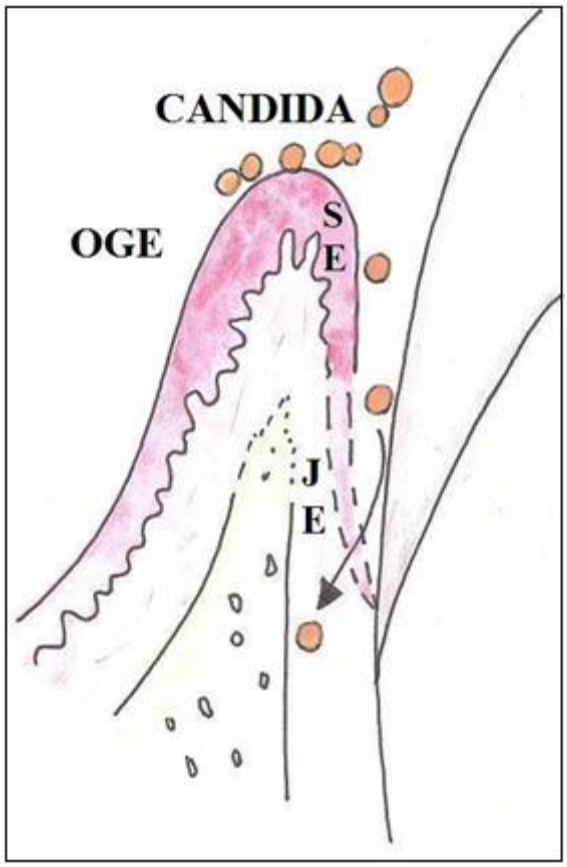


Figure 5. Inflamed gingiva and proposed hypothetical route for *Candida* invasion through degraded junctional epithelium (seen as broken lines). OGE= oral gingival epithelium, SE= sulcular epithelium, JE= junctional epithelium.

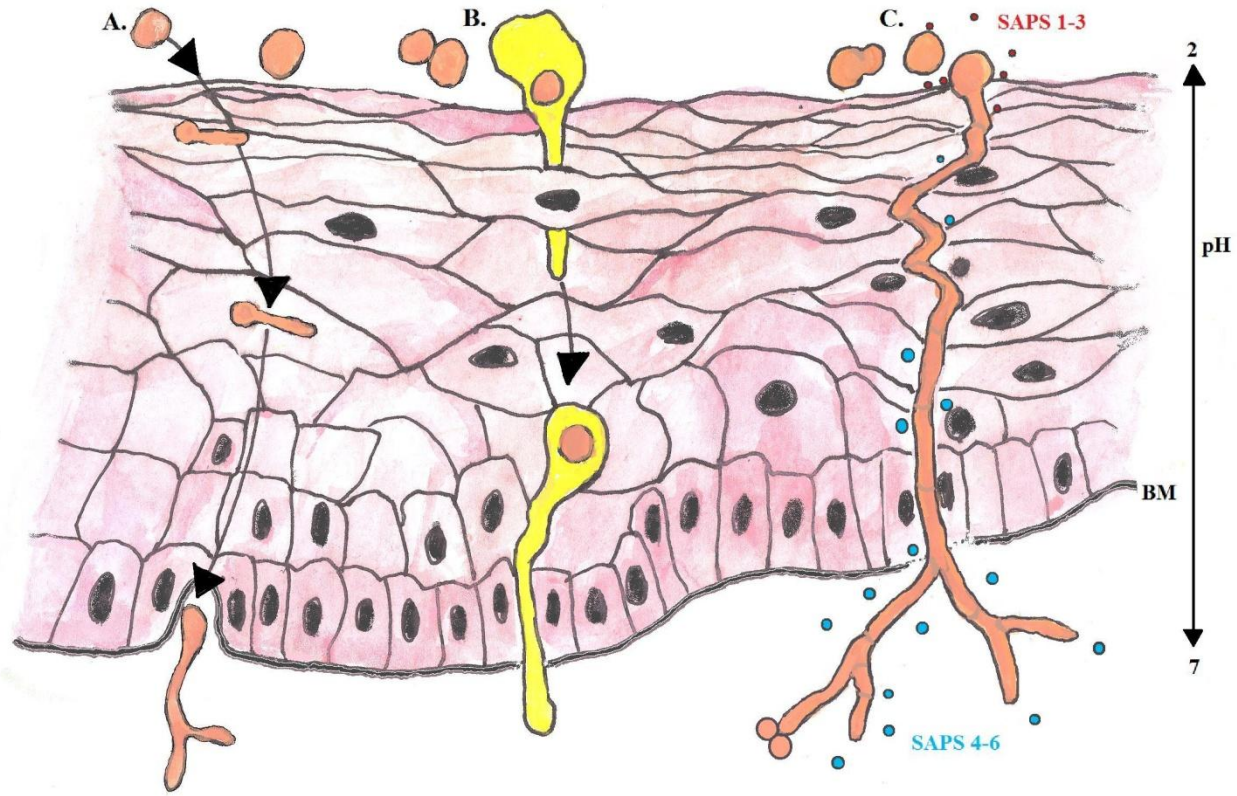


Figure 6. Proposed non-traumatic invasion mechanisms of *Candida* through the oral epithelium. A. endocytosis by epithelial cells, B. endocytosis by leukocytes, C. invasion between epithelial cells. The invasion of *Candida* between oral epithelial cells (C) by degrading E-Cad in adherens junctions is proposed based on the work presented in this thesis. A pH gradient across the epithelial layer favours expression and function of certain secreted aspartic proteinases (Saps). Blastospore-associated Saps(red dots) have an optimal acidic activity area of pH 2-5, and hyphal Saps (blue dots) have a slightly more basic optimal activity area of pH 3-7.

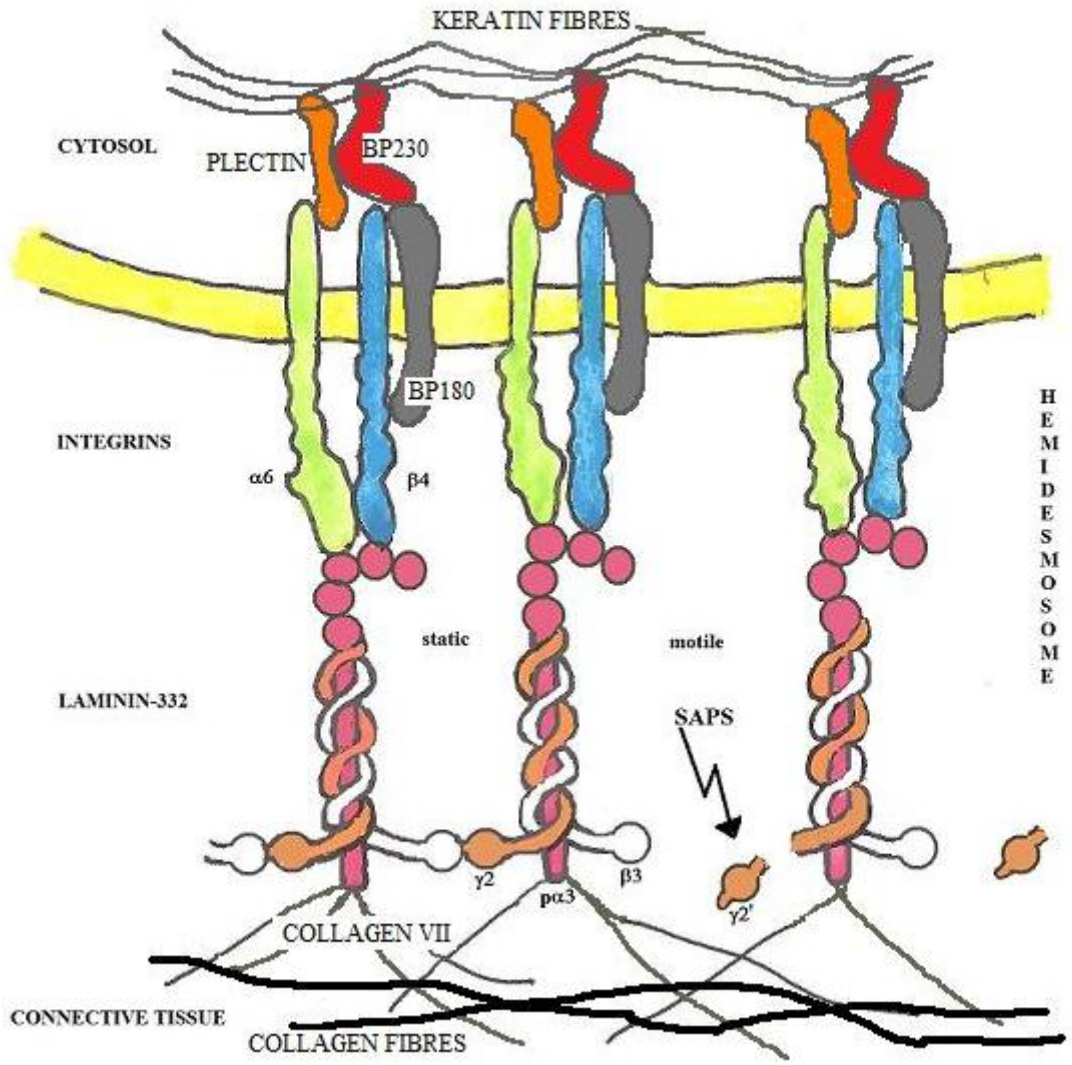


Figure 7. Degradation of the laminin-332 network in the basement membrane by *Candida* proteinases in oral acidic microniches causes destabilization of the hemidesmosomal structure. This proposed event may cause functional and motility alterations in the epithelium and create a possible invasion route for *Candida*. Saps= secreted aspartic proteinases of *Candida* species.

7. MATERIALS AND METHODS

7.1. *Candida* isolates

The yeasts used in these studies are given in the tables of each separate manuscript (I-IV) and are briefly described here in Table 1. Yeast sonicated cells and secreted proteinases from the growth medium were used to identify the fraction causing degradation. NCAC species were included in the studies to compare degradative ability of different *Candida* species. *C. albicans* SC5314 is a well-known clinical isolate from a patient with disseminated candidosis and was chosen for these studies as the most representive, a highly active invasive strain (Gillum *et al*., 1984). Hyphal mutants were used to evaluate the role of yeast-to-hyphal transition in degradation capability. Sap deletion mutants were used to identify the degradative proteinases.

The *Candida* were grown in YPG (yeast peptone glucose) medium at 35ºC for 24 hours in a water bath with shaking. This was considered to be the appropriate time to obtain the highest possible proteinase concentration. Cells were concentrated to 10^7 cells/ml. To obtain the cell fraction,1 ml of this suspension was washed twice (14000 rpm, 5 min, 4ºC) with TNC buffer (50 mM Tris-HCl, 0.2 M NaCl, 1 mM $CaCl_2$, pH 7.6), and the cells were left in 1 ml TNC. The cell suspension was sonicated with the Branson 250 sonicator (Branson, Danbury, CT) on an ice bath until over 50% of the cells were disrupted. Disruption and morphology prior to sonication (blastospore /hyphal form) were verified by phase contrast microscopy. To obtain the cell-free fraction, the *Candida* growth medium was filtrated using a 0.45-µm filter (Millipore, Billerica, MA), dialysed (12 to14 kDa cut- off, Medicell, London, UK) against distilled water for 2 h at 4ºC and concentrated 10x by lyophilization.

Table 1. Yeast strains used in this thesis

C. albicans CCUG 32723
C. albicans B1134
C. albicans SC5314
C. albicans JKC19 (*cph1*/*cph1*)
C. albicans HLC52 (*efg1*/*efg1*)
C. albicans HLC54 (*cph1*/*cph1*, *efg1*/*efg1*)
C. albicans SC5314 Sap 1-3 mutant
C. albicans SC5314 Sap 4-6 mutant
C. dubliniensis Cd3
C. dubliniensis Cd4
C. guilliermondii ATCC 6260T
C. guilliermondii B75B
C. glabrata CCUG 32725
C. glabrata ATCC 90030
C. glabrata 5WT
C. glabrata G212
C. parapsilosis Cp2
C. parapsilosis Cp3
C. krusei ATCC 6258
C. krusei D206B
C. tropicalis ATCC 750
C. tropicalis D213

7.2. *Candida* proteinase activity and inhibition assays

The activity of the *Candida* proteinase cell and cell-free fractions was evaluated by MDPF (2-methoxy-2, 4-dephenyl-3(2H)-furanone; Fluka, Buchs SG, Switzerland) - gelatin zymography (Study I). Fifteen mikrolitres of cell and cell free fractions of *Candida* were incubated with non-reducing Laemmli's sample buffer (RT, 2h) and run on 11% SDS-PAGE with MDPF – gelatin (1 mg/ml) as the substrate. The gels were first washed for 30 min with 50 mM Tris-HCl-0.25% Tween 80-0.02% NaN_3, then for 30 min in the aforementioned buffer with added 0.5 mM $CaCl_2$ and 1 µM $ZnCl_2$. Finally, the gels were incubated for 7 days at 37 ºC in the third buffer, which was otherwise identical to the second buffer, but lacked Tween. The optimal incubation time was evaluated by UV detection. After a 7-day incubation, the gels were stained with 0.2% Coomassie Brilliant Blue and scanned with a Bio-Rad GS-700 Densitometer. Zymography with different buffer pH values was used to define the effect of pH on the degradative activity of the proteinases.

In addition, the gelatinolytic and caseinolytic activity of 15 µl of *P. gingivalis* ATCC 33277 (10^8 cells/ml), 15 µl of *S. salivarius* 13419 (10^8 cells/ml), 15 µl of *L. casei* 921 (10^8 cells/ml), 20 µl of *A. actinomycetemcomitans* (10^8 cells/ml), 0.5 µl of *Clostridium histolyticum* collagenase type IA (2.5 U/mg; 1mg/ml, Sigma, MO), 0.5 µl of bovine trypsin (1mg/ml, Sigma, MO) and 4 µl of human neutrophil elastase (1 mU/ml, Merck, Darmstadt, Germany) were evaluated by the method described previously, with the exception of using several incubation times (2 h to 7 days) thus dividing the proteinase activities into three classes: high (2 h), medium (12 h) or low (7days) activity. Also, we used only neutral test pH.

Several typical proteinase inhibitors were chosen to evaluate their ability to inhibit the proteinase activity of *Candida*. Fifteen mikrolitres of the *Candida* fractions were incubated for 2 h at 37ºC with Pefabloc (Boehringer Mannheim GmbH, Mannheim, Germany), Ilomastat (Chemicon International Inc., Temecula, CA), EDTA (Merck KGaA, Darmstadt, Germany), CMT3 and CMT308 (Collagenax Inc., Newtown, PA) and CTT2 (GRENYHGCTTHWGFTLC, Heikkilä, 2006). The final concentration of the inhibitors was 0.2 mM.

7.3. Immunoprecipitation and fluorography of laminins -332 and -511 and E-cadherin

Degradation of the oral mucosal tissue components was detected by fluorography. This method is semiquantitative, but very sensitive in visualizing protein chain structures with a 35 S label. The separate chains of Lm-332, Lm-511 and E-Cad monomer can be seen as bands and degradation is visualized as alterations in molecular weights.

All of the proteins studied were derived from an immortal human oral epithelial cell line (I-III). Lm-332 and Lm-511 were secreted into the growth medium. The epithelial cell line used was a spontaneously immortalized gingival cell line (Salo *et al*., 1991). The cells were grown at 37ºC in Keratinocyte growth medium 2 (KGM-2, Promocell, Heidelberg, Germany) in a 5% CO_2 atmosphere. The cells were labelled with ^{35}S (25 µCi/ml, GE Health Sciences, Uppsala, Sweden). Growth medium was precleared with non-immune rabbit serum and Lm-332 was immunoprecipitated with polyclonal anti-rabbit antiserum specific to Lm-332 (Filenius *et al*., 2001) or monoclonal antibody 4C7 specific to Lm-511 bound to GammaBind™Sepharose™ beads (Amersham BioSciences, Piscataway, NJ).

Fifteen mikrolitres of the *Candida* fractions were incubated with Lm-332 at 37ºC for 24 h at two different pH values. The samples were run on reducing SDS-PAGE (6.5%/ Lm-332; 5%/ Lm-511). The degradation of Lm-332 was detected by fluorography; after electrophoresis, the gels were washed twice for 30 min with dimethyl sulfoxide (DMSO, Riedel deHaen, Seelze, Germany), then for 3 h with DMSO/2, 5-diphenyloxazole (Sigma, St. Louis, MO) and finally washed with water for 30 min. The gels were dried (80ºC, 2 h), placed on x-ray film (Kodak BioMax MS Film, Rochester, NY) into an intensifying screen (BioMax TransScreen LE, Kodak) and stored at -70ºC (4-7 days). The films were scanned with the Bio-Rad GS-700 Densitometer.

E-Cad is an integral cell-surface membrane protein. Keratinocytes were grown in KGM-2 as above and labelled with ^{35}S (25 µCi/ml). After washing twice with cold phosphate-buffered saline (PBS), 0.5 ml RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) was added per plate (4 ml). Cells were scraped off the plate and lysed on ice for 10 min and centrifuged (13000 rpm, 10 min, 4ºC). Solubilized E-Cad was immunoprecipitated from the supernatant by using 3.5 µg of mouse anti-human-E-Cad antibody (BD Biosciences, San Jose, CA) bound to GammaBind™ Sepharose beads™ (Amersham). Non-reducing SDS-PAGE and fluorography were performed as above.

7.4. Fibronectin assay

Fn was isolated from serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) of blood donors according to Engvall and Ruoslahti (1977). The structure of the Fn monomer is identical in blood and tissues, and this isolate could be tested as a representative tissue Fn molecule. Degradation could be detected by SDS-PAGE (II). Fifteen mikrolitres of *Candida* fractions were incubated with 11.5 µl (690 µg/ml) of Fn at two different pH values at 37ºC for 24 h. The samples were then run on a 5% SDS-PAGE, stained with 0.2% CBB and photographed.

7.5. ProMMP-9 activation

The ability of the *Candida* fractions to convert inactive proMMP-9 (IV) to active form was assessed by Western blot and visualized by enhanced chemoluminesence (ECL)-technique (IV). Two mikrolitres of human recombinant proMMP-9 (20 ng/µl, Invitek, Berlin, Germany) was incubated with 15 µl of sonicated yeast cell or 10x concentrated growth media for 24 h at 37°C. The activation of proMMP-9 was visualized as a fragment of 86 kDa compared with the intact proMMP-9 (92 kDa) and an aminophenylmercuric acid- positive control (APMA, 86 kDa) and detected by ECL. The incubated samples were first run on 8% SDS-PAGE and then blotted onto a nitrocellulose membrane (Protran, Dassel, Germany). Primary antibody against human proMMP-9 (rabbit polyclonal, EMD BioSciences, La Jolla, CA) and secondary antibody (anti-rabbit IgG from donkey, GE Healthcare, Buckinghamshire, UK) were used to visualize the activation.

7.6. TIMP-1- and -2 degradation assay

Human recombinant TIMP-1 and TIMP-2 were incubated with the *Candida* fractions and the degradation was seen as fragmentation of the molecule (IV). 0.5 µl of human recombinant TIMP-1(30 ng/µl, Calbiochem, Darmstadt, Germany) and 3 µl of human recombinant TIMP-2 (30 ng/µl, Calbiochem) were incubated with 15 µl of the yeast cell fractions for 7 days at 37°C. Nonreducing SDS-PAGE (8%) was performed and the proteins were blotted onto a

nitrocellulose membrane (Protran), and detected ECL using rabbit polyclonal primary antibody against TIMP-1 (Chemicon, Temecula, CA) and mouse monoclonal primary antibody (Oncogene, San Diego, CA) against TIMP-2, anti-rabbit IgG from a donkey (BD BioSciences, Buckinghamshire, UK) as a secondary antibody against TIMP-1 and anti-mouse IgG from sheep (BD Biosciences) as a secondary antibody against TIMP-2.

7.7. Casein assay for residual TIMP-1 activity evaluation

The residual activity of TIMP-1 after *Candida* degradation was evaluated by a modified casein assay (Sorsa *et al*., 1997). The degraded TIMP was seen as reduced inhibition on MMP-9 caseinolysis (IV). First TIMP-1 was incubated with *Candida*, as described previously in the TIMP degradation assay. The degraded TIMP-1 was incubated with 3 µl of the proteinase inhibitor Pefabloc (1 mM) for 2 h at 37°C. The samples were then incubated with 2 µl of human recombinant proMMP-9 (20 ng/µl, Invitek) and 5 µl of β-casein (52µM) at 37°C for 1 h. Intact B-casein, uninhibited APMA-induced MMP-9 caseinolysis and intact TIMP-1 were used as controls. Finally, 13 % SDS-PAGE was performed, the gels were stained with 0.2% CBB and analysed using Bio-Rad GS-700 Densitometer.

7.8. Fluorimetric assay

The fluorimetric assay (MMP-14 Fluorescent Assay Kit for Drug Discovery-AK 417, Biomol, PA) was used to compare the enzymatic activities of 15 µl of the microbial fractions and typical commercial proteolytic enzymes to MMP-14 (study IV) according to the manufacturer's instructions in black 96-well microtiter plates (Costar, Corning, NY). Substrate with assay buffer and heat- denatured fractions of the microbial enzymes were used as controls. As typical commercial proteolytic enzymes, we used 0.5 µl of bovine trypsin (1 mg/ml, Biomol), 4 µl of human neutrophil elastase (1 mU/ml, Merck), and 2 µl of *Clostridium histolyticum* collagenase type IA (1 mg/ml, Sigma). Human recombinant MMP-14 (Biomol, PA) was used as a positive control). The assay was performed with a 340-nm excitation wavelength and 394-nm emission wavelength for 2 h with 2- min intervals. Degradation of the Omni-MMP ^{TM} substrate was detected (Tecan Spectrafluor Plus, Hombrechticon, Switzerland) and visualized (X Fluor 4 Plus- program) as increased fluorescence emission values.

The *C. parapsilosis* Cp2 cell fraction was investigated further. The ability of the Cp2 cell fraction to degrade the Omni-MMP ^{TM} synthetic peptide was compared to the ability of MMP-14 to degrade this substrate. The above-mentioned fluorimetric assay was used. Inhibition of Cp2 by 1 µl of Pepstatin A (1 mg/ml, Sigma, MO) and 5 µl of the synthetic inhibitor CTT-2 was measured. Denatured Cp2 was used as a negative control.

8. RESULTS

The results of this series of studies are given in detail in the original publications (I-IV). In the following, only a brief summary of the results is presented.

Lm-332

At neutral pH (7.6), none of the strains degraded Lm-332. At pH 5.0, cell fractions of *C. albicans, C. dubliniensis, C. tropicalis, C. guilliermondii* and *C. glabrata* degraded Lm-332 γ2-chain into a processed 100-kDa form. *C. krusei* caused degradation of Lm-332 γ2-chain into three 100 to130- kDa fragments. The 100-kDa fragment is known as γ2′ (Koshikawa *et al*., 2005). Secreted proteinases of *C. albicans, C. dubliniensis* and *C. tropicalis* also caused degradation of the γ2-chain into 100-kDa form. The degradation varied between reference and clinical strains in most species investigated and the exact strains capable of Lm-332 degradation are given in Study I.

Lm-332 is the only protein in JE. It does not contain E-Cad. This makes JE more vulnerable to degradation by specific proteinases. Weakening or loss of integrity of the JE provides a pathway for *Candida* invasion.

Lm-511

None of the strains caused Lm-511 degradation under the present study conditions. This may indicate that laminin degradation is a highly specific process and dependent on the molecular structure of the substrate and specificity of the proteinase.

Fibronectin

Degradation of human Fn by secreted proteinases of most of the strains evaluated occurred both at physiological and lower pH and was more pronounced at pH 4. This indicates a role of acid- activated secreted proteinases in the degradation process. The cell fractions of *C. tropicalis* and *C. parapsilosis* also degraded Fn at pH 7.8, and this may be the effect of cell membrane- bound or intracellular proteinases, which are activated at physiological pH. *C. tropicalis* cells showed hyphal structures prior to sonication, and *C. parapsilosis* was in the blastospore form, indicating that hyphal structure formation is not necessary for Fn degradative capability.

Fn is the linking protein in the cell-ECM adhesion system and its degradation causes changes in the substructure by unlinking proteins from each other. This may cause alterations in cell signalling pathways.

E-Cad

In study III sonicated cell fractions of hyphal-formed *C. albicans* JKC-19 (*cph1/cph1*) and parental strain SC5314 degraded E-Cad at pH 4, and this was not Cph1-dependent. This result indicates that hyphal cell-associated proteinases are capable of E-Cad degradation at lower pH values. Transcription factors other than cph seem to be related to E-Cad degradation.

Furthermore (Study III) blastospore (yeast form) 10x concentrated secreted proteinases of *C. albicans* HLC-52 (*efg1/efg1*), HLC-54 (*cph1/cph1 efg1/efg1*), ATCC 32723 and clinical strain B1134 were able to degrade E-Cad at pH 4; HLC-52 and HLC-54 Saps also at pH 6, and this was not Efg1-, or Cph1-dependent. In this study setting, Sap1-3 mutant cell fraction did not degrade E-Cad. Saps 1-3 are known to be related to blastospores and may cause destruction at pH 3-5.

C. glabrata did not degrade E-Cad in our study setting. *C. glabrata* does not form true hyphae.In the light of the absent degradation capability, hyphal formation and associated proteinases could be speculated to be elementary to E-Cad degradation.

E-Cad degradation indicated that *Candida* are able to destroy adherent junctions between epithelial cells in the oral epithelium. This then makes invasion of the yeasts plausible.

MMP-9 and TIMP-1

The activation of MMP-9 occurred by cell fractions from both the reference and clinical strains of *C. parapsilosis*. Cell fractions of both strains of *C. albicans* and *C. krusei*, *C. parapsilosis* Cp2 and *C. glabrata* reference strain degraded TIMP-1. TIMP-2 was not degraded by any of the strains tested. The degradation of TIMP-1 caused an elevation of the activity of MMP-9, detected as more effective caseinolysis.

Only *C. parapsilosis* caused MMP-9 activation. Thus it seems that *Candida* has little effect on MMP-9 action. However, TIMP-1 was degraded by several *Candida* species, indicating that *Candida* may modulate MMP-9 action by weakening the action of its inhibitor TIMP-1.

Fluorimetric assay

Fluorimetric comparative analysis of samples tested revealed activation of the substrate in descending order by MMP-14, *P. gingivalis*, *C. parapsilosis* Cp2 , *A. actinomycetemcomitans*, *C. albicans* CCUG 32723, *L. casei* 921, *Clostridium histolyticum* collagenase type IV, *C. albicans* B1134, *C. krusei* ATCC 6258, *S. salivarius* 13914, elastase, bovine trypsin, *C. glabrata* CCUG 32725 and *C. krusei* D206B. All of the microbial samples studied were cell fractions.

Of the *Candida* species, only *C. parapsilosis* Cp2 and Cp3 showed increased specificity towards the synthetic OMNI-MMP substrate. This feature could make it a suitable candidate for developing a diagnostic test to detect *C. parapsilosis* infection.

9. DISCUSSION

In contrast to most of the studies in the literature, which were conducted on mouse-derived or non-oral proteins, we used human-derived proteins and proteins produced by a human oral keratinocyte cell line. *Candida* species are apparently able to degrade, in addition to mouse proteins, also human basement membrane zone proteins Lm-332, E-Cad, Fn and TIMP-1.

Role of Saps

Saps 2-, 5- and -9 seem to be the most important aspartic proteinases of *Candida* capable of causing tissue destruction (Naglik *et al*., 2008). A study with neutropenic mice showed that intraperitoneal injection of pepstatin A, an aspartic proteinase inhibitor, can protect against disseminated candidosis caused by *C. albicans* (Fallon *et al*., 1997). This protective effect was not noticed in intravenous *Candida* challenge. This finding was proposed to indicate the role of *Candida* aspartic proteinases as an early event in the dissemination prior to the entry into circulation. Saps 1-6 are secreted proteinases, while Sap 9 is cell surface-bound. The fractions used in our studies (I-IV) were concentrated growth media (containing Saps 1-6) and sonicated cells (contain cell-bound proteinases), and these represented the separate proteinase groups in the present series. The morphology of the yeasts prior to sonication contained mainly either blastospore or hyphal forms. By using these fractions, we could trace the morphology of the yeast associated with the degrading proteinase. The Sap and hyphal mutants served this purpose as well. The possible activating effect of pH was investigated by conducting the experiments at lower and higher pH values.

Role of pH

The low pH in oral microniches may trigger proteolysis by *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei* acidic proteinases, as has been shown by Samaranayake *et al.* (1994). The activating effect of pH was also observed in our studies (I-III). The internal organs (liver, heart, lung, thyroid) have a non-acidic pH, and both blastospore and hyphal forms of *C. albicans* have been detected by immunostaining in these organ specimens. The tongue, esophagus, intestine and most skin areas are considered non-acidic, and hyphal forms are predominant in them (Monteagudo *et al*., 2005). Our results (I-III) indicate a crucial role of pH as a triggering factor of the expression and function of proteinases; hyphal forms of *Candida* seem to express certain proteinases in less acidic environments, and blastospore forms express proteinases at acidic pH values.

Acidic microniches have been shown to be related to Sap expression in immunoelectrical studies by Schaller *et al.* (1999). Wagner *et al.* (1995) also demontrated that denaturing of proteinases occurs if the pH conditions are unsuitable for the proteins in question. A body temperature of 37°C shifts the denaturing pH one pH unit towards acidity in *C. parapsilosis*. Thus, the *in vitro* studies conducted at room temperature should be interpreted as only rough estimates of proteinase function compared with *in vivo* studies.

Interepithelial invasion/E-Cad degradation

Recent studies (Lermann and Morschhäuser, 2008; Naglik *et al*., 2008) on the role of Saps 1-6 have yielded contradictory results compared with earlier studies from several groups, which have shown that Saps 1-6 would have a role in *Candida* invasion by for instance degrading epithelial proteins. Elevated expression of Saps has been detected in candidosis. Surprisingly,

studies by Lermann and Morschhäuser (2008) have revealed that secreted aspartic proteinases 1-6 of strain SC5314 are not required for invasion of reconstituted human epithelia by *C. albicans* by measuring lactate dehydrogenase (LDH) as an indicator of damage to the epithelium. They considered that this may be due to differences in the study settings compared with other studies. Schaller *et al.* (2008) obtained partially similar results. The role of Saps in the invasion process is still unclear. From the results Studies III, we could speculate that *Candida* invade initially between epithelial cells by degrading E-cadherin and possibly other intercellular adhesion molecules. If this invasion does not cause epithelial cell lysis, there might not be any LDH release. This was also noticed by Naglik *et al.* (2008). Filler *et al.* (1995) have shown that both live and dead *C. albicans* cells were phagocytosed by vascular endothelial cells, but only viable *C. albicans* could induce cell damage. Naglik *et al.* (2008) concluded that hypha formation is the main cause of tissue damage, *SAP5* gene expression can be hypha-independent and is controlled by both Efg1 and Cph1 pathways.

The presence of nutrients at the infection site probably affects the pathogenicity of *Candida*. This would be the case for hyphal forms of *Candida*; glucose is crucial for the formation of hyphae (Brown *et al*., 2006), and in our study hyphal forms of *Candida* species grown in YPG were able to degrade E-Cad. In addition, secreted aspartic proteinases of the studied blastospore formed *C. albicans* strains, only Sap1-3 and Sap 4-6 mutants were able to degrade E-Cad. This suggests the possible involvement of Sap2 and Sap 5 in degrading E-Cad. *C. glabrata*, which does not form hyphae, did not degrade E-Cad. Thus, the pathological mechanism for *C. glabrata* invasion remains unknown. It could be speculated, however, that *C. glabrata* always persists in mixed *Candida* infections containing *C. albicans*, exploits the degradaded pathway created by *C. albicans* and then could use this route for invasion.

The infiltration of hyphae into connective tissue has been suggested to be caused either by *C. albicans* pathogenicity (proteinases) or tissue destruction caused by inflammation, e.g. in periodontitis. For instance, a histological study of chronic periodontitis shows *C. albicans* hyphae in deep gingival pockets, and the infiltration site into the connective tissue is near the border of the sulcular epithelium (Järvensivu *et al*., 2004). The role of *Candida* proteinases in the invasion process is supported by our degradation studies. More studies are needed, however, that use several strains of *Candida* species and also test degradation of other adhesion proteins of the oral epithelium.

Basement membrane degradation

If invasion of *Candida* is possible between the epithelial cells by the hyphal cells, they must first degrade other junctional proteins between the epithelial cells and eventually degrade the BM structure to access deeper layers and disseminate. In our study settings, Lm-332 was degraded by certain *Candida* species at specific pH environments *in vitro*. Thus, local pH may play an essential role in triggering the invasion cascade by activating certain proteinases.

In the studies of Claveau *et al.* (2004) showing paradoxically elevated BM protein production by human oral epithelial cells, two explanations have been proposed: 1) *C. albicans* induces elevated production of these proteins to aid adhesion prior to invasion and 2) *C. albicans* degrades BM proteins, inducing the host cells to produce more proteins to maintain tissue integrity. Our results (I-III) of gelatin, Lm-332 and Fn degradation by several *Candida* species support the latter hypothesis.

JE is different from other parts of the epithelium because it lacks of a true BM and can be considered as a loosely built entity similar to the connective tissue. The only protein found in the BM is Lm-332, and this makes it vulnerable (Oksanen *et al*., 2001). Our studies reveal that *Candida* are able to degrade Lm-332; then they may use this as a second possible route deeper into tissue in addition to E-Cad degradation-related invasion between epithelial cells.

Lm-511 was not degraded by the strains used in the present study, suggesting that laminin degradation is a specific process and depends on the substructure of the substrate molecule.

Plasma-derived Fn produced by hepatocytes is an abundant connective tissue extracellular matrix protein located in all tissues. In addition, gingival tissue also appears to contain locally produced EDA domain-containing Fn closely associated with basal keratinocytes and the BM (Walsh *et al*., 2007). *C. albicans* appears to adhere to gingival Fn (Klotz and Smith, 1991), and this together with its ability to degrade Fn, as shown in the present study, also augments the movement of *Candida* through the gingival BM and connective tissue.

Interestly that elderly toothless patients have higher oral yeast counts than dentate patients, but only little difference in the infection parameter values such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) of white blood cell count (WBC) (Meurman *et al*., 1997). This is speculated to be caused by less portals of entry for infection in toothless patients. In light of the studies findings of this thesis, this might indeed be the case in gingival; the destruction of the junctional epithelium by yeasts is thought to be a route for *Candid*a dissemination. Toothless patients have no junctional epithelium and so these are less portals of infection.

Host protein modulation

Host MMPs and TIMPs play a role in tissue destruction. MMP-9 and MMP-2 are the major MMPs involved in destructive processes in gingival tissue. TIMP-1 and TIMP-2 balance the action of the destructive proteinases by binding and inhibiting them. Claveau *et al.* (2004) proposed that decreased TIMP-2 and MMP-2 levels aid the elevated MMP-9 levels in degrading tissue. Our study (IV) supports this finding; we found *in vitro* activation of MMP-9 and degradation of TIMP-1. In our settings, TIMP-2 was not, however, degraded. The balance of matrix destructive proteinases and their inhibitors plays a major role in tissue remodelling. Imbert *et al.* (2002) reported that *C. albicans* 95 kDa metalloproteinase caused degradation of ECM proteins, and this possibly aids in *Candida* invasion. Hence, the combinations of host and similarly acting *Candida* proteinases have the ability for ECM protein destruction.

When comparing bacterial and candidal proteinase activity, it was surprising to detect fluorescence from samples other than MMP-14; the substrate proved to be non-specific. *P. gingivalis* caused the most fluorescence of the microbial species tested, as expected, because this species has a known strong proteolytic capability. Surprisingly, *C. parapsilosis* Cp2 ranked second regarding the proteolytic degrading ability of *Candida* species for this substrate. The other samples showed expected values of degradation, which were too low for any clinical significance. High Cp2 values may be useful in developing a rapid diagnostic tool for the detection of *C. parapsilosis* infection.

10. CONCLUSIONS

Studies I-IV lead to the following conclusions:

1. Lm-332 degradation was species- and fraction-dependent. Most strains studied had the ability to degrade Lm-332. E-Cad degradation was investigated with two species; *C. albicans* was able to degrade E-Cad, but *C. glabrata* was not. Fibronectin was degraded by most of the strains investigated, and this may cause alterations in cell structure and signalling events. MMP-9 was activated only by clinical species of *C. parapsilosis,* which also showed high proteinase levels measured by the fluorimetric assay. TIMP-1 was degraded by several, but not all, of the species. As the methods used were semi-quantitative, we could measure only crude degradation phenomena, and the exact degree of degradation cannot be estimated.

2. *Candida* is able to adapt to environmental challenges, such as pH, temperature or nutrient availability in the mucosa, and respond to these stimuli in order to survive. Local microniche circumstances affect the morphology of the fungus and the forms best-fitted to survival. This is a fine-tuned mechanism that merits more research. Both morphological forms of *Candida* seem to have a role in the development and progression of candidosis through the mucosal epithelial layer, initially by blastopores and later in the infection dissemination stage in hyphal forms. *C. glabrata*, which does not form hyphae, did not degrade E-Cad in our study settings, but still was nonetheless able to degrade Lm-332 of the basement membrane. This controversy requires further examination.

3. E-Cad is degraded by both blastopore and hyphal forms of *C. albicans*. The blastospore proteinases are best-suited to act in more acidic environment and begin the degradation in the invasion process. As the penetration of yeast proceeds deeper into the epithelium, the hyphal forms start to secrete proteinases best suited for the more physiological basic conditions. Because some studies indicate no epithelial damage by invasion of hyphae , as measured by LDH activity, invasion is likely to occur by hyphal forms between epithelial cells by degrading the main adhesive junctional protein E-Cad. In addition, a second invasion route through degraded basement membrane Lm-332, particularly in the JE, is proposed.

4. The *in vitro* degradation capability of *Candida* species indicates that the proteinases can cause *Candida* dissemination pH-dependently. By activating MMP-9 and degrading TIMP-1, some *Candida* species are able to modify host responses, resulting in easier *Candida* invasion. As more studies are conducted in the controversial field of the role of aspartic proteinases the understanding and prevention of candidosis will improve. At a molecular level development of anticandidal agents may be possible based on the knowledge of enzyme-substrate interaction and its inhibition.

Future studies

On the basis of these results future studies of *Candida* should include: characterization and comparison of individual proteinases from *C. albicans* and NCAC species to gain knowledge of the factors that may trigger (environmental challenge such as pH, nutrients, oxidative stress) and enable *Candida* invasion. The main focus should be the search for a proteinase inhibitor for use as a drug against candidosis. Development of rapid fluorimetric tests to detect *Candida* from patient samples may also be possible.

I conclude with the words of three distinguished yeast scientists, on elucidating the mechanism of pathogenic yeast invasion, thoughts with which I am in full agreement:

"We need now to break down disease processes into temporal events (such as colonisation, penetration, dissemination, organ invasion and tissue necrosis), and reassess how morphogenesis impacts at each of these stages."(Gow, Brown & Odds 2002).

11. ACKNOWLEDGEMENTS

This thesis is based on studies conducted in 2007-2009 at the Research Laboratory of the Institute of Dentistry, University of Helsinki, and at the Department of Anatomy of the Institute of Biomedicine, University of Helsinki.

I particularly thank the following persons for their contributions:

Professor Jukka H. Meurman for his innovative scientific encouragement, and for providing international collaboration possibilities, financial support and the facilities to conduct the research. I hope these studies will make a difference in the quest to enlighten the connection between oral disease and general health.

Professor Ismo Virtanen for wisely guiding me in the world of epithelial proteins and providing facilities and sharing techniques that could be applied to *Candida* studies. Without his expertise, this kind of interdepartmental work would not have been possible.

The official faculty-appointed reviewers of my thesis, Professors Erik Dabelsteen and Malcolm Richardson, for taking on the task of structural criticism and improving the scientific content of this manuscript.

The author-editor Carol Ann Pelli, Language Centre, University of Helsinki, for revising the manuscript.

My co-authors, Professor Lakshman P. Samaranayake, for expertise and advice in the field of *Candida*; Professor Timo Sorsa for the useful advice in the field of molecular oral biology techniques; and Kirsti Kari, The Head of the Research Laboratory of the Institute of Dentistry, for teaching me everything I know about yeasts: the many conversations and techniques that I learned among the years have been most helpful.

From the department of Anatomy, Institute of Biomedicine: Pipsa Kaipainen, for patiently teaching me laboratory skills needed in the studies; Marja-Leena Piironen for helping in culturing cells and Reijo Karppinen for fluorographic data processing.

Ritva Keva and Jukka Inkeri, Institute of Dentistry, Research Laboratory, for advising and helping me in everyday laboratory work.

Marjo Kostia, Institute of Dentistry, for advice and help in preparing the manuscripts for publication and producing the great posters I have had the honour of presenting in several international congresses.

Special thanks to University Lecturer Pirjo Nikula-Ijäs, Department of Biochemistry, University of Helsinki, for advising me in the field of protein chemistry.

Research colleagues from the years spent at the Research Laboratory of the Institute of Dentistry: especially Taina Tervahartiala, Helen Bao, Ulvi Gursoy, Iva Stamatova, Heidi Kuula and Juho Suojanen for sharing knowledge and pleasant moments at the lab.

My parents Pirkko and Heikki for love and support and for providing the best possible education opportunities. Veli-Matti, my life companion, for encouragement and patience

throughout these years. My children Piia, Simo and Leo for putting up for all the hours I should have spent with you instead of at the lab.

Helsinki, March 2010

Pirjo Pärnänen

12. REFERENCES

Albrecht A, Felk A, Pichova I, Naglik J R, Schaller M, deGroot P, MacCallum D, Odds F C, Schäfer W, Klis F, Monod M, Hube B. Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. J Biol Chem 2006; 281: 688-694.

Ashman R B, Farah C S, Wanasaengsakul S, Yan H, Pang G, Clancy R L. Innate versus adaptive immunity in *Candida albicans* infection. Immunol Cell biol 2004; 82: 196-204.

Aumailley A, Bruckner-Tuderman L, Carter W G, *et al.* A simplified laminin nomenclature. Matrix Biol 2005; 24**:** 326-332.

Bair E L, Chen M L, McDaniel K, *et al.* Membrane type 1 matrix metalloprotease cleaves laminin-10 and promotes prostate cancer cell migration. Neoplasia 2005; 7: 380-389.

Barnes S M, Lane S J, Sogin M L, Bibeau C, Weisburg W G. Evolutionary relationships among pathogenic *Candida* species and relatives. J bacteriol 1991; 173: 2250-2255.

Bartie K L, Williams D W, Wilson M J, Potts A J C, Lewis M A O. Differential invasion of *Candida albicans* isolates in an in vitro model of oral Candidosis. Oral Microbiol Immun 2004; 19: 293-296.

Bartold P M and Narayanan A S. Molecular and cell biology of healthy and diseased periodontal tissues. Periodontol 2000 2006; 40: 29-49.

Blankenship J R, Wormley F L, Boyce M K, Schell W A, Filler S G, Perfect J R, Heitman J. Calcineurin is essential for *Candida albicans* survival in serum and virulence. Eukaryotic cell 2003; 2: 422-30.

Blot S, Dimopoulos G, Rello J, Vogelaers D. Is Candida really a threat in the ICU? Curr Opin Crit Care 2008; 14: 60-604.

Bonassoli L A, Bertoli M, Svidzinski T I E. High frequency of *C. parapsilosis* on the hands of healthy hosts. J Hosp Infect 2005; 59: 159-162.

Borradori L and Sonnenberg A. Structure and function of hemidesmosomes: more than simple adhesion complexes. J Invest Dermatol 1999; 112: 411-418.

Bosshardt D D and Lang N P. The junctional epithelium: from health to disease. Crit Rev Oral Biol Med 2005; 84: 9-20.

Bouchara J-P, Tronchin G, Annaix V, Robert R, Senet J-M. Laminin receptors on *Candida albicans* germ tubes. Infect Immun 1990; 58: 48-54.

Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. BBA – Protein Struct M. 2000; 1477**:** 267-283.

Brown A and Gow N A R. Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol 1999; 7: 333-338.

Brown V, Sexton J A, Johnston M. A glucose sensor in *Candida albicans*. Euk Cell 2006; 5: 1726-1737.

Buffo J, Herman M A, Soll D R. A characterization of pH-regulated dimorphism in *Candida albicans.* Mycopathologia 1984; 85: 21-30.

Calderone R, Suzuki S, Cannon R, Cho T, Boyd D, Calera J, Chibana J, Herman D, Holmes A, Jeng H-W, Kaminishi H, Matsumoto T, Mikami T, O´Sullivan J M, Sudoh M, Suzuki M, Nakashima Y, Tanaka T, Tompkins G R, Watanabe T. *Candida albicans*: adherence, signaling and virulence. Med Mycol 2000; 38: 125-137.

Calderone R A, Fonzi W A. Virulence factors of *Candida albicans*. Trends Microbiol 2001; 9:327-35.

Cetinkaya Z, Kiraz N. In vitro investigation of antifungal activities of phenotypic variation *Candida albicans* strains against fluconazole, itraconazole and voriconazole. Jpn J Med Mycol 2005; 46: 197-201.

Chen C G, Yang Y L, Cheng H H, Su C L, Huang S F, Chen C T, Liu Y T, Su I J, Lo H J. Non-lethal *Candida albicans* cph1/cph1 efg1/efg1 transcription factor mutant establishing restricted zone of infection in a mouse model of systemic infection. Int J ImmunoPath Ph 2006; 19: 561-565.

Chitaev N A and Troyanovsky S M. Adhesive but not lateral E-cadherin complexes require calcium and catenins for their formation. J Cell Biol 1998; 142: 837- 846.

Claveau I, Mostefaoui Y, Rouabhia M. Basement membrane protein and matrix metalloproteinase deregulation in engineered human oral mucosa following infection with *Candida albicans*. Matrix Biol 2004; 23: 477-486.

Colognato H, Yurchenco P D. Form and function: The laminin family of heterotrimers. Dev Dyn 2000; 218: 213-234.

Crowe J D, Sievwright I K, Auld G C, Moore N R, Gow N A R, Booth N A. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol 2003; 47: 1637-1651.

De Bernardis F, Muhlsschlegel F A, Cassone A, Fonzi W A. The pH of the host niche controls gene expression in and virulence of Candida albicans. Infect Immun 1998; 66: 3317-3325.

DeMuri G P & Hostetter M K. Evidence for a beta (1) integrin fibronectin receptor in *Candida tropicalis*. J Infect Dis 1996; 174: 127-132.

Dieterich C, Schandar M, Noll M, Johannes F-J, Brunner H, Graeve T. Rupp S. *In vitro* reconstructed human epithelia reveal contributions of *Candida albicans* EFG1 and CPH1 to adhesion and invasion. Microbiol 2002; 148: 497-506.

Doi M, Homma M, Chindamporn A, Tanaka K. Estimation of chromosome –number and size of pulsed-field gel-electrophoresis (PFGE) in medically important *Candida* species. J Gen Microbiol 1992; 138: 2243-2251.

Dong X G, Shi W Y, Zeng Q Y, Xie L X. Roles of adherence and matrix metalloproteinases in growth patterns of fungal pathogens in cornea. Curr Eye Res 2005; 30: 613-620.

Dongari-Bagtzoglou A and Kashleva H. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. Nature Protocols 2006; 1: 2012- 2018.

dos Santos A L S, de Araujo Soares R M. *Candida guilliermondii* isolated from HIV-infected human secretes a 50 kDa serine proteinase that cleaves a broad spectrum of proteinaceous substrates. FEMS Immunol Med Mic 2005; 43: 13-20.

Emingil G, Atilla G, Sorsa T, Savolainen P, Baylas H. Effectiveness of adjunctive low-dose doxycycline therapy on clinical parameters and gingival crevicular fluid laminin-5 gamma2 chain levels in chronic periodontitis. J Periodontol 2004; 75: 1387-1396.

Engvall E, Ruoslahti E. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int J Cancer 1977; 20: 1-5.

Fallon K, Bausch K, Noonan J, Huguenel E, Tamburini P. Role of aspartic proteases in disseminated *Candida albicans* infection in mice. Infect Immun 1997; 65: 551-556.

Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, Sanglard D, Korting H C, Schäfer W, Hube B. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required fot the invasion of parenchymal organs. Infect Immun 2002; 70: 3689-3700.

Ferletta Maria. The laminins and their receptors [dissertation]. Uppsala University; 2002.

Filler S G, Swerdloff J N, Hobbs C, Luckett P M. Penetration and damage of endothelial cells by *Candida albicans*. Infect Immun 1995; 63: 976-983.

Filler S G & Sheppard D C. Fungal invasion of normally non-phagocytotic host cells. PLoS Pathogens 2006; 2: 1099-1105.

Fine J, Mellerio J E. Extracutaneous manifestations and complications of inherited epidermolysis bullosa: Part I. Epithelial associated tissues. JAAD 2009; 61: 367-384.

Fonzi W A, Irwin M Y. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 1993; 134: 717-728.

Forsyth C B, Mathews H L. Lymphocyte adhesion to *Candida albicans.* Infect Immun 2002; 70: 517-527.

Frank C F & Hostetter M K. Cleavage of E-cadherin: a mechanism for disruption of the intestinal epithelial barrier by *Candida albicans*. Translational Research 2007; 149: 211-222.

Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Direct association of occluding with ZO-1 and its possible involvement in the localization of occluding at tight junctions. J Cell Biol 1994; 127: 1617-1626.

Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 1998; 141: 1539-1550.

Germaine G R, Tellefson L M. Effect of pH and human saliva on protease production by *Candida albicans*. Infect Immun 1981; 31: 323-326.

Ghosh S, Stack M S. Proteolytic modification of laminins: functional consequences. Microsc Res Tech 2000; 51: 238- 246.

Gilfillan G D, Sullivan D J, Haynes K, Parkinson T, Coleman D C, Gow N A R. *Candida dubliniensis:* phylogeny and putative virulence factors. Microbiology 1998; 144: 829-838.

Gillum A M, Tsay E Y H, Kirsch D R. Isolation of the *Candida albicans* gene for orotidine-5´-phosphate decarboxylase by complementation of *S. cerevisiae* ura3 and *E. coli pyrF* mutations. Mol Gen Genet 1984; 198: 179-182.

Gow N A R, Brown A J P, Odds F C. Fungal morphogenesis and host invasion. Curr Opin Microbiol 2002; 5: 366-371.

Gozalbo D, Gil-Navarro I, Azorin I, Renau-Piqueras J, Martinez J P, Gil M L. The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein. Infect Immun 1998; 66: 2052-2059.

Grubb S E W, Murdoch C, Sudbery P E, Saville S, Lopez-Ribot J L, Thornhill M H. *Candida albicans*-endothelial cell interactions: a key step in the pathogenesis of systemic Candidosis. Infect Immun 2008; 76: 4370-4377.

Gu J, Nishiuchi R, Sekiguchi K. Matrix metalloproteinase-2 is involved in A549 cell migration on laminin-10/11. Biochem Bioph Res Co 2002; 296: 73-77.

Hatakeyama S, Yaegashi T, Oikawa Y, Fujiwara H, Mikami T, Takeda Y, Satoh M. Expression pattern of adhesion molecules in junctional epithelium differs from that in other gingival epithelia. J Periodont Res 2006; 41: 322-328.

Heikkilä P, Suojanen J, Pirilä E, Väänänen A, Koivunen E. Human tongue carcinoma is inhibited by selective antigelatinolytic peptides. Int J Cancer 2006; 118: 2202-2209.

Hintermann E, Quaranta V. Epithelial cell motility on laminin-5: regulation by matrix assembly, proteolysis, integrins and erbB receptors. Matrix Biol 2004; 23: 75-85.

Hormia M, Owaribe K, Virtanen I. The dento-epithelial junction: cell adhesion by type I hemidesmosomes in the absence of a true basal lamina. J Periodontol 2001; **72**: 788- 797.

Hormia M, Ylänne J, Virtanen I. Expression of integrins in human gingiva. J Dent Res 1990; 69: 1817-1823.

Hormia M, Virtanen I, Quaranta V. Immunolocalization of integrin-alpha-6-beta-4 in mouse junctional epithelium suggests an anchoring function to both the internal and the external basal lamina. J Dent Res 1992; 71: 1503-1508.

Hostetter M K. Integrin-like proteins in *Candida* spp. and other microorganisms. Fungal Genet Biol 1999; 28: 135-145.

Hube B, Monod M, Schofield D A, Brown A J P, Gow N A R. Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. Mol Microbiol 1994; 14: 87-99.

Hube B, Sanglard D, Odds F C, Hess D, Monod M, Schafer W, Brown A J P, Gow N A R. Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. Infect Immun 1997; 65: 3529-3538.

Hube B. Possible role of secreted proteinases in *Candida albicans* infections. Rev Iberoam Micol 1998; 15: 65-68.

Hube B & Naglik J. *Candida albicans* proteinases: resolving the mystery of a gene family. Microbiology 2001; 147: 1997-2005.

Huovinen P, Meri S, Peltola H, Vaara M, Vaheri A, Valtonen V. Mikrobiologia ja infektiosairaudet 2003, kirja I. Kustannus Oy Duodecim.

Indyk J A, Chen Z L, Tsirka S E, Strickland S. Laminin chain expression suggests that laminin-10 is a major isoform in the mouse hippocampus and is degraded by the tissue plasminogen activator/plasmin protease cascade during excitotoxic injury. Neuroscience 2003; 116: 359-371.

Jakab E, Paulsson M, Ascencio F, Ljungh A. Expression of vitronectin and fibronectin-binding by *Candida albicans* yeast cells. APMIS 1993; 101: 187-193.

Janeway C A Jr, Travers P, Walport M, Schlomchik M J. Immunobiology 2001, 5th edition, Garland Publishing, NY, USA.

Jayatilake J A M S, Samaranayake Y H, Cheung L K, Samaranyake L P. Quantitative evaluation of tissue invasion by wild type, hyphal and *SAP* mutants of *Candida albicans*, and non-*albicans Candida* species in reconstituted human oral epithelium. J Oral Pathol Med 2006; 35: 484-491.

Jayatilake J, Samaranayake L P, Lu Q, Jin L J. IL-Ialpha, IL-Ira and IL-8 are differentially induced by *Candida* in experimental oral Candidosis. Oral Dis 2007; 13: 426-433.

Jayatilake J A M S, Samaranayake Y H, Samaranayake L P. A comparative study of *Candidal* invasion in rabbit tongue mucosal explants and reconstituted human oral epithelium. Mycopathologia 2008; 165: 373-380.

Jones JCR, Hopkinson S B, Goldfinger L E. Structure and assembly of hemidesmosomes. BioEssays 1998; 20: 488- 494.

Järvensivu A, Hietanen J, Rautemaa R, Sorsa T, Richardson M. *Candida* yeasts in chronic periodontitis tissues and subgingival microbial biofilms in vivo. Oral Dis 2004; 10: 106-112.

Kaminishi H, Tanaka M, Cho T, Maeda H, Hagihara Y. Activation of the plasma kallikrein-kinin system by *Candida albicans* proteinase. Infect Immun 1990; 58: 2139-2143.

Kaminishi H, Hamatake H, Cho T, Tamaki T, Suenaga N, Fuji T *et al*. Activation of blood clotting factors by microbial proteinases. FEMS Microbiol Lett 1994; 176: 1702-1710.

Kaminishi H, Miyaguchi H, Tamaki T, Suenaga N, Hisamatsu M, Mihashi I *et al*. Degradation of humoral host defence by *Candida albicans* proteinase. Infect Immun 1995; 63: 984-988.

Katayama M, Sanzen N, Funakoshi A, Sekiguchi K. Laminin γ2-chain fragment in the circulation: a prognostic indicator of epithelial tumor invasion. Cancer Res 2003; 63: 222-229.

Katayama M & Sekiguchi K. Laminin-5 in epithelial tumour invasion. J Mol Histol 2004; 35: 277- 286.

Katz J, Sambandam V, Wu J H, Michalek S M, Balkovetz D F. Characterization of *Porphyromonas gingivalis*-induced degradation of epithelial cell junctional complexes. Infect Immun 2000; 68: 1441-1449.

Katz J, Yang Q, Zhang P, Potempa J, Travis J, Michalek S M, Balkovetz D F. Hydrolysis of epithelial junctional proteins by *Porphyromonas gingivalis* gingipains. Infect Immun 2002; 7: 2512-2518.

Kaur R, Domerque R, Zupancic M L, Cormack B P. A yeast by other name: *Candida glabrata* and its interaction with the host. Curr Opin Microbiol 2005; 8: 378-384.

Klotz S A. Fungal adherence to the vascular compartment- a critical step in the pathogenesis of disseminated Candidosis. Clin Infect Dis 1992; 14: 340-347.

Klotz S A. Plasma and extracellular matrix proteins mediate in the fate of *Candida albicans* in the human host. Med Hypotheses 1994; 42: 328-334.

Klotz S A & Smith R L. Gelatin fragments block adherence of *Candida albicans* to extracellular matrix proteins. Microbiology-UK 1995; 141: 2681-2684.

Korting H C, Hube B, Oberbauer S, Januschke E, Hamm G, Albrecht A, Borelli C, Schaller M. Reduced expression of the hyphal-independent *Candida albicans* proteinase genes *SAP1* and *SAP3* in the *efg1* mutant is associated with attenuated virulence during infection of oral infection. J Med Microbiol 2003; 52: 623-632.

Koshikawa N, Giannelli G, Cirulli V, Miyazaki K, Quaranta V. Role of cell surface metalloproteinase MT1-MMP in epithelial cell migration over laminin-5. J Cell Biol 2000; 148: 615-624.

Koshikawa N, Minegishi T, Sharabi A, Quaranta V, Seiki M. Membrane-type matrix metalloproteinase-1 (MT1-MMP) is a processing enzyme for human laminin γ2 chain. J Biol Chem 2005; 280: 88-93.

Krcmery V & Barnes A J. Non-*albicans Candida* spp. causing fungaemia: pathogeneicity and antifungal resistance. J Hosp Infect 2002; 50: 243-260.

Kreidberg J A. Functions of α3β1 integrin. Curr Opin Cell Biol 2000; 12: 548-553.

Laffey S F and Butler G. Phenotype switching affects biofilm formation by *Candida parapsilosis*. Microbiology 2005; 151: 1073-1081.

Laine P, Lindqvist J C, Pyrhönen S O, Teerenhovi L M, Syrjänen S M, Meurman J H. Lesions of the oral mucosa in lymphoma patients receiving sytostatic drugs. Oral Oncol 1993; 29B: 291-294.

Lane S, Birse C, Zhou S, Matson R, Liu H P. DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. J Biol Chem 2001; 276: 48988-48996.

Lermann U and Morschhäuser J. Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. Microbiology 2008; 154: 3281-3295.

Leslie K M, McCann B G, Levell N J. Candidal echtyma gangrenosum with a patient with malnutrition. B J Dermatol 2005; 153: 847-848.

Li L, Redding S, Dongari-Bagtzoglou A. *Candida glabrata*, an emerging oral opportunistic pathogen. J Dent Res 2007; 86: 204-215. (a)

Li L, Kashleva H, Dongari-Bagtzoglou A. Cytotoxic and cytokine-inducing properties of *Candida glabrata* in single and mixed oral infection models. Microb Pathogenesis 2007; 42: 138-147. (b)

Litjens S H M, de Pereda J M, Sonnenberg A. Current insights into the formation and breakdown of hemidesmosomes. Trends Cell Biol 2006; 16: 375-383.

Liu H, Köhler J, Fink G R. Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. Science 1994; 266: 1723-1726.

Lo H. Nonfilamentous *C. albicans* mutants are avirulent. Cell 1997; 90: 939-949

Lopez-Ribot J-L & Chaffin W L. Binding of the extracellular matrix component entactin to *Candida albicans.* Infect Immun 1994; 62: 4564-4571.

Lu Q, Jayatilake J A M S, Samaranayake L P, Jin L J. Hyphal invasion of *Candida albicans* inhibits the expression of human beta-defensins in experimental oral Candidosis. J Invest Dermatol 2006; 126: 2049-2056.

Malic S, Hill nK E, Ralphs J R, Hayes A, Thomas D W, Potts A J, Williams D W. Characterization of *Candida albicans* infection of an in vitro oral epithelial model using confocal laser scanning microscopy. Oral Microbiol Immunol 2007; 22: 188-194.

Marinkovich M P. Lm-332 in squamous-cell carcinoma. Nature 2007; **7:** 370-380.

Matsuo A L, Tersariol I I L, Kobata S I, Travassos L R, Carmona A K, Puccia R. Modulation of the exocellular serine-thiol proteinase activity of *Paracoccidioides brasiliensis* by neutral polysaccharides. Microbes Infect 2005; 8: 84-91.

Mavor A L, Thewes S, Hube B. Systemic fungal infections caused by *Candida* species:epidemiology, infection process and virulence attributes. Current drug targets 2005; 6: 863-874.

McNulty K M, Plianrungsi J, Leigh J E, Mercante D, Fidel Jr P L. Characterization of CD8+ T cells and microenvironment in oral lesions of human immunodeficiency virus-infected persons with oropharyngeal *Candidosis*. Infect Immun 2005; 73: 3659- 3667.

Mendez-Giannini M J S, Soares C P, da Silva J L M, Andreotti P F. Interaction of pathogenic fungi with host cells: molecular and cellular approaches. FEMS Immunol Med Mic 2005; 45: 383-394.

Menzin J, Meyers J L, Friedman M, Perfect J P, Langston A A, Danna R P, Papadopoulos G. Mortality, length of hospitalization, and costs associated with invasive fungal infections in high-risk patients. Am J Health Syst-Pharm 2009; 6: 1711-1717.

Meurman J H, Pajukoski H, Snellman S, Zeiler S, Sulkava R. Oral infections in home-living elderly patients admitted to an acute geriatric ward. J Dent Res 1997; 76: 1271-1276.

Meyer J, Squier C A, Gerson S J (eds). The structure and function of oral mucosa. Pergamon Press, New York, 1984.

Miner J, Lewis R M, Sanes J R. Molecular –cloning of a novel laminin chain, alpha-5, and widespread expression in adult-mouse tissues. J Biol Chem 1995; 270: 28523-28526.

Miner J H, Yurchenco P D. Laminin functions in tissue morphogenesis. Annu Rev Cell Dev Biol 2004; 20: 255-284.

Miner J H. Laminins and their roles in mammals. Microsc Res Techniq 2008; 71: 349-356.

Miyazaki K. Laminin-5 (laminin-332): unique biological activity and role in tumor growth and invasion. Cancer Sci 2005; 97: 91-98.

Monod M, Capoccia S, Lechenne B, Zaugg C, Holdom M, Jousson O. Secreted proteases from pathogenic fungi. Int J Med Microbiol 2002; 292: 405-419.

Monteagudo C, Viudes A, Lazzell A, Martinez J P, Lopez-Ribot J L. Tissue invasiveness and non-acidic pH in human Candidosis correlate with “in vivo” expression by *Candida albicans* of the carbohydrate epitope recognized by new monoclonal antibody 1H4. J Clin Pathol 2004; 57: 598-603.

Moon J L, Shaw L N, Mayo J A, Potempa J, Travis J. Isolation and properties of extracellular proteinases of *Penicillium marneffei*.J Biol Chem 2006; 387: 985-993.

Morschhäuser J, Virkola R, Korhonen T K, Hacker J. Degradation of human subendothelial extracellular matrix by proteinase-secreting *Candida albicans*. FEMS Microbiol Lett 1997; 153: 349-55.

Mäkelä M, Salo T, Larjava H. MMP-9 from TNFα-stimulated keratinocytes binds to cell membranes and type I collagen: a cause for extended matrix degradation in inflammation? Biochem Bioph Res Co 1998; 253: 325-335.

Naglik J, Albrecht A, Bader O, Hube B. *Candida albicans* proteinases and host/pathogen interactions. Cell Microbiol 2004; 6: 915-926.

Naglik J R, Moyes D, Makwana J, Kanzaria P, Tsichlaki E, Weindl G, Tappuni A R, Rodgers C A, Woodman A J, Challacombe S J, Schaller M, Hube B. Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal Candidosis. Microbiology 2008; 154: 3266-3280.

Nanci A and Bosshardt D D. Structure of periodontal tissues in health and disease. Periodontol 2000 2006; 40**:** 11-28.

Nguyen H, Peacock Jr J E, Morris A J, Tanner D C, Nguyen M L, Snydman D R, Wagener M M, Rinaldi M G, Yu V L. The changing face of candemia: emergence of non- *Candida albicans* species and antifungal resistance. Am J Med 1996; 100: 617-623.

Nishiuchi R, Takagi J, Hayashi M, Ido H, Yagi Y, Sanzen N, Tsuji T, Yamada M, Sekiguchi K. Ligand-binding specifities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant α3β1, α6β1, α7β1 and α6β4 integrins. Matrix Biol 2006; 25: 189-197.

Odenthal U, Haehn S, Tunggal P, Merkl B, Schomburg D, Frie C, Paulsson C, Smyth N. Molecular analysis of laminin N-terminal domains mediating self-interactions. J Biol Chem 2004; 279: 44504-44512.

Oksanen J, Sorokin L M, Virtanen I, Hormia M. The junctional epithelium around murine teeth differs from gingival epithelium in its basement membrane composition. J Dent Res 2001; 80: 2093-2097.

Oliveira Crema V, Antunes Teixeira Vde P, Reis M G, Marinho Ede O, Dos Santos V M. Morphometric study of desmosimes from oral squamous cell carcinoma. Ultrastruct Pathol 2005; 29: 349-355.

Pakkala T, Virtanen I, Oksanen J, Jones J C R, Hormia M. Functions of laminins and laminin-binding integrins in gingival epithelial cell adhesion. J Periodontol 2002; 73: 709-718.

Pasqualotto A C, Nedel W L, Machado T S, Severo L C. Risk factors and outcome for nosocomial breakthrough candidaemia. J Infection 2006; 52: 216-222.

Patarroyo M, Tryggvason K, Virtanen I. Laminin isoforms in tumor invasion, angiogenesis and metastasis. Semin Cancer Biol 2002; 12: 197-207.

Penn C and Klotz S A. Binding of plasma fibronectin to *Candida albicans* occurs through the cell-binding domain. Microb Pathogenesis 1994; 17: 387-393.

Pendrak M L, Yan S S, Roberts D D. Hemoglobin regulates expression of an activator of mating-type locus alpha genes in *Candida albicans*. Eukaryotic Cell 2004; 3: 764-775.

Pfaller M A, Jones R N, Doern G V, Sader H S, Hollis R J, Messer S A, International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the Unites States, Canada, and South America for the SENTRY Program. J Clin Microbiol 1998; 36: 1886-1889.

Phan Q T, Myers C L, Fu Y, Sheppard D C, Yeaman M R, Welch W H, Ibrahim A S, Edwards Jr J E, Filler S G. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. PLoS Biology 2007; 5 e64: 0543-0557.

Pirilä E, Sharabi A, Salo T, Quaranta V, Tu H, Heljasvaara R, Koshikawa N, Sorsa T, Maisi P. Matrix metalloproteinases process the laminin-5 γ2-chain and regulate epithelial cell migration. Biochem Bioph Res Co 2003; 303: 1012-1017.

Puccia R, Carmona A K, Gesztesi J L, Juliano L, Travassos L R. Exocellular proteolytic activity of *Paracoccidioides brasiliensis*: Cleavage of components associated with the basement membrane. Med Mycol 1998; 36: 345-348.

Pulimood S, Ganesan L, Alangaden G, Chandrasekar P. Polymicrobial candidemia. Diag Micr Infect Dis 2002; 44: 353-357.

Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. Our current understanding of fungal biofilms. Crit Revs Microbiol 2009; 35: 340-355.

Rauceo J M, De Armond R, Otoo H, Kahn P C, Klotz S A, Gaur N K, Lipke P N. Threonine-rich repeats increase fibronectin binding in the *Candida albicans* adhesin Als5p. Eukaryotic cell 2006; 5: 1664-1673.

Rodier M-H, El Moudni B, Kauffmann-Lacroix C, Daniault G, Jacquemin J-L. A *Candida albicans* metallopeptidase degrades constitutive proteins of extracellular matrix. FEMS Microbiol Lett. 1999; 177: 205-10.

Rodrigues M L, dos Reis F C G, Puccia R, Travassos L R, Alviano C S. Cleavage of human fibronectin and other basement membrane-associated proteins by a *Cryptococcus neoformans* serine proteinase. Microb Pathogenesis 2003; 34: 65-71.

Rollenhagen C, Wöllert T, Langford G M, Sundstrom P. Stimulation of cell motility and expression of late markers of differentiation in human oral keratinocytes by *Candida albicans*. Cellular microbiology 2009; 11: 946-966.

Rüchel R, Böning B, Borg M. Characterization of a secretory proteinase of *Candida parapsilosis* and evidence for the absence of the enzyme during infection. Infect Immun 1986; 53: 411-419.

Salo T, Lyons J G, Rahemtulla F, Birkedal-Hansen H, Larjava H. Transforming growth factor –β1 up-regulates type IV collagenase expression in cultured human keratinocytes. J Biol Chem 1991; 266: 11436-11441.

Sanglard D, Hube B, Monod M, Odds F C, Gow N AS R. A triple deletion of the secreted aspartyl proteinase genes SAP4, SAP5, and SAP6 of *Candida albicans* causes attenuated virulence. Infect Immun 1997; 65: 3539-3546.

Sapone A, de Magistris L, Pietzak M, Clemente M G, Tripathi A, Cucca F, Lampis R, Kryszak D, Carteni M, Generoso M, Iafusco D, Prisco F, Laghi F, Riegler G, Carratu R, Counts D, Fasano A. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. Diabetes 2006; 55: 1443-1449.

Saville S P, Lazzell A L, Monteagudo C, Lopez-Ribot J L. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryotic cell 2003; 2: 1053-1060.

Saville S P, Thomas D P, Lopez Ribot J L. A role for Efg1p in *Candida albicans* interactions with extracellular matrices. FEMS Microbiol Lett 2006; 256: 151-158.

Schaller M, Korting H C, Schäfer W, Bastert J, Chen W, Hube B. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral Candidosis. Mol Microbiol 1999; 34: 169-180. (a)

Schaller M, Hube B, Ollert M W, Schäfer W, Von Zepelin-Borg M, Thoma-Greber E, Korting H C. In vivo expression and localization of *Candida albicans* secreted aspartyl proteinases during oral Candidosis in HIV-infected patients. J Invest Dermatol 1999; 112: 383-386. (b)

Schaller M, Borelli C, Korting H C, Hube B. Hydrolytic enzymes as virulence factors of *Candida albicans*. Mycoses 2005; 48: 365-377.

Schaller M, Zakikhany K, Naglik J R, Weindl G, Hube B. Models of oral and vaginal Candidosis based on in vitro reconstituted human epithelia. Nature protocols 2006; 1: 2767-2773.

Schaller M, Zakikhany K, Weindl G, Borelli C, Korting H C, Hube B. Characterization of the interaction between *Candida albicans* and host cells in vitro model using reconstituted human skin and mucosa. Hautarzt 2008; 59: 964-970.

Schmidt P, Walker J, Selway L, Stead D, Yin Z, Enjalbert B, Weig M, Brown A J P. Proteomic analysis of the pH response in the fungal pathogen *Candida glabrata.* Proteomics 2008; 8: 534-544.

Slutsky B, Buffo J, Soll D R. High frequency switching of colony morphology in *Candida albicans*. Science 1985; 230: 666-669.

Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll D R. White-opaque transition: a second high-frequency switching system in *Candida albicans.* J Bacteriol 1987; 169: 189-197.

Snoek-van Beurden P A M & Von den Hoff J W. Zymographic technique for the analysis of matrix metalloproteinases and their inhibitors. Biotechniques 2005; 38: 73-83.

Soll D R. The ins and outs of DNA fingerprinting the infectious fungi. Clin Microbiol Rev 2000; 13: 332-370.

Sorokin L M, Pausch F, Frieser M, Kroger S, Ohage E, Deutzmann R. Developmental regulation of the laminin alpha 5 chain suggests a role in epithelial and endothelial cell maturation. Dev Biol ; 189: 285- 300.

Sorsa T, Salo T, Koivunen E, Tyynelä J, Konttinen Y T, Bergmann U, Tuuttila A, Niemi E, Teronen O, Heikkilä P, Tschesche H, Leinonen J, Osman S, Stenman U-H: Activation of type IV procollagenases by human tumor-associated trypsin-2. J Biol Chem 1997; 34: 21067-21074.

Sorsa T, Tjäderhane L, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. Oral Dis 2004; 10**:** 311-318.

Sorsa T, Tjäderhane L, Konttinen Y T, Lauhio A, Salo T, Lee H-S, Golub L M, Brown D L, Mäntylä P. Matrix metalloproteinases: Contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. Ann Med. 2006; 38**:** 306-321.

Stoldt V R, Sonnebom A; Leuker C E, Ernst J F. Efg1p, an essentila regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO Journal 1997; 16: 1982-1991.

Sullivan D J, Westerneng T J, Haynes K A, Bennet D E, Coleman D C. *Candida dubliniensis* sp nov.: phenotypic and molecular characterization of a novel species associated with oral Candidosis in HIV-infected individuals. Microbiology 1995; 141: 1507-1521.

Sullivan D, Haynes K, Bille J, Boerlin P, Rodero L, Lloyd S, Henman M, Coleman D. Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. J Clin Microbiol 1997; 35: 960-964.

Sweet S P, Ballsdon A E, Harris J C, Roberts G J, Challacombe S J. Impaired secretory immunity in dystrophic epidermolysis bullosa. Oral Microbiol Immunol 1999; 14: 316-320.

Tamkun J W, Hynes R O. Plasma fibronectin is synthesized and secreted by hepatocytes. J Biol Chem 1983; 7: 4641-4647.

Tani T, Lehto V-P, Virtanen I. Expression of laminins 1 and 10 in carcinoma cells and comparison of their roles in cell adhesion. Exp Cell Res. 1999; 248: 115-121.

Thewes S, Reed H-K, Grosse-Siestrup C, Groneberg D A, Meissler M, Schaller M, Hube B. Haemoperfused liver as an ex vivo model for organ invasion of *Candida albicans*. J Med Mycol 2007; 56: 266-270.

Thorup A K, Dabelsteen E, Schou S, Gil S G, Carter W G, Reibel J. Differential expression of integrins and laminin-5 in normal oral epithelia. APMIS 1997; 105: 519-530.

Timpl R & Rohde H. Laminin- a glycoprotein from basement membranes. J Biol Chem 1979; 254: 9933-9937.

Tronchin G, Bouchara J P, Larcher G, Lissitzky J C, Chabasse D. Interaction between aspergillus fumigatus and basement membrane laminin: Binding and substrate degradation. Biol Cell 1993; 77: 201-208.

Tsubota Y, Mizushima H, Hirosaki T, Higashi S, Yasumitsu H, Miyazaki K. Isolation and activity of proteolytic fragment of laminin-5 alpha 3 chain. Biochem Bioph Res Co 2000; 278: 614-620.

Uittamo J, Siikala E, Kaihovaara P, Salaspuro M, Rautemaa R. Chronic Candidosis and oral cancer in APECED- patients: production of carcinogenic acetaldehyde from glucose and ethanol by *Candida albicans*. Int J Cancer 2008; 124: 754-756.

Uitto V-J, Overall C M, McCulloch C. Proteolytic host cell enzymes in gingival crevice fluid Periodontol 2000. 2003; 31**:** 77- 104.

Van den Steen P E, Dubois B, Nelissen I, Rudd P M, Dwek R A, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol 2002; 37**:** 375-536.

van Roy F & Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci 2008; 65: 3756-3788.

Vartio T, Laitinen L, Närvänen O, Cutolo M, Thornell L-E, Zardi L, Virtanen I. Differential expression of the ED sequence –containing form of cellular fibronectin in embryonic and adult human tissues. J Cell Sci 1987; 88: 419-430.

Vidotto V, Accattatis G, Zhang Q, Campanini G, Aoki S. Glucose influence on germ tube production in *Candida albicans.* Mycopathologia 1996; 133: 143-147.

Villar C C, Kashleva H, Nobile C J, Mitchell A P, Dongari-Bagtzoglou A. Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. Infect Immun 2007; 75: 2126-2135.

Wagner T, Borg-von Zepelin M, Ruchel R. pH-dependent denaturation of extracellular aspartic proteinases from *Candida* species. J Med Vet Mycol 1995; 33: 275-278.

Walsh P, Häkkinen L, Pernu H, Knuuttila M, Larjava H. Expression of fibronectin-binding integrins in gingival epithelium in drug-induced gingival overgrowth. J Periodontal Res 2007; 42: 144-151.

Wang W, Uzzau S, Goldblum S E, Fasano A. Human zonulin, a potent modulator of intestinal tight junctions. J Cell Sci 2000; 113: 4435-4440.

Wang J, Ohshima T, Yasunari U, Namikoshi S, Yoshihara A, Miyazaki H, Maeda N. The carriage of *Candida* species on the dorasal surface of the tongue: the correlation with the dental, periodontal and prosthetic status in elderly subjects. Gerodontology 2006; 23: 157-163.

Weig M, Jänsch L, Groß U, De Koster C G, Klis F M, De Groot P W J. Systematic identification in silico of covalently bound cell wall proteins and analysis of protein-polysaccharide linkages of the human pathogen *Candida glabrata*. Microbiology 2004; 150: 3129-3144.

Weindl G, Naglik J R, Kaesler S, Biedermann T, Hube B, Korting H C, Schaller M. Human epithelial cells establish direct antifungal defence through TLR4-mediated signaling. J Clin Invest 2007; 117: 3664-3672.

White T and Agabian N. *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J Bacteriol 1995; 177: 5215-5221.

Wosniok I, Hornbach A, Schmitt C, Frosch M, Einsele H, Hube B, Loffler J, Kurzai O. Induction of ERK-kinase signaling triggers morphotype- specific killing of *Candida albicans* filaments by human neutrophils. Cell Microbiol 2008; 10: 807-820.

Yan S, Rodrigues R G, Cahn-Hidalgo D, Walsh T J, Roberts D D. Hemoglobin induces binding of several extracellular matrix proteins to *Candida albicans.* J Biol Chem 1998; 5638-5644.

Yang Y L. Virulence factors of *Candida* species. J Microbiol Immunol Infect 2003; 36: 223-228.

Yang Y L, Wang C V, Chen C T, Wang M H, Hsiao C F, Lo H J. Non-lethal *Candida albicans* cph1/cph1 efg1/efg1 mutant partially protects mice from systemic infections by lethal wild-type cells. Mycol Res 2009; 113: 388-390.

Yoshiyuki A. Role of 1,3-beta-D-glucan receptor Dectin-1 in fungal infection and activation of innate and anti-tumor immunity. Trends Glykosci Glyc 2007; 19: 195-207.

Zakikhany K, Naglik J, Schmidt-Westhausen A, Holland G, Schaller M, Hube B. In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. Cell Microbiol 2007; 9: 2938-2954.

Zaugg C, Borg-von Zepelin M, Reichard U, Sanglard DMonod M. Secreted aspartic proteinase family of *Candida tropicalis*. Infect Immun 2001; 69: 405-412.

Zepelin M Borg-von, Beggah S, Boggian K, Monod M. The expression of secretesd aspartyl proteinases Sap4 to Sap6 from Candida albicans in murine macrophages. Mol Microbiol 1998; 28: 543- 554.

Zink S, Nass T, Rosen P, Ernst J F. Migration of the fungal pathogen *Candida albicans* across endothelial monolayers. Infect Immun 1996; 64: 5085-5091.