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Characterisation of *Candida albicans* populations associated with chronic hyperplastic candidosis (*Candida* leukoplakia) and non-*Candida* leukoplakia lesions in patients presenting with oral leukoplakic lesions in Ireland

A thesis submitted to the University of Dublin, Trinity College, in fulfilment of the requirements for the degree Doctor of Philosophy by

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Declaration

I herby declare that this thesis has not previously been submitted for a degree at this or any other University and that it represents my own work, except where duly acknowledged in the text. I agree that this thesis maybe lent or copied at the discretion of the librarian, Trinity College Dublin.

Mohammed H. Abdulrahim

This thesis is dedicated to the memory of my parents.

Summary

Oral squamous cell carcinoma is a significant global health problem, affecting about a half of a million people worldwide annually. Oral leukoplakic lesions have been reported to have significantly increased potential for malignant change. Leukoplakias infected with *Candida* (chronic hyperplastic candidosis, CHC) are regarded as a high-risk lesions compared to uninfected, non-candidal leukoplakia (NCL). Whether *Candida* is the cause of the hyperkeratosis and dysplasia seen in these lesions, or only a superimposed infection of a pre-existent leukoplakic lesion has been open to debate. The main objectives of the present study were to investigate the role of *Candida* species in the aetiopathogenesis of biopsy-proven CHC, to characterise oral *Candida* species and their relative abundance isolated from CHC lesions compared to NCL, to investigate the cellular adhesion properties of these isolates and their genetic population structure compared to commensal isolates from healthy carriers. Seventy eight patients (designated the OL group) were enrolled in the study, 31 with biopsy-proven CHC and 47 with NCL. Clinical and histopathological data were gathered on the patients along with oral rinse and lesional swab samples to correlate clinical findings with *Candida* culture findings.

CHC was more common in patients over 50 years of age and more prevalent in females (2:3, M:F) compared to previous reports. *Candida* species were recovered from oral rinse and lesional swab samples of both CHC and NCL lesions with highly significant densities from swab samples from CHC ($P<0.001$). *Candida albicans* was the predominant *Candida* species isolated. Other non-*C. albicans* species were isolated from CHC and NCL, but rarely and in low abundance with insufficient consistency to play a significant role in CHC pathogenesis. *Candida* isolates were recovered from oral rinse samples in 55/78 (70.5%) of the OL patients; (24/31 (77.4%) CHC and 33/47 (70%) NCL). *Candida* was recovered from 48/78 (61.5%) lesional swabs from the OL cohort; (27/31 (87.1%) CHC and 21/47 (44.7%) NCL). A predisposing factor for CHC was upper denture wear and although smoking and alcohol use were not found to be predisposing for candidal carriage, heavy smokers had increased oral candidal loads. Unsurprisingly, lesional swabs had better discriminatory capacity than the rinse samples for CHC, but also could distinguish between CHC and NCL, with a high degree of sensitivity at various *Candida* colony forming unit (cfu) limits, despite *Candida* being isolated from almost half of the NCL lesions. There was no excess of degrees of dysplasia (none, mild, moderate and severe) in the NCL and CHC groups. Significant lesional *Candida* density ($\geq$100 cfu) correlated with moderate and severe dysplasia grading in the CHC group, compared to the NCL group, ($P<0.001$), supporting the concept that *Candida* induces dysplasia in these lesions. All 10 CHC lesions exhibiting severe dysplasia had significant candidal density scores (all $\geq$150

cfu). Two CHC patients developed oral cancer in a short follow-up period, compared to none in the NCL group, confirming that CHC is a high-risk lesion.

Since *C. albicans* was ubiquitously associated with CHC the relative adherence properties of *C. albicans* isolates recovered from oral leukoplakic lesions to human buccal epithelial cells (BECs) compared to oral and disseminated candidiasis reference strains and comparative adherence to both human normal (NOK) and dysplastic (DOK) oral keratinocytes grown in cell culture monolayers, was investigated. The population structure of isolates from CHC, NCL and from volunteer healthy oral carriers (HV) was investigated using multilocus sequencing typing (MLST) and by ABC genotyping. The adherence properties of 18 isolates of *C. albicans* recovered from OL lesions was compared together with the 132A (oral) and SC5314 (systemic) reference strains. The relative adherence was variable with a wide range of adherence capacity. Seven of CHC isolates and one NCL isolate were significantly more adherent than the oral reference strain 132A ($P<0.05$). In contrast, only one CHC isolate (CL109) was significantly more adherent than SC5314. No significant difference was noted of the adherence properties of *C. albicans* to both cultured NOK and DOK cells.

MLST analysis and ABC genotyping of 72 *C. albicans* isolates (CHC, n=20; NCL, n=15; HV, n=35; 132A and SC5314) was undertaken. Fifty-six (77.8%) isolates (14/20 (70%) CHC, 11/15 (73.3%) NCL and 31/37 (83.8%) HV), yielded a novel allelic profile or diploid sequence type (DST), but all of the isolates investigated were assigned to known MLST clades and 25/72 (34.7%) belonged to clade 1 (including 14 from OL patients), the most common MLST clade worldwide, 13/72 (18%) belonged to clade 2 and 10/72 (13.9%) belonged to clade 4. ABC genotyping revealed that the majority belonged to genotype A (50/72 (69.4%), followed by genotype C (14/72) (19.4%) and genotype B 8/72 (11.1%). However, significant enrichment of genotype C was observed among the CHC *C. albicans* isolates (8/20, 40%) ($P\leq0.02$). Previous studies showed that genoytpe C isolates are a minority (~10%) among *C. albicans* isolates.

The ubiquitous presence of *C. albicans* on the CHC lesions, often in high density, and the correlation with severe dysplasia in CHC but not NCL, strongly implicates this species in the aetiology of CHC. The variability in the *in vitro* adherence of *C. albicans* isolates recovered from oral leukoplakic lesions is likely due to the effect of expressed epithelial cell receptors and phenotypic and genetic regulation of the yeast. The significant enrichment of *C. albicans* genotype C in CHC, might indicate the CHC isolates are distinct from isolates not associated with CHC, however, this was not supported in the MLST analysis as genotype C isolates were evenly distributed among a range of MLST clades. Further investigation with larger numbers of isolates recovered from different sources (lesional and non-lesional and also different geographic locations) is required to assess the significance of this finding.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALS	Agglutinin-like sequence
APECED	Autoimmune polyendocrinopathy, candidosis and ectodermal dystrophy
BECs	Human buccal epithelial cells
bp	Base pair
BURST	Based upon related sequence types
cfu	Colony forming unit
CHC	Chronic hyperplastic candidosis
CIN	Cervical intraepithelial neoplasia
cpd	Cigarette per day
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetracetic acid
e.g.	For example
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
et al.	And others
FCS	Foetal calf serum
Fig	Figure
g	Gramme
g	Gravitational force
h	Hour (s)
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HNSCC	Head and neck squamous cell
HPV	Human papilloma viruses
HV	Human volunteers

HWP1	Hyphal wall protein
Inc.	Incorporated
in situ	In place
in vitro	Performing a given procedure in a controlled environment outside of a living organism – artificial environment outside the living organism – usually undertaken in a laboratory
IU	International unit
kb	Kilobase pair
KGM	Keratinocytes growth medium
l	Litre
LOH	Loss of heterozygosity
M	Molar
mg	Milligram
min	Minute (s)
ml	Millilitre
mV	Megavolt
n	Number
NCL	Non-candidal leukoplakia
ng	Nanogram
OL	Oral leukoplakia
PAS	Periodic acid Schiff stains
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Second (s)
SCC	Squamous cell carcinoma
TBE	Tris-borate EDTA

Tris	Tris (hydroxymethyl) aminoethane
UK	United Kingdom
UPGMA	Unweighted pair group methods according to arithmetic average
USA	United States of America
u/w	Units of alcohol per week
v/v%	Percentage of volume in volume
WHO	World Health Organization
w/v%	Percentage of weight in volume
YEPD	Yeast extract peptone dextrose
µg	Microgram
µl	Microliter
°C	Degree Celsius or centigrade
<	Less than
>	Greater than
~	Approximately
≤	Less than or equal to

Chapter 1

Introduction

1.1. Introduction

Oral chronic hyperplastic candidosis (CHC) - also called chronic hyperplastic candidiasis or candidal leukoplakia - is a benign lesion of the oral mucosa which is recognised as having significant malignant transformation potential (Cawson, 1966; Cawson and Lehner, 1968; Williamson, 1969; Eyre and Nally, 1971). Head and neck cancer is a significant problem with a global annual incidence of about half a million cases (Warnakulasuriya, 2009). The role of yeast species of the genus *Candida* in chronic hyperplastic candidosis and oral cancer development is far from clear and the scientific literature on this subject area is relatively sparse. There are less than 40 scientific research papers in the literature on CHC specifically (although there is an extensive literature addressing oral leukoplakia), which is surprising, considering that the entity has been recognised for nearly 50 years (Lehner, 1964) and the serious implications of the diagnosis and the potentially catastrophic, life-threatening outcome. The purpose of the present study was to characterise the *Candida* species and their relative abundance associated with CHC lesions and other types of oral leukoplakia lesions and to investigate the role of oral *Candida* species in CHC lesions.

1.2. Oral Leukoplakias

1.2.1. Definitions

Oral leukoplakia (oral mucosal white lesions or patches), which may precede oral cancer, is the most common premalignant oral mucosal lesion. It was first described by the Hungarian dermatologist Ernst Schwimmer in 1877 and since then, the term "leukoplakia" has been used for different types of white lesions of the mucous membranes.

In 1978 The World Health Organisation (WHO) (Kramer *et al.*, 1978) first defined oral leukoplakia as "a white patch or plaque that could not be characterised clinically or pathologically as any other disease". Therefore, conditions such as lichen planus, candidiasis, and white sponge nevus, for example, were excluded. However, this definition emphasised clinical diagnosis and had no reference to the importance of the variable histopathological features, particularly dysplasia, of oral leukoplakia. At an international conference in 1983, Axéll *et al.* (1984) suggested that the term *leukoplakia* should be avoided where there was a known aetiology, except in cases where tobacco

was the known causative factor. This new definition proposed that "leukoplakia is a whitish patch or plaque that cannot be characterised clinically or pathologically as any other disease, and is not associated with any physical or chemical causative agent, except the use of tobacco". Axéll *et al.* (1996) proposed a further definition of oral leukoplakia in an attempt to overcome the practical difficulties that could be encountered in reaching an accurate diagnosis of oral leukoplakia. These difficulties include the subjective nature of the degree of whitening of the lesion, "leukoedema", the need for histopathological diagnosis (which is not practical in epidemiological studies), borderline lesions where even after histopathological examination there is still uncertainty in the diagnosis and the tobacco-induced change in the oral cavity, stomatitis nicotina or smoker's palate. The latter, although exclusively induced by smoking is not considered as a true leukoplakia and has almost no malignant potential except in lesions developed in reverse smokers (Gupta *et al.*, 1984). Based on this, Axéll's group suggested the following revised definition, that leukoplakia is "a predominantly white lesion of the oral mucosa that cannot be characterised as any other definable lesion".

At a workshop coordinated by the WHO in 2006, the original 1978 WHO definition was further refined to "the term leukoplakia should be used to recognise white plaques of questionable risk having excluded other known diseases or disorders that carry no increased risk for cancer" (Warnakulasuriya *et al.*, 2007). Based on this definition, the diagnosis of oral leukoplakia is by exclusion of other oral white patches that include, simple keratoses (such as frictional keratosis), leukoplakias of possible infective origin (e.g. syphilitic, candidal and hairy leukoplakia, (see section 1.2.6), papillomavirus lesions, lichen planus and lichen sclerosus et atrophicus, lupus erythematosus, dermatomyositis, oral submucous fibrosis, frank carcinomas, drug burns, skin grafts, white sponge naevus, leukoedema, pachyonychia congenita, tylosis, hereditary benign intra epithelial dyskeratosis, dyskeratosis follicularis and dyskeratosis congenita/Fanconi anaemia spectrum. Although all of these latter conditions, except frictional keratosis and possibly stomatitis nicotina, carry an increased risk for malignant development (however high or low), they are defined clinical entities, diagnosed by clinical phenotype or special tests such as histopathology, serology or genetic testing, or in the case of carcinoma, are already a malignancy. Thus, "pure" leukoplakia becomes a diagnosis of exclusion. Although this will be useful for future studies of malignant potential of true leukoplakia, it will confound previous studies,

particularly epidemiological studies, since sophisticated laboratory-based diagnosis is not available in field studies of oral white lesions on which there is an extensive historical literature (Mehta *et al.*, 1969 and 1972; Axéll, 1987; Bánóczy and Sugár, 1972) often on exceptionally large cohorts of patients (Pindborg, 1965; Gupta *et al.*, 1980; Bouquot and Gundlach, 1986). Therefore, the terms "candidal leukoplakia" and "syphilitic leukoplakia" were misnomers, since the inference is that the infection is causative and the term leukoplakia infers that the lesion is idiopathic. However, they have historically been included in the leukoplakia category, since it is unclear whether "syphilitic leukoplakia" was an idiopathic leukoplakia in a person who happened to be infected with syphilis or whether the leukoplakia was a manifestation of the underlying syphilitic condition. Equally, candidal leukoplakia, although reported to have a greater malignant potential than "idiopathic" leukoplakia and may represent a specific entity caused by invasive candiosis, or might also be a pre-existing leukoplakic patch which has been infected with *Candida.*

Under the current definition (WHO, 2007) CHC/candidal leukoplakia is undoubtedly a white lesion of reported questionable risk for cancer development, which may, or may not be *caused* by *Candida,* and therefore should be regarded not only as a leukoplakia, but also as a specific clinical entity. The "questionable risk" for cancer development is reported to be higher than that for "idiopathic" leukoplakia by some orders of magnititude (Cawson, 1966; Eyre and Nally, 1971). Indeed, most leukoplakias are in fact tobacco-induced keratoses, and it could be argued that the expression of keratosis represents a reaction to smoking by the oral mucosa, which is exhibiting a self-protective reaction to the chronic irritant effect of tobacco smoke. Clinicians have long been aware that those hyperkeratotic oral mucosal white patches, which exhibit dysplasia on histopathology in *non-smokers,* and without other identifiable cause, appear to be higher risk lesions for cancer development than leukoplakia in smokers, although this is not well documented in the world literature (Kramer *et al.*, 1970; Bouquot, 1987).

1.2.2. Chronic Hyperplastic Candidosis

Lehner (1964) described chronic oral *Candida* infection presenting in the form of leukoplakia and introduced the term candidal leukoplakia. The association of *Candida* species with leukoplakia was also reported by Cernea *et al.* (1965) and Jepsen and Winther (1965). More recently the term chronic hyperplastic candidosis appears to be

preferred by pathologists after there was confusion between the oral lesion and variants of chronic mucocutaneous candidosis with histopathologically identical oral white patches and also skin lesions and lesions at other mucosal sites such as the vagina, also described as chronic hyperplastic candidosis. One of the differences between CHC in the oral cavity and the mucocutaneous form is that the onset of the former occurs in adulthood, while the latter seen usually in childhood secondary to subtle inherited immune defects or endocrinopathy (see section 1.3.2).

Oral CHC manifests as white raised plaques that cannot be removed by rubbing, mostly in the commissural areas of the oral mucous membranes. An intermediate form, between acute pseudomembranous candidosis (which can be removed by rubbing) and established CHC may exist (Cawson, 1969). The palate, buccal mucosa and tongue may also be affected, although less frequently (Williamson, 1969).

The term chronic hyperplastic candidosis will be used preferentially throughout this thesis and used synonymously and interchangeably with the terms chronic hyperplastic candidiasis and candidal leukoplakia, found in the literature.

1.2.3. Leukoplakia in other Sites

Leukoplakia can also affect vulval and uterine cervical mucosae, however the term leukoplakia has generally been discontinued in gynaecology and lesions are now classified based on their histopathological features. Cervical white lesions can be benign hyperkeratosis or hyperparakeratosis and can be potentially premalignant and termed cervical intraepithelial neoplasia (CIN). CIN or cervical dysplasia is mostly caused by chronic infection with human papilloma viruses (HPVs) 16 or 18. CIN is graded into 3 categories: CIN I mild (low grade) dysplasia, CIN II moderate dysplasia and CIN III severe dysplasia. Grades II and III are considered as high grade CIN and have a higher risk of malignant transformation. Unlike oral lesions, where degree of dysplasia (unfortunately) does not reliably predict malignant progression (Holmstrup, 2006), the aetiopathogenesis of cervical carcinoma is less complicated. In cervical cancer, there is a close association with persistent papillomavirus infection and HPV-associated molecular events involving papilomavirus E6 and E7 gene expression, and how these proteins interact with DNA replication events and p53 and Rbp has been elucidated (Mammas *et al.*, 2008). HPV-E6 down regulates p53 activity by promoting its degradation through an E6-associated protein (Dalal *et al.*, 1996), while interaction of HPV-E7 with Rb leads to disruption of growth-suppression activity of Rbp (Strati and

Lambert, 2007). Recent evidence indicating that HPV vaccination prevents cervical cancer supports these findings (No *et al.*, 2011). Furthermore, the cervical epithelium is not subjected to a daily challenge from dietary and habit-related carcinogens such as those found in alcohol, tobacco smoke and food.

Vulval white lesions are classified based on histopathological findings into two groups depending on the presence or absence of epithelial atypia to non-neoplastic epithelial disorders and intraepithelial neoplasia. The non-neoplastic epithelial group is further divided into 3 main categories, (i) squamous cell hyperplasia, (ii) lichen sclerosus (iii) and other dermatoses e.g. Paget's disease.

Vulval intraepithelial neoplastic (VIN) lesions can be subdivided into undifferentiated (classic form) or differentiated, the former lesions are highly associated with HPV type 16 and 18 with high similarity to CIN (McCluggage, 2009). As previously stated, unlike cervical dysplasia, categorising oral dysplasia into either low or high risk according to severity, is a not a consistent predictor for malignant transformation of the oral dysplastic lesion. Oral dysplastic lesions, irrespective of grade, do not necessarily progress into malignancy and some, even severely dysplastic lesions, may even regress (Bánóczy, 1977; Pindborg *et al.*, 1977; Gupta *et al.*, 1980; Holmstrup, 2006).

1.2.4. Epidemiology of Oral Leukoplakia

The prevalence of oral leukoplakia is widely variable in different populations. In India, Mehta *et al.* (1969 and 1972) recorded a range between 0.2% - 4.9% in the population aged over 15 years of age. Axéll (1987) reported a prevalence of 3.6% in 20,333 adult Swedes, while in Hungary, Bánóczy and Sugár (1972) reported a range of between 0.6% - 3.6%. This variability in the prevalence of oral leukoplakia can be attributed to several factors including the criteria used for diagnosing oral leukoplakia, and variations in the sampling technique, which may under or over estimate the true representation in the population. Petti (2003) in a systematic review aimed to estimate pooled global leukoplakia prevalence using inverse weighting and random effect methods estimated a global prevalence rate of 1.49% and this is now the generally accepted crude population prevalence. Gupta *et al.* (1980) followed up over 30,000 individuals for 10 years and reported an annual incidence rate ranging from 1.1 - 2.4 per 1000 in men and 0.2 to 0.03 in women in the same geographical areas of India.

The sex distribution of oral leukoplakia varies widely from one study to another and this variation is generally attributed to differences in tobacco habits. In a study conducted by Waldron and Shafer (1975), an increasing frequency of oral leukoplakia in women was reported during the period from 1961 to 1975, with the suggestion that this may reflect an increasing smoking habit among women. Men were more affected than women in a ratio of 4:1 to 85:1 in different geographical regions of India (Gupta *et al.*, 1980), 6:1 to 20:1 in Sweden (Axéll *et al.*, 1976 and 1987) and 3:1 in Hungary (Bánóczy and Rigo, 1991). The age of onset of oral leukoplakia ranges from 30-80 years of age. Most of the patients are more than 50 years old (Arendorf *et al.*, 1983).

1.2.5. Epidemiology of Chronic Hyperplastic Candidosis

The epidemiological data on CHC is very limited. CHC prevalence is usually linked to that of oral leukoplakia. Although between 7-50% of oral leukoplakia lesions are "infected" with *Candida* (Roed Petersen *et al.*, 1970; Lehner, 1971; Daftary *et al.*, 1972; Krogh *et al.*, 1987), in that *Candida* can be isolated from the leukoplakic lesions, only about 10% fulfil the clinical and histological criteria of Cawson and Lehner (1968) and Arendorf *et al.* (1983), namely, demonstration of hyphal invasion of the superficial epithelium.

1.2.6. Aetiology of Oral Leukoplakia

Tobacco is considered as the major risk factor for the development of oral leukoplakia under the current definition. However, other co-factors have also traditionally been implicated in the development of subsequent malignancy, or have the term "leukoplakia" in their clinical designation. These include alcohol and diet, and infections (viruses e.g. HPV), bacteria (e.g. *Treponema pallidum*) and fungi (*Candida albicans*). However, no aetiological factors can be identified in some oral leukoplakic lesions and these latter lesions are termed idiopathic oral leukoplakia.

1.2.6.1. Tobacco

The use of tobacco in its several forms has been strongly associated with the development of oral leukoplakia. The relationship between tobacco use and oral leukoplakia had been proven based on evidence from epidemiological and intervention studies. Epidemiologically, the prevalence of oral leukoplakia was higher among smokers. Pindborg *et al.* (1965) examined 10,000 Indian patients and found that 10% of

tobacco users had oral leukoplakic lesions. Pindborg *et al.* (1972) found that 32% of 345 Danish female patients with oral leukoplakia were smokers. Smokers were also highly represented (86.5%) among 104 Hungarian patients with oral leukoplakia lesions (Bánóczy and Rigo, 1991). In a large population survey of 50,915 Indian villagers, Mehta *et al.* (1969) found that the locations of oral leukoplakia were consistent with the tobacco chewing and smoking habit involved. The aetiological role of tobacco use has been confirmed by a decrease in the prevalence of oral leukoplakia upon smoking cessation. Gupta *et al.* (1986) in an interventional study aimed at prevention of oral cancer among 36,000 Indian tobacco users, found that the incidence rate of oral leukoplakia was four to six times lower among men and women who received an educational programme encouraging smoking cessation than in the non-intervention group. Martin *et al.* (1999) in a study conducted on a group of military trainees where the prevalence of oral leukoplakia was 9.9%, reported that regression of oral leukoplakia lesions was observed in 97.5% of individuals after six weeks cessation of smoking. Schepman *et al.* (2001) retrospectively examined the anatomical site of oral leukoplakia in relation to chewing tobacco usage. They found that the use of tobacco in men can result in the development of oral leukoplakia in the buccal mucosa, however they did not note this in women. They also noted that the oral leukoplakia of the floor of the mouth (sublingual keratosis) almost exclusively occurs in smokers.

1.2.6.2. Alcohol

Tobacco and alcohol consumption are both well documented to be the most important risk factor for oral cancer and precancerous lesions, however the carcinogenic role of alcohol consumption independent of tobacco use remains unclear, but is currently the subject of a large international study (the ARCAGE study). Different mechanisms have been suggested; alcohol may act synergistically with tobacco by increasing the permeability of the oral mucosa to nitrosamines and polycarbonates (Squier *et al.*, 1986). A carcinogenic action of ethanol metabolites has also been suggested, one of the most important of which is acetaldehyde. Acetaldehyde is directly mutagenic to DNA and inhibits DNA repair enzymes in oral epithelial cells (Timmons *et al.*, 2002). Acetaldehyde, which is one of the first ethanol metabolites, and the aldehydic products of lipid peroxidation like malondialdehyde and 4-hydroxynonenal, can bind to protein to form protein adducts that may interfere with their cellular function or modify the immunological response toward these modified proteins. Warnakulasuriya *et al.*, (2008)

demonstrated a high level of these protein adducts in the oral tissue from cancerous and precancerous lesions and also demonstrated expression of the ethanol-induced cytochrome P450 2E1 which in conjunction with acetaldehyde, resulted in a state of oxidative stress in the cells. Interestingly, Yokoyama *et al.* (1996) reported an increased risk of oesophageal cancer in Japanese people harbouring a mutant alcohol dehydrogenase 2 (*ADH2*) gene. Some of normal the microflora are capable of metabolising ethanol to acetaldehyde. Miyakawa *et al.* (1986) found that oral commensal bacteria including *Streptococcus salivarius* can produce acetaldehyde from ethanol in human secretions. Local oral microbial production of acetaldehyde has been reported in patients with poor oral hygiene (Homann *et al.*, 2001). *Candida albicans* has also been implicated in this process, as increased colony forming units of the yeast have been observed in high acetaldehyde containing saliva (Tillonen *et al.*, 1999). Recent data suggest that all *Candida* species can produce significant amounts of acetaldehyde from ethanol and glucose (Neiminen *et al.*, 2009).

1.2.6.3. Candida Infection

The relationship between *Candida* and the risk for development of malignancy originates from the finding of an association of *Candida* with speckled leukoplakia (Jepsen and Winther, 1965; Renstrup, 1970; Bánóczy and Sugar, 1972). This will be discussed in detail in section 1.10 of this chapter.

1.2.6.4. Syphilitic Leukoplakia

Syphilitic leukoplakia is a large homogenous white patch that usually affects the dorsum of the tongue in patients with tertiary syphilis. Cawson (1969) stated that patients with syphilitic leukoplakia have a higher chance of developing a carcinoma of the dorsum of tongue even if the syphilis infection has been treated and patients have a negative Wassermann reaction. Weisberger (1957) described 14 patients with syphilitic leukoplakia, every one of whom developed carcinoma within the leukoplakic patch.

1.2.6.5. Oral Hairy Leukoplakia (OHL)

Oral hairy leukoplakia (Greenspan *et al.*, 1984) is a white lesion predominantly on the lateral border of the tongue of human immunodeficiency virus (HIV)-infected or immunosuppressed patients and is associated with Epstein-Barr virus infection. OHL usually reflects a decreased number of CD4+ cells and increased HIV viral load

(Mabruk *et al.*, 2000). Although OHL bears the pathological term leukoplakia in its name, it appears to have no potential for malignant transformation.

1.2.6.6. Nutritional Factors

An association between vitamin A deficiency and increased susceptibility to carcinogenesis was reported with an increased risk for developing different epithelial carcinomas of the lung, colon, pharynx, larynx, and esophagus (DeVita *et al.*, 1993). Vitamin A analogue retinoids have been used in the treatment of oral leukoplakia. Retinoids have the ability to maintain an adequate balance between growth and differentiation of the epithelial cell and cell death. However, these agents have a severe adverse effect on the liver and are teratogenic (Epstein and Gorsky, 1999). It has been suggested that topical retinoids may have a role in the treatment of resistant oral candidosis (Scardina *et al.*, 2009).

Diet may also play a role in the development and/or prevention of oral cancer. The role of diet in the development of oral cancer was recognised from the high risk of oral and oesophageal cancer in patients with Paterson-Kelly Brown or Plummer Vinson syndrome, where iron deficiency (sideropenia) is one of the disease features (Larsson *et al.*, 1975). Although hyperplastic post-cricoid web in the pharynx and post-cricoid carcinoma are a feature of this syndrome, the oral manifestations of prolonged, persistent iron deficiency are candidosis and epithelial atrophy ("glossitis"), rather than leukoplakia and so, the oral susceptibility to carcinoma in this condition may be due to increased permeability/susceptibility to dietary carcinogens due to epithelial barrier compromise. The role of oral candidosis in this condition, however cannot be ignored but has not, to date, been explored. Other nutritional elements like vitamins A, C and E may also influence the risk of oral cancer. A diet highly enriched with vitamins A, C and E and increased consumption of fruits and vegetables may reduce the risk of oral cancer (Chainani *et al.*, 2011).

1.2.6.7. Other Factors

Human papilloma virus (HPV) has been widely accepted as one of the most prominent causative agent of cervical cancer. However, the role of the HPV in the pathogenesis of oral cancer and precancer is questionable, but probably accounts for, and appears to have a major role in a subset of oropharyngeal disease (Gillson *et al.*, 2000 and 2008), affecting the posterior tongue, pharynx and larynx, and may indicate improved

prognosis as HPV associated oropharyngeal tumour showed better response to the treatment (Fakhry *et al.*, 2007). Studies have reported a wide prevalence rate of HPV in premalignant (dysplastic) oral lesions ranging from 0% (Zeuss *et al.*, 1991) to 85% (Bouda *et al.*, 2000) with HPV 16 and 18 being the most extensively investigated. This wide variability may be due to the sampling (biopsy, rinse or cytology smears) and molecular assays used (*in situ* hybridisation, nested PCR). HPV has been demonstrated in only a *minority* of oral squamous cell carcinomas, but may prove to be an important subset (Toner and O'Regan 2009a and 2009b), and can be detected particularly in carcinomas of the tongue base.

Oral cancer and oropharyngeal cancer therefore appear to have different aetiopathogeneses and molecular models of the disease as several risk factors such as tobacco, HPV, etc., has been implicated in the aetiology of oral cancer with different genetic and molecular markers such as chromosomal aberration, p53, etc., has been suggested.

1.2.7. Clinical Features of Oral Leukoplakia

Clinically, oral leukoplakia is classified as homogenous and non-homogenous variants (Pindborg *et al.*, 1963; Axéll *et al.*, 1984). Homogenous oral leukoplakia is a flat white lesion which might exhibit a wrinkled or corrugated smooth surface with uniformly consistent texture throughout the lesion. Non-homogenous oral leukoplakia (speckled leukoplakia, erythroleukoplakia) is an irregularly white and red lesion that can be further subclassified into nodular (speckled) and verrucous leukoplakia. The concept of nodular leukoplakia was introduced by Pindborg *et al.* (1963) and is clinically important, since this form of oral leukoplakia may carry a higher risk of malignant transformation than the homogenous form. Pindborg referred to this lesion as the "fingerprint of tobacco" (S. Flint, personal communication). Conceptually, this appears to represent a mucosal field where an ordered, reactive, hyperkeratotic process becomes more dysregulated than in homogeneous regions. Red areas in leukoplakia may represent localised areas of erythroplakia and be more dysplastic than white, homogeneously hyperkeratotic regions. Erythroplakia is a fiery red velvety patch that cannot be characterised clinically or pathologically as any other definable lesion (Pindborg *et al.*, 1997). This type of lesion has the highest risk of malignant transformation in comparison to other premalignant lesions. Moreover, erythroplakia can be histologically invasive carcinoma, carcinoma *in situ* or severe dysplasia (Shafer

and Waldron, 1975). Oral leukoplakia can affect any site of the oral cavity, the buccal mucosa, soft palate and ventro-lateral tongue being the most common site (Jaber *et al.*, 2003). However, the affected site may reflect the form and the method of tobacco usage.

1.2.8. Clinical Presentation of Chronic Hyperplastic Candidosis

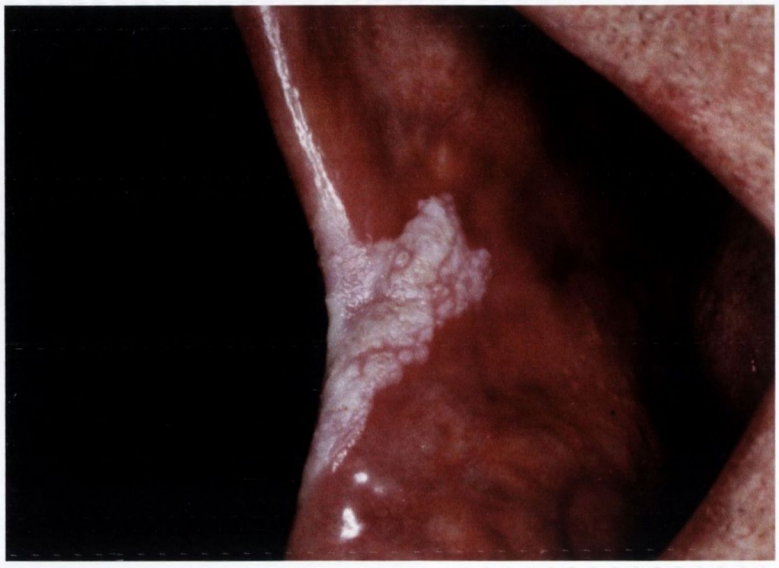
Chronic hyperplastic candidosis presents clinically as a well demarcated, rough, raised, white plaque-like lesion that cannot be rubbed off. The lesion may have a homogenously white surface or non-homogeneous areas with erythematous components along with the white areas that may have a nodular characteristic clinically identical to nodular leukoplakia (Fig. 1.1a). Indeed, although CHC may be discriminated from leukoplakia histopathologically, it almost certainly represents a leukoplakic patch actively infected with *Candida.* Whether the leukoplakia is caused by the invasive candidosis (Fig. 1.1b) or the hyperkeratosis provides a micro-environment which promotes *Candida* growth and invasion, will be discussed later. The commissure is the most common site and it may also affect the palate and tongue. Arendorf *et al.* (1983) observed that in patients with CHC, other forms of candidosis (see section 1.3.2) occur concurrently in about one third of cases. Holmstrup and Besserman (1983) described a chronic multifocal candidosis tetrad of commissural CHC in association with median rhomboid glossitis, denture stomatitis and angular cheilitis.

1.2.9. Histopathology of Oral Leukoplakia (OL)

There are no specific histological features for OL as the histological findings on examination of OL ranges from epithelial hyperkeratosis either ortho- or para- keratosis (or both), with hyperplasia to atrophy that may or may not demonstrate epithelial dysplasia in its various degrees; mild, moderate, severe or carcinoma *in situ.*

Pindborg *et al.* (1997) defined oral epithelial dysplasia as "a precancerous lesion of stratified squamous epithelium characterised by cellular atypia and loss of normal cellular maturation and stratification, short of carcinoma *in situ*" whereas carcinoma *in situ* involves the full epithelial thickness without invasion of the epithelial basement membrane, which is the distinct feature of frank invasive carcinoma. Histopathological features of dysplastic epithelium are listed in Table 1.1. The degree of dysplasia reported relates to the proportion of dysplastic cells in the whole epithelium. If less than one third is affected the term mild dysplasia is used. Moderate and severe dysplasia

(a)



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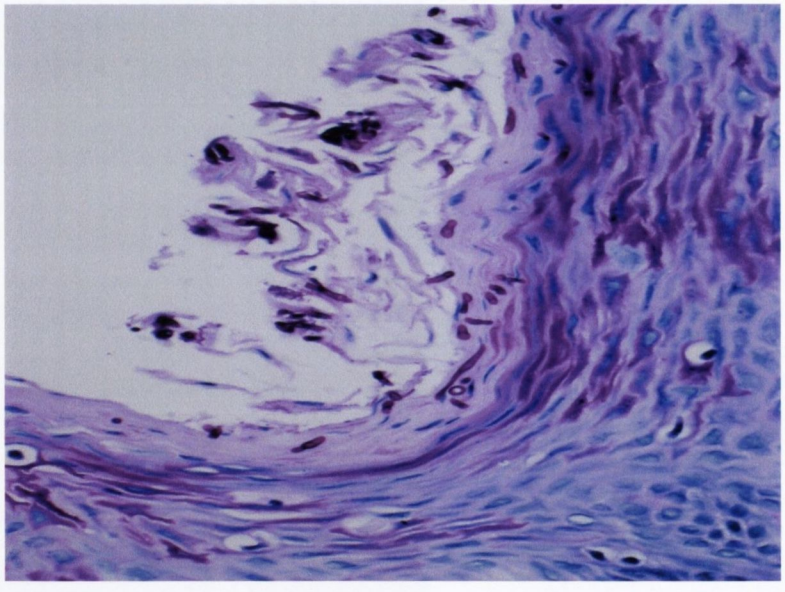


Figure 1.1 Panel a), Clinical photograph showing the appearance of a chronic hyperplastic candidosis lesion at the commisure of the mouth.
Panel b), Light micrograph of a histological section of tissue from a chronic hyperplastic candidosis lesion stained with periodic acid Schiff stain lesion showing invasive candidal hyphae in the superficial layer of the epithelium (X100).

Table 1.1 Criteria used for diagnosis of epithelial dysplasia

Cellular changes	Architectural changes
Abnormal variation in nuclear size and shape (anisonucleosis)	Loss of epithelial stratification
Abnormal variation in nuclear (pleomorphism)	Loss of polarity of the basal cells
Abnormal variation in cell size (anisocytosis)	Basal cell hyperplasia
Abnormal variation in cell shape (pleomorphism)	Premature keratinization and keratin pearls deep in epithelium (dyskeratosis)
Increased nuclear-cytoplasmic ratio	Drop shaped rete pegs
Increased nuclear size	
Hyperchromatic nuclei	
Increased mitotic figures	
Atypical mitotic figures	
Increased number and size of nucleoli	

Barnes *et al.* (2005)

involves the other two thirds and carcinoma *in situ* (intraepithelial carcinoma) is diagnosed when the entire epithelium is dysplastic, thickened and there is marked proliferation in the basal layer, but without evidence of invasion through the basal lamina (see Fig. 1.2). Histopathological grading of dysplastic epithelium is markedly subjective (see section 1.2.11).

1.2.10. Histopathology of Chronic Hyperplastic Candidosis

The histopathology of chronic hyperplastic candidosis varies according to the clinical type as in oral leukoplakia lesions, whether homogenous or speckled, and the presence of dysplasia. The original description is assigned to Lehner (1964) and Cawson and Lehner (1968). Homogenous CHC may be hyperortho- or hyperparakeratinised (Pindborg, 1980) and epithelial dysplasia may be absent, but is commonly present in the speckled type. Various degrees of inflammatory infiltrate are noted in the lamina propria and microabscesses may be seen in the upper epithelium with migrating neutrophils. Polymorphonuclear leukocytes are also often present in the lamina propria, along with a chronic inflammatory infiltrate. The infiltrate comprises mostly of T-lymphocytes (54%), macrophges (14%) and there are generally few B-cells (8%), (Williams, 1977) since humoral immunity plays a relatively minor role in fighting fungal infections, other than opsinisation. *Candida* hyphae invading the epithelium from the keratinised surface cannot be visualised on routine haematoxylin and eosin staining and must be demonstrated with special stains (routinely periodic acid Schiff (PAS) but also Grocott's methenamine silver or Gridley's stain can be used) to confirm the diagnosis (Fig. 1.1b). Parakeratosis is always seen in the nodular type with variable thickness, corresponding to the depth of *Candida* hyphal invasion. In areas where active *Candida* invasion is demonstrated, the hyperkeratinisation may be less prominent, although invasion past the stratum spinosum is rare. The stratum spinosum may be acanthotic, rete ridges bulbous and separation of the surface epithelium is commonly seen.

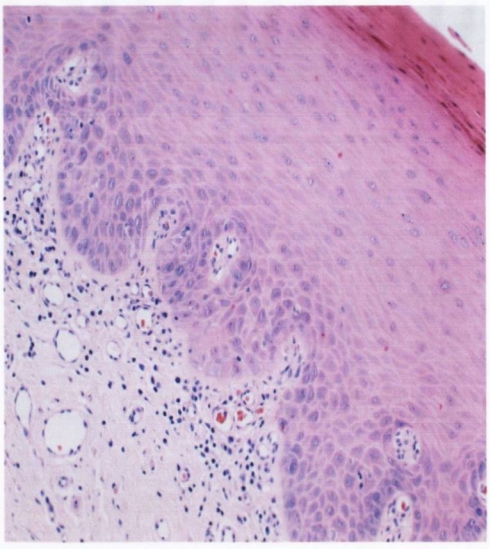
1.2.11. Malignant Transformation of Oral Leukoplakia

The potential for malignant transformation of oral leukoplakia has been examined in several studies. Great emphasis has been placed on the validity of using epithelial dysplasia as a prognostic indicator of malignant transformation. The histological assessment of oral epithelial dysplasia can be subjective, with significant intra-observer

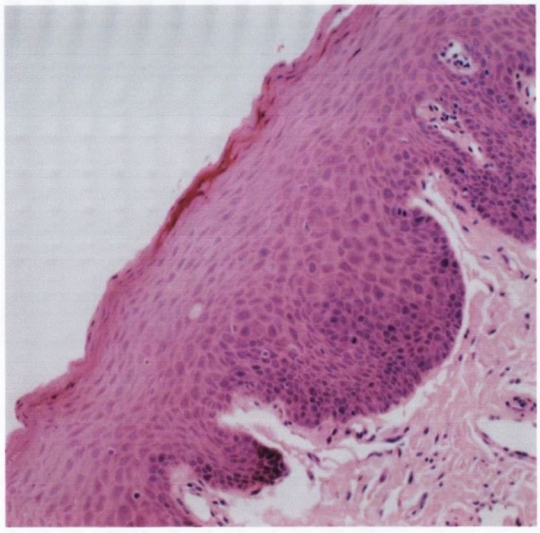
and inter-observer variability. Several studies have examined the inter-observer reliability of diagnosis of oral dysplasia in precancerous lesions. Pindborg *et al.* (1985) at a scientific meeting for oral pathologists showed nine photomicrographs of different oral lesions ranging from no dysplasia to invasive carcinoma to 72 participants; there was great variability in the proffered diagnoses ranging from 1 to 78% of agreement. Abbey *et al.* (1995) evaluated the intra-observer and the inter-observer variability on 60 oral lesions examined by 6 experienced oral pathologists and found poor agreement. These findings were confirmed by Warnakulasuriya (2008). Fischer *et al.* (2004) suggested that the presence of inflammation, site of the lesion and the biopsy technique used would greatly modify the reliability of the histopathological diagnosis of the lesion.

However a recent seminal study by Holmstrup (2006) on a large group of leukoplakia patients in Denmark, with long-term follow up reported that the odds ratio for carcinoma development was not influenced at all by grade of dysplasia. Since no definitive, validated criteria exist for predicting which dysplastic lesions will progress to malignancy, research initiatives are currently focusing on the genetics and epigenetics of oral premalignancy, in a search for predictive markers (Lingen *et al.*, 2011).

Clinically, the rate of malignant transformation of oral leukoplakia is variable. In India, large-scale community based studies with long follow-up periods have estimated the rate of malignant transformation to be between 0.13-2.2% (Mehta *et al.*, 1972; Pindborg *et al.*, 1975, Silverman *et al.*, 1976; Gupta *et al.*, 1980). This variability has been attributed to the difference in group selections, mode of tobacco use and follow-up periods (Napier and Speight, 2008). In the Western world, the rate was somewhat higher where the studies were based on hospital patient selected samples. The estimated rates ranged widely from 1.1 to 17.5 (Pindborg *et al.*, 1968; Bánóczy, 1977; Silverman *et al.*, 1984; Schepman *et al.*, 1998). Petti (2003) calculated the pooled estimated rate of malignant transformation in oral leukoplakia of 1.3% per year. He also commented that the oral cancer transformation rate might be more prevelant as many oral cancer were underreported in developing countries. The potential role of *Candida* in the induction of oral mucosal hyperkeratosis, epithelial dysplasia and proposed mechanisms of malignant transformation of CHC will be discussed in section 1.4



(a)



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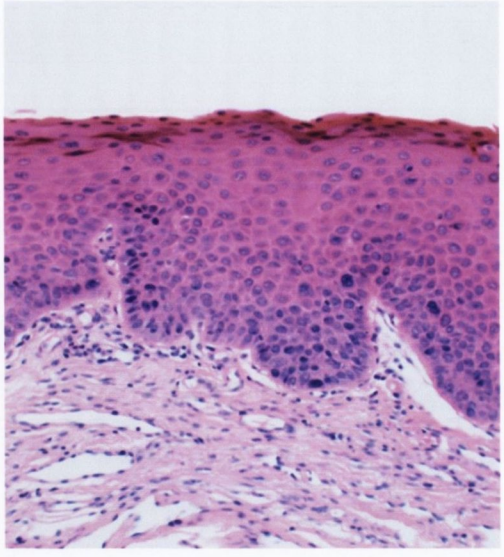

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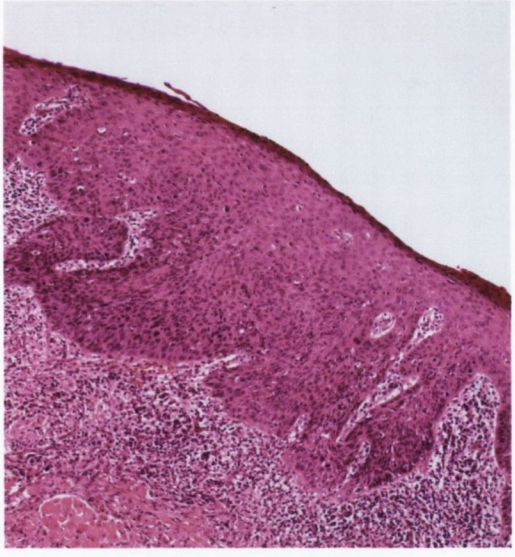


(d)

Figure 1.2 Light photomicrographs showing the various degree of epithelial dysplasia in oral leukoplakic lesions stained with haematoxylin and eosin. (a) mild dysplasia (X40), (b) moderate dysplasia (X20), (c) severe dysplasia (X40) and (d) carcinoma *in situ* (X20).

1.2.12. Carcinogenesis, Molecular Markers and Epigenetics

Oral carcinogenesis is a multistep process that results from accumulation of genetic mutations in oral epithelial cells due to mutagenic effects of various carcinogens (polycyclic hydrocarbons in tobacco, alcohol, nitrosamines, etc.). Numerous genetic mutations had been detected in oral squamous cell carcinoma (SCC); these genetic events lead to the disruption of the normal regulatory pathways that control basic cellular functions including cell division, differentiation and programmed cell death (apoptosis). The genetic markers for determining malignant transformation of an oral premalignant lesion have been described in four main themes: (a) genetic markers; DNA content (ploidy), chromosome aberrations (allelic loss or gain), and changes in the expression of oncogenes and tumour suppressor genes; (b) proliferation and anti-apoptotic markers; (c) differentiation markers; cell surface carbohydrates and keratin and (d) epigenetic events. A recent comprehensive review of the genetics and epigenetics of oral premalignancy has been carried out by Lingen *et al.* (2011).

1.2.12.1. Oncogenes

Oncogenes are altered growth promoting regulatory genes, which are present in normal tissue as proto-oncogenes that control cellular signal transduction pathways. Mutation of these genes may lead to excessive proliferation of keratinocytes. A single genetic alteration is rarely sufficient for the development of a malignant tumour, however, oncogenes are an important initiation factor in the process of malignant transformation (Sidransky, 1995). The mechanisms of oncogene activity include mutation, chromosomal translocation, gene amplification and retroviral insertion (Klein and Klein, 1985). Several oncogenes have been implicated in the process of oral carcinogenesis and can be grouped based on the function into; (a) growth factors and their receptors such as epidermal growth factor (EGF) and receptor (*EGFR*); (b) intracellular signal transducer; *ras* gene family; (c) transcription factors; *c-myc*, (d) cell cycle regulator Cyclin D1, and apoptosis inhibitors *bcl-1* and *myc* (Sidransky, 1995).

(a) c-myc

These are a family of proto-oncogenes comprising three members: *c-myc*, *n-myc* and *l-myc*. (Henriksson and Luscher, 1996). *c-myc* is frequently over-expressed in oral cancer

as result of gene amplification (Field, 1995). Over expression is associated with loss of differentiation of oral SCC (Todd *et al.*, 1997).

(b) Epidermal growth factor (EGF) and receptor (EGFR)

The epidermal growth factor receptor gene (*EGFR*) encodes a transmembrane tyrosine-specific phosphokinase that is expressed primarily in cells of epithelial origin. Binding of activating ligands (epidermal growth factor (EGF), tumour growth factor-β (TGF-β)) to the extra-cellular domain induces homo or hetero-dimerisation and phosphorylation of the receptor, resulting in activation of the downstream signalling pathways that lead eventually to cell proliferation and inhibition of apoptosis (Zhong *et al.*, 1994). Over-expression of EGF receptors is common in oral cancer, (Todd *et al.*, 1991; Partridge *et al.*, 1988), and is associated with advanced tumour stage, diffuse invasive mode and high incidence of neck metastasis (Kusukawa *et al.*, 1996). A significant correlation has been shown for EGFR expression and shortened patient survival with the strongest association for HER-2/neu, a member of the EGFR family (Xia *et al.*, 1999). In oral leukoplakias increased EGFR expression and increased copy number has been associated with risk of cancer development (Taoudi Benchekroun *et al.*, 2010). Beside the role of EGFR in carcinogenesis, this molecule has become an attractive target for anti-cancer treatment (Nagatsuka *et al.*, 2001).

(c) mdm-2

The *mdm-2* gene is present on human chromosome 12q (Marchetti *et al.*, 1996). When over-expressed, it may increase oncogenic potential. Binding of mdm-2 proteins to the p53p is responsible for the export of the p53p out of the nucleus to the cytoplasm, where p53p is inactivated through an auto-regulatory feedback loop (Haupt *et al.*, 1997). Mdm-2 therefore inhibits p53p function (Sanchez-Beato *et al.*, 1996). Agarwal *et al.*, (1999) investigated the expression of mdm-2p in oral malignancies and found over-expression in both oral SCC (in 78%) and premalignant lesions (in 52%), suggesting that over-expression of mdm-2p is an early change in the pathogenesis of oral neoplasia. They also showed a significant association of combined *P53* and *mdm-2* over-expression with advanced tumour stage and lymph node metastasis. Yanamoto *et al.*, (2002) found that *mdm-2* expression might be associated with tumour proliferation. However, when compared with *mdm-2* expression alone, combined *p53* and *mdm-2* expression was more closely related to tumour proliferation, suggesting that inactivation

of wild type p53p by mdm-2p affects tumour proliferation. *P53* is expressed in the final phase of the cell cycle and effectively inhibits mitosis temporarily (cell cycle arrest) to allow time for the replicated DNA to be checked and repaired if there is a transcription error by sophisticated DNA repair mechanisms. Inhibition of this crucial stage in cell replication allows mitosis to proceed too rapidly for repair to occur and the daughter cells carry through to the next cell cycle transcription error mutations.

1.2.12.2. Tumour Suppressor Genes

Tumour suppressor genes encode proteins involved in transduction of negative growth-regulatory signals (Bishop, 1991). These genes are involved in cell-cycle regulation, including cell-cycle arrest and apoptosis. Tumour suppressor genes can be inactivated by several mechanisms including point mutation and/or deletion and rearrangements in both alleles of the gene in a "two-hit" manner (Knudson, 1977), subsequent to this inactivation the cells are predisposed to uncontrolled growth and division (Levin, 1997).

(a) P53

*TP*53 or P53 is a tumour suppressor gene located on chromosome 17, which encodes the p53p. It is a 53-kD nuclear phosphoprotein. P53p is not demonstrable immunohistochemically in normal tissue because of its very short half-life. P53p is involved in maintaining genome stability and plays an important role in cell cycle progression, cellular differentiation, DNA repair and apoptosis. It is involved in the detection of DNA damage, after which it can stop the cell cycle while the damage is repaired or can trigger apoptosis. If the *TP53* gene is non-functional, or DNA repair mechanisms faulty, epithelial carcinomatous transformation may occur (such as is seen in di Fraumini syndrome, the Fanconi anaemia-dyskeratosis congenita spectrum and Peutz-Jegher syndrome). Mutation of the *P53* gene is claimed to be the most common genetic alteration associated with human cancer. Mutations of the *P53* gene have been reported in about 60% of oral SCC, and are strongly associated with immunohistochemically detectable accumulation of the p53p product (Ahomadegbe *et al.*, 1995). The results of sequence analysis of the whole *P53* gene (11 exons) indicate that *P53* gene alterations are present in almost 100% of head and neck SCC (Kropveld *et al.*, 1999). Over- expression of p53p is seen more often in head and neck squamous cell carcinoma (HNSCC) patients who are heavy smokers and heavy drinkers (Field *et al.*, 1992). It may also be seen in apparently normal epithelium of smokers (Colucci *et*

al., 1997). Cruz *et al.* (1998) in a follow-up study of 35 histologically defined premalignant lesions with different degrees of epithelial dysplasia found that expression of supra-basal p53p had a high predictive value in progression of these lesion toward carcinoma. Vora *et al.* (2006) examined *P53* expression in oral leukoplakia and found that 79% of the lesions were positively stained for p53p, and noted that the patients with *P53* expression have a higher risk of developing tongue cancer than those who do not show *P53* expression. In a review of biomarkers in oral dysplasia and cancer progression, Smith *et al.*, reported a relative risk of cancer progression from 1.42 to 6.82 in p53 positive dysplasias. In patients with early glottic carcinoma, *P53* over-expression was found to be associated significantly with poor patient outcome (Jin *et al.*, 1998).

(b) Retinoblastoma (Rb) gene and p16

Loss of retinoblastoma tumour suppressor gene function can contribute to hyperproliferation of oral keratinocytes. Simultaneous deactivation of both *p53* and *Rb* is probably necessary for tumourigenesis in head and neck cancer (Shah *et al.*, 2003). *P16* and *P14* genes are key tumour suppressor genes involved in control of the cell cycle. Mutation and genetic alteration of *P16* that causes inactivation of *P53* is the second most common genetic event in human tumours (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). Decreased expression of p16 correlates with histologic grade of dysplasia (Bradley *et al.*, 2006), although the data are conflicting as a single study (Gologan *et al.*, 2005) has reported an increased expression of p16 in increasing dysplasia grade. A decreased immunohistochemical expression of p16p detected in up to 89% of cases of oral cancer is associated with reduced survival rate, increased recurrence rate and nodal metastasis (El-Naggar *et al.*, 1999).

(c) Vascular endothelial growth factor (VEGF)

VEGF stimulates the proliferation and physiologic function of vascular endothelial cell growth (Ferrara, 1996). VEGF has been identified recently as one of importance in carcinogenesis (Keck *et al.*, 1989; Ferrara, 1996). It is a multifunctional cytokine that acts as a highly specific mitogen on endothelial cells (Keck *et al.*, 1989). It can open calcium channels to increase calcium influx and elicit a signalling cascade. Tumour expression of VEGF results in the development and maintenance of a vascular network. Increased VEGF expression has been demonstrated in progression from normal mucosa

to dysplasia and malignancy (Johnstone and Logan, 2006 and 2007). Smith *et al.* (2000) suggested that VEGF is an important prognostic indicator in different types of tumours including HNSCC.

1.2.12.3. Genetic Abnormalities

(a) DNA content

Abnormal DNA content is a chromosomal aberration due to loss or gain of one or more chromosomes is called aneuploidy. Aneuploidy has been associated with malignant and premalignant changes (Sen, 2000). However, the role of aneuploidy in carcinogenesis is still unclear whether it is a non-specific and spontaneous change that resulted during the tumour formation or whether it is a part of the process of tumorigenesis. Evidence such as the presence of tumour specific aneuploidy, aneuploidy in preneoplastic lesions, higher frequency of genetic instability in aneuoploid cells compared with diploid cells and mutation of mitotic check point genes, suggest that aneuploidy plays an important role in carcinogenesis (Dey, 2004). However an increase in aneuploidy can be an inhibitor of the tumourigenesis process (Weaver *et al.*, 2007). Aneuoploidy is not necessary for the malignant state and it is most likely involved in the development of a more aggressive cancer phenotype (Dey, 2004). Many preneoplastic lesions show aneuploidy. These include Barrett's oesophagus dysplasia (Menke-Pluymers *et al.*, 1994), large cell liver dysplasia (Thomas *et al.*, 1992) and intraepithelial prostatic (Montironi, *et al.*, 1992). In oral premalignacy, Torres-Rendon *et al.* (2009) investigated the status of DNA ploidy in oral dysplastic cells and its association with malignant transformation in these lesions. They found that aneuploidy was present in 33% of the oral dysplastic lesions that progressed into cancer, while it was only present in 11% of lesions that had not progressed. Furthermore, dysplastic lesions with abnormal DNA content developed SCC in a shorter period of time than diploid lesions. Aneuploid oral dysplastic lesions had significantly higher risk of malignant transformation (Högmo *et al.*, 1998), however, they have also reported that aneupolidy was not correlated with the degree of dysplasia (Högmo *et al.*, 1996). Allelic imbalance, signalling increased cancer risk has been reported by many groups worldwide, most recently by Garnis *et al.* in 2009.

(b) Loss of heterozygosity

Loss of heterozygosity (LOH) in a cell represents a loss of normal function of one allele of a gene in which the other allele is already inactive (often a tumour suppressor gene) by which an important mechanism of regulation of cell proliferation is lost, is frequently seen in oral cancers and dysplasias.

An early observation by Solomon *et al.* (1987) who showed that about 20% of human colorectal cancers had undergone allelic loss on chromosome 5q started the search for LOH in cancers. Loss of heterozigosity in cancer cells frequently involved allelic loss that occurs in regions containing tumour- suppressor genes; p53, brca1, rb, brca2, apc, vhl, and p16. Loss of heterozigosity is an early event and may occur at the stage of dysplasia or carcinoma in situ, including lung (Boyle *et al.*, 2001), colorectal polyps (Allen, 1995) and Barrett's oesophagus (Prasad *et al.*, 2010). Oral dysplastic lesions with LOH have a greater risk for malignant transformation (Partridge *et al.*, 2000). Rosin *et al.* (2000) investigated hyperplastic and mild and moderate dysplastic oral lesions and compared lesions that progressed to carcinoma and those that not progress using microsatellite LOH as a biomarker. They reported a 33 fold elevated cancer risk for lesions that progressed to cancer when LOH at 3p and/or 9p combined with additional losses at 4q, 8p, 11q, or 17p. Similar observations were reported by Partridge *et al.* (1998).

It is noteworthy, that despite the advances in molecular detection methods of the genetic abnormalities, there is no single or set of molecular markers that can be reliably used to predict the malignant transformation in oral dysplasia. Smith *et al.* (2009) systematically reviewed the effectiveness of biomarkers in predicting progression of oral dysplasia into malignancy. They noted that various anomolies in the data could be attributed to methodological limitations, including studied sample size, quality of follow-up data and lack of consistent histological and clinical definitions. They suggested that multicentre collaboration and standardisation of both clinical and histological definitions is necessary to allow further appropriate comparison.

1.2.12.4. Epigenetic Events

DNA hypermethylation, RNA-associated post translational silencing and histone modification are the molecular events associated with malignancy (Lingen *et al.*, 2011), although only hypermethylation has, to date, been studied in oral cancers and dysplasia.

Hypermethylation of p15 and 16 is a frequent finding in oral SCC and may be seen in over 50% of cancers (Kato *et al.*, 2006), In oral dysplastic lesions, increased hypermethylation of p14,15 and p16 correlates with degree of dysplasia (Takeshima *et al.*, 2008), although this is an emerging field of study where there is currently limited data.

The role of *Candida* species in causing, inducing or promoting the genetic changes seen in oral dysplasia and cancer is unclear at present. That *Candida* can induce hyperkeratosis and dysplasia in the mouth can be deduced from genetic anomalies where patients have increased susceptibility to oral candidosis, specifically CHC, as in autoimmune polyendocrinopathy, candidosis and ectodermal dystrophy (APECED) syndrome, epidemiological and histopathological studies, animal experimental models, effects of antifungal treatment on CHC and studies of carcinogen elaboration by *Candida* species and is discussed in detail in section 1.4.

1.3. *Candida* and Candidiasis

The genus *Candida* is composed of an extremely heterogeneous group of yeast and consists of about 200 species that are considered to be unable to reproduce sexually. Most of the *Candida* species can produce a filamentous type of growth that is called pseudohyphae, but only *Candida albicans* and *Candida dubliniensis* can also produce true hyphae. *Candida albicans* is the most common *Candida* species isolated from the oral cavity as both a commensal and pathogenic organism in healthy and diseased individuals. Other species isolated from the mouth more rarely include *C. glabrata, C. tropicalis, C. dubliniensis, C. parapsiilosis, C. krusei, C. guilliermondii* and *C. kefyr*. Few of these species are medically important and most of them have the ability to produce the pseudohyphae (Claderone, 2002).

1.3.1. Oral Carriage

The oral carriage rate of *Candida* in the general populatiation varies from 20-70%. *Candida albicans* is the most common species and alone accounts for 50% of *Candida* organisms recovered followed by *C. tropicalis, C. glabrata, C. parapsilosis* and *C. krusei* (Odds, 1988; Cannon and Chaffin, 1999). *Sacchromyces cerevisiae* and *Rhodotorula glutinis* are yeasts rarely isolated from the oral cavity and are often transient and not known to cause oral pathological infection (Stenderup, 1990). The oral *Candida* carriage rate may be associated with various host factors, including reduced salivary rate (McFarlane, 1990; Torres *et al.*, 2003), low pH of saliva (Arendorf and Walker, 1979; Kadir, *et al.*, 2002), increased salivary glucose concentration (Knight and Fletcher, 1971; Sashikumar and Kannan, 2010), high carbohydrate diet (Samaranayake, MacFarlane, 1985) smoking (Arendorf and Walker, 1980; Soysa and Ellepola, 2005), blood group O and non secretion of blood group antigens (Burford-Mason *et al.*, 1988; Ben-Aryeh *et al.,* 1995) and denture wearing (Odds, 1988; Lyon *et al.*, 2006).

Candida albicans can colonise anywhere in the oral cavity. However, *C. albicans* is most frequently isolated from the mid-dorsum of the tongue. The persistence of *C. albicans* strains in the mouth have been reported by several studies (Williamson, 1972; Odds, 1987; Williams *et al.*, 2001; Vargas and Joly, 2002; Odds *et al.*, 2006). In a few cases, more than one species of *Candida* and different strains of the same species can be isolated from the same individual (Kam and Xu, 2002).

1.3.2. Oral Candidiasis

Candida are opportunistic organisms that can cause infection when the local or the systemic balance between the host and the microorganism is disturbed. Odds (1988) classified the predisposing factors as generalised factors, dietary and mechanical factors (Table.1.2). Oral candidiasis can present in various clinical forms but most commonly seen as "thrush" or acute pseudomembranous candidiasis, acute and chronic atrophic and erythematous candidiasis, angular cheilitis and, more rarely, chronic hyperplastic candidiasis (Holmstrup and Axell, 1990; Samaranyake, 1991). More recently, the chronic mucocutaneous candidosis syndromes with a genetic basis, have been grouped under the entity APECED (autoimmune polyendocrinopathy candidosis and ectodermal dysplasia) a polyendocrinopathy subset caused by mutations (up to 5) in the *AIRE* gene (Finnish-German APECED consortium, 1997). The term Polyglandular autoimmune syndromes (PGA) I-III has also been used. See Table 1.3.

1.3.2.1. Clinical Variants of Oral Candidosis

The clinical variants of oral *Candida* infection, as opposed to the commensal state are well described in textbooks of oral medicine and pathology, such as Scully *et al.* (2011). Clinical examples of the various lesions are presented in Figs. 1.3 to 1.8

(a) Acute pseudomembranous candidosis (thrush)

Acute pseudomembranous candidosis (Fig. 1.3) is a "disease of the diseased". In order for *Candida* to cause this severity of oral infection the local defences of the host must be compromised. Thus, although thrush is seen in patients related to antibiotic use, local or systemic steroid use, xerostomia, or uncontrolled diabetes mellitus, a search for an underlying systemic immunoparesis should also be undertaken. Acute pseudomembranous candidosis is also seen in the new-born, particularly if they are premature, since the immune system is underdeveloped. The advent of HIV disease has highlighted the importance and pathogenic potential of this micro-organism. It is the most common oral manifestation of HIV disease (dos Santos Pinheiro *et al.*, 2009). Oro-pharyngeal thrush is AIDS defining (Mocroft *et al.*, 1998).

Clinically, the pseudomembranes are said to resemble cottage cheese or milk curds as a confluent white or creamy plaques or spots that can cover small or extensive areas of the mouth. They can be wiped off the mucosa with gauze or a cotton bud

leaving an erythematous, raw surface (Fig. 1.3). Pin-point bleeding may occur, but is uncommon. The pseudomembrane comprises bacteria, leukocytes, necrotic epithelial cells, debris, and matted mycelial *Candida* hyphae. The hyphae are seen to penetrate the epithelial cells, which acts to hold the psuedomembrane on the mucous membrane (Samaranyake and MacFarlane, 1990). Patients complain of soreness and dysgeusia and it may be possible to detect an odour on the breath resembling brewer's yeast.

(b) Acute atrophic candidosis.

This rare condition has also been called "antibiotic sore mouth", although it may also be caused by systemic or topical steroid therapy. As implied, the condition is of acute onset, (usually following broad spectrum antibiotics, particularly tetracyclines) and painful. Clinically, the oral mucosa, particularly the tongue appears extremely erythematous (Fig. 1.4). There may be depapillation of the tongue, particularly affecting the filiform papillae. It may represent a situation where an acute pseudomembranous candidiasis, has lost the pseudomembranes.

(c) Chronic atrophic candidosis

This condition encompasses three variants: denture-induced stomatitis, erythematous candidosis and median rhomboid glossitis. Denture–induced stomatitis rarely affects the lower arch. It has also been called "denture sore mouth", and although it appears very florid, patients are usually completely unaware of the condition, i.e. soreness is not a feature. The pathogenesis relates to denture hygiene, or wearing the denture at night. Placing an upper denture in the mouth creates a micro-environment under the fitting surface where *Candida* species and bacteria have a biological advantage. *Candida* that adhere readily to denture material and are held in close contact with the palatal mucosa and are difficult to remove even with vigorous mechanical cleaning or chemical agents. (Fig. 1.5) In the lower arch, denture-induced stomatitis is only seen under partial dentures, which are close fitting, such as cobalt chrome partial dentures, or on removal of implant retained prostheses in patients with inadequate oral hygiene. Denture-induced stomatitis does not occur under lower complete dentures, unless implant-retained, since they are relatively mobile and saliva can periodically bathe the mucosal support surface. Clinically, the lesions can be graded; starting as pin-point, erythematous lesions, progressing to erythematous plaques, until eventually the entire mucosal surface appears oedematous and erythematous (Barbeau *et al.*, 2003). There is

Table 1.2 Predisposing factors to oral candidosis

I - Generalised factors

- Age extremities
- Pregnancy
- Diabetes mellitus "hyperglycaemia"
- Defective immunity (either congenital or acquired)
- Altered phagocytosis (defective myeloperoxidase system)
- Malignancies especially leukaemia and lymphoma
- HIV infection
- Xerostomia

II - Dietary factors

- Carbohydrates-rich diet
- Vitamin deficiencies
- Iron deficiency

III - Mechanical factors

- Denture wearing
- Damaged mucosal barrier

IV - Iatrogenic factors

- Long-term use of broad-spectrum antibiotics
- Corticosteroids use
- Radiotherapy
- Immunosuppressant and cytotoxic drug use
- Smoking

Modified from Odds (1988).

Table 1.3 Clinical classifications of candidosis

Group I

Superficial associated with hereditary immunodeficiency.

- -Hereditary thymic aplasia
- -Di George syndrome
- -Severe combined immunodeficiency
- -Chronic granulomatous diseases
- -Chediak-Higashi syndrome
- -Myeloperoxidase deficiency

Group II

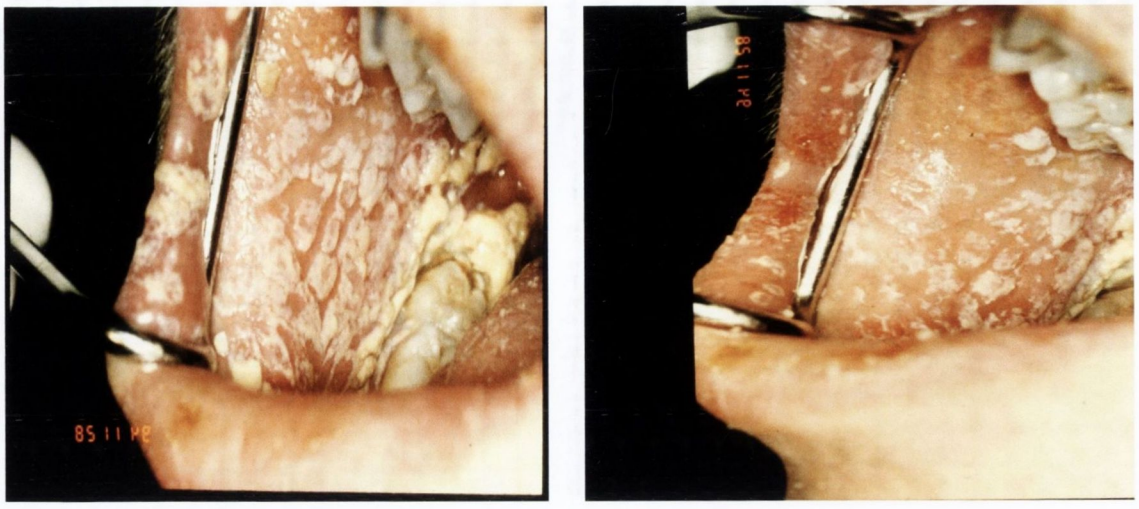
- -Familial chronic muco-cutaneous candidosis
- -Diffuse chronic muco-cutaneous candidosis
- -*Candida* endocrinopathy syndrome (now termed autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, APECED)
- -Candidosis-thymoma syndrome (Good's syndrome)

Group III

Primary oral candidosis:

- -Acute pseudomembranous candidosis
- -Acute atrophic candidosis
- -Chronic atrophic candidosis, erythematous candidosis and median rhomboid glositis
- -Chronic hyperplastic candidosis
- -Angular cheilitis

Samaranyake, (1991)



(a) (b)

Figure 1.3 (a) Clinical photograph showing the appearance of acute pseudomemberanous candidosis in the oral cavity. Pseudomemberanous candidiasis presents with the apperance of confluent "milk curds" or white plaques. The photograph shown in panel (b) shows the same lesions shown in panel (a) after the lesions were wiped off leaving erythematous, raw surfaces.

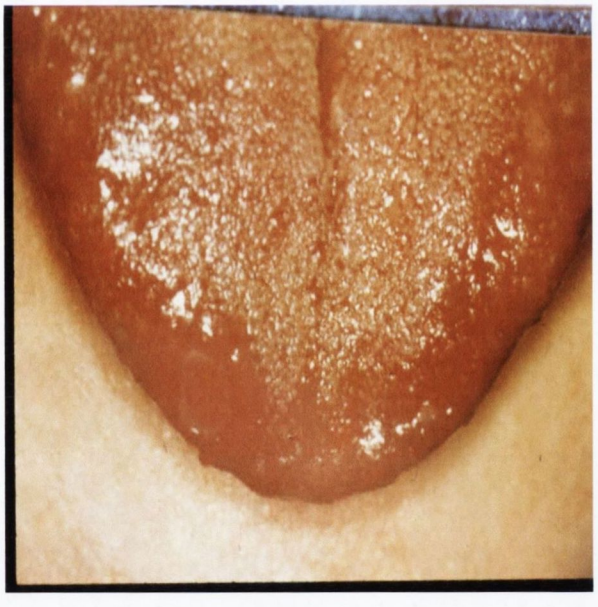
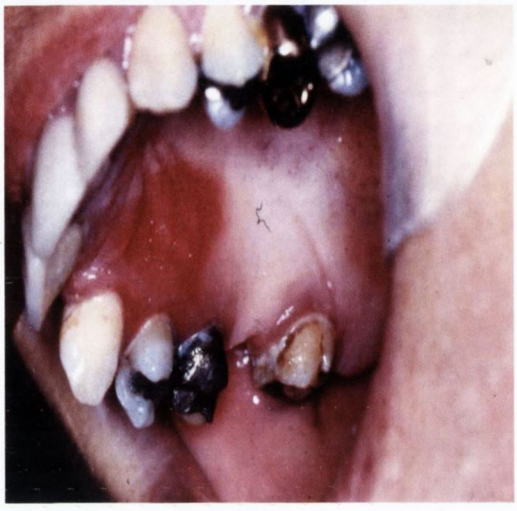
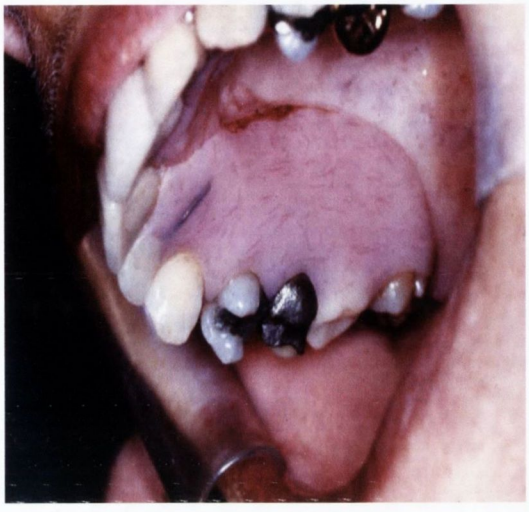


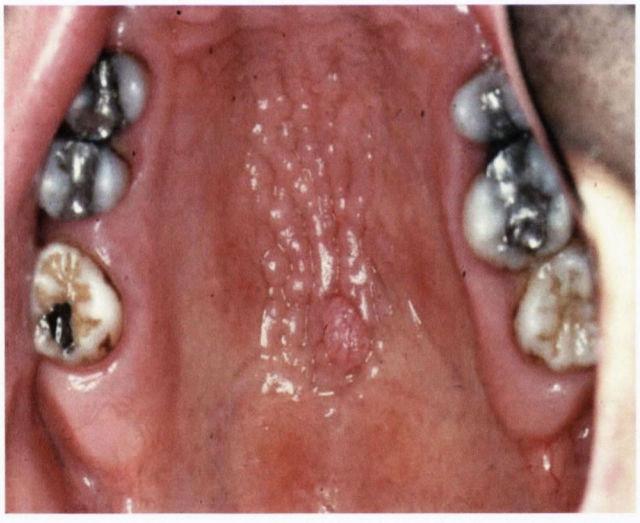
Figure 1.4 Clinical photograph showing acute atrophic candidosis on the tongue (antibiotic sore mouth). Note, depapillation affecting mainly the filiform papillae.



(a)



(b)



(c)

Figure 1.5 Clinical photographs showing examples of chronic atrophic candidosis or denture stomatitis, (a and b). Note the abrupt change to normal mucosa where the denture covering the anterior palate ends. Panel (c) shows an example of papillary hyperplasia of the palate caused by an upper partial denture replacing the upper anterior teeth.

an abrupt change to normal appearing mucosa, where the denture ends (Fig. 1.5). The best treatment is to completely remove the denture (although this is rarely practical), but denture hygiene instruction along with antifungal therapy leads to complete resolution in most cases. Long-standing cases can show a degree of mucosal hyperplasia and ultimately, this may culminate in denture-induced papillary hyperplasia, which is irreversible (Fig. 1.5c).

Erythematous candidosis is a condition which was originally described in the mouths of heavy smokers. The presentation is of asymptomatic, well-defined, discrete, erythematous macules, which particularly affect the soft and hard palate and tongue (Fig. 1.6). Erythematous candidosis has been described in untreated HIV infection: indeed it is by far the most common presentation of candidiasis in this highly susceptible group (Felix and Wray, 1993). As with median rhomboid glossitis, when erythematous candidosis affects the tongue and palate, there may be "kissing lesions". The EC Clearing House on Oral Problems Related to HIV Infection and WHO Collaborating Centre on Oral Manifestations of HIV have defined definitive and presumptive diagnostic criteria for this lesion (WHO, 1993).

Median rhomboid glossitis (Fig. 1.7) represents an area of erythematous candidosis at the junction of the anterior two thirds and posterior third of the tongue in the mid-line anterior to the circumvallate papillae. It was once thought to be caused by the persistence of the embryonic tuberculum impar. However, since it is not seen in children, this suggestion is unlikely. *Candida* species can almost invariably be cultured from the lesion, although response to antifungal therapy is variable. Clinically, it appears as a round to diamond-shaped lesion, often some centimetres in size. The surface appears depapillated and ranges from a smooth to a nodular appearance (Fig. 1.7). Despite the dramatic appearance of the nodular variant, malignant change at this site is extremely rare. Often there is a corresponding contact infection (erythematous candidosis) in the roof of the mouth. The lesion is asymptomatic and spontaneous resolution or persistence may occur.

(d) Chronic hyperplastic candidosis (Chronic hyperplastic candidiasis, Candidal leukoplakia)

Discussed in detail in section 1.4.

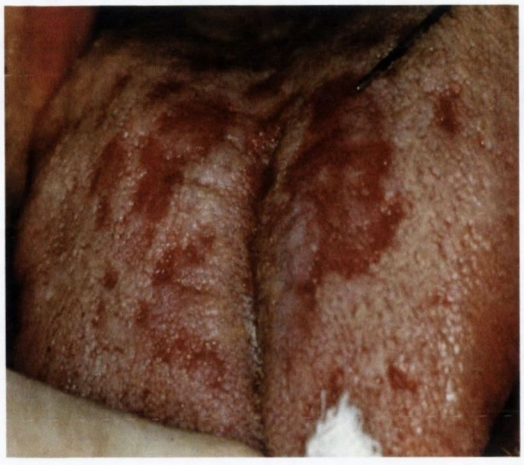
(e) Angular cheilitis

Angular cheilitis (angular stomatitis, perleche) is usually a disease of the elderly. It has been quoted that in edentate patients, with dentures constructed at a reduced vertical dimension, deepening of the angular fold (which leads to lack of aeration of the skin and local maceration) predisposes to *Candida* superinfection. There is a similarity to the pathogenesis of intertrigo. Clinically, the lesions may be symptomatic, or not, there is fissuring, erythema, maceration of the skin at the angular fold, occasional crusting and bleeding. There is usually an associated chronic atrophic candidiasis. The disease is predisposed by anaemia, haematinic (B12, folate and iron) deficiency and vitamin B complex deficiency (Fig. 1.8). It is often seen in patients with Crohn's disease or oro-facial granulomatosis, (where a microbial aetiology is often lacking and the disease responds to topical steroids). It is commonly seen in HIV disease. The microbiology of angular cheilitis is variable. Although it can be caused by *Candida* species alone, *Staphylococcus aureus* (clinically these patients often present with yellow crusts at the angle of the mouth) and *Streptococcus pyogenes* have also been implicated (Dias and Samaranayake, 1995).

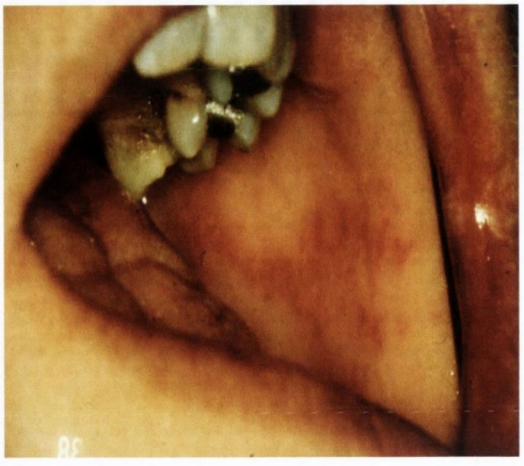
Chronic hyperplastic candidosis and angular cheilitis caused by *Candida* species are presenting features of APECED (Fig. 1. 9 and 1.10).

1.3.3. Treatment of oral candidiasis

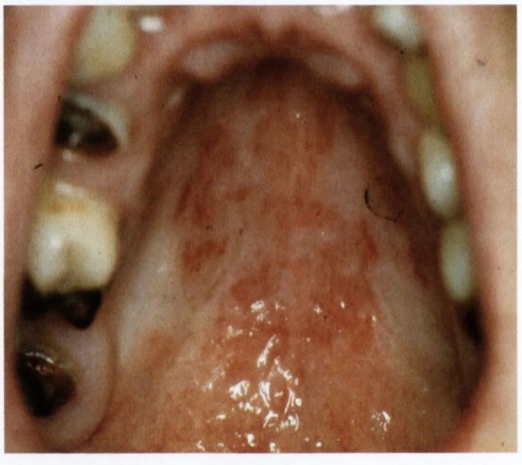
Oral candidiasis is well recognised as a disease of the diseased and reflects the opportunistic nature of several *Candida* species, especially *C. albicans* (Cannon *et al.*, 1995). Based on this, the management of oral candidiasis requires the identification of underlying predisposing factors in individual patients and correction of these factors where possible (e.g. improved denture hygiene in cases of *Candida*-induced denture stomatitis; restoration of the normal oral commensal flora following broad-spectrum antibiotic therapy in patients with antibiotic induced oral thrush). However, in circumstances where predisposing factors can not be readily rectified (e.g. HIV infection, radiation induced xerostomia) management of oral candidiasis by treatment with antifungal drugs is necessary. A limited range of antifungal drugs is available to treat oral candidiasis (Table 1.4) and these are described briefly below.



(a)



(b)



(c)

Figure 1.6 Clinical photographs showing examples of erythematous candidosis lesions in a HIV-infected patient. (a) tongue lesion, (b) buccal mucosa lesion and (c) palatal "kissing lesion" from erythematous candidosis on the dorsal tongue.

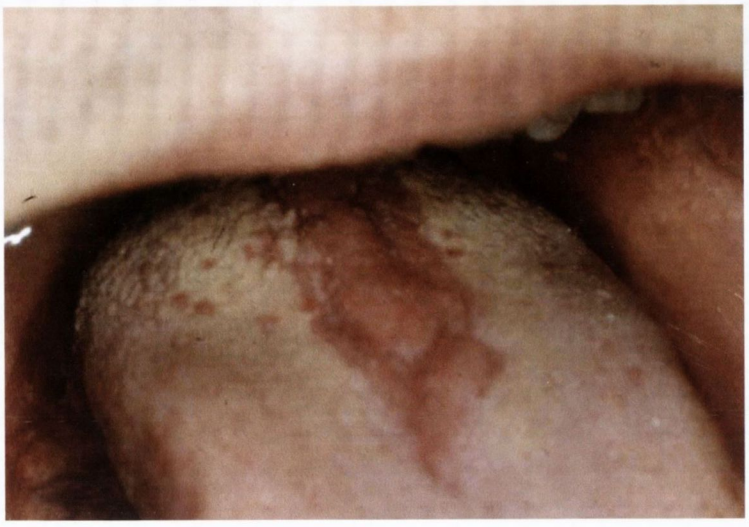


Figure 1.7 Clinical photograph showing an example of median rhomboid glossitis

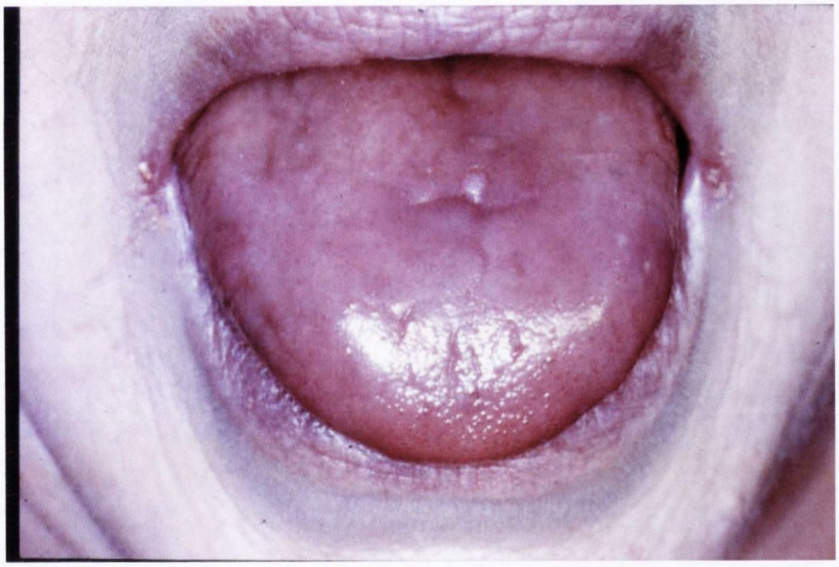


Figure 1.8 Photograph showing angular cheilitis in haematinic deficiency. Note depapillation of the tongue

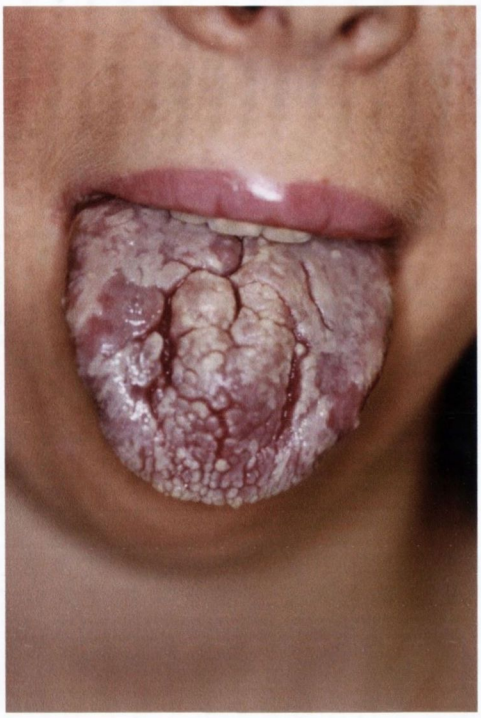


Figure 1.9 Clinical photograph showing chronic hyperplasic candidosis in APECED

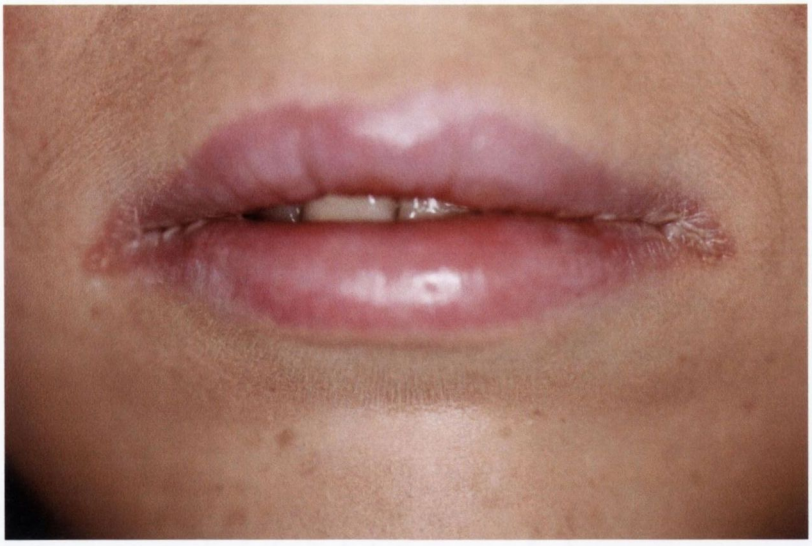


Figure 1.10 Photograph showing angular cheilitis in APECED

Table 1.4 Antifungal drugs used for treating oral candidiasis

I. TOPICAL TREATMENTS (and drug class)

Nystatin (Polyene macrolide)

Clotrimazole (Polyene)

Miconazole (Imidazole)

Amphoteracin B (Polyene)

II. SYSTEMIC (Oral)

Miconazole (Imidazole)

Ketoconazole (Imidazole)

Fluconazole (Triazole)

Itraconazole (Triazole)

III. SYSTEMIC (Intra-venous)

Amphoteracin B (Polyene)

Liposomal Amphoteracin B (Polyene)

Fluocytosine (Pyrimidine)

None of the echinocandins antifungals are indicated for treatment of oral candidiasis (Reijden, 2011).

1.3.3.1. Azoles

The azoles are a group of synthetic antifungal agents, generally with broad-spectrum fungistatic activity and low toxicity. Members of the azole group have either an imidazole or triazole ring with N carbon substitution. Azoles with an imidazole ring have a five-membered ring structure containing two nitrogen atoms (e.g. ketoconazole, miconazole and clotrimazole), whereas triazoles have a five-membered ring structure containing three nitrogen atoms (e.g. fluconazole, itraconazole and the second-generation agents voriconazole, posaconazole and ravuvonazole). Azoles inhibit fungal P450 cytochrome enzymes responsible for the synthesis of ergosterol, the principal sterol found in fungal cell membranes, resulting in the accumulation of ergosterol precursors, some of which become incorporated into the cell membrane resulting in membrane perturbation (Ghannoum and Rice, 1999). Azoles are poorly soluble in water, with the exception of the triazoles flucoanzole and itraconazole, both of which are consequently well absorbed through the gastrointestinal tract. Azole antifungal agents have become important in the treatment of oropharyngeal candidiasis in AIDS and other patients predisposed to oropharyngeal or oral candidiasis (Patton *et al.*, 2001). However, the emergence of acquired resistance to azoles has been described in several *Candida* species, such as *C. albicans* (White *et al.*, 1998) and *C. dubliniensis* (Moran *et al.*, 1997), while other *Candida* species such as *C. glabrata* are inherently less susceptible to azoles (Vermitsky and Edlind, 2004) or inherently resistant to azoles such as *C. krusei* (Johnson *et al.*, 1995).

1.3.3.2. Polyenes

Polyenes are a class of antibiotics produced by a variety of *Streptomyces* species, some of which have antifungal activity. There are around 100 polyene antibiotics, but only a few such as amphotericin B and nystatin have been developed for antifungal treatment. Polyene antifungals are broad spectrum drugs that exert their fungicidal effect by inducing cell membrane porosity following selective binding to ergosterol present in fungal cell membranes. Nystatin was the first successful antifungal drug to be developed, and it is still in general use. However, due to the poor systemic absorption of nystatin, its use is limited to topical applications such as superficial mucosal infections, including oral candidiasis. In contrast, the polyene amphotericin B is one of the main antifungal drugs that is used for the treatment of serious fungal infections, and prior to

the introduction of the azole antifungal agents, it was the only available broad-spectrum antifungal drug. However, amphotericin B is poorly soluble in water, which makes it unsuitable for oral and intramuscular administration (Calderone, 2002). Early formulations of amphotericin B were associated with nephrotoxicity, neurotoxicity and hepatotoxicity due to its ability to bind to cholesterol in mammalian cell membranes, resulting in a low maximum tolerable dose (Chabot *et al.*, 1989). Reformulation of amphotericin B preparations using liposomes (e.g. AmBisome) has permitted the use of significantly larger doses of drug therapeutically with significantly reduced side-effects (Yardley and Croft, 1997). Polyenes are generally used to treat life-threatening systemic infections caused by fungi such as *Aspergillus* species and generally are not used extensively to treat candidal infections, although nystatin lozenges and topical gels may still be used to treat oral candidiasis. Resistance to polyenes is rare.

1.3.3.3. Echinocandins

Echinocandins are semi-synthetic lipopeptide compounds produced from natural fungal products (Eschenauer *et al.*, 2007). Echinocandins interfere with the synthesis of β(1,3)-D-glucan, a major fungal cell wall component, through non-competitive inhibition of the activity of the β-1,3-D-glucan synthase enzyme complex, leading to loss of osmotic integrity and cell lysis (Denning, 2003). These agents include caspofungin, micafungin and anidulafungin. Caspofungin is fungicidal against *Candida* species but shows poor absorption by the oral route limiting its use to intravenous therapy. Resistance to echinocandins among clinical isolates of *Candida* species is low (Pfaller *et al.*, 2006; Baixench *et al.*, 2007). Echinocandin-resistant yeast isolates investigated to date usually have point mutations that lead to single amino acid substitutions in the *FKS1* and *FKS2* genes in *S. cerevisiae* and in the *GSC1* in *C. albicans* (Katiyar *et al.*, 2006). These genes encode β-1,3-D-glucan synthase and render the enzyme less susceptible to inhibition by echinocandins (Baixench *et al.*, 2007)

1.3.3.4. Antimetabolites

Flucytosine (also known as 5-flucytosine or 5FC) is a fluorinated pyrimidine used as a systemic antifungal agent in the treatment of severe candidal and cryptococcal infections. Flucytosine inhibits DNA and RNA synthesis and has a good therapeutic effect against *Candida* species. It is well absorbed through the gastrointestinal tract. However, monotherapy for the treatment of *Candida* infection is not indicated due to

the frequent development of drug resistance (Modgal and Sobel, 2010).

1.3.3.5. Antifungal Resistance

Resistance of *Candida* species to antifungal drugs can have potentially serious implications for the management of systemic infections infections. This arises from the fact that only a small number of antifungal agents are available compared to the wide range of antibiotics available to treat bacterial infections. Fortunately, resistance to antifungal drugs by *C. albicans*, the most frequently isolated causative organism of oral candidiasis, is relatively rare. Resistance to polyenes is very uncommon among *Candida* species, and where encountered has been attributed to a decreased ergosterol content within polyene-resistant *Candida* isolates (Dick *et al.*, 1980). However, azole resistance has been reported frequently in *Candida* species, especially *C. albicans*, recovered from patients treated extensively with azole antifungal drugs. Azole resistance has been shown to be caused by a variety of mechanisms including alterations in the azole target enzyme lanosterol demethylase resulting in reduced affinity for azole drugs, removal of intracellular azole from the cell by multidrug transporter pumps and compensation by other sterol synthesis enzymes in membrane biosynthesis. Other *Candida* species such as in *C. krusei* exhibit intrinsic resistance to azoles such as fluconazole (Johnson *et al.*, 1995).

1.3.4. *Candida* Virulence

Candida is an opportunistic pathogen. In order to cause infection, the organism must have the ability to evade the host defence system, survive and divide in the host environment and spread into the host tissues. *Candida albicans* is the most pathogenic *Candida* species and this is attributed to its ability to adhere strongly to host surfaces, produce hydrolytic enzymes like phospholipases and also a family of related secreted aspartyl proteinases (Calderone and Fonzi, 2001; Yang, 2003). Other putative factors include phenotypic switching, which is accompanied by changes in antigenic expression and the ability to produce hyphae, may play a role in pathogenesis. Phenotypic switching is thought to provide adaptation of the organism to adverse environments (Soll, 2004).

1.3.4.1. Pathogenicity of Candida

Candida species and even biotypes and strains within a species have variable ability to cause disease in a host (Allen, 1983; Hellstein *et al.*, 1993; Samaranyake *et al.*, 1998; Arendrup *et al.*, 2002; Soll, 2002; Ruhnke, 2006). Pathogenicity will depend on factors associated both with the organism and also the host, since in predisposed hosts opportunistic infection can occur. The infective process starts with adhesion of the microorganism to the oral epithelial cells. This is achieved by interaction between epithelial surface glycoproteins and mannoproteins on the *Candida* cell wall. There is evidence that salivary secretor status of blood group antigens may assist this process. *Candida* will also adhere effectively to the acrylic of dentures (Panagoda, *et al.*, 2001; Lou and Samaranayke, 2002) and biofilm formation on catheter surfaces can occur (Hawser and Douglas, 1994; Estivill *et al.*, 2011). *Candida* tends to exhibit pathogenicity when in the hyphal form (O'Connor *et al.*, 2010). Fungal factors associated with pathogenicity include ability to produce various enzymes. These include phospholipases at the invading tip of the hypha that facilitate host cell penetration (Ghannoum, 2000), acid proteinases, such as secreted aspartyl proteinases (SAPs) (section 1.3.4.2).

Host defences against *Candida* are both specific (immunological) and non-specific or innate (non-immunological). Salivary non-specific factors include; lysozyme (Samaranayake *et al.*, 2001), iron, lactoferrin (Hibino *et al.*, 2009), histatins (Edgerton and Koshlukova, 2000; Konopka *et al.*, 2010), lactoperoxidase (Edgerton and Koshlukova, 2000), glycoproteins and calmodulin (Kleinegger *et al.*, 2001). The immunological defences tend to be of two major types. The humoral wing of the immune system does not appear to play a major role except through salivary IgA and opsinisation (Challacombe and Naglik, 2006). The myeloperoxidase killing system in phagocytes is very important. In myeloperoxidase deficiency there is a particular susceptibility to candidosis (Kalinski, *et al.*, 2007). The cell-mediated wing of the immune system, through cytokine-mediated activation of other T cells and phagocytes plays a key role in controlling *Candida* and maintaining it in a commensal relationship in those individuals who are chronic carriers of the micro-organism (Dongari-Bagtzoglou and Fidel, 2005).

1.3.5. Adhesion

Adherence of *Candida* to the host cells is the first essential step for colonisation and subsequently progression to infection. The interaction between *C. albicans* and the host cells are controlled by both non-specific and specific mechanisms.

1.3.5.1. Non-Specific Mechanisms

Non-specific *Candida* adherence to the host cells are in form of electrostatic forces, aggregation and cell surface hydrophobicity.

(a) Cell surface hydrophobicity

Cell surface hydrophobicity or electrodynamic forces (van der Waal's interactions) is defined as 'a hydrophobic interaction as a reaction between two or more compounds in an aqueous phase, with subsequent elimination of water molecules associated with the interfacing particles' (Jones *et al.*, 1991). Cell surface hydrophobicity properties of *C. albicans* has been linked to the cell wall mannoprotein, alteration of mannoprotein conformation is contributed to cell surface hydrophobicity switching (Masuoka and Hazen, 2004). Cell surface hydrophobicity can also be shifted with growth temperature and the growth phase of *C. albicans* (Hazen and Hazen, 1988). Cell surface hydrophobicity of *C. albicans* may contribute to the adherence to both epithelial and plastic surfaces (Hazen, 1989). It is believed to affect adherence by promoting the specific adherence of *C. albicans* to host cells proteins such as collagen type IV, fibrinogen, laminin and fibronectin (Silva *et al.*, 1995). Hazen, (1989) demonstrated that the shift of the yeast cell from hydrophilic to hydrophobic cells greatly affected adherence of the yeast to HeLa monolayer cells.

1.3.5.2. Specific Mechanisms

The specific mechanism of adhesion of *Candida* to epithelial cells is influenced by host cell factors, yeast factors and environmental factors.

(a) Epithelial Cell factors

The components of the epithelial cell surface that are recognised by the *Candida* adhesins are diverse and represent different classes of biomolecules.

Fibronectin: is a plasma and extracellular matrix glycoprotein of 37 to 120 kDa. It is involved in human cell adhesion and migration (Castelleni *et al.*, 1986). The role of binding of *C. albicans* to the fibronectin containing RGD tripeptide (arginine-glycine-aspartic acid) was detected by inhibition of the yeast adherence by monoclonal antibodies and RGD-containing peptides (Santoni *et al.*, 1994).

Integrins: integrin and integrin receptors are membranous proteins that have different functions including cellular adhesion. *Candida* have antigenic and functional similarity to human complement receptor 3 and 4 (CR3 and CR4) which are known as complement analogues because of the placement of CR3 and CR4 within the integrin supergene family (Hostetter, 1994). The CR3 receptor is present on the surface of those *C. albicans* that share homology with the iC3b subunit receptor on the surface of neutrophils and that mediate the adherence of *C. albicans* to the CR3 receptor (Lee *et al.*, 1997). The iC3b receptor is present on both yeast and hyphal forms of *C. albicans* (Heidenreich and Dietrich, 1985). Inhibition of *C. albicans* adherence has been demonstrated with integrin monoclonal antibodies and RGD tripeptides (Bendel and Hostetter, 1993).

(b) Yeast Factors

(i) Adhesins

Mannoproteins are widely believed to play a major role in mediating adherence of *Candida* to the host cells (Douglas, 1985). Mannoproteins are cell wall components (20%-30%) found in both the outer and the inner electron dense area of the cell wall and play major virulence and immunogenic roles with both their mannan and protein moieties. The protein moieties of the mannoprotein (lectin-like molecules) recognise the glycosides containing $_{L}$-fucose or *N*-acetyl-$_{D}$-glucosamine on the epithelial cell surface and function like receptor (Fukazawa and Kagaya, 1997). The adhesion of *C. albicans* is greatly inhibited with fucose-binding protein (Cameron and Douglas, 1996). The mannan moiety of the mannoprotein defines the two serotypes of the *C. albicans*; serotype A and serotype B (Tsuchiya *et al.*, 1984).

(ii) Agglutin-Like Sequence (ALS)

The Agglutin-Like Sequence (*ALS*) gene family of *C. albicans* includes eight genes that encodes large cell-surface glycoproteins, they are divided into 3 subgroups based on

cross-hybridization of the central tandem repeats; *ALS1-ALS4* are in one group *ALS5-ALS7* in a second group and *ALS9* in separate group (Hoyer *et al.*, 2008). In *C. albicans* the expression of *ALS* genes are affected by the stage of growth (*ALS4*), morphology (*ALS3*) and change of growth medium (*ALS1*) (Hoyer, 2001). Als1p protein products in *C. albicans* have sequence similarity to the *S. cerevisiae* cell surface glycoprotein α-agglutinin, the function of this protein is to facilitate the mating between haploid cells (Hauser and Tanner, 1989).

(iii) Hyphal wall protein (HWP1)

Hyphal-specific cell wall proteins the adhesins include Als1p, Als3p and Hwp1p. Hwp1p is a glycosylphosphatidylinositol modified glycoprotein that found on surfaces of germ tubes, but not yeasts or pseudohyphae of *C. albicans* (Sundstrom, 2002). Hwp1p protein is a substrate for keratinocyte transglutaminase (TGase) and covalently cross-linked to the buccal epithelial cells (Staab *et al.*, 1996; Sundstrom *et al.*, 2002), therefore, Hwp1p tightly binds the *C. albicans* cells to the host cells (Staab *et al.*, 1999). In stratified squamous epithelia, TGase mediates the formation of innate host barrier defence by cross-linking substrate proteins such as small proline-rich protein; involucrin, loricrin and keratins that produce an insoluble cornified envelope (Steinert *et al.*, 1998).

Candida albicans mutants with disruptions in the *HWP1* gene can produce normal germ tubes but are defective in stable adherence to buccal epithelial cells (Staab *et al.*, 1999). The ability of the organism to cause endothelial cell injury was also attenuated in an *HWP1* mutant (Tsuchimori *et al.*, 2000). Invasion of the murine dorsal tongue by *HWP1* mutant strains was limited to a small shallow area around the foramina cecum. Similar observations were noted in the oesophagus, where the extent of microabscess formation was limited with the mutant strains (Sandstrom *et al.*, 2002). The Hwp1 plays a pivotal role in establishing infection by resisting the dislodgement of *C. albicans* from the surface epithelium and enabling the fungus to cause host cell injury and maintain a deep seated infection (Tsuchimori *et al.*, 2000).

(iv) Secreted aspartyl proteinases (Saps)

These are a group of hydrolytic enzymes that encoded by a family of 10 *SAP* genes. Mature Sap (1-10) enzymes are between 35 to 48 kD and contain two highly conserved regions with reactive aspartic residues of the active site of the pepsin-like proteinases

and a third conserved region at the C-terminus of the protein. It also contains four cysteine residues that form two disulphide bridges that may be responsible for maintaining the three dimensional structure (Schaller *et al.*, 2005). The role of Saps in the adherence of *C. albicans* was suggested from *in vitro* studies that show the *C. albicans* strains that have strong proteolytic activity are adhering more to the human buccal epithelial cells (BECs) than the stains that produced less proteinases (Ghannoum and Abu Elteen, 1986). The exact role of the Saps in the adherence of *C. albicans* is not clear. It has been suggested that Saps could act as ligands to the surface proteins on the host cells or Saps may act as an enzyme to modify the host cells surface proteins or fungal cell surface (Monod and Borg-von Zeplin, 2002).

1.3.6. Morphogenesis

The ability of *C. albicans* to switch from yeast form to filamentous growth forms of both hyphae and pseudohyphae is a defining property of *C. albicans* which is believed to be necessary for virulence (Whiteway *et al.*, 2007). Of all *Candida* species only *C. albicans* and *C. dubliniensis* are capable to grow in both filamentous forms. The morphological transition of *C. albicans* can be induced by several environmental condition that including growth medium e.g. serum, temperatures and pH. The morphogentic yeast-hyphal transition of *C. albicans* has been linked to pathogenicity (Calderone and Fonzi, 2001). Mutants that are locked in the yeast form have been found to be avirulent (Lo *et al.*, 1997). However, many filamentous mutant *C. albicans* strains were also attenuated (Limjindaporn *et al.*, 2003). Two signal transduction pathways that regulate morphogenesis in *C. albicans* has been identified (Calderone and Fonzi, 2001) including the mitogen-activated protein (MAP) kinase signalling pathway, which is an external pH sensing and medium sensing pathways, and the MAP kinase signalling pathway that regulates *C. albicans* transcription factor Cph1 (Lo *et al.*, 1997). Deletion of genes encoding MAP kinase pathways proteins resulted in mutants with depressed hyphal development in specific agar media (Czank *et al.*, 1997). Another morphogenesis pathways is mediated by the transcription factor Egf1 (Ramage *et al.*, 2002). This pathway includes Ras, adenyl cyclise and cAMP-dependent protein kinase. Cells with defective adenyl cycline Cdc35 are able to grow as yeasts but unable to form hyphae under certain conditions (Rocha *et al.*, 2001). This pathway is independent but parallel to that mediated by Cph1.

1.3.7. Phenotypic Switching of *C. albicans*

Phenotypic switching in fungi is a reversible phenomenon that is defined as the ability of colonies to spontaneously and reversibly emerge with altered colony morphology at high-frequency (Soll, 1992). This high-frequency switching enables the micro-organisms to adapt to changing micro-environments and switching antigenicity to evade host defences. Slutsky *et al.*, (1985) demonstrated that reference strain *C. albicans* 3153A can switch reversibly between a number of variant colony morphologies. The strain WO-1 switches between a white colony phenotype and opaque colony phenotype which is called as white-opaque switching, this switching also distinguishable at microscopic level. In white colonies, the cells exhibit the round-to-oval budding yeast form while cells in opaque phase are elongated or bean-shaped cells form with rough surface that also proliferates by budding (Slutsky *et al.*, 1987). White and opaque cells also expressed different genes (Soll, 2004). High-frequency phenotypic switching can affect many virulence traits, including hyphal formation (Anderson *et al.*, 1990), proteinase secretion (Morrow *et al.*, 1994, Naglik *et al.*, 2003), adhesion (Kennedy *et al.*, 1988), antigenicity (Anderson *et al.*, 1990) and antifungal susceptibility (Velegraki, 1995). White-opaque switching has been linked to the sexual reproduction (recombination) in *C. albicans* (Soll, 2004). In *S. cerevisiae*, sexual mating is controlled by genes encoded at a single genetic locus (*MAT* locus). The *S. cerevisiae MAT* locus consists of two alleles: *MAT*a and *MAT*α. Mating type-like (*MTL*) locus is the homologue of *MAT* in *C. albicans*. The ability to switch to the opaque form depends on whether the cells are homozygous for the *MTL* locus (*MTLa* or *MTLα*), whereas heterozygous cells are unable to switch (Lockhart *et al.*, 2002). Opaque phase hemizygous (a/- and α/-) cells mate more efficiently than the same cell type in the white phase (Miller and Johnson, 2002).

1.4. The Role of *Candida* in Chronic Hyperplasic Candidosis

1.4.1. Role of *Candida* in CHC

There is some controversy regarding the initiation of epithelial hyperplasia by *Candida*. Jepsen and Winther (1965) proposed that *Candida* invades a pre-existing hyperplastic lesion rather being the cause of it. In contrast, Cawson and Lehner (1968) suggested that *Candida* infection is the primary cause of chronic hyperplastic candidosis, supporting evidence coming from the response of some CHC lesions to antifungal therapy (Lamey *et al.*, 1989) (Figs. 1.11 and 1.12). Holmstrup and Besserman (1983) demonstrated reversion of non-homogenous chronic hyperplastic candidosis to the homogenous type after the use of topical antifungal agents. Further evidence came from increased thickness of epithelium in response to cutaneous *Candida* infection as a result of increased basal cell turnover (Sohnle and Kirkpatrick, 1978). Similarly, Jennings and MacDonald (1990) demonstrated increased thickness of epithelium in patients with chronic atrophic candidiasis (denture stomatitis lesions).

In animal studies, both Partridge *et al.* (1971) and Cawson (1973) demonstrated that *C. albicans* can produce hyperplasia on chick chorioallantoic membrane. Russell and Jones (1975) found that chronic *Candida* infection of the rat's tongue can produce hyperplasia, some of these lesions showed epithelial atypia, but none of these progressed to carcinoma *in situ* or invasive carcinoma. Franklin and Martin (1986) demonstrated that induced hyperplasia in hamster cheek pouches showed epithelial dysplasia similar to that seen in human CHC after inoculation with *C. albicans*. Shakir *et al.* (1986) investigated the effect of *C. albicans* infection on the mitotic activity and thickness of palatal epithelium of Wistar rats. They found that a significant rise in the mitotic activity and thickness of the epithelium compared with the control animals. Reed *et al.* (1990) reported that *C. albicans* products can induce increased proliferative activity and epithelial thickness by both a direct effect on the epithelium or indirectly by modulating the host immune response.

The presence of *Candida* hyphae in biopsies of oral leukoplakia lesions were generally considered as an indication for infection. Renstrup (1970) found that 61% of speckled leukoplakia lesions were infected with *Candida* and 71% of these lesions showed epithelial dysplasia compared with only 3% of homogenous lesions that contained the fungal hyphae. Roed-Peterson *et al.* (1970) reported *that Candida* was present in only 40% of all oral leukoplakia lesions, however, *Candida* infection was

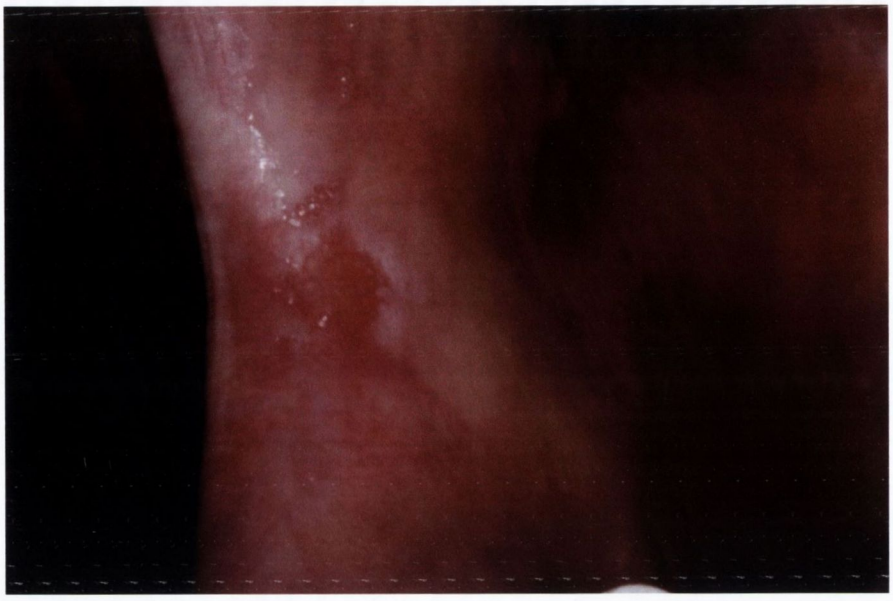


Figure 1.11 Commissural chronic hyperplastic candidosis presenting as speckled leukoplakia

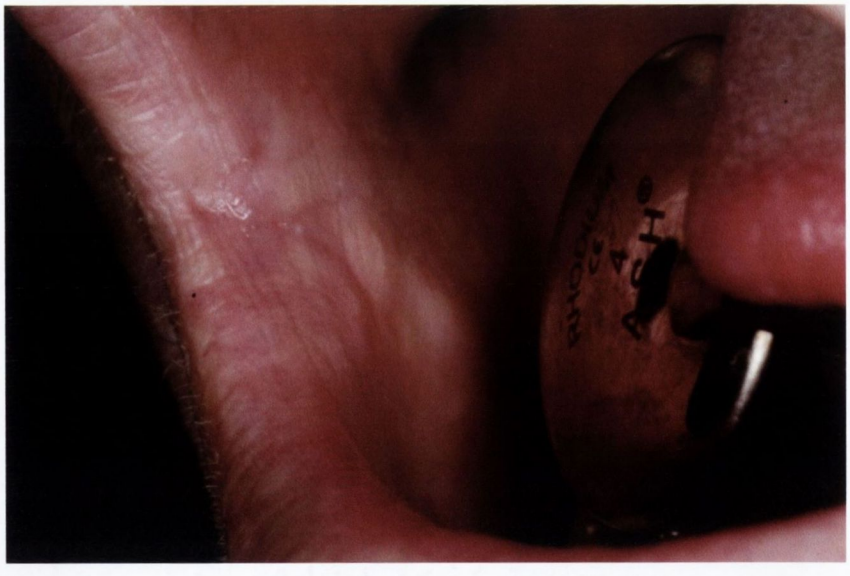


Figure 1.12 Same patient as above showing resolution of lesion following a course of systemic antifungal therapy (fluconazole)

evident in 67% of lesions which exhibited cellular atypia. Banoczy (1977) reported that 61% of erosive (speckled by her definition) leukoplakia and 63% of oral SCC were positive. Barrett *et al.* (1998) retrospectively examined pathology reports of 223 biopsies stained with PAS-positive fungal hyphae and found a statistically significant association between lesions with moderate and severe dysplasia and they also noted that fungal-infected dysplastic lesions were almost three times more likely to worsen in histological severity. McCullough *et al.* (2002) described a strong statistical association between increased numbers of oral yeast and oral epithelial dysplasia and oral squamous cell carcinoma, and the degree of epithelial dysplasia correlated with higher amounts of oral yeast.

Candida albicans biotypes isolated from lesions with more advanced precancerous changes exhibit the highest rates of carcinogenic nitrosamine production from nitrite precursor compounds which are readily available in the mouth (Krogh *et al.*, 1987). Nitrosamine particularly, benzylmethylnitrosamine (BMN), had been implicated in the carcinogenesis of oesophageal cancer (Magee and Barnes *et al.*, 1967), Hasi *et al.* (1981) investigated the role of *C. albicans* in enhancing the formation of BMN from it is precursor nitrates. They reported that *C. albicans* was able to catalyse BMN from its precursors. Nitrosamines produced by *Candida*, could result in changes in proto-oncogenes, and consequently initiate the development of malignancy (Field and Martin, 1989). O'Grady and Reade (1992) compared the ability of induction of neoplastic changes by *C. albicans* and carcinogen 4-nitroquinoline-1-oxide on mucosal models of rats. They observed that cancer developed in rats that were infected with *C. albicans* similar to that promoted by the carcinogen stimulus. Furthermore, studies from China indicate that *Candida* can be isolated from 65% to 90% of patients with oesophageal carcinoma (Xia and Zhan, 1978 and Annual Report of Chinese Academy of Medical Sciences, 1978). This result was replicated in India, *Candida* infection being detected in 76% of oesophageal carcinomas, however, *Candida* hyphae were detected in only 25% of the PAS sections (Bhatia *et al.*, 1989). The high incidence of oesophageal cancer, which is considered as endemic in certain areas of both countries, could be also attributed to a high environmental nitrate exposure and ingestion of mycotoxins and nitrosamines, in addition to nutritional deficiency that is considered as an important aetiological factor in cancer development. Recently, a series of case reports of increased incidence of oral and oesophageal cancers in a group of patients with chronic mucocutaneous candidasis (now termed autoimmune polyendocrinopathy associated

ectodermal dysplasia or APECED) (Rautemaa *et al.*, 2007; Rosa *et al.*, 2008; Domingus-Ferreira *et al.*, 2009; Böckle *et al.*, 2010), Whitaker syndrome or polyglandular autoimmune syndrome type 1. Rautemaa *et al.* (2007) reported 10% of 56 patients with APECED had oral and oesophageal carcinoma. Interestingly, most of the patients had malignant transformation at a relatively young age compared with "normal" individuals. The tumours were usually an aggressive form and patients had more than one primary with high recurrence. High production of carcinogenic acetaldehyde by *C. albicans* isolated from APECED patients has been implicated as a possible causative factor (Uittamo *et al.*, 2009). However, other risk factors cannot be excluded such as smoking (as two thirds of the reported patients in the Rautemaa *et al.*, case series were smokers) and dysregulation of the anti-tumour immune response.

The role of *Candida* in the development of chronic hyperplastic candidosis can be summarised as follows:

- *Candida* invasion of epithelium can cause hyperplasia (Cawson, 1965).
- Intermediate forms between thrush and CHC may exist (Cawson 1969) in the development of established CHC.
- Increased oral cancer risk and early onset of oral cancer is seen in mucocutanous candidiasis syndromes (Rautemaa *et al.*, 2007) (especially in APECED).
- Regression of *Candida*-infected non-homogenous leukoplakia lesions or resolution upon antifungal treatment (Holmstrup and Bessermann, 1983; Lamey *et al.*, 1989).
- Experimental *Candida* infection in rats can produce white lesions with epithelial hyperplasia and epithelial atypia (Franklin and Martin, 1986; Russell and Jones, 1975).
- A strong statistical association between increased numbers of oral yeast and oral epithelial dysplasia and oral squamous cell carcinoma, and the degree of epithelial dysplasia correlates with higher amounts of oral yeast (McCullough *et al.*, 2002).
- *C. albicans* biotypes isolated from lesions with more advanced precancerous changes exhibit the highest rates of carcinogenic nitrosamines production Krogh *et al.* (1987).

- Production of the carcinogen acetaldehyde from ethanol by oral microflora including *C. albicans* (Tillonen *et al.*, 1999; Meurman and Uittamo 2008). *C. albicans* can also produce acetaldehyde in the absence of ethanol (Uittamo et al., 2009).

- The role of *Candida* in induction of cellular atypia, however, is not clear. Renstrup (1970) reported that 40% of CHC show epithelia atypia.

- *Candida* can induce dysplasia when it is inoculated into the murine buccal mucosa (Dwivedi *et al.*, 2009).

1.5. Aims of the Study

The purpose of the present study was to investigate the role of *Candida* species in the aetiopathogenesis of chronic hyperplastic candidosis and characterise oral *Candida* species and their relative abundance isolated from chronic hyperplastic candidosis lesions compared to (non-*Candida*) oral leukoplakia and to investigate the *C. albicans* population structure of isolates from chronic hyperplastic candidosis lesions compared to other leukoplakia lesions and to commensal isolates from healthy carriers.

The following areas were studied:

- To evaluate the role of *Candida* species in oral leukoplakia and CHC lesions.

- To characterise the oral *Candida* species isolated from oral leukoplakia and CHC lesions.

- To correlate *Candida* species and strains with the degree of dysplasia exhibited by oral leukoplakia and CHC lesions.

- To investigate the adherence properties of *C. albicans* isolates recovered from oral leukoplakia and CHC lesions in comparison with reference *C. albicans* strains.

- To determine if a specific genotype(s) of *C. albicans* is associated with the development of oral leukoplakia and CHC lesions.

Chapter 2

General Materials and Methods

2.1. General Microbiological Methods

2.1.1. Patients

Ethical approval for these and the following studies was obtained from the St. James's Hospital (SJH) and Adelaide and Meath Hospitals including the National Children's Hospital (AMNCH) Research Ethics Committee in 2008 (see Appendix 1).

Patients were were recruited from Oral Medicine and Oral Dysplasia clinics in Dublin Dental University Hospital (DDUH). The patients were examined by two oral medicine consultants and an oral and a maxillofacial surgeon, a medical and dental history was recorded and a comprehensive oral examination undertaken. Clinical and histopathological details were recorded on the data sheet for each patient. Oral rinse Oral rinses and swab samples were collected from each selected patients with oral leukoplakia lesions (either CHC or NCL lesion) (Ch.3).

2.1.2. *Candida albicans* Isolates and Culture Media

All clinical isolates used in the adherence assays and molecular typing experiments were isolated from patients with oral leukoplakia by swab sampling and oral rinse sampling. Swab samples were taken by swabbing leukoplakia lesions using Copan Venturi Transystem® swabs that consisted of a cotton swab applicator and a plastic tube containing Amies transport medium (Copan Italia s.p.a, Brescia, Italy). Oral rinse sampling was undertaken by providing each patient with a plastic container with 10 ml of Milli-Q® ultra-purified water (Millipore™, Carrigtwohill, Cork, Ireland). Patients were requested to rinse their mouths for 1 min and to return the rinse solution into the container. The well characterised *C. albicans* strains SC5314 (originally recovered from a case of systemic infection) (Gillum *et al.*, 1984) and 132A (originally recovered from an oral infection) (Gallagher *et al.*, 1992) were used as laboratory reference strains.

Clinical isolates were recovered on the chromogenic medium CHROMagar™ *Candida* for the presumptive identification of clinically important yeasts (CHROMagar, Paris, France). All cultures were incubated at 37°C for 48 h. *Candida* species were presumptively identified on the basis of colony colour and/or morphology (section 2.1.2). The density of *Candida* colonies recovered on individual CHROMagar plates was counted using a Flash & Go automatic colony counter (IUL Instruments, Barcelona, Spain). *Candida* density data were recorded in colony forming units (cfus). The relative abundance of different types (i.e. different colours and/or morphologies) of

colony present in individual samples was also recorded. Samples that yielded mixed cultures with confluent and subconfluent yeast growth were serially diluted to identify the different colonies in the culture and to obtain a quantitative estimate of *Candida* cell density. All isolates were subcultured on Potato Dextrose Agar (PDA) that consisted of potato extracts 4 g/l, glucose 20 g/l, and agar 15 g/l (Oxoid Ltd., Basingstoke, Hampshire, UK) pH 5.6 at 37°C for 48 h. For liquid culture, Yeast Extract Peptone Dextrose (YEPD) broth was used (1% (w/v) yeast extract (Sigma-Aldrich DMEM or Oxoid), 2% (w/v) bacterial peptone (Oxoid) and 2% (w/v) glucose (Sigma-Aldrich Ltd, Tallaght, Dublin, Ireland), at 37°C overnight in an orbital incubator at 200 rpm (Gallenkamp, New Burnswick Scientific Company Incorporated, Edison, New Jersey, USA).

2.1.3. Identification of *Candida* Species

2.1.3.1. CHROMagar™ Candida Medium

Presumptive identification of *Candida* isolates was undertaken following primary culture from clinical specimens on the chromogenic medium CHROMagar™ Candida (CHROMagar) (Fig. 2.1), which contains chromogenic substances that allow colonies of some *Candida* species to be presumptively identified on the basis of colony colour and/or morphology as follows: *C. albicans* colonies appear light blue green, *C. glabrata* colonies appear pink, *C. tropicalis* colonies appear dark blue with a pink halo, *C. krusei* colonies appear as large rough spreading pale pink colonies, *C. parapsilosis* colonies appear white or pale pink, *S. cerevisiae* colonies appear white-purple (Odds and Bernaerts, 1994). *Candida dubliniensis* colonies often appear as small dark green colonies on CHROMagar medium on primary isolation but frequently lose this trait upon subculture (Coleman *et al.*, 1997).

2.1.3.2. Biotyping

Candida isolates were definitively identified using the API ID 32C yeast identification system (BioMérieux) (Fig. 2.2). The system is used to identify *Candida* isolates to a species level based on a series of carbohydrate assimilation tests contained in 32 separate cupules on a plastic strip. The tests were carried out according to the manufacturer's instructions. Briefly, an inoculum was prepared from a few colonies cultured on PDA agar for 48 h in 2 ml of PBS to the turbidity equivalent to a 2

McFarland standard (BioMérieux). A 250 µl aliquot of this homogenised suspension was then used to inoculate a tube of API C medium supplied by the manufacturer. Each of the cupules in the strip was then inoculated with 135 µl of the homogenised API C medium suspension and incubated for 48 h at 30°C. Growth in each cupule was visually assessed and compared to that of a control cupules lacking substrate, which was also included in the strip. Any cupules that are more turbid than the control after 48 h incubation are recorded as positive. Results from each cupule were recorded on a results sheet supplied by the manufacturer, and the substrate assimilation profile of the isolate was converted into an eight digit numerical profile. This profile was then used to identify the test organism using the profile database available at https://www.apiweb.bioMérieux.com. Each profile is listed along with the percentage of identification (%id), with an estimate of how closely the profile corresponds to a taxon relative to all other taxa in the database and the T index, which is an estimate of how closely the profile corresponds to the most typical set of reactions for a particular taxon. Based on these parameters, a set of reactions which closely resemble those of a particular taxon will be classified as "excellent" or "good" and yield identification to the species level, whereas atypical results will be classed as "poor" and the latter are unusually unable to yield a positive identification.

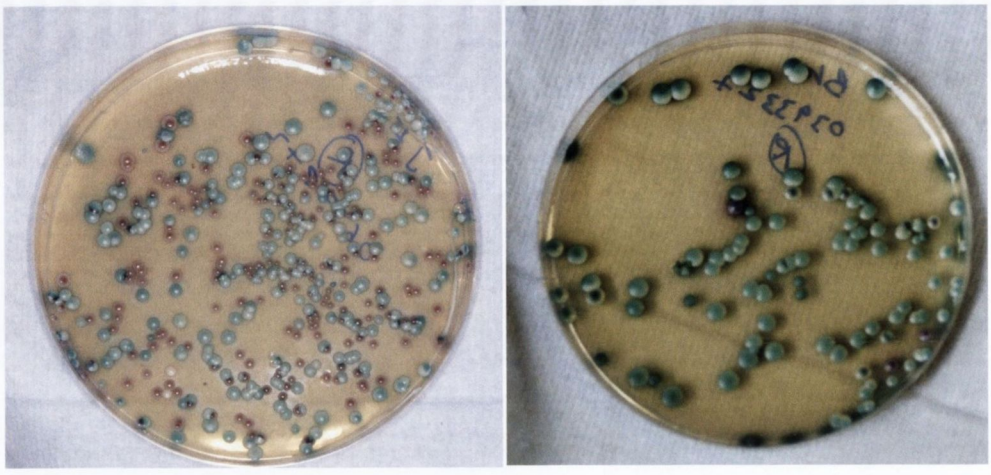
2.1.3.3. Storage of Candida Isolates

Candida isolates were stored in Microbank® cryovials (Pro-Lab Diagnostics, Ontario, Canada) that contain a cryopreservative liquid and porous plastic beads at -80°C. Stored *Candida* isolates were reactivated by subculture on PDA or YEPD growth media and incubated for 48 h at 37°C.

2.1.4. Chemicals, Buffers, Dyes and Solutions

Analytical-grade, molecular biology-grade or cell culture-grade chemicals were purchased from Sigma-Aldrich or Fisher Scientific Ltd. (Loughborough, UK). Phosphate buffer saline (PBS) at a concentration 1x was prepared from tablets (Sigma-Aldrich) and consisted of 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. Accustain® Gram stain (Sigma-Aldrich) consisted of crystal violet solution (2.3% (w/v) certified crystal violet, 01% (w/v) ammonium oxalate and 20% (w/v) ethyl alcohol), Gram's iodine solution (0.33% (w/v) iodine and

(a) (b)



(c)

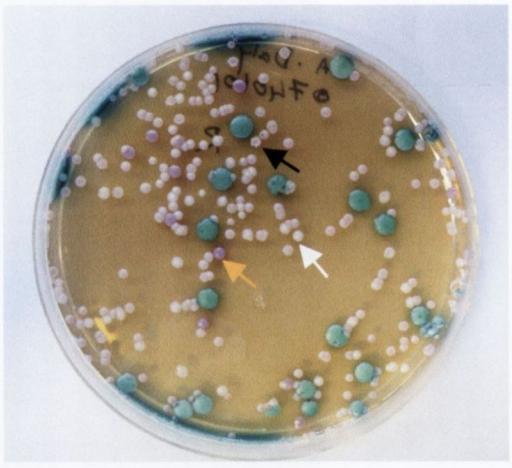


Figure 2.1 Photographs of CHROMagar Candida agar plates containing *Candida* colonies. CHROMagar Candida is useful for the presumptive identification of several clinically important *Candida* species and is particularly effective at identifying the presence of mixed species in individual clinical samples. Panel a) contains three colony types; *C albicans* (green colonies), *C. glabrata* (Pink colonies) and *S. cerevisiae* (creamy colonies), panal b) contains two colonies type; *C. albicans* (green colonies) and *C. tropicalis* (blue colonies) and panel c) contains four types of colonies *C. albicans* (green colonies), *C. parapsiilosis* (white arrow; smooth creamy pink colonies), *C. guilliermondii* (black arrow; small rough pinkish colonies) and *C. glabrata* (orange arrow; pink colonies).

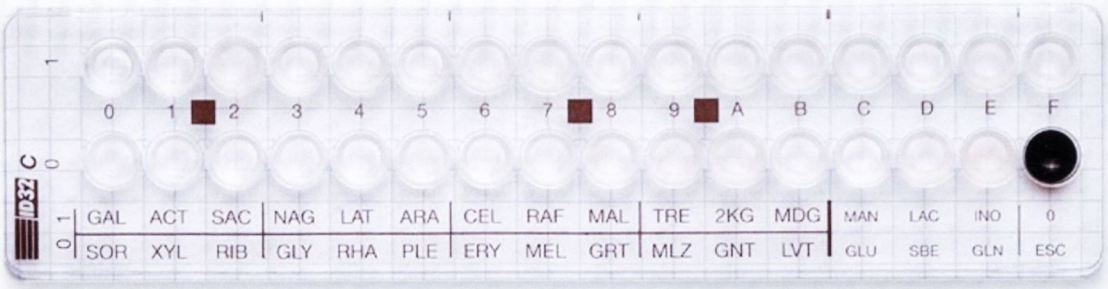


Figure 2.2 Photograph of an individual ID 32C strip used in the present study for the definitive identification of *Candida* species isolates. Isolate identification is based on the ability of a test isolate to assimilate a series of carbohydrate substrates contained in 32 separate cupules. Growth in each cupule is visually assessed and compared to that of a control cupules lacking substrate (Cupule 1-F). The pattern of test assimilations is then converted into a digital code and identification is made relative to the profiles contained in www.apiweb.com.

0.66% (w/v) potassium iodide [Sigma-Aldrich]) and decolouriser (75% (w/v) isopropyl alcohol and 25% (w/v) acetone [Sigma-Aldrich]).

2.1.4.1. Tissue Culture Media and Supplements

Dulbecco's Modified Eagle's Medium (DMEM) used as a basic tissue culture medium (supplemented with 4.5 g/l glucose, sodium pyruvate, L-glutamine and sodium bicarbonate), Hams F-12 nutrient medium, heat inactivated foetal calf serum (FCS), penicillin-streptomycin, fungizone (amphotricin B), epidermal growth factor, hydrocortisone, insulin, L-glutamine, adenine and cholera toxins all were purchased from Sigma-Aldrich. Trypsin-EDTA 1x (0.25 % (v/v)) and Dispase enzyme was purchased from Sigma-Aldrich. Hank's balanced salt solution (HBSS) without calcium and magnesium was prepared in a volume of 1 litre from 8 g/l sodium chloride, 0.4 g/l potassium chloride, 0.06 g/l potassium phosphate (monobasic), 0.048 g/l sodium phosphate (dibasic), 0.35 g/l sodium bicarbonate, 1 g/l D-glucose and 0.01 g/l phenol red, pH adjusted to 7.0 to7.4, solution were then sterile filtered. XTT solution (contained 0.6 mM XTT sodium salt (sodium 3′-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), minimum 90%) and 0.27 mM coenzyme Q (2,3-Dimethoxy-5-methyl-*p*-benzoquinone) in PBS. Triton X-100 (t-octylphenoxypolyethanol) was purchased from Sigma-Aldrich.

2.1.4.2. Oligonucleotides and Molecular Biology Reagents

Custom-synthesised oligonucleotides were purchased from Sigma Life Science (Sigma-Aldrich). DNA molecular weight markers, deoxynucleotide triphosphates (dNTPs), magnesium chloride and *Taq* DNA polymerase and *Taq* reaction buffer were purchased from the Promega Corporation (Madison, WI, USA) and used according to the manufacturers' instructions. Zymolase 20T (21,600 U/g) was purchased from the Seikagaku Corporation (Tokyo, Japan). Zymolase buffer consisted of 1 M sorbitol, 100 mM EDTA and 14 mM β-mercaptoethanol. Cell lysis buffer consisted of 10 mM Tris-HCl 2% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulphate (SDS), 1 mM ethylenediaminetetracetic acid (EDTA) and 100 mM NaCl, pH 8.0. Liquefied phenol was purchased from Fisher Scientific Ltd., and was used in preparation of phenol: chloroform: isoamyl alcohol (24:24:1). Tris-EDTA (TE) buffer consisted of 1 mM EDTA and 10 mM Trizma® base pH 8.0. Tris-borate-EDTA (TBE) buffer was prepared at 10x concentration and consisted of 0.89 M Trizma® base (Sigma-Aldrich), 0.89 M

boric acid (Sigma-Aldrich) and 0.02 EDTA (Sigma-Aldrich). Prior to use, TBE 10x was diluted in Milli-Q® ultra-purified water (resistivity 18.2 MΩ.cm) (Millipore™) to a final concentration of 0.5% (v/v) and was used as the buffer for agarose gel electrophoresis. DNA templates and agarose gels were prepared at the required concentration by dissolving agarose (Sigma-Aldrich) in 0.5x TBE buffer containing 0.013 M ethidium bromide (Sigma-Aldrich)/GelRed (Biotium Inc. Hayward, CA, USA). Samples for electrophoresis were loaded into agarose gels in 6x blue/orange loading dye (Promega). PCR products were visualised on an UV transilluminator (AphaImger®, Medical Supply Company, Dublin, Ireland).

2.1.5. Kits

The ID32C yeast identification kit (BioMérieux, Marcy l'Etoile, France) was used for definitive identification of yeast isolates (section 2.1.2). Yeast genomic DNA extraction were performed using the DNeasy Blood & Tissue Kit (Qiagen, Crawley, West Sussex UK) (see Section 2.2.1). PCR products were purified using the Qiaquick 96 PCR Purification Kit (Qiagen) and the Sigma GenElute™ PCR CleanUp kit (Sigma-Aldrich).

2.1.6. Disposable Laboratory Plasticware

Microfuge tubes (0.2 ml, 0.5 ml, and 1.5 ml) and 96-well microplates were purchased from Eppendorf AG (Hamburg, Germany). Fifteen ml screw cap tubes, 50 ml universal tubes with a conical bottom, 5 ml, 10 ml, and 25 ml pipettes, 96-well flat bottomed tissue culture plates with lids, 90 mm diamater triple-vented Petri dishes and 90 mm treated tissue culture plates were purchased from Greiner Bio-One GmbH., (Solingen, Germany). Oral rinse sampling disposable containers with screw cap, screw cap tubes of 2 ml capacity with conical bottom and skirt and collagen coated75 cm^2 tissue culture flasks (Cell + growth surface) were purchased from Sarstedt AG & Co. (Drinagh, Wexford, Ireland). Corning® Cellbind® 75 cm^2 cell culture flasks were purchased from Sigma- Aldrich. Pipette tips with and without filters were purchased from Starlab GmbH (Ahrensburg, Germany).

2.2. DNA Isolation and Purification

2.2.1. Genomic DNA Extraction

Two methods of genomic DNA extraction from *Candida* isolates were employed. The concentration of DNA samples was determined by measuring their absorbance at 260 nm using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Dublin, Ireland).

2.2.1.1. DNA Extraction by Bead-Beating

One or two colonies from *C. albicans* isolate grown on PDA agar for 48 h were inoculated into 5 ml of yeast extract-peptone-dextrose broth YEPD (YEPD broth (1% (w/v) yeast extract (Sigma-Aldrich), 2% (w/v) bacterial peptone (Oxoid) and 2% (w/v) glucose (Sigma-Aldrich) and grown overnight at 37°C in an orbital shaking incubator (Gallenkamp) at 200 rpm. Cells from 1.5 ml of culture were harvested by centrifugation in Eppendorff 5417C microfuge (Eppendorf AG) at 12000 x *g*. The resulting pellet was suspended in cell lysis buffer (Section 2.1.2) and then transferred to a 2 ml screw-capped tube (Sarstedt) that contained 0.3 g of acid washed glass beads of 425-600 µm in diameter (Sigma-Aldrich), 200 µl of phenol: chloroform: isoamyl alcohol (24:24:1) mixture was added and the cells were vortexed in a BIO101 FastPrep instrument (Qbiogene, Cambridge, UK) for 1 min at maximum speed. The mixture was then centrifuged at 14,000 x *g* for 10 min. The aqueous phase was transferred to a new tube and extracted with 200 µl of chloroform: isoamyl alcohol (24:1) solution. DNA was precipitated by the addition of 400 µl absolute ethanol (Sigma-Aldrich) at -20°C. Purified DNA was pelleted by centrifugation for 10 min at 14,000 x *g*, washed with 70% (v/v) ethanol, dried and suspended in 50 µl of Milli-Q ultrapurified water.

2.2.1.2. DNA Extraction Using the DNeasy Blood and Tissue Kit

Candida albicans isolates were grown and harvested as previously described in section 2.3.1.1. Cell walls were digested by resuspended the pellet in 200 U of Zymolase enzyme in sorbitol buffer and incubated at 37°C for 30 min. Spheroplasts were harvested by centrifugation at 300 x *g* for 10 min and were treated with 20 µl of 10 mg/ml of proteinase K and incubated at 56°C until completely lysed. DNA was extracted from the lysed cells by using a DNeasy® blood and tissue Kit for DNA extraction (Qiagen) according to the manufacturer's instructions.

2.2.2. Polymerase Chain Reaction

Custom-synthesised oligonucleotides were purchased from Sigma Genosys Biotechnologies Europe Ltd. (Pampisford, Cambridgeshire, UK) and were used in PCR amplification experiments (MLST and ABC typing). Each PCR reaction required specific sets of conditions that are described in Chapter 6.

2.2.3. Purifications of PCR Products

Amplified PCR products for DNA sequencing were purified to remove salt, unincorporated primers and nucleotides. Purification of PCR amplimers were performed using both the Qiaquick® 96 well purification kit (Qiagen) and the Sigma GenElute® PCR CleanUp kit (Sigma-Aldrich) according to the manufacturers' instructions.

2.2.3.1. Gel Electrophoresis

Amplified PCR products were separated by conventional agarose gel electrophoresis. Briefly, agarose gels were prepared at the required concentration by dissolving agarose (Sigma-Aldrich) in 0.5x TBE buffer containing 0.013 M ethidium bromide (Sigma-Aldrich) or 10 µl of 10,000X GelRed (Biotium Inc). in 100 ml of agarose gel. Agarose gel were usually prepared at a range of concentrations; 2% (w/v) for visualising PCR amplimers for multilocus sequencing typing (MLST) and 3% (w/v) for ABC typing. Samples for electrophoresis were loaded into agarose gels in 6x blue/orange loading dye (Promega). Electrophoresis was carried in horizontal agarose gels in 25x15x6 cm electrophoresis tanks (Medical Supply Ltd, Dublin, Ireland) using 0.5% (v/v) TBE as the electrophoresis buffer, at 5 V/cm for 0.5-3 h . PCR products were visualised on an UV transilluminator (AphaImager®).

2.2.4. DNA Sequencing

DNA sequencing reactions were performed commercially either by Co:Genics (Essex, UK), Eurofins MWG Operon (Ebersberg, Germany) or Geneservice (Source Bioscience, Dublin, Ireland) using an ABI 3730*xl* DNA analyzer and dye-labeled terminators (Applied Biosystems, Foster City, CA, USA). Primers used in PCR amplification were also used in DNA sequencing.

2.3. Statistical Analysis

Statisical analyses were performed using contigency tables tests: Fisher's exact test and Chi square test GraphPad (GraphPad Software Inc. La Jolla, CA, USA). Multivariant and regression analysis using JMP® (SAS Instititute Inc. NC, USA) (see Ch. 3), one way analysis of variance (ANOVA) were performed using Prism® software version4.00 (GraphPad software Inc.) (see Ch. 4).

Chapter 3

Candida Species Associated with Leukoplakic and Chronic Hyperplastic Candidosis Oral Lesions

3.1. Introduction

The concept and term leukoplakia as an oral mucosal white patch or plaque was first introduced nearly 150 years ago by the dermatologist Ernst Schwimmer in 1877. The importance of the lesion relates to the fact that some leukoplakias will transform into cancer with time, although identifying which lesions will transform has been the subject of extensive research. As yet, there are no definitive clinical or laboratory features which will accurately and reliably predict that an individual leukoplakic patch will definitely transform into cancer in a given timeframe. Predictors of malignant potential have been identified, but only identify risk, and are of only moderate positive predictive value. Clinical, histopathological, genetic and infectious predictive factors have been identified.

The definition of oral leukoplakia has been the subject of much debate, modification and refinement since the first attempt at definition in 1978 by the World Health Organisation (Kramer *et al.*, 1978). The original definition was of "a white patch or plaque that could not be characterised clinically or pathologically as any other disease" was modified in 1983 by Axéll *et al.* to "a whitish patch or plaque that cannot be characterised clinically or pathologically as any other disease, and is not associated with any physical or chemical causative agent, except the use of tobacco". This definition was further modified by Axéll *et al.* (1996) to "a predominantly white lesion of the oral mucosa that cannot be characterised as any other definable lesion" and finally, revised to the current definition "the term leukoplakia should be used to recognise white plaques of questionable risk having excluded other known diseases or disorders that carry no increased risk for cancer" (Warnakulasuriya *et al.*, 2007). The reason for all these modifications was to try to take account of clinical and histopathological features and malignant potential which may affect prognosis and exclude recognised oral conditions and lesions which also present as oral white patches. Clinical and family history, clinical lesional features, histopathological diagnosis or other laboratory tests, such as serology may then further define specific entities presenting as oral white patches but which are not leukoplakia. Oral leukoplakia therefore becomes a diagnosis of exclusion. These non-leukoplakic conditions and diseases include acquired, hereditary and genetic conditions such as: simple keratoses (such as frictional keratosis), "leukoplakias" of putative infective origin (e.g. syphilitic, hairy leukoplakia), papillomavirus lesions, lichen planus and lichen sclerosus et

atrophicus, lupus erythematosus, dermatomyositis, oral submucous fibrosis, carcinomas, drug burns, skin grafts, white sponge naevus, leukoedema, pachyonychia congenita, tylosis, hereditary benign intra epithelial dyskeratosis, dyskeratosis follicularis and dyskeratosis congenita/Fanconi anaemia spectrum.

Lehner (1964) described chronic oral *Candida* infection presenting in the form of leukoplakia and introduced the term "candidal leukoplakia", although histopathologist currently prefer the term chronic hyperplastic candidosis. The importance of this lesion arises from reports of increased malignant potential (Cawson and Lehner, 1968) compared to simple leukoplakia. The oral lesions of chronic hyperplastic candidosis almost invariably cannot be differentiated clinically from "classical" leukoplakia (although there are subtle clinical features, such as the site of the lesions) but must be definitively diagnosed histopathologically with special staining techiques. The presence of invading *Candida* hyphae in superficial layers of epithelium, intraepithelial inflammatory infiltrates of polymorphonuclear leukocytes and usually accompanying dysplasia are the hallmark histopathological features of CHC lesions (Cawson and Lehner, 1968; Sitheeque and Samaranayake, 2003).

However, the role and types of *Candida* inducing these keratotic changes and cellular atypia is not clear and there is some controversy regarding the initiation of epithelial hyperplasia by *Candida*. Jepsen and Winther (1965) suggested that *Candida* invades the pre-existing hyperplastic lesion rather than being the cause of it. In contrast, since Cawson and Lehner (1968) proposed that *Candida* infection is the primary cause of CHC, evidence of the role of *Candida* in the development of the lesion and dysplastic change in the lesions came from the observation of the resolution of some CHC lesions in response to antifungal therapy, the presence of the CHC in childhood as a part of mucocutaneous syndromes such as APECED and laboratory animal studies, (Russell and Jones, 1975; Holmstrup and Besserman, 1983; Franklin and Martin, 1986; Lamey *et al.*, 1989; Dwivedi *et al.*, 2009).

McCullough *et al.* (2002) reported a strong statistical association between increased numbers of oral yeast cells detected by oral rinse sampling and oral epithelial dysplasia and oral squamous cell carcinoma, and the degree of epithelial dysplasia correlated with increased oral yeast numbers. This study demonstrated not only the presence of high levels of yeast carriage in the mouth of patients with CHC, but for the first time *in vivo*, the correlation with epithelial dysplasia suggested a causative role for *Candida* in patients with dysplastic CHC. This finding is supported by the laboratory

findings reported by Barrett *et al.* (1998,) where analysis of 223 PAS-positive biopsies showed increased representation of moderate and severe dysplasia. There is very limited data in the world literature, however, examining *Candida* isolates from histopathologically diagnosed CHC lesional tissue where, by definition, pathological hyphal invasion is occurring and the number and characteristics of these isolates compared to commensal isolates present in saliva cultured from established leukoplakic lesions. With leukoplakic lesions that are not CHC, the inference is that *Candida* species isolated from these lesions are non-pathogenic, commensal blastospore contaminants on the leukoplakic mucosa originating from saliva containing planktonic *Candida* species, but this has not been investigated.

3.1.1. Aims

The aims of this part of this study were:

- To isolate, identify and compare *Candida* species recovered from non-*Candida* leukoplakia (NCL) and chronic hyperplastic candidosis (CHC) lesions

- To investigate *Candida* species prevalence and relative abundance recovered from swabs sampling of oral NCL and CHC lesions

- To compare cell densities of *Candida* from NCL and CHC isolates obtained by lesional swab sampling with oral rinse isolates from the same individuals

- To correlate *Candida* cell density from swab and oral rinse samples with the degree of dysplasia associated with NCL and CHC lesions

- To investigate the potential influence of tobacco and alcohol usage and denture wearing on oral candidal carriage and dysplasia status in patients with NCL and CHC.

3.2. Materials and Methods

3.2.1. Patients

Ethical approval for these and the following studies was obtained from the St. James's Hospital (SJH) and Adelaide and Meath Hospitals including the National Children's Hospital (AMNCH) Research Ethics Committee in 2008 (see Appendix 1).

Oral rinses and swab samples were collected from patients with oral white patches (either NCL or CHC) attending the Oral Medicine and Dysplasia clinics at the DDUH. Patients were selected, in the NCL group, by site of lesion and an attempt was made to control for age and sex. Since most CHC affects the buccal and commissural mucosa and tongue, patients in the NCL group with lesions at this site were enrolled selectively. Site matching was achieved in 60% of cases. The patients were examined by two oral medicine consultants and an oral and a maxillofacial surgeon, (in a dedicated Dysplasia clinic), where a medical and dental history was recorded and a comprehensive oral examination undertaken. Incisional biopsies were undertaken in all cases. Clinical and histopathological details were recorded on the data sheet for each patient. Thirty one patients with CHC and 47 with NCL were identified for study making a total of 78 patients with oral leukoplakic lesions (OL).

3.2.2. Oral Sampling for *Candida*

3.2.2.1. Oral Rinse

Oral rinse samples were taken with 10 ml of sterile MilliQ ultra-purified water (Millipore), provided in a 50 ml sterile plastic container (Sarstedt Ltd.). Each patient rinsed for 60 s with the 10 ml water aliquot, and expectorated into the same container which was then transported to the hospital's Microbiology Laboratory for processing within 2 h. One millilitre of each sample was transferred aseptically into a sterile 1.5 ml Eppendorf tube (Eppendorf AG), centrifuged in an Eppendorf 5417C centrifuge at 20200 x g for 1 min. Following centrifugation, the supernatant was discarded and 100 µl of sterile ultrapure water was added to the pellet. This was then mixed with a mini vortexer Whirlimixer (Fisher Scientific) for 15 s and plated onto CHROMagar™ Candida culture medium (CHROMAgar Company) using sterile plastic L-shaped spreaders (Greiner Bio-One GmbH), followed by incubation at 37° C for 48 h. Following incubation, the plates were examined and the density of yeasts present in

each sample was recorded in colony forming units per millilitre (cfu/ml). The relative abundance of different coloured colonies and colonies with distinct morphologies was also recorded. Some yeast colonies were presumptively identified based on characteristic colony colours on CHROmagar Candida medium as describe in Chapter 2, General Material and Methods, section 2.1.2 and Fig. 2.1.

3.2.2.2. Lesion Swab Sampling

Swab samples were taken using sterile cotton transport swabs and then transferred to tubes containing alginate gel (Venturi Transystem). Swabbing was performed by rubbing the entire surfaces of the lesion for 30 s; the swabbed areas sampled depended on the size of the lesion. Swabs samples were then transferred to the hospital's Microbiology Laboratory within 2 h and plated immediately on to CHROMagar™ Candida medium, followed by incubation at 37° C for 48 h for presumptive identification (see Chapter 2, section 2.1.2.1). The density of yeasts present in each sample was recorded in cfu per swab. The relative abundance of each colony type present on each plate was also recorded.

Samples from both oral rinse and lesional swabs that yielded mixed cultures with confluent and subconfluent yeast growth were serially diluted to identify the different species present in the samples and to obtain a quantitative estimate of *Candida* cell density.

A representative colony of each colony colour and/or morphology isolated on CHROMagar Candida agar for each sample was selected at random, purified by subculture on PDA agar and stored at -80°C on cryobeads to await subsequent detailed analysis (see Chapter 2, section 2.1.2.3). Definitive identification of all stored isolates was undertaken following 48 h growth on PDA agar using the API ID 32C yeast identification system (BioMérieux) according to the manufacturer instructions (see Chapter 2, section 2.1.2.2).

The cell density of *Candida* species recovered from lesional swabs that yielded ≥100 cfu/swab were considered as microbiological evidence of *Candida* infection. This threshold was defined based on clinical and microbiological analysis of oral candidiasis in more than 20,000 specimens that were mainly taken from the mid-dorsum of tongue (McManus *et al.*, 2011). Other cut-off points at ≥30, and ≥50 cfu/swab were also examined and are reported to assess correlations of *C. albicans* density in the lesions with the clinical and histopathological diagnosis.

3.2.3. Histopathology

Incisional lesional biopsies were performed by an oral surgeon at the Oral Medicine and Oral Surgery clinics in DDUH. Histopathological investigation of biopsy samples taken from leukoplakia lesions was performed at the Central Pathology Laboratory at St. James's Hospital by the head and neck pathologist (Dr. M. Toner). Briefly, labelled biopsy specimens were sent in CellStor™ Pots (Cell Path Ltd., Newtown Powys, UK) containing 10% (v/v) neutral buffered formalin (4% (v/v) formaldehyde) and forwarded to the pathology laboratory. Briefly, fixed tissues were dehydrated by a series of washes in 70-100% (v/v) ethanol, followed by clearing the samples with xylene. The samples were then oriented and infiltrated with paraffin wax. Tissue samples were then cut into 6-8 μm thickness sections and stained with haematoxylin and eosin stains followed by periodic acid Schiff stains (PAS) if necessary (Samaranayake and MacFarlane, 1990). Following staining, stained sections were examined by an oral and maxillofacial pathologist who then recorded a diagnosis based on the features of each specimen. The degree of dysplasia of the lesions was also noted.

3.2.4. Identification of *Candida* Isolates

For detailed methods see section (Chapter 2, section 2.1.2). Briefly, yeast isolates were initially presumptively identified on the basis of colony colour and/or morphology following 48 h growth on CHROMagar™ Candida medium. Definitive identifications of yeast isolates was undertaken using the API ID 32C yeast identification system (BioMérieux) which identifies yeast isolates based on their carbohydrate assimilation profile.

3.2.5. Statistical Analysis

Statistical analysis were performed by Contingency table tests; Fisher's exact test and Chi square test GraphPad (GraphPad Software, Inc. La Jolla, CA, USA) (http://www.graphpad.com/quickcalcs/index.cfm) and multivariate and regression analysis performed on JMP® software, (SAS Institute Inc. NC, USA).

3.3. Results

3.3.1. Patients and Samples

Seventy eight (OL) patients were studied in detail based on the availability of definitive clinical and histopathological diagnosis of their leukoplakic lesions. The lesions in 31/78 (39.7%) patients were diagnosed histopathologically as CHC by demonstration of hyphal invasion (and polymorphonuclear leukocyte invasion) of the surface epithelium on PAS staining. Forty-seven (60.3%) patients were diagnosed with oral leukoplakia. The latter are referred to hereafter as having non-candidal leukoplakia (NCL) throughout this study. None of the 47 NCL patients showed histopathological evidence of hyphal invasion following histopathological analysis. The same oral and maxillofacial pathologist examined and reported on all the specimens, thus minimising inter-observer error. As has been previously discussed, wide inter-observer variation can occur between different pathologists reporting on the same specimens, with respect to grading of dysplasia (Pindborg *et al.*, 1985). Full details of the cases are presented in Tables 3.1 to 3.4).

3.3.1.1. Age and Gender

The age range of the patient cohort was 29-87 years (mean of 58.7 years). The age range of patients with CHC 29-77 years (mean 57.5 years) and patients diagnosed with NCL had an age range of 35-87 years (mean, 59.4 years). There were a higher number of females in the cohort than expected. Thirty eight (49%) patients were female and 40 (51%) were male in the OL group, with 13/31 (41.9%) in the CHC group and 25/47 (53.2%) in the NCL group (Table 3.1 and 3. 2).

3.3.1.2. Ethnic Groups

The majority of the patients were Irish 75/78 (96.1%). The remaining three were German, Indian and East African.

3.3.1.3. Medical History

The medical histories of the patients were analysed. There was no excess of medical predisposing factors for oral candidosis such as diabetes mellitus, xerostomia (or history of radiotherapy) corticosteroid use, such as inhalers, or known immunosuppression between the CHC and NCL groups.

Table 3.1 History and clinical features of 31 cases of *Candida* leukoplakia investigated for oral *Candida*

Case No.	Lesion	Lesion site	Smoker	CPD	Alcohol consumption u/w	Upper denture
1.1	Chronic hyperplastic candidosis	Palate	Yes	20	10	Yes
1.2	Chronic hyperplastic candidosis	Buccal mucosa	Yes	20	5	Yes
1.3	Chronic hyperplastic candidosis	Tongue and soft palate	No	0	4	Yes
1.4	Chronic hyperplastic candidosis	Buccal mucosa	Yes	20	20	No
1.5	Chronic hyperplastic candidosis	Buccal mucosa	Yes	10	8	Yes
1.6	Chronic hyperplastic candidosis	Tongue and plate	No	0	0	No
1.7	Chronic hyperplastic candidosis	Alveolar ridge	Yes	50	3	Yes
1.8	Chronic hyperplastic candidosis	Palatal gingiva	No	0	0	No
1.9	Chronic hyperplastic candidosis	Tongue and buccal mucosa	No	0	4	No
1.10	Chronic hyperplastic candidosis	Buccal mucosa	Yes	15	10	Yes
1.11	Chronic hyperplastic candidosis	Buccal mucosea	Yes	15	5	Yes
1.12	Chronic hyperplastic candidosis	Buccal mucosa	Yes	10	12	No
1.13	Chronic hyperplastic candidosis	Buccal mucosa and floor of the mouth	No	0	0	Yes
1.14	Chronic hyperplastic candidosis	Buccal mucosa	Yes	20	15	No
1.15	Chronic hyperplastic candidosis	Buccal mucosa	No	0	10	No

Continued overleaf

Table 3.1 Continued

Case No.	Lesion	Lesion site	Smoker	CPD	Alcohol consumption u/w	Upper Denture
1.16	Chronic hyperplastic candidosis	Buccal mucosa	Yes	20	4	No
1.17	Chronic hyperplastic candidosis	Buccal mucosa	Yes	20	12	No
1.18	Chronic hyperplastic candidosis	Buccal mucosa	Yes	10	4	No
1.19	Chronic hyperplastic candidosis	Buccal mucosa and tongue	No	0	0	Yes
1.20	Chronic hyperplastic candidosis	Palate and tongue	No	0	0	No
1.21	Chronic hyperplastic candidosis	Tongue	No	0	0	No
1.22	Chronic hyperplastic candidosis	Buccal mucosa	Ex	0	4	Yes
1.23	Chronic hyperplastic candidosis	Buccal mucosa	Yes	30	20	Yes
1.24	Chronic hyperplastic candidosis	Buccal mucosa	No	0	3	No
1.25	Chronic hyperplastic candidosis	Buccal mucosa	Yes	10	10	No
1.26	Chronic hyperplastic candidosis	Buccal mucosa	Yes	10	6	No
1.27	Chronic hyperplastic candidosis	Buccal mucosa	Yes	30	4	Yes
1.28	Chronic hyperplastic candidosis	Buccal mucosa	Yes	30	4	Yes
1.29	Chronic hyperplastic candidosis	Tongue and soft palate	Yes	10	30	No
1.30	Chronic hyperplastic candidosis	Buccal mucosa	Yes	10	20	No
1.31	Chronic hyperplastic candidosis	Buccal mucosa	Yes	10	6	Yes

M, male; F, female; CPD, cigarettes per day; u/w units per week

Table 3.2 History and clinical features of 47 cases of non-*Candida* leukoplakia investigated for oral *Candida*

Case No.	Lesion	Lesion site	Smoker	CPD	Alcohol consumption u/w	Upper denture
2.1	Non candidal leukoplakia	Buccal mucosa	Ex	0	No	Yes
2.2	Non candidal leukoplakia	Buccal mucosa	Ex	0	4	Yes
2.3	Non candidal leukoplakia	Buccal mucosa	Yes	60	No	No
2.4	Non candidal leukoplakia	Tongue	Ex	0	6	No
2.5	Non candidal leukoplakia	Alveolar ridge	Yes	10	No	Yes
2.6	Non candidal leukoplakia	Buccal mucosa, palate and alveolar ridge	Yes	15	6	No
2.7	Non candidal leukoplakia	Tongue	Yes	10	10	Yes
2.8	Non candidal leukoplakia	Soft and hard palate	Yes	40	8	No
2.9	Non candidal leukoplakia	Buccal mucosa	No	0	3	No
2.10	Non candidal leukoplakia	Tongue, palate	Ex	0	10	No
2.11	Non candidal leukoplakia	Tongue	No	0	No	No
2.12	Non candidal leukoplakia	Buccal mucosa	No	0	8	No
2.13	Non candidal leukoplakia	Palate	Yes	20	50	No
2.14	Non candidal leukoplakia	Buccal mucosa, palate and alveolar ridge	Yes	6	4	No
2.15	Non candidal leukoplakia	Buccal mucosa and tongue	Yes	Pipe	10	No
2.16	Non candidal leukoplakia	Alveolar ridge	Yes	10	4	No

Continued overleaf

Table 3.2 Continued

Case No.	Lesion	Lesion site	Smoker	CPD	Alcohol consumption u/w	Upper denture
2.17	Non candidal leukoplakia	Alveolar ridge	Yes	25	15	No
2.18	Non candidal leukoplakia	Floor of the mouth	Yes	20	20	Yes
2.19	Non candidal leukoplakia	Floor of the mouth and buccal mucosa	Yes	40	5	No
2.20	Non candidal leukoplakia	Buccal Mucosa	Yes	20	12	Yes
2.21	Non candidal leukoplakia	Floor of the mouth	Yes	15	3	No
2.22	Non candidal leukoplakia	Retromolar	Yes	40	30	No
2.23	Non candidal leukoplakia	Floor of the mouth	Ex	0	5	No
2.24	Non candidal leukoplakia	Buccal mucosa	Yes	10	12	No
2.25	Non candidal leukoplakia	Buccal mucosa	Ex	0	4	No
2.26	Non candidal leukoplakia	Tongue	No	0	NR	Yes
2.27	Non candidal leukoplakia	Alveolar ridge	No	0	8	Yes
2.28	Non candidal leukoplakia	Buccal mucosa	Ex	0	NR	No
2.29	Non candidal leukoplakia	Buccal mucosa and floor of the mouth	Yes	40	5	No
2.30	Non candidal leukoplakia	Soft palate	Yes	10	NR	No
2.31	Non candidal leukoplakia	Buccal mucosa and palate	Yes	20	4	Yes

Continue overleaf

Table 3.2 Continued

Case No.	Lesion	Lesion site	Smoker	CPD	Alcohol consumption u/w	Upper denture
2.32	Non candidal leukoplakia	Tongue	Yes	10	12	Yes
2.33	Non candidal leukoplakia	Tongue and palate	Ex	0	NR	No
2.34	Non candidal leukoplakia	Buccal mucosa	Yes	10	10	No
2.35	Non candidal leukoplakia	Soft palate	Yes	15	15	No
2.36	Non candidal leukoplakia	Buccal mucosa	Ex	0	NR	yes
2.37	Non candidal leukoplakia	Buccal mucosa	Yes	10	5	No
2.39	Non candidal leukoplakia	Tongue	Yes	10	10	No
2.40	Non candidal leukoplakia	Buccal mucosa	No	0	5	No
2.41	Non candidal leukoplakia	Floor of the mouth	Yes	20	10	No
2.42	Non candidal leukoplakia	Alveolar ridge	No	0	10	No
2.43	Non candidal leukoplakia	Tongue	Yes	20	No	No
2.44	Non candidal leukoplakia	Palate	Yes	10	4	No
2.45	Non candidal leukoplakia	Alveolar ridge and floor of the mouth	Yes	10	5	No
2.46	Non candidal leukoplakia	Gingiva	No	0	NR	No
2.47	Non candidal leukoplakia	Buccal mucosa	No	0	4	No

Ex, ex-smoker; CPD, cigarette per day; u/w, unit per week; NR data not recorded.

Table 3.3. Histopathological findings, *Candida* cell density and species recovered from the 31 cases of CHC investigated

Case no.	Lesion	Sex	Age	Degree of dysplasia	*Candida* cell density		*Candida* species isolated
					Rinse[1] cfu/ml	Swab[2] cfu	
1.1	Chronic hyperplastic candidosis	M	58	Moderate	40	420	*C. albicans*
1.2	Chronic hyperplastic candidosis	F	52	None	70	35	*C. albicans*
1.3	Chronic hyperplastic candidosis	M	74	Severe	0	415	*C. albicans*
1.4	Chronic hyperplastic candidosis	M	38	Moderate	157	42	*C. albicans*
1.5.	Chronic hyperplastic candidosis	M	60	Moderate	C	380	*C. albicans*
1.6	Chronic hyperplastic candidosis	F	77	Mild	1450	120	*C. albicans*
1.7	Chronic hyperplastic candidosis	M	73	None	0	7 2	*C. albicans* *C. glabrata*
1.8	Chronic hyperplastic candidosis	F	58	Severe, SCC	0	480	*C. albicans*
1.9	Chronic hyperplastic candidosis	F	47	Severe	0	1525	*C. albicans*
1.10	Chronic hyperplastic candidosis	M	55	Mild	20 60	130	*C. albicans* *C. dubliniensis*
1.11	Chronic hyperplastic candidosis	F	73	Moderate	120 10 25	84 4 7	*C. albicans* *C. parapsilosis* *C. guilliermondii*
1.12	Chronic hyperplastic candidosis	F	44	Moderate	2	15	*C. albicans*
1.13	Chronic hyperplastic candidosis	M	29	Severe	225 60 35	441 9 1	*C. albicans* *C. tropicalis* *S. cerevisiae*
1.14	Chronic hyperplastic candidosis	M	63	Severe, SCC	SC	166	*C. albicans*
1. 15	Chronic hyperplastic candidosis	F	64	Severe	C	SC	*C. albicans*

Continued overleaf

Table 3.3 Continued

Case no.	Lesion	Sex	Age	Degree of dysplasia	*Candida* density		*Candida* species isolated
					Rinse[1] cfu/ml	Swab[2] cfu	
1.16	Chronic hyperplastic candidosis	F	57	Severe	450	400	*C. albicans*
1.17	Chronic hyperplastic candidosis	M	41	Moderate	160	10	*C. albicans*
1.18	Chronic hyperplastic candidosis	M	69	Moderate	27	0	*C. albicans*
1.19	Chronic hyperplastic candidosis	F	76	Severe	SC	200	*C. albicans*
1.20	Chronic hyperplastic candidosis	M	72	Moderate	290	0	*C. albicans*
1.21	Chronic hyperplastic candidosis	F	63	Severe	C	SC	*C. albicans*
1.22	Chronic hyperplastic candidosis	M	77	Moderate	0	0	None
1.23	Chronic hyperplastic candidosis	M	45	Moderate	10	2	*C. albicans*
1.24	Chronic hyperplastic candidosis	F	60	Moderate	240	125	*C. albicans*
1.25	Chronic hyperplastic candidosis	M	47	Severe	Con 130	440 6	*C. albicans* *C. glabrata*
1.26	Chronic hyperplastic candidosis	M	63	Moderate	SC	SC	*C. albicans*
1.27	Chronic hyperplastic candidosis	F	60	Moderate	280	92	*C. albicans*
1.28	Chronic hyperplastic candidosis	F	46	Mild	0	250	*C. albicans*
1.29	Chronic hyperplastic candidosis	M	46	None	293	220	*C. albicans*
1.30	Chronic hyperplastic candidosis	F	40	Moderate	215	185	*C. albicans*
1.31	Chronic hyperplastic candidosis	M	56	Mild	0	0	None

[1] oral rinse samples with 10 ml sterile ultra-purified water that cultured on CHROMagar® media; [2]Lesional swab samples were cultured on CHROMagar media; cfu, colony forming unit; C, confluent growth; SC semiconfluent growth

Table 3.4 Histopathological findings, *Candida* cell density and species recovered from the 47 cases of NCL investigated

Case no.	Lesion	Sex	Age	Degree of dysplasia	*Candida* density		*Candida* species isolated
					Rinse[1] cfu/ml	Swab[2] cfu	
2.1	Non candidal leukoplakia	F	79	Severe	20	Con	*C. albicans*
2.2	Non candidal leukoplakia	M	55	Severe	50	0	*C. albicans*
2.3	Non candidal leukoplakia	M	62	Moderate	0	2	*C. albicans*
2.4	Non candidal leukoplakia	M	46	Moderate	150	0	*C. albicans*
2.5	Non candidal leukoplakia	F	82	Moderate	30 10	0 7	*C. albicans* *C. glabrata*
2.6	Non candidal leukoplakia	F	41	Severe	48	18	*C. albicans*
2.7	Non candidal leukoplakia	M	83	Moderate	172	150	*C. albicans*
2.8	Non candidal leukoplakia	M	68	None	4	0	*C. albicans*
2.9	Non candidal leukoplakia	F	39	Moderate	10	0	*C. albicans*
2.10	Non candidal leukoplakia	M	59	Severe,	16	9	*C. albicans*
2.11	Non candidal leukoplakia	F	49	Severe	SC	0	*C. albicans*
2.12	Non candidal leukoplakia	F	44	Severe	83	32	*C. albicans*
2.13	Non candidal leukoplakia	M	51	Moderate	79	8	*C. albicans*
2.14	Non candidal leukoplakia	F	64	Severe	39	10	*C. albicans*
2.15	Non candidal leukoplakia	M	76	Mild	10	9	*C. albicans*
2.16	Non candidal leukoplakia	F	55	Mild	50	11	*C. albicans*
2.17	Non candidal leukoplakia	M	40	Mild	0	3	*C. albicans*

Continued overleaf

Table 3.4 Continued

Case no.	Lesion	Sex	Age	Degree of dysplasia	*Candida* density		*Candida* species isolated
					Rinse[1] cfu/ml	Swab[2] cfu	
2.18	Non candidal leukoplakia	F	35	Moderate	485 113	0 1	*C. albicans* *C. krusei*
2.19	Non candidal leukoplakia	F	65	Moderate	20	0	*C. albicans*
2 .20	Non candidal leukoplakia	M	67	Moderate	0	0	None
2.21	Non candidal leukoplakia	F	62	Moderate	100	0	*C. albicans*
2.22	Non candidal leukoplakia	M	73	Moderate	0	0	None
2.23	Non candidal leukoplakia	M	58	Moderate	110	13	*C. albicans*
2.24	Non candidal leukoplakia	M	78	Moderate	40	12	*C. albicans*
2.25	Non candidal leukoplakia	F	51	Moderate	0	203	*C. albicans*
2.26	Non candidal leukoplakia	F	55	Severe	260	107	*C. albicans*
2.27	Non candidal leukoplakia	F	87	Mild	108	0	*C. albicans*
2.28	Non candidal leukoplakia	M	66	None	3040	0	*C. albicans*
2.29	Non candidal leukoplakia	F	66	Mild	20	0	*C. albicans*
2.30	Non candidal leukoplakia	M	60	Mild	477	16	*C. albicans*
2.31	Non candidal leukoplakia	F	78	None	150	41	*C. albicans*
2.32	Non candidal leukoplakia	F	60	Moderate	40	0	*C. abicans*
2.33	Non candidal leukoplakia	M	38	Severe	120	60	*C. albicans*
2.34	Non candidal leukoplakia	M	47	Moderate	180	5	*C. albicans*
2.35	Non candidal leukoplakia	F	48	Moderate	43	2	*C. albicans*

Continued overleaf

Table 3.4 Continued

Case no.	Lesion	Sex	Age	Degree of dysplasia	*Candida* density		*Candida* species isolated
					Rinse[1] cfu/ml	Swab[2] cfu	
2.36	Non candidal leukoplakia	M	81	Severe	C SC	270 120	*C. albicans* *C. tropicalis*
2.37	Non candidal leukoplakia	F	73	Severe	0	0	None
2.38	Non candidal leukoplakia	F	56	Severe	0	0	None
2.39	Non candidal leukoplakia	F	62	Severe	0	0	None
2.40	Non candidal leukoplakia	F	56	Severe	0	0	None
2.41	Non candidal leukoplakia	M	62	Severe	0	0	None
2.42	Non candidal leukoplakia	F	61	Moderate	0	0	None
2.43	Non candidal leukoplakia	F	61	Severe	0	0	None
2.44	Non candidal leukoplakia	F	40	None	0	0	None
2.45	Non candidal leukoplakia	M	51	None	0	0	None
2.46	Non candidal leukoplakia	M	51	Severe	0	0	None
2.47	Non candidal leukoplakia	M	54	Severe	0	0	None

[1] oral rinse samples with 10 ml sterile ultra-purified water that cultured on CHROMagar® media;[2]Lesional swab samples were cultured on CHROMagar media; cfu, colony forming units; C, confluent growth; SC semiconfluent growth

3.3.1.4. Tobacco Use

Forty nine (62.8%) patients were smokers of which 20/31 (64.5%) were in the CHC group and 29 (61.7%) were in the NCL group. Ten patients were previous tobacco users (12.8%), 1/31 (3.2%) in the CHC group and 9/47 (19.1%) in the NCL group. The remaining 19 (24.3%) of patients were non-smokers, 10/31 32.2%) in the CHC group and 9/47 (19.1%) in the NCL group. Mean number of self reported cigarettes per day in smokers was 17.6 cigarettes per day for the NCL cohort and mean tobacco use for patients with CHC lesions was 16.6 cigarettes per day. For the entire OL cohort the mean was 17.5 cigarettes per day.

3.3.1.5. Alcohol Use

Sixty patients (76.9%) regularly consumed alcohol, of which 25/31(67.7%) were in the CHC group and 35/47 (74. 4%) were in the NCL group. The mean consumption fof alcohol or NCL and CHC patients was 6.9 units per week and 7.5 units per week, respectively, and the mean was 7.1 units per week for the entire OL cohort.

3.3.1.6. Dentures

Fifteen patients (45.5%) with CHC lesions were upper denture wearers, while only 11 (23.4%) patients with NCL were upper denture wearers.

3.3.2. Anatomical Distribution of Leukoplakic Lesions

The buccal mucosa was the most commonly affected site for CHC lesions, affecting 24 patients (77.4%), followed by the tongue in four (12.9%) patients and the palatal mucosae in three (9.7%) patients. Six CHC patients had leukoplakic lesions in several intraoral sites (Table 3.1). Leukoplakic lesions in the NCL group were distributed on the buccal mucosae in 20 patients (42.5%), followed by the tongue in nine patients (19.1%), the floor of the mouth in six patients (12.8%), the palatal mucosae and alveolar ridge in five patients (10.6%) each and the retromolar area and the gingivae in one patient (2.1%) each (Table 3.2). Intra oral distribution at multiple sites was seen in 6/31 (19.4%) of CHC and 10/47 (21%) NCL patients.

3.3.3. Prevalence of *Candida* Species and Oral Leukoplakia Lesions

Oral carriage of *Candida* in patients with all oral leukoplakias (OL) was investigated by oral rinse sampling and lesional swab sampling. Samples were cultured on CHROMAgar™ Candida agar for the presumptive identification and differentiation of different *Candida* species. Definitive identification of *Candida* species was carried out using the API ID32 C yeast identification system.

3.3.3.1. Oral Rinse Sampling

Candida isolates were recovered from oral rinse samples in 55/78 (70.5%) OL patients. *Candida albicans* was the most predominant *Candida* species isolated (n=55) and was recovered from all the CHC group of patients and was also present in all cases where *Candida* species were isolated (n=31) from the NCL group. Several non-*C. albicans* *Candida* species were also recovered from a small number of patients including *C. glabrata* (n=3), *C. tropicalis* (n=2), *C. parapsilosis* (n=1), *C. dubliniensis* (n=1), *C. guilliermondii* (n=1) and *C. krusei* (n=1) (Table 3.5) but at very low abundance compared to *C. albicans*. In the CHC group mixed species were recovered from 5 patients and from 3 patients in the NCL group. The yeast *Saccharomyces cerevisiae* (n=1) was isolated isolated from a single CHC patient (Table 3.5).

Oral rinse samples yielded *Candida*-positive results in 24/31 (77.4%) CHC cases (Table 3.3). The remaining seven CHC patients were *Candida* culture-negative following oral rinse sampling even though these seven patients had been histopathologically diagnosed as having CHC. Three of these seven patients were *Candida*-positive on lesional swab sampling (see section 3.3.3.2).

Oral rinse sampling is a representative sampling technique used to assess the oral candidal load in patients who carry *Candida* in their mouths. Regression analysis was performed to ascertain if the clinical parameters: age, sex, case (CHC or NCL), smoking, alcohol use and upper denture wear, predisposed patients to oral candidal carriage or increased candidal load in the total group (OL) and NCL and CHC subsets. Although there was no association between the presence of lesions and smoking in the OL, CHC and NCL groups, the statistics did approach significance ($P=0.07$). However, in terms of candidal load in the total OL group, smokers had a significant predisposition to harbour higher candidal loads in their mouths ($P<0.040$). Patients with CHC ($P<0.001$), almost by definition had a highly significant association with candidal

Table 3.5 Distribution of *Candida* species recovered from oral leukoplakic lesions from the rinse and swab samplings.

Species	NCL		CHC	
	Rinse	Swab	Rinse	Swab
C. albicans	31	21	24	27
C. dubliniensis	0	0	1	0
C. glabrata	0	0	1	2
C. parapsilosis	0	0	1	1
C. krusii	1	1	0	0
C. tropicalis	1	1	1	1
C. guilliermondii	0	0	1	1
S. cerevisiae	0	0	1	1

carriage, but not load. Age, sex and denture wear in this data set were not independently significant variables for higher candidal load in the OL group.

In the CHC group, denture wearers had increased candidal loads ($P<0.007$) as did patients over 50 years ($P<0.007$). In the NCL group, smoking was significantly associated with higher oral candidal loads ($P=0.04$), but not with *Candida* carriage ($P=0.07$).

3.3.3.2. Lesional Swab Sampling

Candida albicans was cultured from swabs taken from 48/78 of the total OL patient cohort. Of the 31 CHC patients, 27/31 (87.1%) yielded positive cultures for *Candida* species and swabs from 4 CHC lesions yielded mixed cultures with more than one species. Of these, *C. albicans* was present in all cases as the predominant species. *Candida glabrata* was co-isolated with *C. albicans* in two cases, *C. parapsilosis* and *C. guillermondii* were co-isolated with *C. albicans* in one case and *C. tropicalis* and *S. cerevisiae* were co-isolated with *C. albicans* in one case. In all cases, the relative abundance of the non-*albicans* yeasts was low (≤ 10 cfu/swab). The isolation of *S. cerevisiae* from one patient is of uncertain clinical significance. Almost half (21/47, 44.6%) the swabs from the NCL group yielded *C. albicans* and generally in low abundance (Tables 3.4 and 3.5). In two cases, the only *Candida* species isolated was *C. glabrata* and *C. kruseii*, respectively and in a third case the leukoplakia lesion yielded both *C. albicans* and *C. tropicalis* (Tables 3.4 and 3.5).

Nineteen of the 27 (70.4%) *Candida*-positive CHC patients yielded a significant number of cfus from the swab sample (i.e. ≥100 cfu per swab). A significance cut-off point of ≥100 cfu per swab has been determined in the DDUH Oral Bioscience clinical and laboratory database based on large numbers (20,000) of oral swab samples taken over a 20 year period. The cell density of other *Candida* species were also recorded (Tables 3.3 and 3.4). In the case of three patients *Candida* was recovered by lesional swab sampling but these patients were *Candida* culture-negative on oral rinse sampling. *Candida* isolates were recovered from 23/47 (48.9%) NCL lesions by swab sampling but only 5/47 (10.6%) lesions yielded a significant (i.e. ≥100 cfu per swab) *Candida* cell density (Table 3.4).

Candida levels recovered from lesional swabs were also a better discriminator between CHC and NCL compared to oral rinses (Spearman nonparametric analysis, $P<0.038$). There were no associations between *Candida* density recovered from either

oral rinse samples or lesional swabs and the parameters of age, sex, tobacco and alcohol use and tobacco dose in the CHC group, but denture wear did correlate with higher candidal loads recovered from oral rinse samples.

3.3.4. *Candida* Density in Oral Leukoplakic Lesions from Swab Sampling and Comparison of Rinse and Swab Results

To investigate the significance of *C. albicans* cell density recovered from leukoplakic lesions, three cell density cut-off points in cfu were established to assess correlations of *C. albicans* density in the lesions with the clinical and histopathological diagnosis. These were set at ≥30, ≥50 and ≥100 cfu per swab (Table. 3.6), respectively. The difference in cultured yeast density recovered from lesions diagnosed as CHC and NCL was significant ($P<0.001$) at 30, 50 and 100 cfu per swab using the Fisher's exact test. Thus the use of a candidal swab was discriminatory between CHC and NCL at all levels investigated (see Table 3.6).

Comparing the rinse and swab data there was discordance in the CHC group between swab and rinse results. *Candida* recovery was negative in 2/31 cases of histologically proven CHC by the swab technique. Interestingly, of the 7 patients who yielded *Candida*-negative results by oral rinse sampling, 5 patients grew *Candida* from their swab sample. In 2 cases, the swab was negative but the rinse positive, and *Candida* was not isolated by either technique in 2 cases.

Candida albicans was isolated in all CHC cases but secondary species, (*C. glabrata*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii* and *S. cerevisiae*) were also identified, albeit in low abundance relative to *C. albicans*.

Candida species were cultivated from oral rinse samples of 33/47 (70%) of NCL patients and the swab sample was positive in 23/47 (49%) (Table 3.4). In 13 cases the patients were *Candida* carriers according to the rinse culture data, but their lesional swab was negative. In 3 patients their swab sample was positive, but rinse samples were negative. Both rinse and swab culture was negative in 13 NCL patients.

3.3.5. *Candida* Species Density and Degree of Dysplasia

Degree of dysplasia in oral leukoplakic OL lesions is shown in Table 3.7. There was no significant difference noted in the numbers of patients in the no dysplasia, mild dysplasia, moderate dysplasia or severe dysplasia grades between the NCL and CHC groups (although not significant statistically there was a moderate excess of severe

Table 3.6 *Candida albicans* density recovered from patients with CHC and NCL in all cases from lesional swabs

Lesions	*Candida* cell density in cfu					
	≤30	≥30[a]	≤50	≥50[a]	≤100	≥100[a]
CHC *(n=31)*	8	23	10	21	12	19
NCL *(n=47)*	40'	7	41	6	42	5

[a] The difference in *Candida* cell density thresholds between CHC and NCL lesions was highly significant (*P <0.0001*).

Table 3.7 distribution of degree of dysplasia in chronic hyperplastic candidosis and oral leukoplakia lesions

Lesion	None	Mild	Moderate	Severe
Chronic hyperplastic candidosis	4	3	15	9
Non-candidal leukoplakia	5	7	17	18

Table 3.8 Correlation of degree of dysplasia with the *Candida* density in all lesions of CHC and NCL at three *Candida* density cut-off points a) 100 cfu, b) 50 cfu and c) 30 cfu

a)

Lesions	Non and Mild *n* (%)		Moderate and severe *n* (%)*	
	< 100 cfu	>100 cfu	< 100 cfu	>100 cfu
Chronic hyperplastic candidosis	3 (9.7)	4 (12.9)	9 (29.0)	15 (48.4)
Non-candidal leukoplakia	11 (23.4)	0	29 (61.7)	7 (10.6)

b)

Lesions	Non and Mild *n* (%)		Moderate and severe *n* (%)*	
	< 50 cfu	>50cfu	< 50 cfu	>50 cfu
Chronic hyperplastic candidosis	3 (9.7)	4 (12.9)	7 (22.6)	16 (51.6)
Non-candidal leukoplakia	11 (32.4)	0	30 (63.8)	6 (12.8)

c)

Lesions	Non and Mild *n* (%)		Moderate and severe *n* (%)*	
	< 30 cfu	>30 cfu	< 30 cfu	>30 cfu
Chronic hyperplastic candidosis	2 (9.7)	5 (12.9)	5 (16.1)	18 (58.1)
Non-candidal leukoplakia	10 (21.3)	1 (2.2)	29 61.7)	7 (10.6)

cfu, colony forming units. * *P value <0.001*

Table 3.9 History of tobacco and alcohol consumption and histological data for the 11 non-smoker patients with chronic hyperplastic candidosis

Case	Smoking cpd	Alcohol u/w	Degree of dysplasia	*Candida* density[1]		*Candida* species isolated
				Rinse cfu	Swab cfu	
1.3	No	4	Severe	0	415	*C. albicans*
1.6	No	0	Mild	1450	120	*C. albicans*
1.8	No	0	Severe*	0	480	*C. albicans*
1.9	No	4	Severe	0	1525	*C. albicans*
1.13	No	0	Severe	225	441	*C. albicans*
				60	9	*C. tropicalis*
				35	1	*S. cerevisiae*
1.15	No	10	Severe	C	SC	*C. albicans*
1.19	No	0	Severe	SC	200	*C. albicans*
1.20	No	0	Moderate	290	0	*C. albicans*
1.21	No	0	Severe	C	SC	*C. albicans*
1.22	No	4	Mild	0	0	None
1.24	No	3	Moderate	240	125	*C. albicans*

[1]Oral sample rinse and lesional swab were grown on CHROMagar® media; *lesions progressed to cancer.cpd, cigarette per day; u/w, units per week; cfu, colony forming unit; C, confluent growth; SC, semiconfluent growth.

Table 3.10 History of tobacco and alcohol consumption and histological data for the 20 smoker patients with chronic hyperplastic candidosis

Case	Smoking cpd	Alcohol u/w	Degree of dysplasia	*Candida* density[1]		*Candida* species isolated
				Rinse cfu	Swab cfu	
1.1	20	10	Moderate	40	420	*C. albicans*
1.2	20	5	None	70	35	*C. albicans*
1.4	20	20	Moderate	157	42	*C. albicans*
1.5	10	8	Moderate	C	380	*C. albicans*
1.7	50	3	None	0	7	*C. albicans*
					2	*C. glabrata*
1.10	15	10	Mild	20	130	*C. albicans*
				60	0	*C. dubliniensis*
1.11	15	5	Moderate	120	84	*C. albicans*
				10	4	*C. parapsilosis*
				25	7	*C. guilliremondii*
1.12	10	12	Moderate	2	15	*C. albicans*
1.14	20	15	Severe*	SC	166	*C. albicans*
1.16	20	4	Severe	450	400	*C. albicans*
1.17	20	12	Moderate	160	10	*C. albicans*
1.18	10	4	Moderate	27	0	*C. albicans*
1.23	30	4	Moderate	10	2	*C. albicans*
1.25	10	10	Severe	C	440	*C. albicans*
				130	6	*C. glabrata*
1.26	10	6	Moderate	SC	SC	*C. albicans*
1.27	30	4	Moderate	280	92	*C. albicans*
1.28	30	4	Mild	0	250	*C. albicans*
1.29	10	30	None	293	220	*C. albicans*
1.30	10	20	Moderate	215	185	*C. albicans*
1.31	10	6	Mild	0	0	None

[1]Oral sample rinse and lesional swab were grown on CHROMagar® media; *lesions progressed to cancer. cpd, cigarette per day; u/w, units per week; cfu, colony forming unit; C, confluent growth; SC, semiconfluent growth.

dysplasia lesions in the NCL group). Moderate dysplasia was most commonly found, being reported in 32/78 (41.0%) lesions and severe dysplasia was reported in 25/78 (32.1%) of lesions. The correlation between degree of dysplasia in CHC and NCL lesions and abundance of *Candida* species was investigated (Table 3.8). There was a significant correlation between *Candida* density recovered from CHC exhibiting moderate and severe dysplasia, ($P \leq 0.001$) at 3 cut-off points: 30, 50 and 100 cfu. This was not noted in the non- or mild dysplasia subset, although numbers were very small. Compared to NCL lesions, CHC lesions with moderate and severe dysplasia were more likely to harbour a significant density of *Candida* cells (≥100 cfu) than NCL lesions with the same degree of dysplasia. Interestingly, significant levels of *Candida* cells (≥100 cfu) were recovered from all CHC lesions with severe dysplasia (Table 3.3). Smoking levels in the NCL group approached significance for correlation with degree of dysplasia, ($P=0.07$), by multivariate analysis, but that association was not proven.

In the CHC group of patients studied there was a high representation of non-smokers (11/31, 35%) and none of these patients reported that they drank alcohol to excess. Indeed, 6 of the eleven patients were both non-smokers and abstained from alcohol. Of these 6 patients, all but one also had severe dysplasia on biopsy, the sixth having only mild dysplasia. All but one (patient 1.20) also yielded significant growth (≥100 cfu) of *Candida* from their swab sample (patients data summarised in Table 3.9).

Two patients (1.8 and 1.14, Tables 3.3, 3.9 and 3.10) with CHC with severe dysplasia developed a SCC in the lesions within a two-year follow-up period. One of the patients, a female, was both a non-smoker and abstained from alcohol and the second, a male, both smoked heavily (20 cpd) and consumed a reported 15 units of alcohol per week. Patient 1.8 yielded a significant candidal growth from her swab sample, but the rinse sample showed no growth.

None of the NCL patients developed carcinomatous change in the same follow-up period. There was no statistical correlation in the NCL cohort, between degree of dysplasia and age, sex, denture wear, alcohol and tobacco use and tobacco dose, by regression analysis.

3.4. Discussion

In this study 78 patients were enrolled, 31 with biopsy-proven CHC and 47 with NCL oral leukoplakia, from the Dysplasia clinic at the DDUH. Both lesions have a reported propensity to malignant transformation. CHC is believed to have a higher malignant transformation rate than "simple" oral leukoplakia. Both lesions present clinically as white or speckled red and white lesions and are clinically indistinguishable, apart from subtle clinical features, such as a slight site predilection, in the case of CHC for the commissure of the mouth, but can occur at any non-keratinised oral mucosal site. Indeed, NCL can occur at the commissural site, and it is impossible to differentiate CHC and NCL at sites such as the lateral border of the tongue. Much of the available data on oral white lesions comes from field studies, where laboratory analysis (such as *Candida* culture) and definitive diagnosis (by histopathology and special stains) are unavailable, although there are a number of such reports where extremely large numbers of patients have been studied. What is not clear from this extensive literature is which white oral lesions are CHC and which are NCL. The literature on CHC, *per se*, is sparse.

Clinical and histopthological data were gathered on the patients along with oral rinse and lesional swab samples in an attempt to correlate clinical findings with *Candida* culture findings. At enrolment an attempt was made to control for age, sex and lesional site and no difference was noted in the biopsy data for degree of dysplasia in the NCL and CHC groups.

3.4.1. Demographic, Social and Clinical Data

CHC and NCL has been reported to be a disease of the over 50s (Arendof *et al.*, 1983) and the age demographic data on this cohort is consistent with the literature, with a mean age approaching 60 years in both groups. However, the gender profile is somewhat atypical. NCL is generally believed to have a significant male predominance in the western world of approximately 4:1 but in countries like India, probably due to local tobacco habits, it may greatly exceed this. In this regard the studied cohort is somewhat atypical, since the ratio was approaching 1:1 in the total OL group and NCL groups and 3:2 M:F in the CHC group. Although this finding is inconsistent for NCL, this was due to the control parameters used at enrolment. Due to the lack of demographic data on CHC, the 3:2 M:F prevalence may be a significant clinical

finding, although this is a small cohort. Oral candidal carriage could be postulated to be increased in females, since vaginal candidosis is not uncommon and studies have shown concordance of strains from the mouths and vagina in women, and higher oral candidal loads in women with vulvovaginal candidosis, so that seeding from vagina to mouth would not be implausible (Goncalves e Silva *et al.*, 2008).

No increase in oral candidal carriage could be demonstrated in this study with increasing age in the OL group, or in respect of gender, however there was a significant association with higher oral candidal loads in the CHC group with increasing age ($P<0.007$).

Similarly, the ethnicity of the OL group was overwealmingly Irish, an ethnic group for which oral candidal carriage data is not available, although there is no reason to assume that oral candidal carriage should be any different from reports from the rest of the western world. Age was not associated with degree of dysplasia in either of the CHC or NCL patient groups.

Examination of the medical histories of the cohort showed no excess of diseases which might predispose to oral candidosis such as diabetes mellitus, xerostomia (or history of radiotherapy) corticosteroid use, such as inhalers, or immunosuppression and upper denture wear (which would predispose to *Candida*-induced denture stomatitis) between the CHC and NCL groups.

There was a fairly low prevalence of upper denture wear in the NCL group (at 23%), compared to 46% in the CHC group.

Tobacco use in the OL cohort at over 60% is perhaps a little lower than in other reports for oral leukoplakia, where it approaches 80% in some series, although when used, the mean tobacco dose at 17.5 cigarettes per day was in the heavy smoker range. Tobacco and alcohol usage was not found to predispose to oral *Candida* carriage in this study, although the confidence interval at $P=0.07$ approached significance. Although statistically highly significant, the finding of *Candida* association in smokers in the CHC group is not meaningful since all these patients, whether smokers or not, are known to already have a *Candida*-associated lesion in their mouths.

In the NCL and total OL cohort, statistical analysis of the continuous data in the rinse samples by ANOVA showed a significant association with the amount of cigarettes that were smoked per day ($P<0.04$) and the cfu counts in rinse samples. In other words, although smoking was not a predictive factor for the presence of *Candida*

in the mouth, in smokers, the more a patient smokes the higher the candidal load is likely to be.

Considering the data together, the fact that the confounders for candidosis such as, diabetes mellitus, xerostomia (or history of radiotherapy) steroid use, such as inhalers, or immunosuppression and upper denture wear are not found in excess in the NCL group supports this finding. Perhaps, in some way, the smoking habit creates a favourable micro-environment for *Candida* species to proliferate in those who harbour the microorganism in their mouths. Alternatively, the tars in tobacco smoke when dissolved in saliva, reduce the growth of competitive species like bacteria, or alter the quality or quantity of those species, and it has been proposed that there may be qualitative changes in the oral keratinocytes, Langerhans cell density in the mucosa or salivary anti-microbial agents such as SLP1. There has been a great deal of debate and conflicting findings in the world literature, regarding whether and, if so how, the smoking habit predisposes to *Candida* carriage in the mouth (reviewed by Soysa and Ellepola, 2005).

3.4.2. Anatomical Site

The commonest sites for CHC are recognised to be the buccal mucosa, particularly the commissure, and the tongue. This is confirmed in this series, where 90% of lesions in the CHC group were at these sites (77% buccal/commissural mucosa and 13% tongue). In the NCL group, patients were, whenever possible, actively selected for enrolment in the study when they had lesions at these sites. Over 60% of the patients had buccal mucosal or tongue lesions (42% buccal/commissural mucosa and 19% tongue). Approximately one fifth of patients in both groups had lesions at multiple sites. In the NCL group, all the patients were either smokers (8) or ex smokers (2). This finding is in keeping with the concept of "field cancerisation", which is seen in oral cancer. Since the entire mouth (and aerodigestive tract) is exposed to the carcinogens in the tobacco smoke of smokers, all areas are at risk and the clinical observation that oral cancer patients may have synchronous and metachronous primary malignant disease supports this concept. An unexpected finding in the CHC group was that only one of the six patients who had leukoplakic lesions at more than one site was a smoker, and she smoked relatively lightly, (10 cpd). She did, however, drink 30 units of alcohol per week. If smoking was the predisposing factor for oral *Candida* carriage in this single patient, 3 of the other five patients wore an upper denture which may have contributed

to the presence of *Candida* in their mouths. These findings lend weight to the argument that *Candida* species, are capable of inducing chronic hyperplastic oral lesions in humans, rather than oral leukoplakia providing a favourable ecological or microenvironmental niche for proliferation of the yeast.

3.4.3. Oral Leukoplakia and *Candida*

The association between *Candida* and oral leukoplakic lesions *in vivo* was investigated by taking an oral rinse sample and a lesional sample for analysis from each patient. *Candida albicans* was the predominate species isolated and was present in greater abundance than other species in all cases except 2 in the NCL group, where non-*C. albicans* species (*C. glabrata and C. kruseii)* were present on the lesions. In all cases where *Candida* species were isolated, *C. albicans* was present in the CHC group. Qualitatively, a small number of patients harboured other species in low abundance. In the oral rinse samples from 8 patients, a mixed growth was seen, (5 CHC and 3 NCL) and *C. glabrata, C. tropicalis, C. parapsilosis, C. dubliniensis, C. guilliermondii* and *C. krusei* were isolated, and this result is not unexpected due to the fact that these species are less frequently isolated from oral cavity compared with *C. albicans*.

In the swab samples, mixed growth was observed in 4 cases of CHC and yielded *C. glabrata, C. parapsilosis, C. guilliermondii* and *C. tropicalis*. The clinical significance of isolation of *S. cerevisiae* from both the swab and rinse from one patient is of uncertain clinical significance, since this microorganism has not been associated with disease in humans. Presumably it was commensal or more likely, transient. Since there was no single consistent non-*albicans Candida* species isolated from the lesional tissue, and since all the isolates were in very low abundance, it is unlikely that non-*albicans* species are intimately involved in the pathogenesis of CHC. The ubiquitous presence of *C. albicans* on the CHC lesions, often in high abundance, strongly implicates this particular species as the prime mover in the aetiological process.

The strong association of *C. albicans* with CHC in this series is of interest, because of Krogh's comments on aetiopathogenesis of CHC (Krogh, 1990). In his series, there were pure cultures of non-*albicans* species isolated from CHC patients but they had less nitrosamination potential than *C. albicans* species, many of which belonged to the rarer *C. albicans* biotypes (Krogh *et al.*, 1987).

This observation, in the present study, poses the question whether the strains of *C. albicans* isolates associated with CHC, although part of the *albicans* species, differ in

some way from other oral *C. albicans* isolates not associated with CHC and prompted the further investigations described later in this thesis.

Despite there being a *Candida*-associated oral lesion in all CHC cases, the rinse sample yielded no growth in 7 patients. Three of these yielded a positive *Candida* culture from by swab sampling. Looking at the individual cases, significant growth ($\geq$100 cfu) from the lesional swab was seen in 4 cases where the rinse was negative. In 2 cases the swab was negative but the rinse positive (1.18 and 1.20), although the growth was only above 100 cfu/ml in one of the rinse specimens.

The fact that *Candida* was not isolated from CHCs in 4 patients when the lesion was swabbed is of interest. By definition, histopathologically defined CHC lesions contained hyphae, but not necessarily *Candida* hyphae. It is possible that some of these lesions were infected with other yeasts or fungi present in the oral cavity such as *Pichia* species. However, assuming the CHC lesions were actually infected with *Candida*, since the microorganism is invading the tissues, and firmly adherent, it would not be expected that it could be isolated from the lesion. Any blastospores on the biopsy would presumably be removed by the washing and dehydration steps in the processing of the biopsy, and so would not be observed histopathologically. In these 4 cases, it is also possible that all the yeasts present were in hyphal form or that any blastospores were so firmly attached to the epithelium that it would not be possible to dislodge them with the swabbing technique employed. Certainly, the adhesion of *Candida* (see Chapter 4)) to epithelial cells is a complex process involving many different mechanisms, from weak electrostatic forces to very strong covalent bonds between the two cell surfaces.

In the NCL group *Candida* species were isolated from the lesional tissue in 49% of cases. Generally, *Candida* cells were in very low abundance, although one case demonstrated confluent growth. In three cases the swab result was positive, but rinse negative, although the cfu count from two of the cases were 2 and 3 cfu only. In one case the swab grew 230 cfu, despite the negative rinse culture.

The majority of patients with CHC yielded a significant culture ($\geq$100 cfu) and the sensitivity of the use of a lesional swab to detect CHC versus NCL was explored using three cut-off points, 100 cfu, 50 cfu and 30 cfu. There was a statistically detectable difference at the 95% confidence level between swabs from CHC and NCL. The swabbing technique (compared to rinsing) appears to be a highly discriminatory technique for testing the clinical suspicion that a patient might have CHC rather than

NCL, despite 49% of NCL lesions having *Candida* species. culturable from the lesional surface.

The continuous nonparametric data for *Candida* density in the swab and rinse samples for CHC and NCL were analysed by Spearman multivariate analysis. There was significant correlation for both NCL ($P<0.006$) and CHC ($P<0.037$), suggesting as would be expected that patients with a high oral candidal load also have higher swab counts, presumably due to increased planktonic salivary *Candida* contamination of the lesion.

In the CHC group there were a large proportion of upper denture wearers (46%). Although the data were not recorded, it can be speculated that since upper (as opposed to lower) denture wear predisposes to denture stomatitis, and that the main cause of denture stomatitis is candidosis, wearing a denture predisposes to oral candidal carriage and that this increases the chances of *Candida* either infecting a pre-existing leukoplakic lesion or, causing CHC in the mouths of the denture wearers. Against this, in the current series, of the 11 cases of non-smokers who had CHC, only 4 wore an upper denture.

3.4.4. *Candida* Density and Dysplasia

The association between the degree of dysplasia in lesional tissue and *Candida* was investigated. If *Candida* is causative of the CHC lesion, rather than just being a passenger, it would be expected that there would be more dysplasia associated with increased candidal lesional density. The majority of OL patients 57/78 were diagnosed with moderate dysplasia or worse. Ten out of 47 NCL cases and 7 of the 31 CHC cases had no or mild dysplasia. There was no difference in numbers of patients and degree of dysplasia in the CHC and NCL groups.This excess of moderate and severe dysplasia in both groups probably reflects two clinical situations. Firstly, the clinician in assessing the site for an incisional biopsy usually chooses a site which is either nodular or non-homogeneous, since these features in a leukoplakic lesion are associated with higher grades of dysplasia, giving rise to selection bias at the biopsy site. Secondly, in reporting on biopsies, pathologists will assign a higher dysplasia grade, if there is any doubt as to the exact diagnosis.

Nevertheless, there was a statistically significant association between significant ($\geq$100 cfu) *C. albicans* recovery from the lesional tissue and moderate or severe

dysplasia in the CHC lesions compared to NCL lesions. In fact, every severely dysplastic CHC (10 cases) had a lesional *Candida* density of over 150 cfu.

In patients that smoke, it would be reasonable to assume that the cellular dysplastic changes seen were due to the smoking habit. However, there was no statistical association in this patient group between smoking and dysplasia, although the statistical analysis approached significance. Furthermore, in the CHC non-smoker subgroup, 10/11 patients had moderate or severe dysplasia on their biopsy, of whom 7/11 were graded severe.

It could be argued that the surface candidal infection elevates the dysplasia grade by cellular interaction with keratinocytes inducing dysplasia-like changes in the uppermost epithelium. However, a dysplasia grade of severe infers that the histopathological changes are seen throughout the entire epithelium including the basal layers. Hyphal *Candida* in CHC is rarely seen invading past the stratum spinosum in CHC and the dysplastic features of CHC are identical to those seen in NCL

Further evidence that *Candida* may be causative in CHC comes from the observation that in the small group of 6 patients with CHC, who were neither smokers nor drank alcohol, all, except one, exhibited severe dysplasia on their biopsies. One unfortunate patient in this group with severe dysplasia went on to develop a carcinoma in the lesional tissue. Her only risk factor for malignant change in the lesion was candidal infection. A second patient in the CHC cohort also developed a squamous cell carcinoma within the lesional tissue within a two year timeframe from diagnosis (despite antifungal treatment following diagnosis). He also exhibited severe dysplasia on his CHC biopsy, but was also a 20 cpd smoker and drank a self-reported level of alcohol of 15 units per week. As such, despite smoking cessation advice, he maintained other risk factors for cancer development. The fact that 2/31 (6%) of the CHC cohort developed cancer within a short timeframe from diagnosis but none of the patients in the NCL group developed malignant change adds weight to the evidence that CHC is a high risk lesion for malignant transformation.

3.5. Summary of Key Findings

- CHC a disease of the over 50s and the finding of an increased number of females (3:2) may be significant. It predominantly affects the buccal/commissural mucosa and tongue.
- CHC appears to have increased malignant change potential compared to NCL.
- Upper denture wear not only predisposes to increased oral *Candida* load but also to CHC.
- Smoking does not appear to predispose to oral *Candida* carriage, but in smokers, heavy smoking is associated with higher oral candidal loads.
- *Candida albicans* was by far the most dominant *Candida* species associated with CHC (100%), Although non-*C. albicans* species were isolated from both CHC and NCL, they are unlikely to play a key aetiological role in either CHC or NCL.
- Lesional swabs have better discriminatory capability between CHC and NCL compared to oral rinse samples.
- In CHC, but not in NCL, lesional candidal density, as determined by the lesional swab, correlates with the the higher (moderate and severe) dysplasia histopathological grade. This finding provides support for the concept that *C. albicans* is causative of, rather than simply associated with, CHC.

Chapter 4

Investigation of the Adherence of *Candida albicans* Isolates from Oral Leukoplakia Lesions to Buccal Epithelial Cells and Cultured Normal and Dysplastic Keratinocytes *in Vitro*

4.1. Introduction

Adherence of *C. albicans* to cells is the first critical phase in the infection process. It is essential for both colonisation and subsequent invasion of tissue and subsequently organs. This comes from evidence that less adherent strains of *C. albicans* have a reduced ability to cause infection in animal models (Calderone and Braun, 1991; Franzke *et al.*, 1993; Naglik *et al.*, 2008) and blocking the presumptive *C. albicans* adherence ligand decreased the number of the organisms in various tissues (Klotz *et al.*, 1992). Calderone and Braun (1991) stated that the most pathogenic *Candida* species including *C. albicans* and *C. tropicalis* have a greater ability to adhere to host cells *in vitro* than relatively non-pathogenic species such as *C. krusei* and *C. guillermondi.* Adherence of *C. albicans* to host tissues and surfaces is a survival mechanism and prevents the microorganisms from being cleared by saliva in the oral cavity. Furthermore, saliva does not support the growth of *Candida* unless it is supplemented with glucose. Thus adherence is essential to maintain *Candida* in such an environment (Samaranayake *et al.*, 1982; Cannon and Chaffin, 1999).

4.1.2. Adherence of *Candida albicans* to Human Buccal Epithelial Cells

Human buccal epithelial cells (BECs) have been extensively used as an adherence model for *C. albicans.* Several factors can affect adherence properties of *C. albicans* to BECs including host and microorganism factors and environmental factors. The binding capacity of BECs is affected by the age of the donor; *C. albicans* adherence was found to be higher in BECs from adults and premature newborn infants than that in full-term newborn infants (Cox, 1986). *Candida albicans* was found to be more adherent to buccal epithelial cells obtained from denture wearers than BECs from dentate individuals (Lyon and Resende, 2007). Hormonal influences have been suggested as a factor in increased binding of *C. albicans* to BECs during the menstrual cycle (Theaker *et al.*, 1993). *Candida albicans* adherence to BECs was also found to be greater in both denture wearing non-insulin dependent diabetic patients than to BECs from non-diabetic denture wearers (Dorocka-Bobkowska *et al.*, 1996). Hormonal changes might induce the expression of receptors on the host cells that mediate the adherence of the yeast (Cannon and Chaffin, 1999). The use of antibacterial agents has been also implicated in increased *Candida* adhesion to BECs (Cox, 1983). Antimicrobial agents

probably reduce the levels of adherent bacteria on the surface of BECs making more sites available for *Candida* to adhere to (Liljemark and Gibbons, 1973).

Antifungal treatment of BECs prior to *Candida* adherence testing has been investigated with a variety of agents including subinhibitory concentration of nystatin (Ellepola *et al.*, 1999), amphotericin B (Dorocka-Bobkowska *et al.*, 2003), miconazole (Abu-el Teen *et al.*, 1989), fluconazole (Darwazeh *et al.*, 1991; Lyon and Resende, 2007) and 5-flucytosine (Ellepola and Samaranyake, 1998) and all have been shown to reduce the adherence of *C. albicans* to BECs. Antifungal therapy with fluconazole reduces the binding of *C. albicans* to BECs and Wu *et al.*, speculated that this might be due to changes in BEC cell surface proteins as well as the antifungal effect of fluconazole (Wu *et al.*, 1996). In contrast, *C. dubliniensis* has an increased binding capacity to BECs when exposed to fluconazole (Borg-von Zepelin *et al.*, 2002). Antimicrobial agents such as chlorhexidine can also reduce the adherence of *C. albicans* to BECs (Tobgi *et al.*, 1987), which might be due to reduced cell surface hydrophobicity (Jones *et al.*, 1991). A number of studies have shown that the ability of different *C. albicans* strains to bind to BECs is variable, with isolates recovered from cases of candidiasis being more adherent compared to commensal isolates (Schmid *et al.*, 1995; Bernhardt *et al.*, 2001). This phenomenon may be due to the high proteinase activity associated with isolates recovered from candidiasis (Ghannoum and Abu Elteen, 1986, Yildirim *et al.*, 2010) or due to the possession of specific adhesins with different surface cell receptors (Critchely and Douglas, 1987). For further details refer to section 1.3.4. Adherence of *C. albicans* to BECs *in vitro* can also be affected by the growth conditions of the yeasts. *Candida albicans* adherence to BECs was higher in media containing a high concentration of various sugars compared with media with lower concentrations (McCourtie and Douglas, 1981). Adherence was greatly enhanced when *C. albicans* were grown in media containing maltose compared to lactose, sucrose, galactose and glucose; the latter was the least effective (Samaranayake and McFarlane, 1982). Furthermore, *C. albicans* grown at 25°C was more adherent to BECs than when grown at 37°C, probably due to alterations in the cell surface hydrophobicity that increases at lower temperatures (Hazen and Hazen, 1988; Jabra-Risk *et al.*, 2001).

Candida albicans can express multiple surface molecules, termed adhesins, that promote the adherence of the fungi to host cells (Calderone and Fonzi, 2001). One type of adhesin is encoded by the Agglutinin Like Sequence (*ALS*) gene family (Hoyer, 2001), which encodes eight glycosylphosphatidylinositol cell surface proteins that

mediate binding to host cell receptors (Hoyer, 2001; Zhao *et al.*, 2004). Some of these Als proteins play a key role in hte adhesion of *C. albicans* to various host cells and cellular components. Als1, Als3 and Als5 mediate adherence to epithelial cells (Fu *et al.*, 2002; Zhao *et al.*, 2004). Heterologous overexpresssion of *ALS* genes in *S. cerevisiae* and the use of deletion mutants has been used to investigate the role of *ALS* genes and proteins in adhesion (Hoyer *et al.*, 2008). *Candida albicans als3/als3* mutant derivatives exhibited a reduced binding to BECs and a marked decrease in damage to reconstituted human epithelium (RHE) tissue models while, *als1/als1* mutant strains showed a slight decrease in both adherence to BECs and damage to RHE (Zhao *et al.*, 2004).

Hwp1 (hyphal wall protein) ia another important *C. albicans* adhesin which is found on the surface of *C. albicans* hypha but not on yeast cells and is a substrate of tissue transglutaminase (TG), resulting in a cross-link to TG on the surface of epithelial cells (section 1.3.4).

4.1.3. Adherence of *Candida albicans* to Cultured Keratinocytes

The human BEC assay is a widely used model to study the adhesion of *Candida* to human cells, however it has some limitations. The adherence of *C. albicans* strains to BECs can vary depending on the BEC donor and can also vary for BECs recovered from the same individual at different times (diurnal variation) (Kearns *et al.*, 1983). Adherence of *C. albicans* to BECs can also be modulated by oral bacterial flora on BECs (Nair and Samaranayake, 1996). However, the BEC adherence assay is also time consuming and may be susceptible to operator error (Douglas, 1987; Polacheck *et al.*, 1995). However, despite these limitations, the BEC adherence assay is still widely used due to its ease of use and the speed by which BECs can be obtained. It also facilitates experiments with *Candida* cells grown under different growth conditions, such as temperature.

The use of a uniform model of "viable" human epithelium to assess *Candida* adhesion would have significant advantages over the human BEC model. A variety of different models are available; these include the reconstituted human epithelium (RHE) model, and cultured human oral cancer cell lines such as the TR146, SCC15, SCC4 (Dongari-Bagtzoglou *et al.*, 2003; Moran *et al.*, 2007), Hep-2 and A549 cell lines (Holmes *et al.*, 2002). Using these models has the advantage that the cells originate from one cell line which helps to eliminate any source of variation from host cells as

occurs with the BEC model. Employing these alternative models combined with a molecular cell probe for yeast adhesion removes any intra-or inter-examiner variations. *Candida albicans* adherence to primary epithelial cell lines has been investigated in a few studies with immortalised primary vaginal epithelial cells (VECs) (Hollmer *et al.*, 2006), with the immortalised oral epithelial cell line OKF6/Tert-2 (Dongari-Bagtzoglou *et al.*, 2006, Dwivedi *et al.*, 2011) and primary epidermal keratinocytes (Ollert *et al.*, 1993). However, most of these models are derived from cancer cell lines or transformed primary cell lines that do not have the same normal cellular complexity and most of the oral cancer cell lines show no consistency of malignancy markers and tumourigenicity in nude mice. Furthermore, these cell lines do not reflect the cellular changes in oral leukoplakia lesions. Both normal and dysplastic epithelial cells are more appropriate to assess the adhesion of *Candida*.

In chronic hyperplastic candidosis (CHC), it has been postulated that the oral keratotic change of the oral mucosa precedes *Candida* infection (Jepsen and Winther, 1965). Furthermore, traumatic lesions like frictional keratosis can be a precursor lesion for CHC (Arendorf *et al.*, 1983). Epithelial changes such as hyperkeratosis or dysplasia can provide a suitable environment for *Candida* invasion (Samaranayake and MacFarlane, 1990). In an attempt to investigate this issue, assessment of *Candida* adherence to normal oral epithelial cells and dysplastic oral epithelial cells were investigated in this part of the present study.

4.1.4. Aims

The aims of this part of the present study were:

- To investigate whether *C. albicans* isolates recovered from both CHC and NCL are more adherent than oral and systemic control reference laboratory strains.

- To assess whether *C. albicans* isolates recovered from CHC and NCL lesions are more adherent to oral dysplastic epithelial cells monolayers than normal human oral epithelial cells monolayers.

4.2. Material and Methods

4.2.1. Adherence of *Candida albicans* Leukoplakia Isolates to Human Buccal Epithelial Cells

This assay was based on a modified protocol from Kimura and Pearsall (1978) and Murphy and Kavanagh (2001).

4.2.1.1. Growth Conditions

All *Candida* cultures were grown at 37°C overnight (approximately 16 h) in 50 ml YEPD broth (1% (w/v) yeast extract (Sigma-Aldrich), 2% (w/v) bacterial peptone (Oxoid) and 2% (w/v) glucose (Sigma-Aldrich)), in a 100 ml conical flask in an orbital incubator at 200 rpm (Gallenkamp). Yeast cultures were grown to stationary phase overnight, harvested by centrifugation at 2100 x *g* for 10 min, washed twice with 5 ml of sterile PBS and resuspended in PBS at a cell density of 1 x 10^7 cells/ml using an improved neubauer haemocytometer (Hausser Scientific, Horsham, PA, USA).

Candida albicans isolates were grown in YEPD growth medium, containing 2% (w/v) glucose as the standard growth medium for all isolates. Carbohydrate supplements can alter the adherence properties of *C. albicans*, for instance, galactose can increase the adherence of *C. albicans* to BECs (Samaranyake and MacFarlane, 1982). All isolates were tested at temperature of 37° C.

4.2.1.2. Buccal Epithelial Cells

Buccal epithelial cells (BEC) were collected from healthy adult human volunteers using sterile swabs (Venturi Transystem) by gently rubbing the inside of the buccal cavity. Cells were resuspended in 5 ml PBS (Sigma-Aldrich). BECs were pooled in 10 ml sterile PBS, centrifuged at 760 x g in an Eppendorff 5804 centrifuge (Eppendorff) for 5 min at 18°C, washed twice in sterile PBS and resuspended at a concentration of 2 x 105 cells/ml using an improved Neubauer haemocytometer. A similar group of donors were used during the course of the study to minimise the effect of donor to donor epithelial cell variability

4.2.1.3. Adherence Assay

Yeast cell suspensions (1 ml) and BECs suspensions (1 ml) were pooled (giving a ratio of 50:1, yeast: BEC) and incubated for 2 h at the same temperature that the yeast cells

were grown at (i.e. 37°C) in an orbital incubator at 200 rpm. Following incubation, BECs with adhering yeast were filtered through a polycarbonate membrane containing 12 μm pores (Millipore) and then washed gently with a 3 x 10 ml of sterile PBS in order to remove any non-adherent yeast. The filters were removed from the Nalgene® polysulfone filter holders (Nalgene®) and placed face down on a glass slide (25 x 75 x 1 mm). BECs with adherent yeast were thus transferred to the slide. These slides were allowed to air dry (until completely dry) and stained for 30 s using 2.3% (w/v) crystal violet solution, followed by Grams' iodine solution and decolouriser (Sigma-Aldrich). The number of yeast adhering to each of 200 BECs per treatment was counted using a Nikon Eclipse E400 microscope and photographic images were recorded using a Nikon DXM1200 digital camera. Counting of adherent yeasts was done using the following criteria; a hundred BEC cells were observed for attached yeast cells in a random manner. Clumped, folded or overlapped BECs were excluded. Only single BEC cells were counted and daughter yeast cells smaller than the mother cell were not counted (Tsang and Samaranayake, 1999).

4.2.2. Adherence of *Candida albicans* to Cultured Oral Keratinocytes

4.2.2.1. Patients and Tissue Sampling

Normal oral mucosa biopsy samples used in this study were obtained from consenting patients undergoing a third molar surgical removal. A few millimetres of normal oral tissue were trimmed from the edge of a muco-periosteal flap or the edge of the wound. Biopsies were taken following informed consent using the consent form approved by SJH/AMNCH Research Ethics Committee, (Appendix 1).

Patients were kindly asked to give permission to use part of the biopsy for tissue culture. The procedures were explained to the patients verbally and in writing, and upon approval the patients were asked to sign the consent form for the procedures. The specimen was placed in DMEM (Sigma-Aldrich) supplemented with 100 μl/ml of penicillin/streptomycin (Sigma-Aldrich) and 5 μl/ml amphotericin B (Sigma-Aldrich) and taken immediately for processing.

Twenty *C. albicans* isolates recovered from oral leukoplakia lesions and references *C. albicans* strains 132A and SC5314 were tested for adherence to monolayers of the normal human epithelial cells grown from the edges of the surgical flap of wisdom teeth and the dysplastic cell line DOK (Chang *et al.*, 1992). Normal oral

keratinocytes were cultured from three individuals; tissue samples were taken from the edge of the surgical flap of wisdom teeth.

4.2.2.2. Media and Reagents

All reagents and culture media were purchased from Sigma-Aldrich unless stated otherwise.

(a) Keratinocytes growth medium (KGM)

- Dulbecco's modified Eagle medium (DMEM) 3 parts
- Ham-F12 nutrient medium 1 part
- Foetal bovine serum (FBS) 10%
- Epidermal growth factor (EGF) 10 ng/ml
- Hydrocortisone 0.5 μg/ml
- Insulin 5 μg/ml
- L-Glutamine 2 mM
- Adenine 0.18 mM
- Cholera toxin 10-7 mM
- Penicillin/streptomycin 100 IU/ml/100 μg/ml
- Fungizone (amphotericin B) 0.25 μg/ml

(b) 3T3 fibroblast cell line growth medium

- DMEM
- FBS 10%
- Penicillin/streptomycin 100 IU/ml/100 μg/ml
- Fungizone (amphotericin B) 0.25 μg/ml

(c) DOK cell line growth medium

- DMEM
- FBS 10%
- Hydrocortisone 5 μg/ml
- L-Glutamine 2 mM
- Penicillin/streptomycin 100 IU/ml/100 μg/ml
- Fungizone (amphotericin B) 0.25 μg/ml

4.2.2.3. Culture of Normal Oral Keratinocytes

Normal oral keratinocytes were isolated and amplified according to Rheinwald and Green (1975) with minor modifications.

(a) Swiss albino mouse fibroblast 3T3 cell lines

Growth of epidermal and oral keratinocytes can be enhanced by a non- proliferating feeder layer of Swiss albino mouse fibroblast 3T3 cell line (Rheinwald and Green, 1975). This cell line was established from primary Swiss albino mouse fibroblast by Todaro and Green in the 1960s (Todaro and Green, 1963). Feeder layers of 3T3 fibroblast are prepared by exposing the cells to sub-lethal dose of γ-irradiation or treatment with anti-mitotic agents like mitomycin C. Both treatments prevent the cells from dividing but allow the cells to retain their ability to attach and release growth factors in the culture medium (Booth and O'Shea, 2002). 3T3 fibroblasts were kindly donated by Dr. C. Murdoch, Sheffield School of Dental Science. 3T3 fibroblasts were grown in DMEM supplemented with 10% (v/v) FBS and, 100 µl/ml of penicillin/streptomycin, and 0.25 µg/ml amphotricin B, in an atmosphere of 5% (v/v) CO2 and 98% relative humidity in an incubator equipped with a safeCell™ UV unit (Sanyo Europe Ltd., Watford, UK). The UV unit consists of an ozone- free UV C lamp that is isolated from the tissue culture area of the incubator by a plenum cover that glows for 2 min after the incubator is door closed to destroy airborne contaminants and water-borne organisms without causing any damage to the cells being cultured. Cells were subcultured by exposing the culture to 0.25% trypsin-EDTA (Sigma-Aldrich) solution that consisted of 2.5 g of porcine trypsin, 0.2 g of EDTA in Hank's Balanced Salt solution (HBSS) with phenol red which was filter-sterilised by the manufacturer. Cells were washed twice with 5 ml Ca+2/Mg+2-free HBSS before being rinsed with 5 ml of the trypsin solution at 37°C for 2-4 min. Cells suspensions were centrifuged at 250 x g for 10 min. The supernatant was then discarded and the cells resuspended and counted using a haemocytometer. Cells were reseeded at a density of 1 x 106 in 75 cm2 tissue culture flasks (Sigma-Aldrich) and allowed to grow to subconfluence before serial propagation (to avoid spontaneous transformation). Stocks of 3T3 cells were prepared by suspension of the cells at density of 1 x 106 cells/ml in growth medium supplemented with 10% (v/v) DMSO (Sigma-Aldrich) and then frozen in liquid nitrogen.

(b) Preparing the Swiss 3T3 cells for use as a feeder layer

3T3 fibroblasts were cultured and allowed to reach confluence before being treated with 10 mg/ml mitomycin C (Sigma-Aldrich) in complete medium for 2 h at 37°C. Following treatment, culture plates were washed three times with Ca^{+2}/Mg^{+2} HBSS to remove residual medium. Cells were then trypsinised with 0.25% (w/v) trypsin-EDTA (Sigma-Aldrich) for 1-2 min and then resuspended in the 3T3 fibroblast growth medium. Cells were then centrifuged at 250 x *g* for 10 min and either used immediately or frozen in 1 ml aliquots in liquid nitrogen for subsequent use. Feeder layers were prepared by pre-culturing mitomycin C-treated 3T3 fibroblasts for 24 h before culture of the epithelial cells. Feeder cell layers were cultured to a cell density of 2.5×10^5 cells/ml.

(c) Propagation of normal oral keratinocytes

Human normal oral keratinocytes were grown using two methods. The first method was as described previously by (Moharamzadeh *et al.*, 2008), in which the cells were grown on collagen coated tissue culture flasks (Sarstedt). The second method was carried out using non-proliferative fibroblast feeding layers that were treated by irradiation or with the anti-mitotic drug mitomycin C to enhance the growth of oral keratinocytes (Rheinwald and Green, 1975).

Human biopsy samples tissues from three patients were digested overnight with either 0.1% (w/v) trypsin solution or using the dispase enzyme (Sigma-Aldrich) which is a neutral protease isolated from culture filtrates of *Bacillus polymyxa*. The latter has been shown to be a rapid, effective, and gentle agent for separating intact epidermis from the dermis and intact epithelial sheets in culture from the substratum (Stenn *et al.*, 1989). Tissue samples were placed in dispase enzyme at concentration of 2.5 mg/ml reconstituted in 10 ml DMEM supplemented with 100 µl/ml of penicillin/streptomycin and 0.25 µg/ml amphotericin B and incubated overnight at 4°C and at 37°C for 30 min before processing. Tissues were then washed twice with 5 ml of DMEM supplemented with penicillin/streptomycin and amphotericin B. The fibrous connective tissue was separated from the epithelial tissue using a small amount of the culture medium in a tissue culture dish. Epithelial tissue was cut into small pieces of less than 1-2 mm with a number 15 sterile scalpel balde (Romed®-Holland, Wilnis, Netherland) in keratinocyte growth medium (KGM) that consists of 3:1 DMEM and Ham F12 nutrient medium,

supplemented with 10% FBC, 10 ng/ml epidermal growth factor (EGF) (sigma), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 5 μg/ml insulin, 2 mM L-glutamine, 0.18 mM adenine, 10^{-7} mM cholera toxin, 100 IU/ml/100 μg/ml penicillin/streptomycin and 0.25 μg/ml Fungizone (amphotericin B). Tissue samples were then transferred to a 75 cm^2 collagen coated tissue culture flask with pre-cultured feeder 3T3 fibroblast cells and incubated at 37°C in a 5% (v/v) CO_2 incubator in 98% relative humidity. The medium was changed every 3-5 days until 80% confluence was achieved. The cells were then trypsinised and removed from the tissue culture dishes by decanting and discarding 9 ml of medium from each tissue culture dish. Feeder cell were removed by adding 2-3 ml of 0.25% (v/v) trypsin-EDTA at 37°C for 1 min. Epithelial cells were dissociated by using 5 ml of 0.25% (v/v) trypsin-EDTA incubated for 10-15 min under normal incubation conditions, detached from the tissue culture dish by forcible pipetting and the resultant cell suspension was aliquoted into a 50 ml conical base screw capped centrifuge tubes. The cells were then centrifuged in at 250 *x g* for 10 min at 4°C. The supernatant was then discarded and the cells were resuspended in 5 ml of DMEM prior to counting to determine the cell density. Cell were passaged for amplification and frozen for subsequent experiment at the third passage.

Contaminating fibroblasts were eliminated using either cold (room temperature [16-20°C]) or warm (37°C) 0.25% (v/v) trypsin-EDTA solution, provided that only a few fibroblasts were observed in the culture. Otherwise the whole culture was discarded if there was a heavy growth of fibroblasts.

(d) Propagation of DOK cell line

The DOK cell line was originally established from dysplastic tissue samples adjacent to well-differentiated squamous cell carcinoma (SCC) lesions on the tongues of patients who were heavy smokers. The histolopathology of tissue used to grow the DOK cells showed a mild to moderate degree of dysplasia (Chang *et al.*, 1992). DOK cells were a kind gift from Dr. C. Murdoch, School of Clinical Dentistry, Sheffield, UK. DOK cells were grown in 75 cm^2 treated tissue culture flasks containing 10 ml DMEM supplemented with 10% (v/v) FBS, 2 mM L-Glutamine (Sigma-Aldrich) and 5 μg/ml hydrocortisone (Sigma-Aldrich), and were maintained at 5% CO_2 at 37°C in 98% relative humidity. Culture medium was replaced every 4-5 days until the cells reached 80% confluence before passaged with 0.25% (v/v) trypsin-EDTA. Briefly, cells were washed twice with 5 ml Ca^{+2}/Mg^{+2}-free HBSS, before adding 5 ml trypsin-EDTA

solution to each flask and incubated for 10 min at 37°C in 5% (v/v) CO_2, 98% relative humidity. Cells were then collected in 50 ml cone-based screw capped tubes (Greiner) and centrifuged at 250 x *g* for 10 min. Trypsin solution was then discarded and the cells were resuspended in 5 ml of DMEM prior to counting to determine cell density. Cells were replated if required for propagation or suspended in complete medium with 10% DMSO (v/v) for storage in liquid nitrogen.

4.2.2.4. Effect of Media, Atmospheric Conditions and Triton X-100 on the Integrity of the NOK and DOK Cells

To evaluate the viability of the primary NOK and DOK cell line upon exposure to PBS and 0.1% (v/v) Triton X-100, cells were suspended at density of 2 $x10^5$ cells/ml in 1 ml of PBS and 0.1% (v/v) Triton X-100 and complete growth medium in a standard incubator at 37°C for 3 h. Cells viability were analysed with Trypan blue (Sigma-Aldrich), using the dye exclusion test. Cells with intact cell membranes do not absorb Trypan blue, whereas dead cells and cells with damaged cell membrane stain blue as the dye crosses the cell membrane.

The effect of Triton X-100 was assessed on fully-intact, confluent cells plated in 96-well plates. Cells were exposed to 100 µl of 0.1% (v/v) Triton X-100 in sterile ultra-purified water at room temperature for 8 min followed by repeated aspiration with 200 µl micro-pipette (Gilson, Luton, Bedfordshire UK) of the Triton X-100 solution in an attempt to disrupt the cell line. Trypan blue were used to evaluate the viability of the cells.

4.2.2.5. Effect of 0.1% Triton X-100 on C. albicans Growth

In order to evaluate the effect of the Triton X-100 on *C. albicans* isolates at the concentration of 0.1% used to lyse the NOK and DOK cells, *C. albicans* isolates were tested with 0.1% (v/v) Triton X-100 as follows; cultures of *C. albicans* isolates were grown in 50 ml YEPD in 250 ml conical flasks (Duran) at 37°C overnight (~16 h) in an orbital incubator (Gallenkamp) at 200 rpm. These cultures were washed twice with sterile PBS, resuspended in RPMI-1640 (Sigma-Aldrich) at 1 × 10^8 cells/ml and serial dilutions of *Candida* were inoculated onto PDA (Oxoid) plates. Similar concentrations of *Candida* cells were suspended in 0.1% (v/v) Triton X100 in sterile ultra-purified water, the yeast cells were disrupted by frequent aspiration and left in 0.1% (v/v) Triton X-100 solution for 8 min at room temperature. Serial dilutions of Triton X-100-treated

Candida which corresponded to the non-Triton X100-treated *Candida* dilutions were inoculated onto PDA plates. These plates were incubated for 48 h at 37°C and the colonies counted.

4.2.2.6. XTT Adherence Assay

The vital cell stain XTT ((sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) was used to assess the adherence of *C. albicans* isolates recovered from oral leukoplakia to cultured NOK and DOK cells. XTT is a tetrazolium derivative and can be used to assess cell viability based on the activity of mitochondrion dehydrogenase enzymes, the latter of which are inactivated shortly after cell death. Live cells reduce XTT to a highly water-soluble orange colored formazan compound that can easily be measured by absorbance. This adhesion assay is based on the modified assay described by Alberti-Segui *et al.* (2004).

(a) Growth condition of C. albicans

All cultures were grown at 37°C in 50 ml YEPD broth (1% (w/v) yeast extract (Sigma-Aldrich), 2% (w/v) bacterial peptone (Oxoid) and 2% (w/v) glucose (Sigma- Aldrich) in 250 ml conical flasks in an orbital incubator at 200 rpm (Gallenkamp). Yeast cultures were grown to stationary phase overnight, harvested by centrifugation at 2100 x *g* for 10 min, washed twice with 5 ml of sterile PBS and resuspended in PBS at a cell density of 1 x 10^8 cells/ml.

Serial dilutions of *C. albicans* isolates were performed to elucidate the growth curve of each isolate.

(b) Growth condition of normal oral keratinocytes

Cultured keratinocytes from normal oral mucosa and cells from the oral dysplastic DOK cell line were trypsnised and washed twice in sterile PBS and resuspended at a concentration of 2 x 105 cells/ml. For the 96-well tissue culture plate adhesion assay, the cell line was seeded at 2 x 105 cells/ml densities in a final volume of 100 µl of complete tissue culture medium in each well of a 96-well tissue culture plate (three wells of each cell line; three wells of normal keratinocytes and three wells of DOK cells). A cell density of 2 × 105 cells/ ml in 100 µl final volume complete tissue culture medium was optimal to form a confluent monolayer in each well of a 96-well tissue culture plate when grown overnight (~16 h) at 37°C in 5% (v/v) CO2 in a static

incubator at 98% relative humidity. *Candida* cells were grown overnight (~16 h) at 37°C in 250 ml conical flasks containing 50 ml YEPD in an orbital incubator at 200 rpm. *Candida* cells were washed twice in 10 ml sterile PBS. The cultures were re-suspended in sterile PBS and re-adjusted to a cell density of 1 × 108 cells/ ml. Each well of keratinocyte monolyers was washed twice gently with 100 µl sterile PBS and 100 µl *Candida*-suspension was added to each well, each isolate was tested in three wells of each cell line. Following incubation at 37°C for 1 h in a static incubator in air, non-adherent *Candida* cells were removed by gently washing each well three times with 100 µl sterile PBS. Keratinocytes monolayers were lysed by the addition of 100 µl 0.1% (v/v) Triton X-100 in sterile ultra-purified water and incubated at room temperature for 8 min before aspiration to disrupt the monolayer. The 96-well tissue culture plates were then centrifuged at 1,783 × g for 5 min in a Sorvall® Legend T centrifuge (Thermo Scientific) fitted with a TTH-750 rotor and 4 microplate carriers (Thermo Scientific). The supernatant was discarded and the remaining *Candida* cells were stained with XTT-solution.

Candida cells that were to be stained with XTT dye were re-suspended in a solution of 400 µg/ml XTT in sterile PBS containing 50 µg/ml Coenzyme Q (Sigma-Aldrich). To each well, 200 µl XTT-solution was added and incubated for 60 min at 37°C before 100 µl XTT-solution was removed from each well and transferred to a new 96-well tissue culture plate which was read at 480 nm in a microplate reader (Tecan GENios basic; Tecan Group Ltd., Männedorf, Switzerland). Any signal obtained with XTT-solution from wells that previously contained mammalian cells only was subtracted from the results values prior to data analysis (the OD values of the non-infected NOK and DOK cells were subtracted from the corresponding OD values of kerationcytes to which *Candida* was added).

4.2.3. Statistical Analysis and Graphical Depiction of Results

Data from BEC *Candida* adherence assays were analysed and graphically depicted using GraphPad Prism® Software version 4.00 (GraphPad Softwares Inc.). Data were analysed using the mean and standard error of the mean (SEM), one way analysis of variance (ANOVA) with Tukey's multiple comparison post test.

Data for the optimisation of the cell stain was correlated, analysed and graphically depicted using Excel and GraphPad Prism. Data was analysed in Excel using the mean and standard deviation (SD), and was graphically depicted using a

standard curve with a linear slope. Final data for the optimisation of the cell stain was correlated, analysed and graphically depicted with GraphPad Prism using the mean, SEM and linear regression (polynomial of the 1st order).

Data for the number of lysis washes needed was correlated, analysed and graphically depicted with GraphPad Prism using the mean and SEM. Data on the effect of Triton X-100 on *Candida* viability was correlated, analysed and graphically depicted using GraphPad Prism. Results of the adhesion of *Candida* to the NOK and DOK cell lines were correlated, analysed and graphically depicted with Microsoft Excel and GraphPad Prism using the mean, SEM, two-tailed, unpaired t-tests and one-way ANOVA with Tukey's multiple comparison post-test.

4.3. Results

4.3.1. Adherence of *C. albicans* Isolates Recovered from Oral Leukoplakia Lesions to Buccal Epithelial Cells

The main objectives of this part of the present study was to investigate the adherence properties of *C. albicans* isolates recovered from oral leukoplakia lesions (Table 4.1) to human BECs and epithelial cells grown in culture in comparison to the *C. albicans* reference strains SC5324 and 132A, based on a modified protocol from Kimura and Pearsall (1978) and Kavanagh and Murphy, (2001). Eighteen isolates of *C. albicans* recovered from oral leukoplakia lesions, 14 of which were recovered from CHC lesions and four from NCL lesions and the reference strains SC5314 and 132A were tested for their adherence to human BECs.

The adherence results for each isolate are shown in Fig. 4.1. The data were analysed by one way ANOVA with Tukey's Multiple Comparison post Test. The adherence of the *C. albicans* isolates investigated was variable with a wide range of adherence capacity (Tables 4.1 and 4.2). Seven of CHC isolates (CL101, CL105, CL107, CL109, CL110, CL113 and CL117) and one NCL isolate (OL116) were significantly more adherent than the oral reference strain 132A ($P<0.05$). There was no significant difference in the adherence of the six remaining CHC isolates (CL102, CL103, CL106, CL114, CL115, CL118) or the three remaining OL isolates (OL108, OL128 and OL135) relative to 132A ($P>0.05$) (Table 4.1 and Fig. 4.2). In contrast, the results obtained with reference strain SC5314 were variable (Table 4.2 and Fig. 4.2); SC5314 was significantly more adherent than isolates CL118, CL115, CL114 and OL128. However, only one leukoplakia isolate (CL109) was significantly more adherent than SC5314. For the rest of the isolates there were no significant differences in their adherence to BECs compared to the SC5314 reference strain ($P>0.05$).

4.3.2. Culture of Normal Oral Keratinocytes

To grow NOK cells *in vitro*, two different culture methods were attempted; the first method involved seeding the isolated cells from oral mucosa biopsies on collagen coated tissue culture flask, while in the second method the cells were grown using a non-proliferating fibroblast feeder layer. Initially the cells were grown using the first method, but after several attempts this method was discontinued due to the sparse and slow growth of the cells, despite attempts to improve the yield of cells by increasing the

seeded cell density and increasing the concentration of growth factors in the culture medium (i.e. EGF and hydrocortisone). An alternative technique, which was originally described by Rheinwald and Green, (1975), was then used to grow NOK cells.

Cultured NOK cells (Fig. 4.3) exhibited multiple morphologies with varied sizes and shapes. Irregular, polygonal, and round small and large cells were observed with large nuclei that had one or two prominent nucleoli. Within two to three days after seeding, the cells started to form small colonies or islets of small round cells. After 5-10 days, the cells proliferated at more rapid rates and epithelial colonies extended toward each other. The cells reached a confluent level within 3 weeks. As the colonies grew the cells at the peripheries were more differentiated as they were larger in size and acquired a more polygonal shape with condensed nuclei and a large cytoplasmic space. The number of these polygonal cells increased following sequential passage and became more predominant at the fourth passage, as it might be an indication of cell maturation. Cells at higher passages appeared more flattened or even spindle shaped with cellular vacuoles until the cell desquamated and died. NOK cells survived for five to six passeges.

Fibroblast contamination was the major problem during the culture of NOK cells. Treatment with cold 0.25% (v/v) trypsin-EDTA was more efficient in eliminating contaminating fibroblasts from the culture compared with the use of trypsin-EDTA at 37°C.

4.3.3. Adherence of *C. albicans* to Cultured Oral Keratinocytes

4.3.3.1. Effect of Triton X-100 on C. albicans growth

The effect of the detergent Triton X-100 at a concentration of 0.1% (v/v) on *C. albicans* growth was evaluated. There was no significant difference between the number of colonies growing on Triton X-100-treated and non-Triton X-100-treated plates, indicating that 0.1% (v/v) Triton X-100 in sterile ultra-purified water had no effect on *Candida* viability (Fig. 4.4).

4.3.3.2. Effect of Media, Atmospheric Conditions and Triton X-100 on the Integrity of NOK and DOK Cells

Treatment of primary NOK and DOK cells with PBS for 30 min at 37°C in air had little effect on the cell lines with less than 1% dead NOK and DOK cells observed using the

Table 4.1. Comparative adherence of *C. albicans* isolates recovered from CHC hyperplastic candidosis and non-*Candida* leukoplakia and the reference strain 132A to buccal epithelial cells

Multiple comparison test	Mean diff.	*P* value
132A vs CL101	-4.440	*P < 0.05**
132A vs CL102	-2.242	*P > 0.05*
132A vs CL103	-3.573	*P > 0.05*
132A vs CL105	-5.453	*P < 0.01**
132A vs CL106	-1.690	*P > 0.05*
132A vs CL107	-6.750	*P < 0.001**
132A vs OL108	-2.973	*P > 0.05*
132A vs CL109	-10.11	*P < 0.001**
132A vs CL110	-7.813	*P < 0.001**
132A vs CL113	-8.912	*P < 0.001**
132A vs CL114	2.670	*P > 0.05*
132A vs CL115	0.2433	*P > 0.05*
132A vs OL116	-4.533	*P < 0.05**
132A vs CL117	-5.563	*P < 0.01**
132A vs CL118	1.067	*P > 0.05*
132A vs CL120	-3.853	*P > 0.05*
132A vs OL128	-0.6217	*P > 0.05*
132A vs OL135	-1.862	*P > 0.05*
132A vs SC5314	-5.683	*P < 0.001**

The data was analysed with one way ANOVA with Tukey's multiple comparison post test.

* Significant level of the *P* value

Table 4.2. Comparative adherence of *C. albicans* isolates recovered from CHC hyperplastic candidosis and non-*Candida* leukoplakia and the reference strain SC5314 to buccal epithelial cells

Multiple Comparison Test	Mean diff.	*P* value
SC5314 vs CL101	1.243	*P > 0.05*
SC5314 vs CL102	3.442	*P > 0.05*
SC5314 vs CL103	2.110	*P > 0.05*
SC5314 vs CL105	0.2300	*P > 0.05*
SC5314 vs CL106	3.993	*P > 0.05*
SC5314 vs CL107	- 1.067	*P > 0.05*
SC5314 vs OL108	-2.710	*P > 0.05*
SC5314 vs CL109	-4.430	*P < 0.05**
SC5314 vs CL110	- 2.130	*P > 0.05*
SC5314 vs CL113	-3.228	*P > 0.05*
SC5314 vs CL114	8.353	*P < 0.001**
SC5314 vs CL115	5.927	*P < 0.001**
SC5314 vs OL116	1.150	*P > 0.05*
SC5314 vs CL117	0.1200	*P > 0.05*
SC5314 vs CL118	6.750	*P < 0.001**
SC5314 vs CL120	1.830	*P > 0.05*
SC5314 vs OL128	5.062	*P < 0.05**
SC5314 vs OL135	3.822	*P > 0.05*

The data analysed with one way ANOVA with Tukey's multiple comparison post test

* Significant level of the *P* value

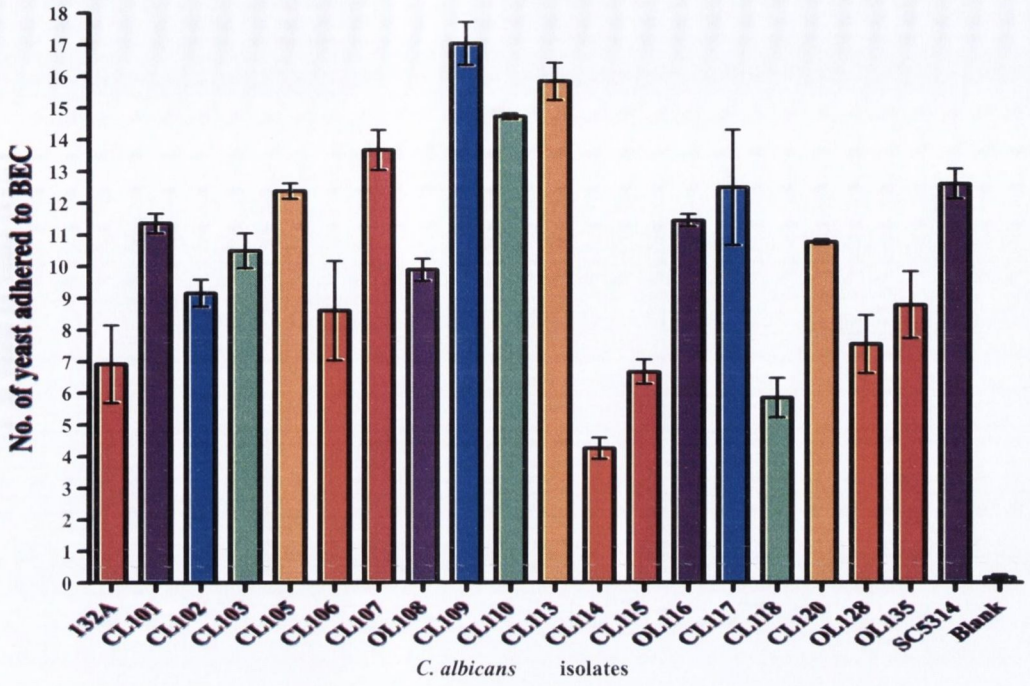
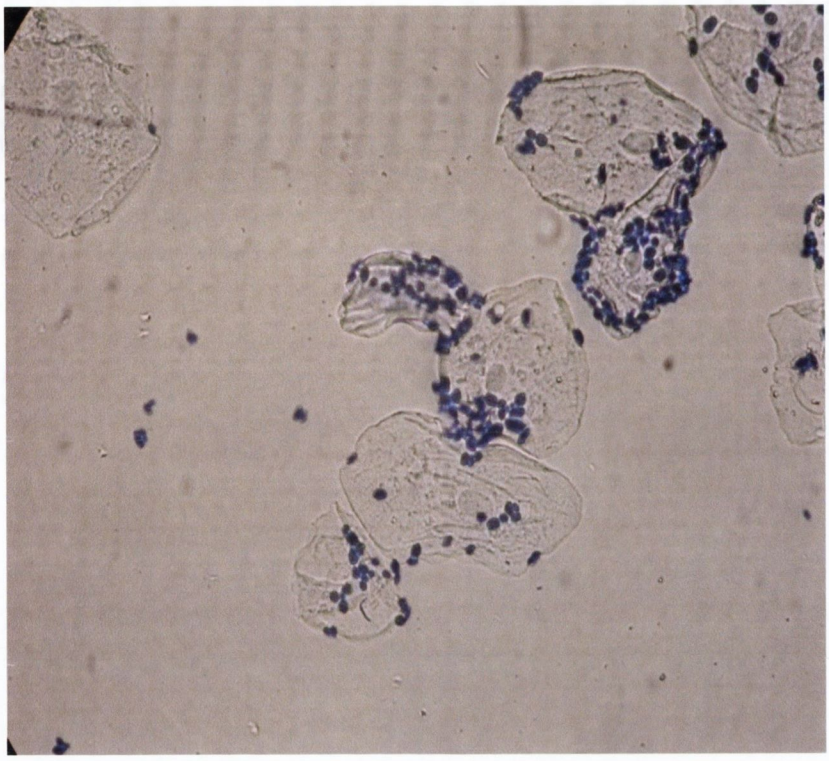
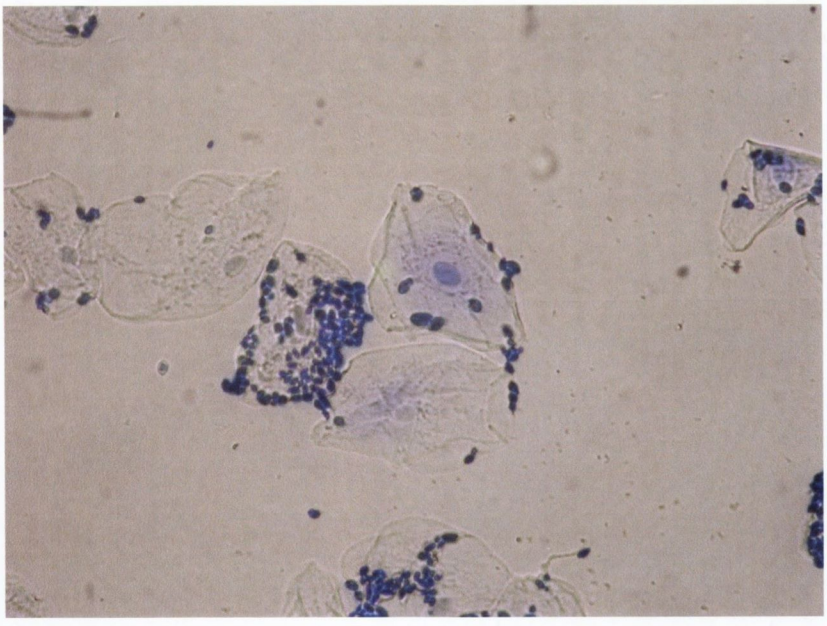


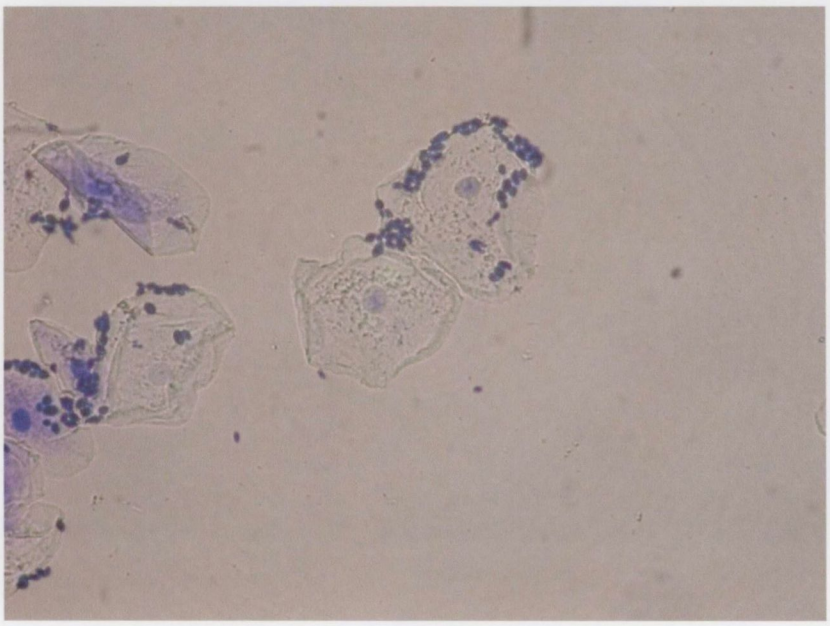
Figure 4.1. Average of adherence of *C. albicans* isolates recovered from non *Candida* leukoplakia (NCL) lesions and chronic hyperplastic candidiasis (CHC) lesions to human buccal epithelial cells (BECs) in comparison to the reference *C. albicans* strains SC5314 and 132A. Seven Isolates CL101, CL105, CL107, CL109, CL110, CL113 and CL117 recovered from CHC lesion and one NCL isolate (OL116) were significantly more adherent than the oral reference strain 132A (*P* value *<0.05*).



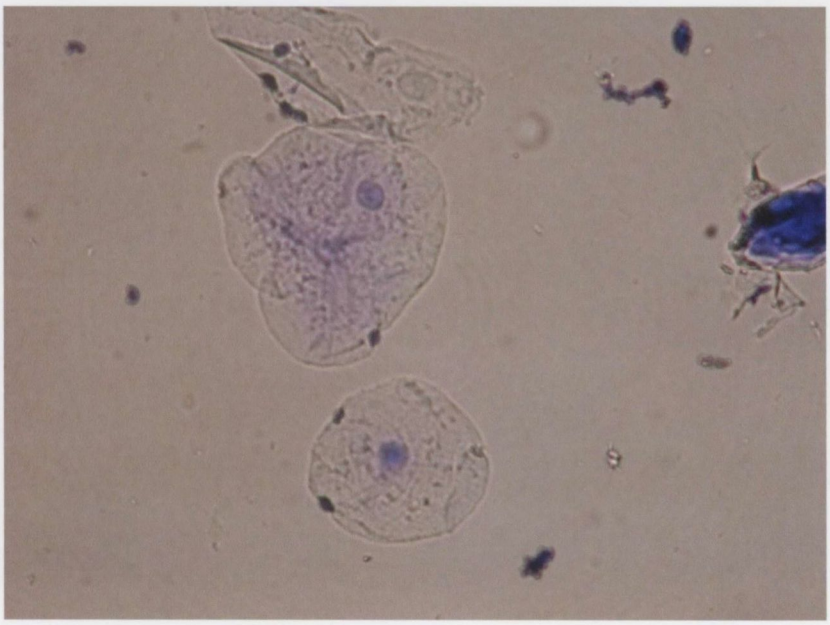
(a) OL109



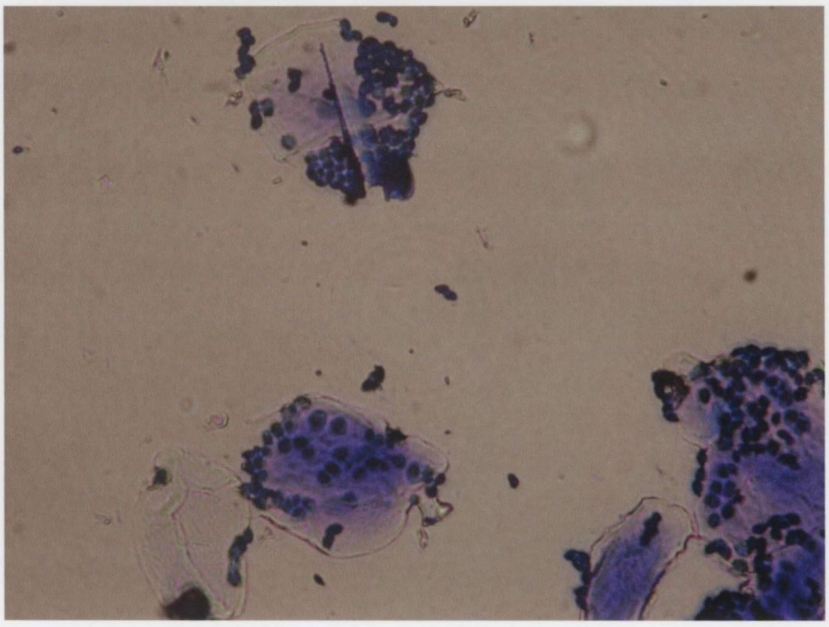
(b) SC5314



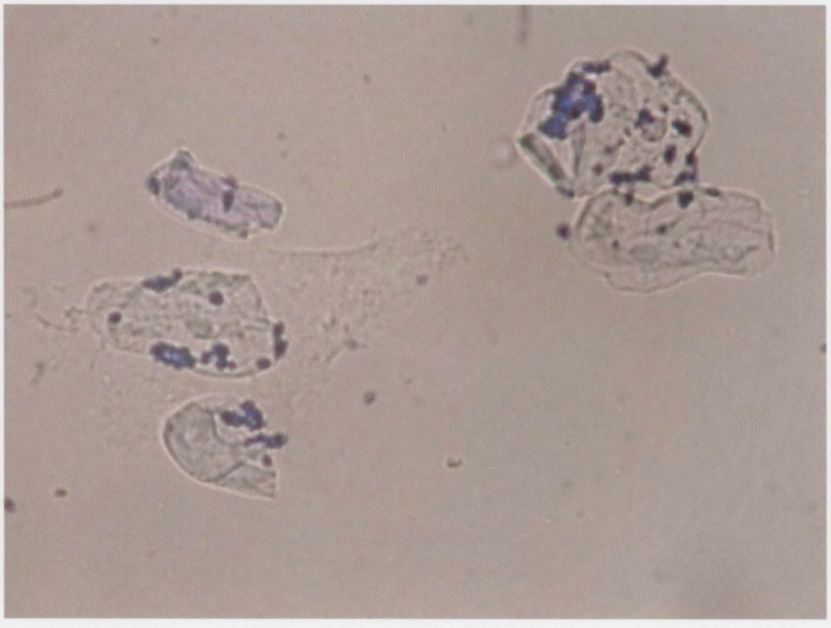
(c)132A



(d) OL114



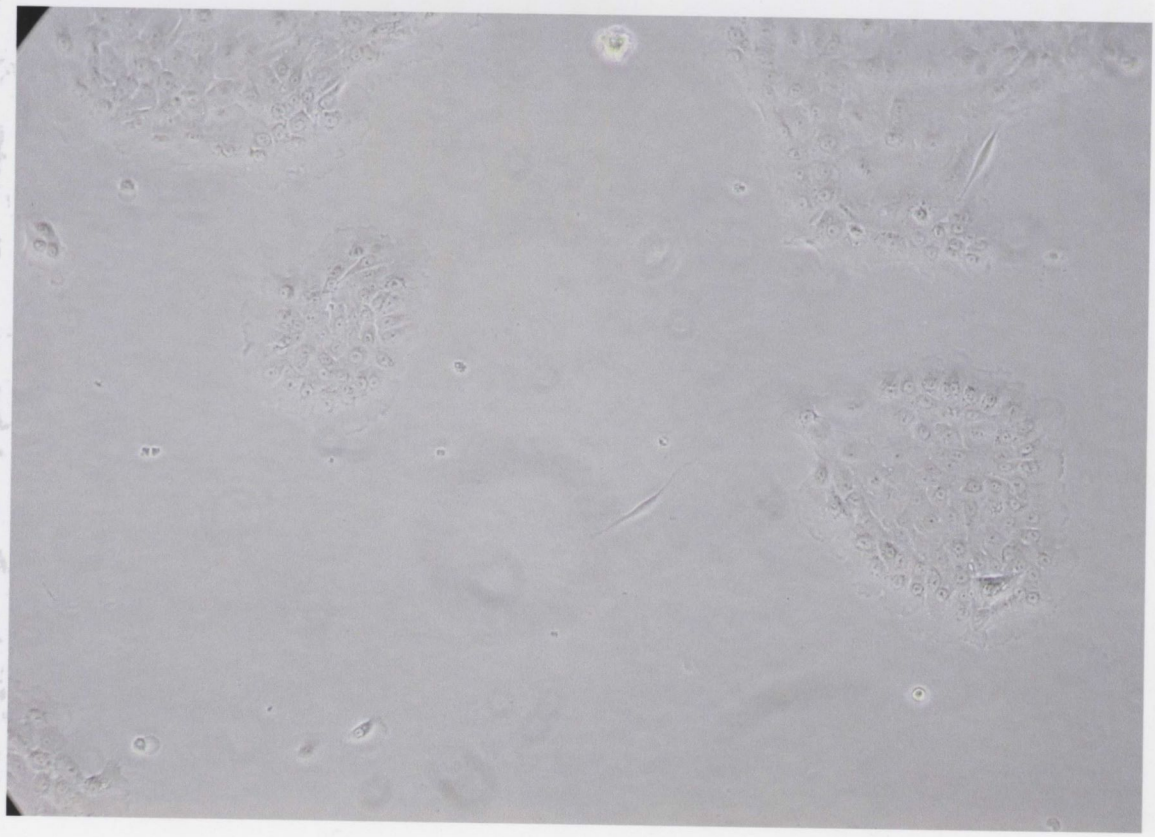
(e) OL1110



(f) OL107

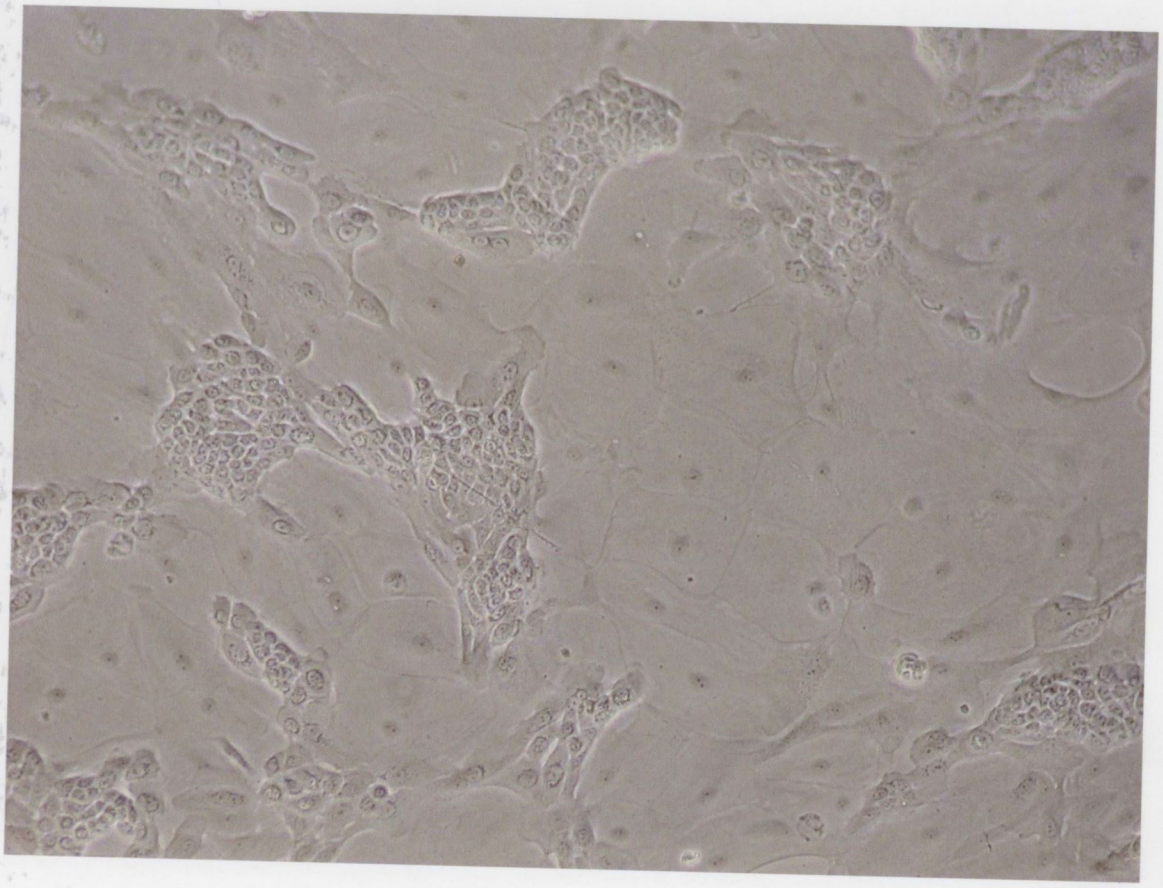
Figure 4.2 Photomicrographs of of *Candida albicans* oral leukoplakia isolates and reference strains adhering human buccal epithelial celles (BEC).

Adherent C. albicans cells are stained with Gram's stain. Isolate OL109; (b) reference strain SC5314; (c) reference strain 132A; (d) isolate OL114; (e) OL110 and (f) isolate OL107 (Magnification X40). Isolates OL109, OL110 and SC5314 reference strain were strongly adherent compared with OL114, OL107 and 132A reference strain.



(a)

(b)



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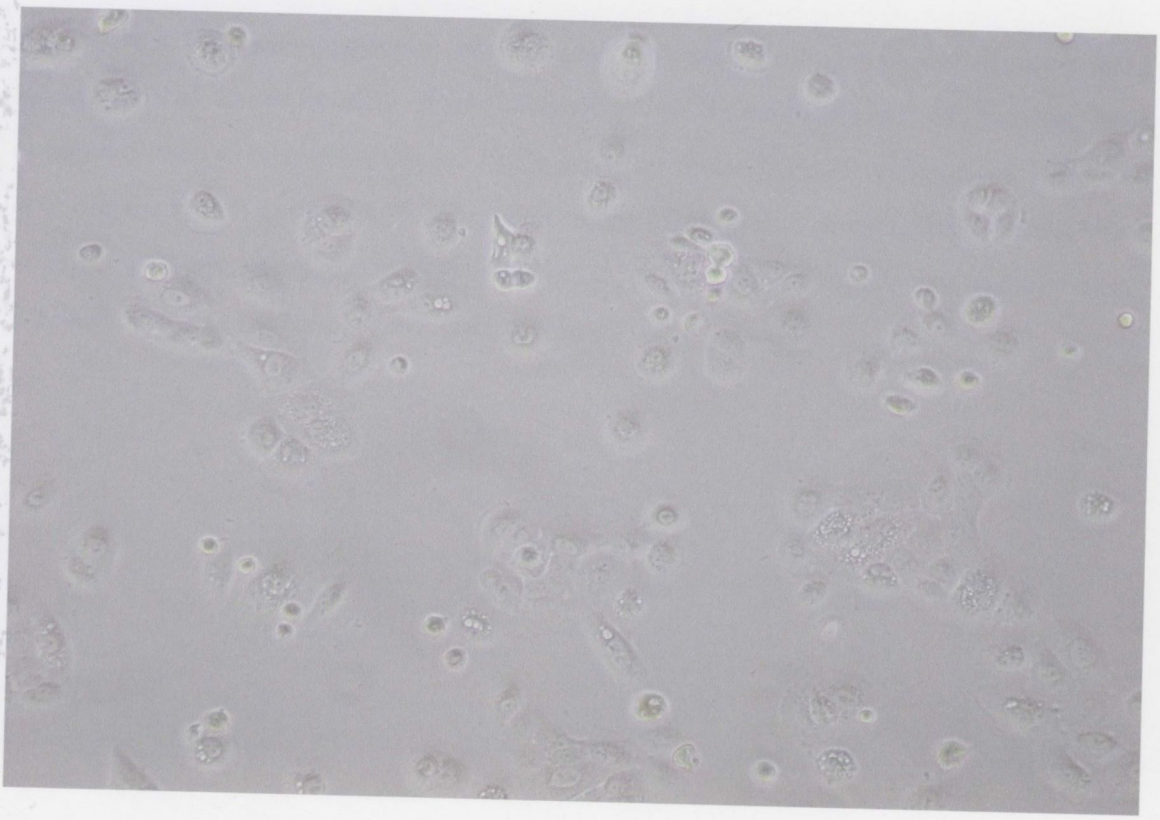
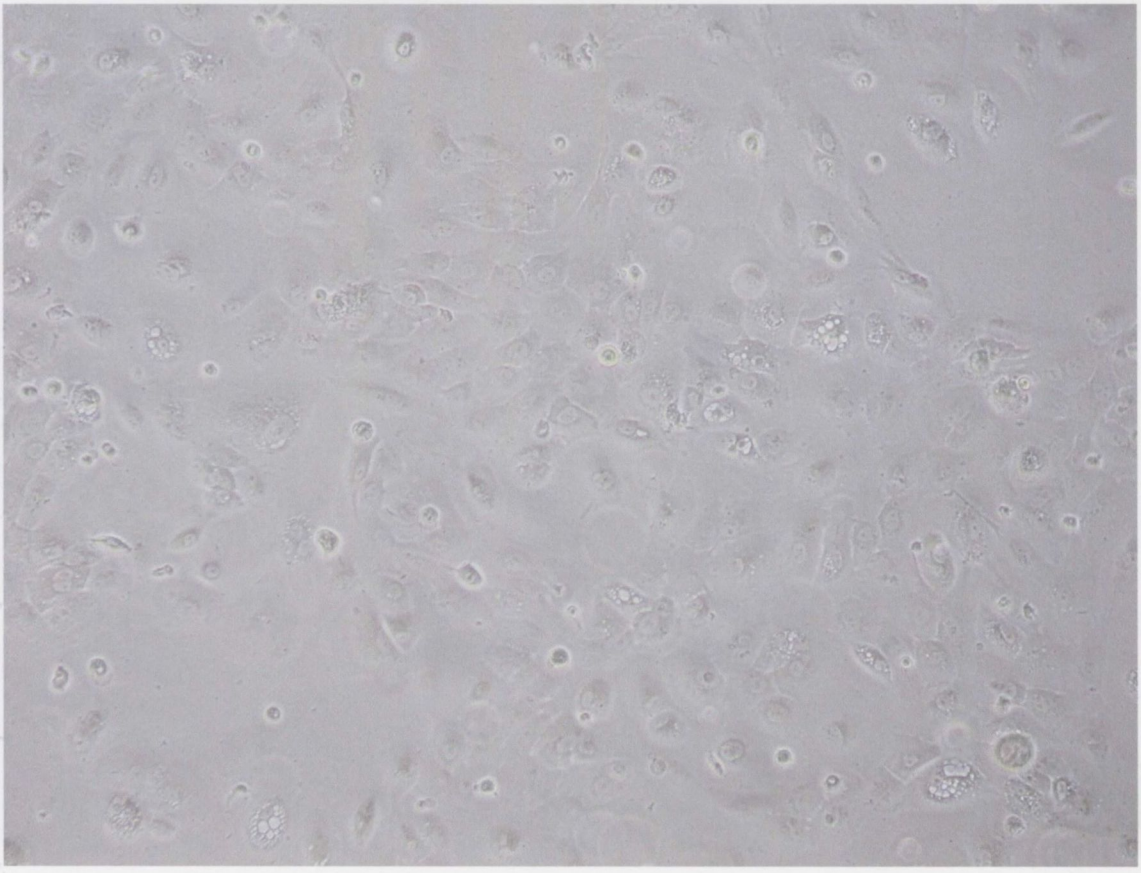


(d)



(e)

Figure 4.3 Photomicrographs of cultured normal and dysplastic human oral keratinocytes used in this study showing the various stages of normal oral epithelial cells differentiation. (a) Early growing epithelial cells colonies or islets; (b) at more confluent growth and (c) at late stage of epithelial cells differentiation were cells became polygonal flat in shape and larger in size. (d) DOK cell line in early stage, (e) DOK cell lines at more confluent growth.

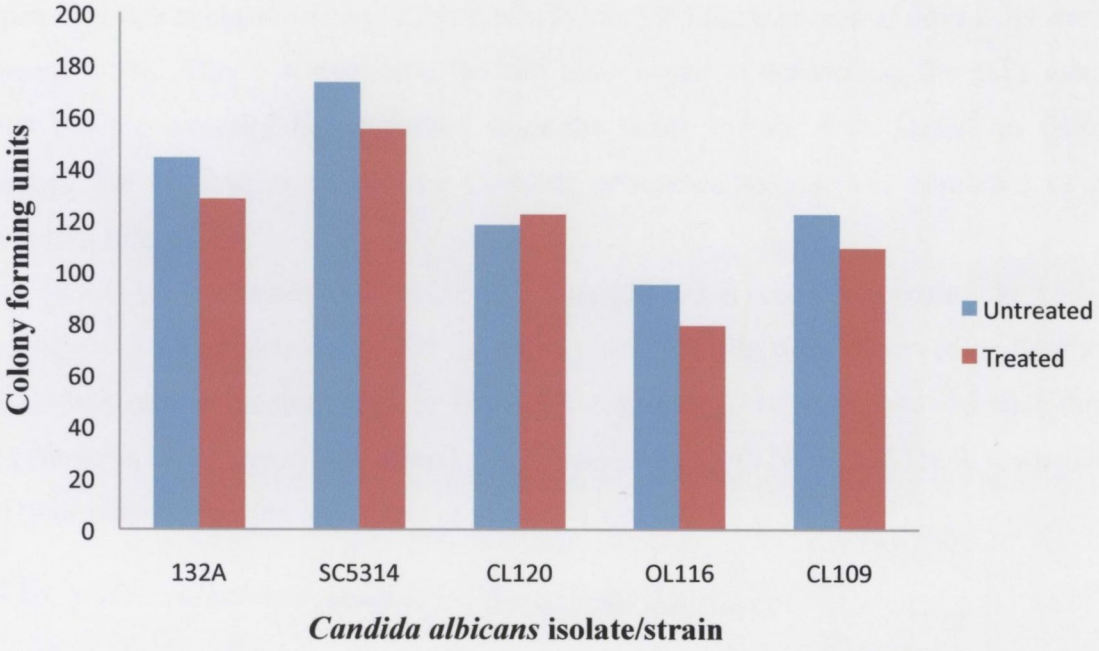


Figure 4.4. The effect of 0.1% (v/v) Triton X-100 on the growth of *C. albicans* reference strains and selected leukoplakia isolates. This concentration of Triton X-100 was used to lyse oral keratinocytes with adherent *Candida* cells in order to quantify the extent of adherence by particular *Candida* isolates. There was no significant effect on the growth of the *C. albicans* isolates and strains tested following treatment for 8 min.

trypan blue dye exclusion assay. After 2 h in sterile PBS the numbers of dead cells were between 1-5%. After 3 h exposure, the cell lines began to deteriorate; the cells were intact but the majority had detached from the tissue culture dish. Based on these findings, the incubation period for *Candida* adherence assays was restricted to a maximum time of 2 h.

Cells were exposed to 0.1% (v/v) of Triton X-100 at room temperature for 8 min with repeated aspirations using 200 µl micro-pipettes. Cells were observed to float to the surface of the medium and to have disintegrated. This result showed that this concentration of detergent was effective for lysis of the both NOK and DOK epithelial cell lines without causing any effect on *Candida* viability.

4.3.3.3. XTT Adherence Assay

The density of adherent yeast cells to both NOK and DOK cell lines for each isolate of *C. albicans* recovered from oral leukoplakia lesions and reference strains were elucidated using the XTT vital stain Both positive and negative controls were included on each clear 96-well tissue culture plate for each isolate tested per plate. Standard curve of all isolates were undertaken in separate 96-well tissue culture plates (Fig. 4.5).

4.3.3.4. Comparative Adherence of C. albicans Isolates

The results of *C. albicans* adherence to NOK and DOK cell lines are shown in Fig. 4.6. *Candida albicans* leukoplakia isolates OL108, CL109, CL110 and the reference strain SC5314 all were more adherent to NOK cells ($P \leq 0.02$) than the other isolates investigated ($P > 0.05$). No significant difference was observed in the adherence of the *C. albicans* leukoplakia isolates and reference strains tested to the DOK cell line ($P > 0.05$).

There was no significant difference in the adherence of individual *C. albicans* leukoplakia isolates and reference strains to the NOK cell line compared with the DOK cell line in each case ($P > 0.05$) (Fig. 4.6 and 4.7). However, there was a general trend that the majority of leukoplakia isolates tended to be more adherent to the DOK cell line.

4.4. Discussion

The adherence of microorganism to host cells is considered to be the first step in the processes of colonisation and infection. *Candida albicans* adherence to epithelial cells has been shown to vary considerably between different strains (Kearns *et al.*, 1983). This has been attributed to the complexity of the adherence process due to the multiplicity of the factors that contribute to adhesion. Several methods have been used previously to investigate the adherence of *Candida* to epithelial cells *in vitro*. Generally, these methods were conducted either with non-viable exfoliated BECs or cultured epithelial cell lines, the latter mostly cancer cell lines. The use of cultured human cultured epithelium as a model system for *Candida* adherence has some advantages over BECs by eliminating many of the variables associated with the use of BECs. Furthermore, the use of molecular markers to quantify the adherence yeast cells with cultured cells can overcome the effect of intra- and inter-observer variability inherent with the use of BECs.

The main objective of this study was to investigate the relative adherence ability of *C. albicans* isolates recovered from oral leukoplakia lesions to human oral epithelium using BECs and cultured normal oral keratinocytes and dysplastic oral keratinocytes *in vitro*. The results of adherence assays with BECs showed a significant ($P<0.05$) greater adherence of 8/18 of *C. albicans* isolates from oral leukoplakia lesions tested (isolates CL101, CL105, CL107, CL109, CL110, CL113 and CL117 that were recovered from CHC lesions and one isolate, OL116, from an NCL lesion) compared to the oral reference *C. albicans* strain 132A, which was originally recovered from a HIV-infected patient (Gallagher *et al.*, 1992). The remaining leukoplakia isolates (CL102, CL103, CL106, and CL120 from CHC lesions and OL108, OL128 and OL135 from NCL lesions) were more also adherent but there were no significant difference ($P>0.05$) (Tables 4.1 and Fig. 4.1). In contrast, only one *C. albicans* leukoplakia isolate (CL109) was significantly more adherent than the *C. albicans* reference strain SC5314 originally recovered from a patient with disseminated candidiasis (Gillum *et al.*, 1984) (Table 4.2 and Fig. 4.1). *Candida albicans* SC5314 was significantly more adherent than leukoplakia isolates CL118, CL115, CL114 and OL128 ($P<0.05$). For the rest of the oral leukoplakia isolates investigated there were no significant difference ($P>0.05$) in adherence to BECs compared to the SC5314 reference strain. These findings suggest that some *C. albicans* isolates recovered from oral leukoplakia had a higher binding

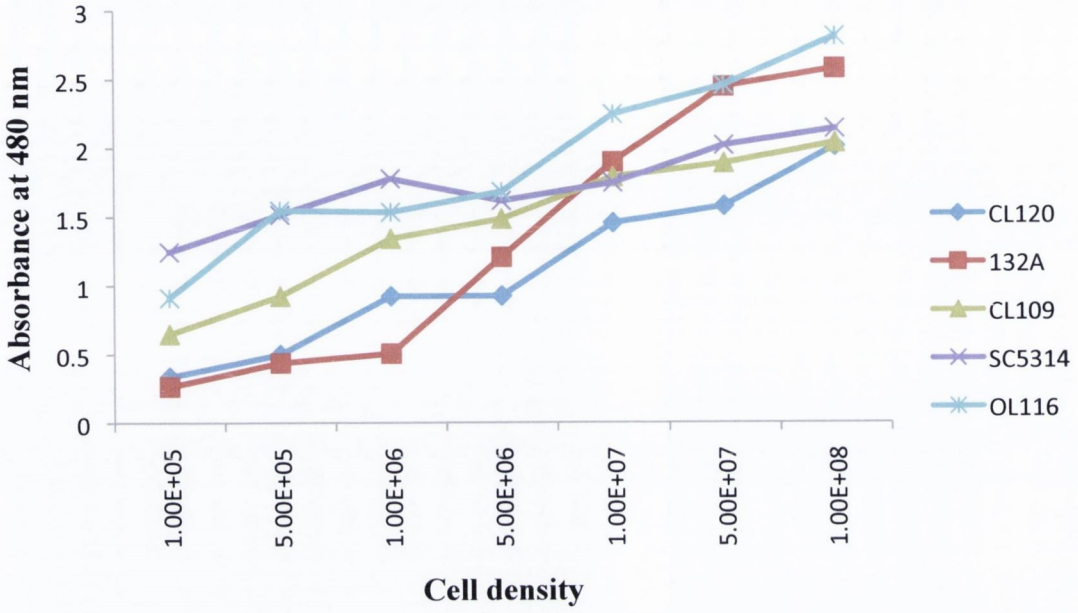


Figure 4.5 Standard growth curve of the *C. albicans* isolates CL109, OL116, OL120 and the reference strains SC5314 and 132A.

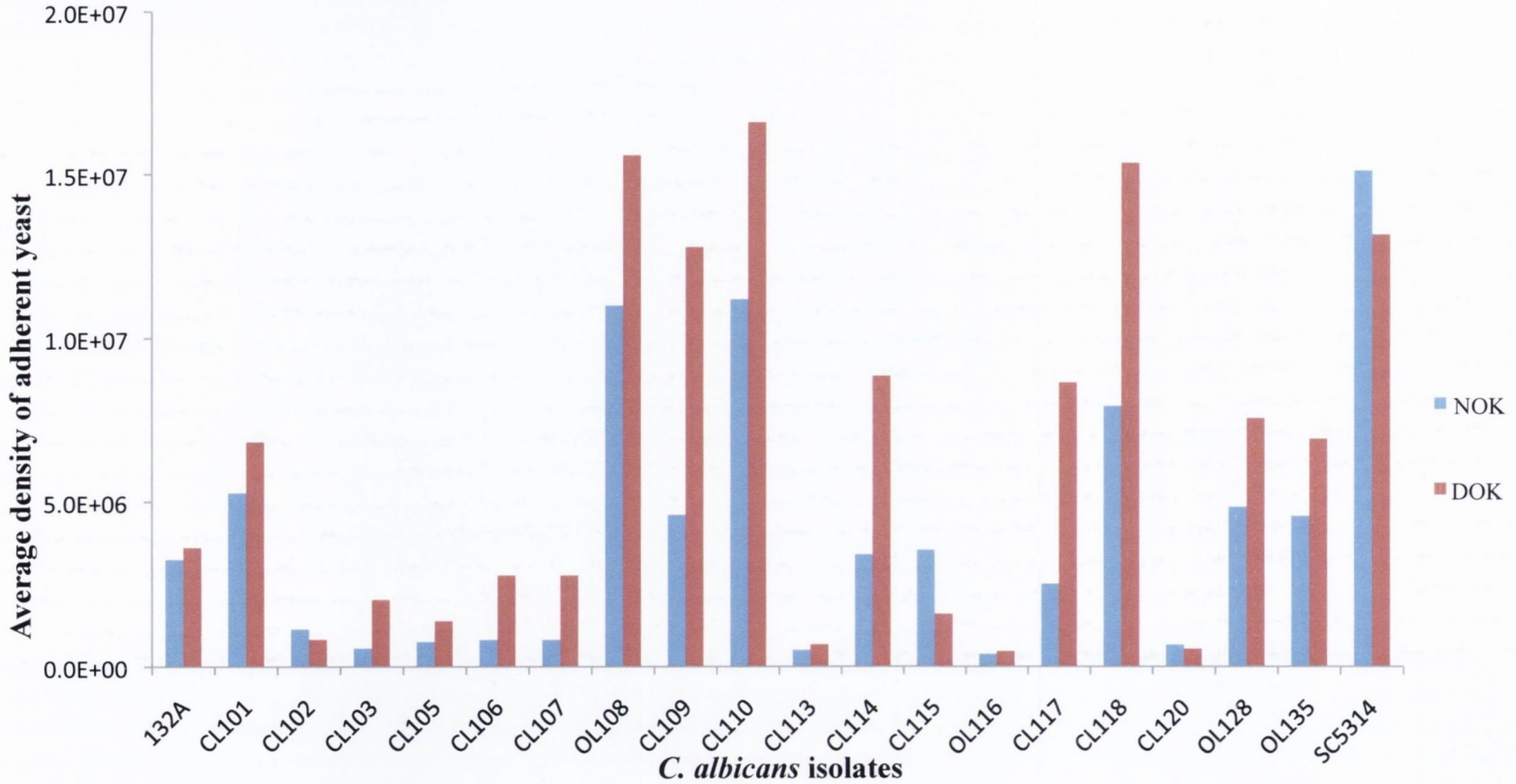
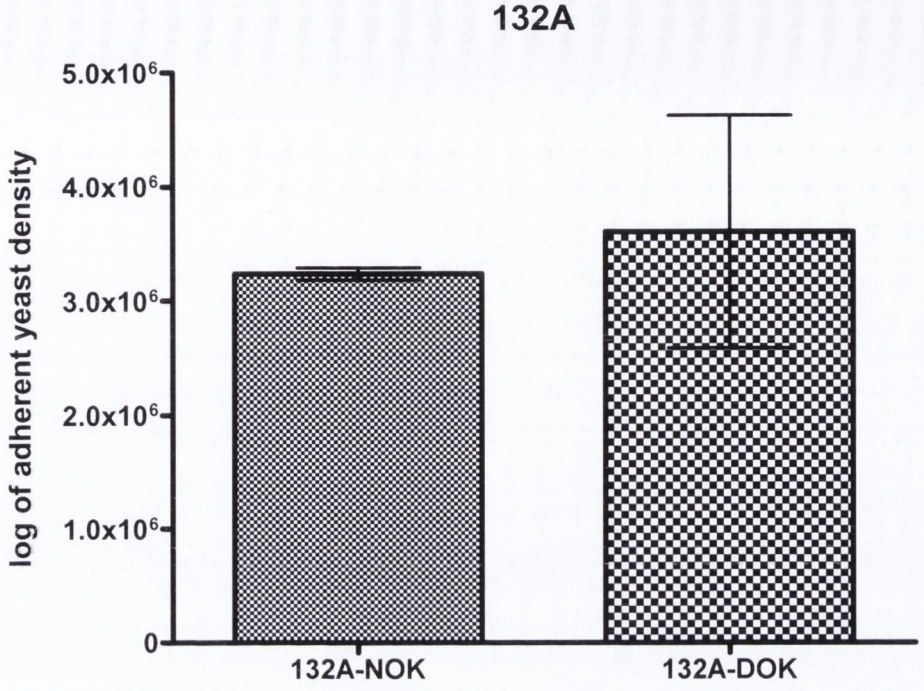
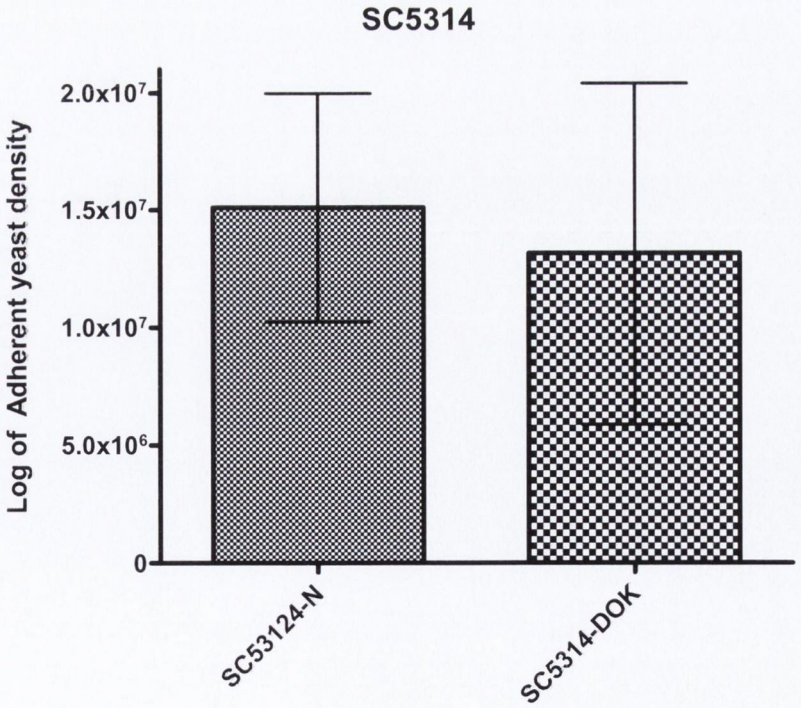


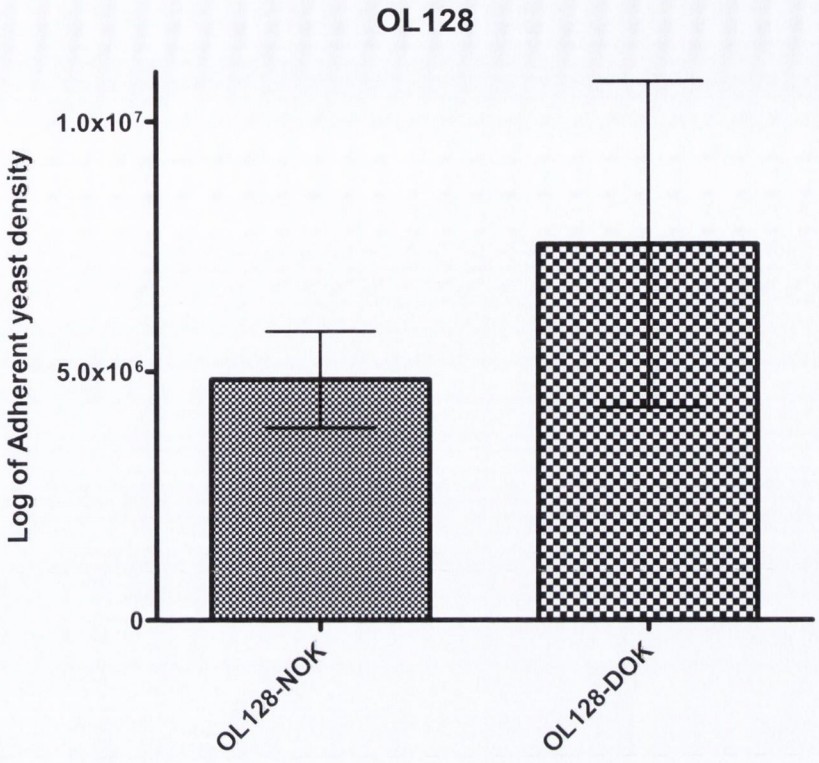
Figure 4.6. Comparative adherence of *C. albicans* isolates recovered from chronic hyperplastic candidiasis (CHC) and non *Candida* leukoplakia (NCL) lesions and reference strains 132A and SC5314 to cultured normal human oral keratinocytes (NOK) and to the DOK cell line. The results shows that log numbers of the average adherent yeasts to both oral normal and dysplastic keratinocytes that achieved with linear regression models of the yeast growth standard curve.



a) 132A



b) SC5314



c) OL128

Figure 4.7 Comparative adhesion of *C. albicans* (a) 132A; (b) SC5314 and (c) OL128 to NOK and DOK cell lines.

capacity to BECs than the oral reference 132A strain and consequently, probably have a better chance of surviving in the oral cavity. Generally, the adherence of *C. albicans* to BECs was variable ranging from a strongly adherent to weakly adherent. However, this result should be interpreted with caution as the 132A strain was first isolated nearly two decades ago and has been subjected to repeated subcultures, which might altered the surface properties related to the adherence (Gallagher *et al.*, 1992). Adherence of *C. albicans* to BECs can be affected by different factors including donor variation in the BECs and *Candida* growth conditions (Kearns *et al.*, 1983). Tsang and Samaranayake (1999) observed a wide difference in the adherence of *C. albicans* to BEC collected from HIV-infected and HIV-negative individuals, the difference was also observed among BEC among different groups of HIV-infection individuals as the yeast were less adherent BECs collected from HIV haemophiliac, heterosexual and bisexual compared with the cells from homosexual. They questioned the difference in life style among the HIV-infected groups. However, intrinsic factors such as the state of immunity suppression and antiretroviral medication cannot be ruled out.

The second part of this study investigated the adherence of *C. albicans* leukoplakia isolates to both normal and dysplastic epithelial cells in culture. The results of this study showed that that *C. albicans* leukoplakia isolates differed in their adherence to NOK and DOK cells and isolates OL108, CL109, CL110 were more adherent than the other isolates ($P<0.02$). However, the differences were not statistically significant compared to results obtained with both reference strains 132A and SC5314. Comparing the adherence of each *C. albicans* isolates to both NOK and DOK cell lines, there was no significant difference in the adherence of individual *C. albicans* leukoplakia isolates and reference strains to the NOK cell line compared with the DOK cell line in each case ($P>0.05$) (Fig. 4.6). However, there was a general trend that the majority of leukoplakia isolates tended to be more adherent to the DOK cell line. Based on these finding, it could be suggested that epithelial cell transformation does not affect the adherence of the *C. albicans*. However, there may be other factors that can promote the adherence of *Candida* to oral leukoplakia lesions, such as surface roughness of the lesion (Arendorf *et al.*, 1983). Other host and yeast factors such as state of immunity and phenotypic switching of the fungi cannot be excluded.

To the author's knowledge, adherence of *C. albicans* isolates recovered from oral leukoplakia to normal and dysplastic epithelial cells has not been compared previously. Although, there was no significant difference between the adherence of *C.*

albicans isolates to normal and dysplastic epithelial cell lines, dysplastic epithelial cells could behave differently in vivo to normal epithelial cells in relation to cellular adhesion to *C. albicans*. Transglutaminases (TG) are a group of enzymes that catalyze a calcium dependent transamination reaction, resulting in protein cross-linking, polyamination or deamination. It plays an important role in terminal differentiation of the mature keratinocytes. Over expression of the TG-3 gene has been demonstrated previously in oral leukoplakia (Ohkura *et al.*, 2005). Staab *et al.*, (1999) recognised the importance of epithelial cell-associated transglutaminase activity in covalently binding to its substrate hyphal wall protein (Hwp1) on *Candida* hyphae. The state of epithelial cell differentiation could play a key role in the initiation of *Candida* infection, in that Hwp1 binds to more terminally differentiated epithelial cells (Ponniah *et al.*, 2007).

Generally, the adherence of *C. albicans* is a multi-factorial process that influenced by combination of the host and yeast cells factors (Henriques *et al.*, 2006). The variability in the adherence of *C. albicans* isolates recovered from oral leukoplakia is likely due to effect of the expressed epithelial cells receptors and phenotypic and genetic regulation of the yeast. In order to assess these factors large number of *C. albicans* isolates from different clinical setting need to be explored for their differential adherence and their molecular interaction with both normal and dysplastic epithelial cell receptors to evaluate the differential adhesin expression

Chapter 5

Genotyping of *Candida albicans* Isolates by Multilocus Sequencing Typing and ABC Typing

5.1. Introduction

Epidemiological studies of microorganisms can facilitate the identification of outbreak related strains and tracing sources and routes of transmission of infection. Ideally, such studies require analytical methods with a high discriminatory power that yield reproducible data and are suitable for screening large number of isolates for epidemiological investigations. Molecular typing approaches have superseded older typing methods of strain differentiation such as serological, morphological and phenotypic methods based on carbohydrate substrate assimilation profile analysis. These latter methods often suffer from shortcomings such as poor discriminatory power and subjectivity (Soll, 2000; Xu *et al.*, 2002).

5.1.1. Molecular Typing of *C. albicans*

A variety of molecular typing methods have been used for typing of *C. albicans* isolates including electrophoretic karyotyping (EK) (Clemons *et al.*, 1997), random amplified polymorphic DNA (RAPD) analysis (Williams *et al.*, 1990), restriction fragment length polymorphism (RFLP) analysis (Trost *et al.*, 2004), genomic DNA fingerprinting (Anderson *et al.*, 1993; Pujol *et al.*, 2002), microsatellite sequence analysis (Fan *et al.*, 2008), multilocus enzyme electrophoresis (MLEE) (Boriollo *et al.*, 2006), and multilocus sequencing typing (MLST) (Bougnoux *et al.*, 2002).

5.1.1.1. Electrophoretic Karyotyping (EK)

Chromosomal-length polymorphisms can be assessed by karyotype analysis of *Candida* chromosomes using pulsed field gel electrophoresis (PFGE), which permits the separation of large DNA molecules in agarose gel using an alternating electrical field. Chromosomes sizes can vary between different *Candida* isolates which results in different banding patterns following PFGE analysis. Several PFGE systems have been described including orthogonal-field-alternation gel electrophoresis (OFAGE), contour clamped homogenous electric field gel electrophoresis (CHEF) and transverse alternating field electrophoresis (TAFE). These methods enable the resolution of yeast chromosomal size DNAs and also permit the detection of chromosomal length polymorphisms (Neppelenbroek *et al.*, 2006). EK has been used extensively to type *C. albicans* strains (Asakura *et al.*, 1991; Tavanti *et al.*, 2004) and to differentiate *C. albicans* isolates from *C. dubliniensis* (Sullivan and Coleman, 1998; Magee *et al.*,

2008). However, EK has significant disadvantages as it requires expensive equipment, is labour-intensive, time consuming and unsuitable for routine analysis of large numbers of isolates.

5.1.1.2. Random Amplified Polymorphic DNA (RAPD)

RAPD involves a PCR reaction that amplifies random DNA segments using one or more short oligonucleotide primers (usually 10 nucleotides in length). The primer sequences are chosen arbitrarily and prior knowledge of the targeted sequence is not required (Welsh and McClelland, 1991). Initially, several different primers have to be tested separately to identify an appropriate primer that provides adequate discrimination between the isolates being investigated. Although RAPD is rapid, inexpensive and technically simple, the reproducibility of the RAPD can be affected by minor differences in experimental conditions. RAPD analysis has been used for identification of clinical isolates of *Candida* species (Melo *et al.*, 1998; Baires-Varguez *et al.*, 2007) and it has also been applied to genotyping of *C. albicans* strains (Samaranayake *et al.*, 2003). Isolates of *C. dubliniensis*, a germ tube- and chlamydospore-positive species closely related to *C. albicans*, was shown to yield distinct RAPD profiles with specific primers compared to *C. albicans* and *C. stellatoidea* strains (Sullivan *et al.*, 1995).

5.1.1.3. Restriction Fragment Length Polymorphism (RFLP)

This technique involves the digestion of genomic DNA by one or more restriction endonucleases followed by separation of the resulting DNA fragments by agarose gel electrophoresis. Following electrophoresis, staining with ethidium bromide, and visualisation by ultraviolet light illumination, the patterns of fragments obtained with different isolates are compared and used as a basis for isolate discrimination. Strain variation results from changes in restriction endonuclease cleavage sites, or as a result of deletions or insertions of DNA sequences between restriction endonuclease cleavage sites. The fragment patterns obtained by RFLP are usually complex and it can be difficult to compare banding patterns between different isolates (Sullivan *et al.*, 1996). RFLP patterns frequently harbour dominant bands generated from digestion of rDNA and mitochondrial DNA sequences (Gil-Lamaignere *et al.*, 2003). The use of Southern blot hybridisation with specific DNA probes following RFLP (see next section) can improve the discriminatory power of RFLP as fewer DNA fragments are recognised by

the probes and the hybridisation patterns obtained are easier to compare between isolates (Soll, 2000).

5.1.1.4. DNA Fingerprinting with the Ca3 Probe

The use of RFLP followed by Southern blot hybridisation with DNA fragments consisting of cloned repetitive sequence-containing genomic DNA used to be one of the most popular methods employed for genotyping *C. albicans* using probes such as Ca3 (Soll, 2000). The Ca3 probe was originally cloned from *Eco*R1-digested genomic DNA of *C. albicans* strain 3153A. Hybridisation analysis of *Eco*R1-digested genomic DNA from *C. albicans* isolates with Ca3 generates patterns of 15-25 hybridisation bands of different intensity. The relatedness of separate isolates can be assessed by comparing the hybridisation patterns, or fingerprint profiles, obtained using computer-assisted systems (Anderson *et al.*, 1993; Soll, 2000). Furthermore, Ca3 fingerprinting analysis can permit the assessment of microevolution based on the patterns of the hypervariable bands of the repetitive sequences, which are located on fragment C of the Ca3 probe (Soll, 2000). Pujol *et al.* (1997) used the Ca3 probe to investigate the relatedness of 29 clinical isolates of *C. albicans* from the United States. The isolates were grouped into three clusters termed I, II and III, respectively. Later, two different groups were added including cluster E, which was predominantly populated with *C. albicans* isolates of European origin (Pujol *et al.*, 2002), and cluster SA from South Africa (Blignaut *et al.*, 2005). These Ca3 clades were later shown to correspond to MLST clades 1-4 (Robeles *et al.*, 2004; Tavanti *et al.*, 2005a; Odds *et al.*, 2007). Although the use of the Ca3 probe for fingerprinting and investigating the genetic relatedness of *C. albicans* isolates has proven to be a reproducible and highly reliable method for epidemiological studies (Pujol *et al.*, 1997), it is a time consuming technique that requires considerable technical expertise and is also expensive.

5.1.1.5. Microsatellites Sequences Analysis

Microsatellites are short tandem repeated (STR) sequences of 1-6 bp that spread randomly in DNA and show polymorphisms and codominant inheritance patterns. Sequence polymorphisms in microsatellite regions occur due to mismatching slippage during DNA replication, that result in an increase or decrease in the number of repeats. PCR amplification of these regions is useful in identifying the genetic relatedness and genotyping of microorganisms. In a diploid organism such as *C. albicans,* microsatellite

length polymorphism analysis can detect the presence of heterozygosity at specific loci (Sampaio *et al.*, 2003; Chavez-Galarza *et al.*, 2010). Microsatellite markers are a useful molecular tool for strain identification as they are known to be highly polymorphic (Boriollo *et al.*, 2010), stable over many generations (Stéphan *et al.*, 2002), easy to use, and have a high discriminatory index (Botterel *et al.*, 2001). However, the use of microsatellites markers may be limited depending on the complexity of the microbial community and prior knowledge of STR region sequences is needed to design appropriate primers (Kirk *et al.*, 2004). Furthermore, microsatellite markers analysis has inferior discriminatory power compared with EK and MLEE (Boriollo *et al.*, 2010), but this might be due to the selection of the STR region tested.

5.1.1.6. Multilocus Enzyme Electrophoresis (MLEE)

MLEE is based on the evaluation of nucleotide polymorphisms in isoenzymes that give rise to amino acid substitutions, possibly altering electrophoretic mobility. In this method protein extracts from cells are separated by electrophoresis on starch gels under non-denaturing conditions and visualised with specific enzyme staining procedures (Calderone, 2002). The main advantage of MLEE is its ability to study the codominant markers for each locus in diploid organisms, which is not achieved by some DNA fingerprinting methods and MLEE can provide enough information for the detection of microevolution among different strains (Boriollo *et al.*, 2010). MLEE has a high discriminatory power when a sufficient number of enzymes are used (Boriollo *et al.*, 2006).

5.1.1.7. Multilocus Sequencing Typing (MLST)

Multilocus sequencing typing (MLST) is based on analysis of variation of nucleotide sequences of unrelated housekeeping genes. Initially MLST was developed for population structure analysis of bacterial species, which are haploid (Maiden *et al.*, 1998). More recently, MLST has also been developed for diploid organisms such as *Candida* species (Bougnoux *et al.*, 2002, 2003; Tavanti *et al.*, 2003, 2005b; Dodgson *et al.*, 2003; Lan *et al.*, 2006; Jacobsen *et al.*, 2007; McManus *et al.*, 2008). MLST is based on the analysis of nucleotide polymorphisms (or single nucleotide polymorphism (SNP)) of the sequences of seven to eight fragments (loci) of individual housekeeping genes. For each locus, a distinct genotype or allelic number is assigned on the basis of the sequence polymorphism of that locus. The combination of the assigned allelic

numbers (seven in case of *C. albicans*) defines the unique allelic profile or the sequence type (ST) or diploid sequence type (DST) in the case of diploid organisms, for each isolate. The use of sequence data has minimised the bias generated from subjectivity inherent in other typing methods and has also offered a high level of accuracy and reproducibility. MLST sequences can be stored in internet databases (e.g. www.mlst.net) that permit an unambiguous global surveillance and comparative system for *C. albicans* strain genotypes.

The sequence hetrozygosity of the PCR products sometimes obtained when applying MLST to *C. albicans* isolates is due to the simultaneous amplification of both alleles (*C. albicans* is diploid), which result in the presence of two coinciding peaks on the sequence chromatogram. Based on this, the hetrozygosity of diploid organisms may present in 10 possible variations at each polymorphic site (i.e. A/A, C/C, G/G, T/T, R: A/G, Y: C/T, M: A/C, W: A/T, S: C/G and K: G/T), while in haploid organisms there are only four possible variations (i.e. A/A, C/C, G/G, T/T). This phenomenon increases the diversity of the genotypes generated by MLST for diploid organisms (Tavanti *et al.*, 2003). The discriminatory power index (DP) of a typing method is its ability to measure diversity, which estimates the probability of two randomly selected isolates being unrelated (Hunter *et al.*, 1990). Tavanti *et al.*, (2005a) analysed 416 isolates of *C. albicans* with MLST and reported a discriminatory power index value of 0.9996 (99.96%). A similar result was achieved by Garcia-Hermoso *et al.*, (2007) who reported a 99.7% for 50 independent *C. albicans* isolates. Odds and Jacobsen, (2008) calculated the discriminatory power of MLST of *C. albicans* using the whole MLST database (1,594 isolates) which was found to be 99.9%.

5.1.2. Population Structure of *C. albicans* based on MLST Analysis

MLST has proved an effective tool for epidemiological screening for many bacterial and fungal species, by determining the genetic relatedness of isolates by analysing nucleotide sequence variation within housekeeping genes. These evolutionary changes can occur by recombination as well as by simple point mutation. MLST analysis can be carried out either by using allelic profile and ST analysis, or by concatenated sequences of MLST loci. An algorithm Based Upon Related Sequence Type (eBURST) subdivides MLST data sets into hypothetical non-overlapping groups of clonal complexes (CCs) that are composed of a single putative founder ST and its descendant STs of closely related isolates. This diversification results from changes of the allelic profile from that

of the founder at only one of the seven loci (i.e. a single locus variant (SLV)). Further changes in MLST loci result in double locus variants (DLV) and then triple locus variants (TLV) (Feil *et al.*, 2004). Dendrograms based on the Unweighted-pair group method with arithmetic average (UPGMA) clustering algorithm is the most widely used analytical tool for MLST data. It has the advantage that it produces a similar grouping of the clades delineated by DNA fingerprinting with *C. albicans* isolates using the C3 probe and also correlates well with eBURST clonal clusters (Odds and Jacobsen, 2008).

The epidemiological and the clinical relevance of these clades were investigated by Odds *et al.* (2007), who analysed 1391 *C. albicans* isolates present in the MLST database and showed that 97% of the isolates can be assigned to one of 17 clades. Four major clades (1 to 4) accounted for approximately 70% of the total isolates and these correlated well with the DNA fingerprinting clades I, II, III and SA previously described by Soll and Pujol (2003) using probe Ca3. The geographical distribution of these MLST clades have also investigated (Chen *et al.*, 2006; Odds *et al.*, 2007; Wrobel *et al.*, 2008; Da Matta *et al.*, 2010). Clades 1 and 3 were highly enriched with North American isolates, two thirds of isolates assigned to clade 2 and 6 were recovered in the UK and other European isolates comprised the majority of clade 10. Clade 14 and 17 were predominantly from Southeast Asia. Isolates from Central and South America comprised about one-third of clade 8. *Candida albicans* isolates exhibiting 5-fluorocytosine resistance were predominantly found in clade 1 (Pujol *et al.*, 2004; Tavanti *et al.*, 2005a). Other phenotypic characteristics such as acid phosphatase activities, salt intolerance and adherence to BEC were also investigated among the four major clades (MacCallum *et al.*, 2009). Salt tolerance was found to be significantly associated with clade 1. However, none of the characteristics investigated was unique to any clade.

5.1.3. 25s rDNA Gene ABC Typing

This technique is based on the presence or absence of an intron in the 25S rDNA region. PCR amplification of this region from *C. albicans* isolates has been shown to yield three different products permitting *C. albicans* isolates to be divided into three different genotypes termed A, B and C, respectively. Genotype B isolates yield the largest amplimer of approximately 840 bp, while genotype A isolates yield the smallest amplimer of approximately 450 bp. Genotype C isolates yield both of these amplimers (McCullough *et al.*, 1999). Genotype D isolates have also been described but these

isolates belong to the *C. dubliniensis* species (McCullough *et al.*, 1999). Several studies have reported an association of genotype A *C. albicans* with increased resistance to the antifungal drug 5-fluorocytosine (Mercure *et al.*, 1993; McCullough *et al.*, 1999). ABC typing analysis has been used as a supportive test that augments MLST data if there is only one or two SNP differences in the MLST tested alleles among the isolates under investigation (Odds *et al.*, 2006).

5.1.5. Aims

The aims of this part of the study are:

- To document oral *Candida* species isolated from both CHC and NCL lesions.

- To determine if a specific genotype(s) of *C. albicans* is associated with CHC and NCL lesions and to determine if these isolates are genetically related.

- To compare the genotypes of *C. albicans* isolates recovered from CHC and NCL lesions to *C. albicans* isolates recovered from the oral cavities of normal healthy individuals.

5.2. Materials and Methods

5.2.1 Isolates

Thirty five *C. albicans* isolates that were selected for study were recovered from oral leukoplakia lesions (OL); 20 isolates were from lesions diagnosed as chronic hyperplastic candidosis (CHC) and the remaining 15 were from non-*Candida* leukoplakia (NCL) lesions. Thirty five oral isolates recovered from healthy volunteers (HV) attending the Accident and Emergency Department of DDH (n=18) and from TCD students (n=17) were included as control isolates. *Candida* albicans SC5314 (Gillum *et al.*, 1984) and 132A (Gallagher *et al.*, 1992) were used as laboratory reference strains (Table 5.1).

5.2.2. *Candida albicans* DNA Isolation

One or two colonies from *C. albicans* isolates grown on PDA agar for 48 h were inoculated into 5 ml of yeast extract-peptone-dextrose broth YEPD (YEPD broth (1% (w/v) yeast extract (Sigma-Aldrich), 2% (w/v) bacterial peptone (Oxoid) and 2% (w/v) glucose (Sigma-Aldrich) and grown overnight at 37°C (~ 16 h) in an shaking orbital incubator (Gallenkamp) set at 200 rpm. Cells from 1.5 ml of culture were harvested by centrifugation in an Eppendorff 5417C benchtop microfuge (Eppendorf) at 12000 x g and the pellet resuspended in sorbitol-EDTA (Sigma Aldrich) buffer containing 200 U of Zymolase enzyme (Seikagaku Corporation) and incubated at 30°C for 30 min. Spheroplasts were then treated with 10 mg/ml Proteinase K and incubated at 56°C until completely lysed. DNA was extracted from the cells by using a Qiagen DNeasy® blood and tissue kit for DNA extraction (Qiagen) according to the manufacturer's instructions.

5.2.3. MLST

5.2.3.1. Selection of Loci for C. albicans MLST Analysis

Candida albicans MLST analysis was performed as described by Bougnoux *et al.* (2003). The seven housekeeping gene loci analysed included AAT1a, ACC1, ADP1, PMIb (formerly known as MPIb), ALA1 (formerly SYA1), VPS13 and ZWF1b (Table 5.1).

5.2.3.2. PCR Amplification

PCR assays were carried out in 50 µl reaction volumes containing a 200 µM concentration of each deoxynucleoside triphosphate, 1.25 U of GoTaq polymerase (Promega), 10 µl (1x) of GoTaq FlexiBuffer (Promega), 5 µM magnesium chloride, 100 pmol of each primer, and 100 ng of the DNA template. PCRs were performed with an initial 5-min denaturation step at 93°C, followed by 30 cycles of 93°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension step of 4 min at 72°C. The PCR products were purified using a QIAquick® 96 PCR purification kit (Qiagen).

5.2.3.3. Sequence Determination

Purified PCR fragments were sequenced on both strands using the same primers that had been used previously for the PCR amplification. DNA sequencing reactions were performed commercially either by Co:Genics, Eurofins MWG Operon or Geneservice (Source Bioscience) using an ABI 3730xl DNA analyzer and dye-labeled terminators (Applied Biosystems).

5.2.3.4. Sequence Analysis

Sequences were analysed using BioNumerics version 5.0 software (Applied Maths NV, Saint-Martens-Latem, Belgium). The resulting loci sequences were uploaded on the *C. albicans* MLST website (http://calbicans.mlst.net) to be compared with sequences in the *C. albicans* database and an allelic number for each locus query was generated. Each *C. albicans* strain is characterised by a diploid strain type (DST) resulting from the combination of the genotypes obtained at the seven housekeeping gene loci *AAT1a*, *ACC1*, *ADP1*, *PMIb*, *ALA1*, *VPS13* and *ZWF1b*. The sequence data of the seven alleles for each isolate was examined for the presence of SNPs. Subsequently, SNP sequences were concatenated in a single sequence as described by Tavanti *et al.* (2005a) and the MEGA software package (version 4) (Tamura *et al.*, 2007) was used to generate UPGMA dendrograms showing the relationships between the isolates investigated. The MEGA software package does not recognise the International Union of Pure and Applied Chemistry (IUPAC) heterozygosity codes (R, Y, M, W, S and K). To deal with this, a heterozygous polymorphic site was replaced with two nucleotides in the concatenated sequence, one nucleotide to represent each allelic state. Homozygous polymorphic nucleotides at the corresponding sites in other isolates were then

duplicated. For example, a codon containing a heterozygous polymorphic site such as AYG was replaced with the sequence AACTGG in the concatenated sequence, and the corresponding homozygous polymorphic codon ACG was thus listed as AACCGG. *C. albicans* isolates included in the present study were assigned to MLST clades by comparing the allelic profile of these isolates to those of the isolates published by Odds *et al.* (2007). Similar concatenated SNP sequences for thirty *C. albicans* isolates were selected from Odds *et al.* (2007) to be used as clade representative to assess the accuracy of the clade designation generated by the UPGMA dendrogram. The eBURST package version 3 (http://eburst.mlst.net/) was used to determine putative relationships between isolates. This software scans pairs of alleles and records isolates as related when six of the seven alleles are identical between a pair. The eBURST algorithm places all related isolates into clonal complexes and, where possible, predicts the founding, or ancestral DST of each complex. The output is a display of the most parsimonious patterns of descent of each DST from the ancestral type. Clonal clusters using allelic profiles and DSTs were generated using START software version 1.0.5 (http://pubmlst.org/software/analysis/), which yields the same results generated using eBurst software but is easier to use as it permits the determinantion of the allelic and profile frequency and it also permits generation of UPGMA dendrograms based on the allelic profile data.

5.2.4. 25s rDNA Gene ABC Typing

This technique is based on the presence or absence of an intron in the 25S rDNA region. This was determined by PCR using the primers CA-INT-L (5'-ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA-3') and CA-INT-R (5'-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3') according to McCullough *et al.* (1999). PCR assays were carried out in 50 µl reaction volumes containing a 200 µM concentration of each deoxynucleoside triphosphate, 1.25 U of GoTaq polymerase (Promega), 10 µl (5x) of GoTaq FlexiBuffer (Promega), 5 µM magnesium chloride, 1 µM of primer, and 1 ng of DNA template. PCRs were performed with an initial 5 min denaturation step at 94° C, followed by 30 cycles of 94° C for 30 s, 58° C for 1 min, and 72° C for 2.5 min, with a final extension step of 10 min at 72°C. Three microliters of the amplified DNA reaction mix were visualised following electrophoresis in 2.5% (w/v) agarose gels (Sigma-Aldrich) using 0.5x TBE buffer. Following electrophoresis, gels were stained with

GelRedTM (Biotium Inc) and amplimers visualised on an UV transilluminator (AphaImger®).

5.2.5. Statistical Analysis

The discriminatory power of MLST was calculated according to Hunter's method (Hunter, 1990). Chi square and Fisher's exact test http://*faculty*.vassar.edu/lowry/fisher.html. used to analyse the distribution of the isolate within the tested population.

5.3. Results

Candida albicans isolates recovered from cases of CHC (n=20), NCL (n=15) and oral carriage isolates from healthy volunteers (HV) (n=35) were analysed by MLST and ABC typing. The *C. albicans* reference strains 132A and SC5314 were also investigated.

5.3.1. MLST

MLST undertaken with the consensus *C. albicans* MLST scheme (Bougnoux *et al.*, 2003) using the seven household gene loci resulted in a data set of 2,883 bp for each isolate. The sequences of the seven genes yielded 86 variable site SNPs (Table 5.1) representing 2.9% of the total data set; 11 (12.8%) in *AAT1a*, 6 (7.0%) in *ACC1*, 18 (21.9) in *ADP1*, 11 (12.8%) in *PMIb*, 11 (12.8%) in *ALA1*, 16 (18.6%) in *VPS13* and 13 (15.1%) in *ZWF1b*. The number of SNPs detected in oral leukoplakia isolates was higher (81 sites, 2.8%) (Table 5.2) than that of the oral carriage isolates and the reference strains (66 sites, 2.30%) sites (Table 5.3). These polymorphisms defined a total of 18 *AAT1a*, 19 *ACC1*, 20 *ADP1*, 13 *PMIb*, 26 *ALA1*, 32 *VPS13* and 25 *ZWF1b* genotypes (Table 5.1, 5.2, 5.3). A total of 56/72 (77.8%) isolates of which, 14/20 (70%) isolates recovered from *Candida* CHC lesions, 11/15 (73.3%) isolates recovered from NCL lesions and 31/37 (83.8%) isolates were recovered from HV individuals, yielded a novel allelic profile or DST not previously described in the *C. albicans* MLST database.

The level of heterozygosity amongst the polymorphic MLST nucleotides were determined for the *C. albicans* isolates investigated in the present study, as significant losses of heterozygosity can be associated with minor genetic switches or microvariation (Odds *et al.*, 2006; Bougnoux *et al.*, 2006). The percentage polymorphic sites exhibiting heterozygosity amongst the seven MLST loci were ranged from 3/86 (3.5%) for isolate OL116, to 28/86 (32.6%) sites for isolate CL113, in *C. albicans* isolates recovered from oral leukoplakia lesions while it was ranged from 1/86 (1.2%) site for isolate HV224 to 21/86 (24.4%) in isolate (HV213) for oral carriage isolates. The average of the heterozygosity in isolates recovered from CHC were higher (15.9 sites) compared to that of NCL and HV isolate (13.1 and 11.4 sites, respectively) (Tables 5.4 to 5.6).

Unsurprisingly, DST 69, the predicted ancestral founding strain of clonal cluster 1 (Fig. 5.1) (see below) and one of the most commonly encountered strains in the

MLST database, was identified in the profile of the isolate OL201. Furthermore, two of its closely related DSTs; DSTs 37 (isolate HV213) and 79 (isolate CL124) were also identified among isolates in this study (Table 5.4). Both DST37 and DST79 are SLVs of DST69 and both DST37 and DST79 are subgroup founders to DLVs strains to DST69 in eBURST clonal cluster 1 (Fig 5.1). DST 1659 was identified in CHC isolates CL129 and the HV isolate HV208, respectively. Of the seven alleles investigated for each isolate, four new alleles (genotypes) were identified; three in *ZWF1b* (genotypes 200, 201 and 202) and one in *PMIb* (genotype 102) present in isolates CL103, CL122, CL102 and HV100, respectively.

5.3.2. Cluster Analysis of the Tested Isolates

eBURST analysis was performed using the software programme available online (http://eburst.mlst.net/v3/enter_data/single/) based on the allelic profiles and resulting DSTs. The analysis was performed with the MLST database of *C. albicans* that currently contains 1987 isolates profiles under the *C. albicans* MLST consensus scheme (Table 5.2). Apart from 33 singletons, all tested isolates from CHC, NCL and HV were distributed among 11 clonal clusters from the total of 85 burst clusters for the whole *C. albicans* MLST database. Seventeen isolates (17/70; 24.29 %) belonged to clonal cluster 1, the most predominant clonal cluster identified amongst the isolates. Eleven of these isolates were recovered from oral leukoplakia lesions (6 CHC and 5 NCL). Eight isolates (8/70; 11.43%) localised to clonal cluster 2. Twenty of the 33 singletons (60.6%) were recovered from healthy individuals and 13 (39.4%) were recovered from patients with oral leukoplakia. eBURST analysis of data for the 72 isolates investigated grouped all isolates into five different clonal clusters with 53 singleton sequence types. Singletons consisted of 22 isolates from oral leukoplakia lesions (13 isolates from CHC and 9 isolates from NCL) and 30 isolates recovered from normal healthy individuals.

A UPGMA dendrogram (Fig. 5.2) was constructed from the concatenated 86 SNP sequences identified amongst the 72 tested isolates using MEGA 4 software. In addition, corresponding SNP sequences from 30 *C. albicans* isolates representative of each MLST clade previously identified by Odds *et al.* (2007) were also included to assess the accuracy of the clade assignment in the present study. This UPGMA dendrogram showed that all of the 72 isolates from the present study were assigned into 11 clades (Fig. 5.2 and Tables 5.4 to 5.6). Clade 1 was the largest with 25 isolates (34.7%), 14 of which were recovered from oral leukoplakia (eight isolates from CHC

Table 5.1 The housekeeping genes and oligonucleotide primers used in *C. albicans* MLST analysis and a summary of resulting genotypes and polymorphic sites identified in the loci with the 72 *C. albicans* isolates investigated

Locus	Putative function of gene products	Amplicon size (bp)	Sequenced fragment size (bp)	Primer	No (%) of SNP	No of resulting genotypes	No of poly-morphic A.A sites
AAT1a	Aspartate aminotransferase	478	349	Fwd 5'-ACTCAAGCTAGATTTTTGGC- 3' Rev 5'-CAGCAACATGATTAGCCC- 3'	11 (12.8%)	19	7
ACC1	Acetyl-coenzyme A carboxylase	519	407	Fwd 5'-GCAAGAGAAATTTTAATTCAATG- 3' Rev 5'-TTCATCAACATCATCCAAGTG- 3'	6 (7.0%)	19	4
ADP1	ATP-dependent permease	537	443	Fwd 5'-GAGCCAAGTATGAATGATTTG- 3' Rev 5'-TTGATCAACAAACCCGATAAT- 3'	18 (21.9%)	20	6
PMI1b	Mannose phosphate isomerase	486	375	Fwd 5'-ACCAGAAATGGCCATTGC- 3' Rev 5'-GCAGCCATGCATTCAATTAT- 3'	11 (12.8%)	13	4
ALA1	Alanyl-tRNA synthetase	543	391	Fwd 5'-AGAAGAATTGTTGCTGTTACTG- 3' Rev 5'-GTTACCTTTACCACCAGCTTT- 3'	11 (12.8%)	26	4
VPS13	Vacuolar protein sorting protein	741	403	Fwd 5'-TCGTTGAGAGATATTCGACTT- 3' Rev 5'-ACGGATGGATCTCCAGTCC- 3'	16 (18.6%)	32	11
ZWF1b	Glucose-6-phosphate dehydrogenase	702	491	Fwd 5'-GTTTCATTTGATCCTGAAGC- 3' Rev 5'-GCCATTGATAAGTACCTGGAT- 3'	13 (15.1%)	25	5

SNP, Single nucleotide polymorphism; A.A, amino acids; Fwd, Forward primers; Rev, Reverse primers.

Table 5.2 Number of polymorphic sites identified in MLST loci of thirty-five *C. albicans* isolates recovered from oral leukoplakic lesions and the number of resulting genotypes identified

Locus	No. (%) of SNP	No. of genotypes	No. of polymorphic A.A
AAT1a	11 (13.6)	15	6
ACC1	6 (7.4)	13	3
ADP1	15 (18.5)	15	5
PMIb	11(13.6)	11	5
ALA1	11 (13.6)	19	4
VPS13	14 (17.3)	21	7
ZWF1b	13 (16.0)	13	3

A.A, Amino acids; SNP, single nucleotide polymorphism

Table 5.3 Number of polymorphic sites identified in MLST loci of thirty-five oral carriage *C. albicans* isolates and reference strains recovered from healthy volunteers and the number of resulting genotypes identified.

Locus	No. (%) of SNP	No. of genotypes	No. of polymorphic A.A
AAT1a	8 (12.1)	13	7
ACC1	5 (7.5)	13	4
ADP1	7 (10.6)	13	7
PMIb	10 (15.2)	8	4
ALA1	10 (15.2)	14	4
VPS13	14 (21.2)	23	9
ZWF1b	12 (18.2)	19	5

A.A, Amino acids; SNP, single nucleotide polymorphism

Table 5.4 MLST allelic profiles, DSTs, eBURST clusters and ABC genotypes for the twenty *C. albicans* isolates recovered from chronic hyperplastic (CHC) lesions

Isolate	Source	DST	*AAT1a*	*ACC1*	*ADP1*	*PMIb*	*ALA1*	*VPS13*	*ZWFb1*	MLST clade	eBURST Cluster	Hetro. SNP*	ABC Genotype
CL110	CHC	1235	2	2	2	31	2	24	5	1	1	14	A
CL109	CHC	1234	2	2	2	27	2	24	5	1	1	14	A
CL129	CHC	1659	2	2	2	2	2	24	5	1	1	15	A
CL101	CHC	408	2	5	5	2	2	20	5	1	1	17	A
CL120	CHC	1400	2	5	5	31	2	6	5	1	1	22	A
CL118	CHC	1239	2	23	5	2	2	63	5	1	S	18	A
CL115	CHC	1237	73	5	5	9	2	127	20	1	S	7	A
CL123	CHC	228	8	5	20	6	43	69	22	8	23	14	A
CL125	CHC	538	62	12	21	1	6	30	4	11	5	23	A
CL117	CHC	1240	13	10	15	24	50	37	15	3	4	21	B
CL121	CHC	1294	21	26	14	18	72	86	84	6	8	21	B
CL122	CHC	1682	4	31	6	4	61	15	201	15	58	11	B
CL124	CHC	79	2	5	5	9	2	6	5	1	1	17	C
CL113	CHC	1236	13	32	15	6	7	140	15	3	4	28	C
CL114	CHC	1681	13	10	83	6	84	32	15	3	S	19	C
CL105	CHC	1232	14	3	6	4	56	3	8	4	S	7	C
CL102	CHC	1432	4	26	61	4	34	60	202	4	S	8	C
CL106	CHC	1233	33	7	38	31	78	122	15	8	S	19	C
CL107	CHC	1680	28	14	38	102	31	47	15	8	S	17	C
CL103	CHC	1431	4	35	6	4	58	15	200	15	S	6	C

*Hetro. SNP, heterozygous sites among the 86 polymorphic sites for the 72 isolates.

Table 5.5 MLST allelic profiles, DSTs, eBURST clusters and ABC genotypes for the fifteen *C. albicans* isolates recovered from non-*Candida* leukoplakia (NCL) lesions

Isolate	Source	DST	*AAT1a*	*ACC1*	*ADP1*	*PMIb*	*ALA1*	*VPS13*	*ZWFb1*	MLST clade	eBURST Cluster	Hetro. SNP	ABC Genotype
OL131	NCL	1648	8	3	2	4	2	6	5	1	1	9	A
OL134	NCL	1047	8	2	5	2	2	6	5	1	1	16	A
OL135	NCL	1651	2	2	5	2	2	63	20	1	1	8	A
OL201	NCL	69	2	5	5	2	2	6	5	1	1	23	A
OL133	NCL	1650	3	3	5	3	57	3	6	1	10	10	A
OL116	NCL	1238	35	2	4	4	49	4	4	2	2	3	A
OL130	NCL	1496	35	2	4	4	49	26	4	2	2	7	A
OL203	NCL	481	8	14	8	4	2	3	8	4	3	4	A
OL126	NCL	1683	62	3	3	3	26	16	95	9	6	19	A
OL128	NCL	1685	67	3	10	1	6	8	20	11	S	8	A
OL127	NCL	1684	28	14	38	2	106	122	15	8	S	22	B
OL108	NCL	1429	3	3	3	3	67	16	95	9	S	13	B
OL132	NCL	1649	8	3	2	2	2	6	5	1	1	16	C
OL202	NCL	1686	11	8	4	3	7	19	4	5	S	23	C
OL204	NCL	1652	32	3	43	3	3	32	94	9	S	15	C

Table 5.6 MLST allelic profiles, DSTs, eBURST clusters and ABC genotypes for the thirty five *C. albicans* isolates recovered from healthy volunteers (HV) and the two *C. albicans* SC5314 and 132A reference strains (ref.).

Isolate	Source	DST	*AAT1a*	*ACC1*	*ADP1*	*PMIb*	*ALA1*	*VPS13*	*ZWFb1*	MLST Clade	eBURST Cluster	Hetro SNP	ABC Genotype
HV211	HV	1653	5	5	5	2	2	78	5	1	1	18	A
HV213	HV	37	2	5	5	2	2	21	5	1	1	21	A
HV208	HV	1659	2	2	2	2	2	24	5	1	1	15	A
HV102	HV	1660	3	5	5	2	2	76	5	1	1	18	A
HV820	HV	1430	8	2	2	2	2	6	5	1	1	7	A
HV100	HV	1428	2	23	5	102	2	20	20	1	S	10	A
HV107	HV	1664	4	5	6	2	2	106	5	1	S	16	A
HV116	HV	1673	2	3	10	2	2	94	2	1	S	16	A
HV815	HV	1427	2	5	5	31	2	106	25	1	S	17	A
HV214	HV	1677	4	60	4	4	34	26	4	2	2	8	A
HV229	HV	194	36	4	14	4	4	4	4	2	2	9	A
HV215	HV	1654	4	7	4	4	4	41	4	2	2	9	A
HV202	HV	194	36	4	14	4	4	4	4	2	2	9	A
HV106	HV	1663	4	5	4	4	139	26	4	2	2	11	A
HV119	HV	275	35	2	4	4	4	4	4	2	2	8	A
HV220	HV	1676	4	3	65	4	7	26	20	2	S	13	A
HV103	HV	1661	40	24	41	21	4	76	27	2	S	13	A
HV109	HV	1666	4	2	14	4	139	41	67	2	S	7	A
HV112	HV	1669	36	2	6	4	49	41	4	2	S	4	A
HV209	HV	1657	13	6	10	6	7	32	15	3	S	16	A
HV110	HV	1667	8	14	8	4	56	10	8	4	3	9	A
HV115	HV	1672	4	60	6	4	4	41	4	2	S	11	A
HV224	HV	1675	8	3	6	4	46	13	22	4	S	1	A

Continue overleaf

Table 5.6 continued

Isolate	Source	DST	*AAT1a*	*ACC1*	*ADP1*	*PMIb*	*ALA1*	*VPS13*	*ZWFb1*	MLST Clade	eBURST Cluster	Hetro. SNP	ABC Genotype
HV225	HV	1678	14	14	8	4	146	44	89	4	S	6	A
HV117	HV	1674	13	3	6	34	62	8	47	5	S	16	A
HV210	HV	1656	28	14	21	4	34	126	15	11	S	15	A
HV108	HV	1665	5	27	37	4	34	105	12	11	S	20	A
HV204	HV	1655	3	7	6	4	99	32	12	15	S	3	A
HV114	HV	1671	4	19	6	4	61	15	201	15	58	8	A
HV105	HV	659	11	26	6	4	34	60	119	4	9	10	B
HV104	HV	1662	3	26	6	4	34	60	55	4	S	4	B
HV111	HV	1668	14	14	30	4	56	3	8	4	S	8	B
HV113	HV	1670	8	7	6	4	56	3	118	4	S	5	C
HV226	HV	1658	33	7	6	2	106	86	15	8	S	14	C
HV207	HV	1679	4	7	16	9	97	19	14	10	S	16	C
SC5314	Ref	52	2	3	5	9	2	24	5	1	1	10	A
132A	Ref	1426	2	5	5	2	2	6	21	1	1	22	A

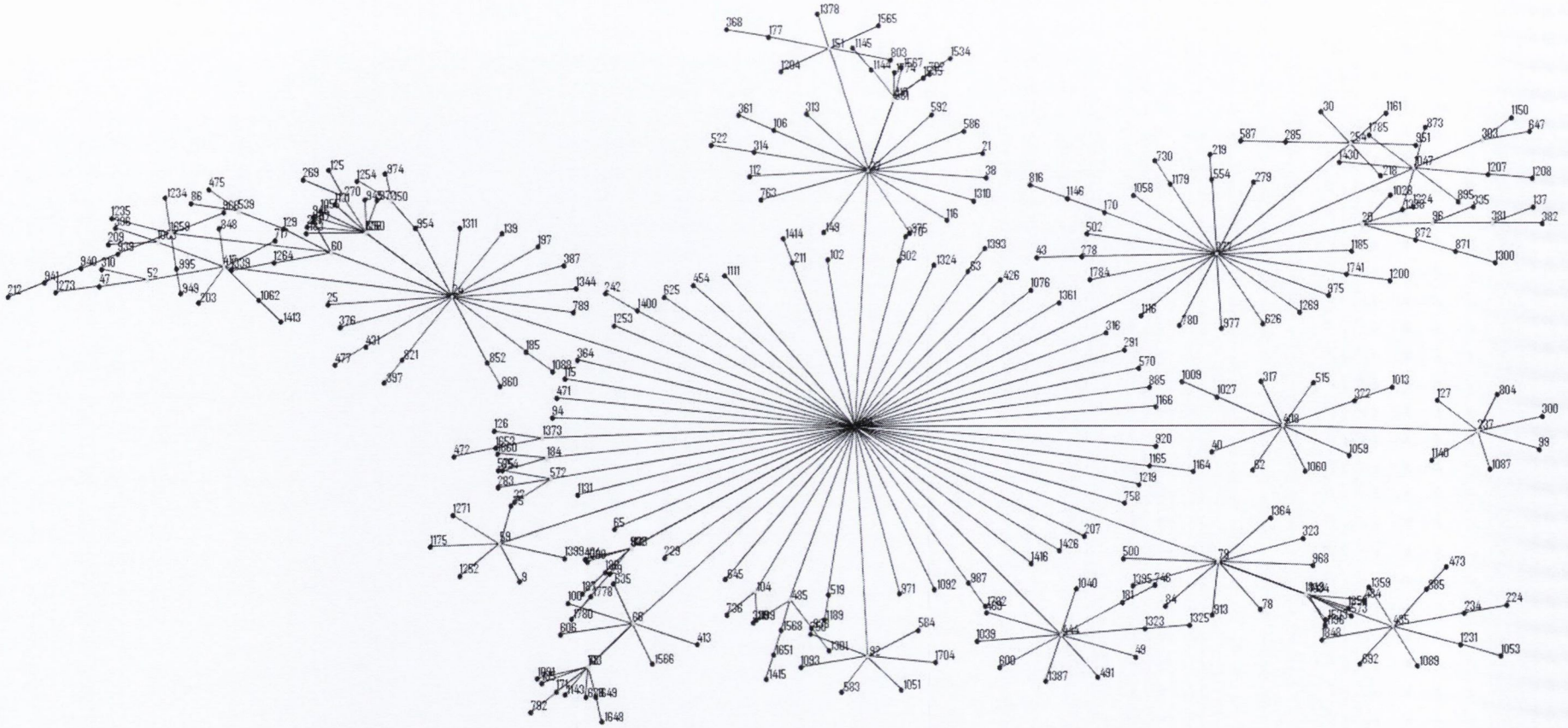


Figure 5.1 eBURSTanalysis of *C. albicans* clonal cluster 1. This illustration was generated from MLST database (MLST.net), including isolates from the present study, using the eBURST software programme version 3.0. DSTs are directly linked by lines when differ by one of the seven MLST loci. The length of the line is not significant. Isolates at nodes shown in yellow are the predicted sub-founder of the group.

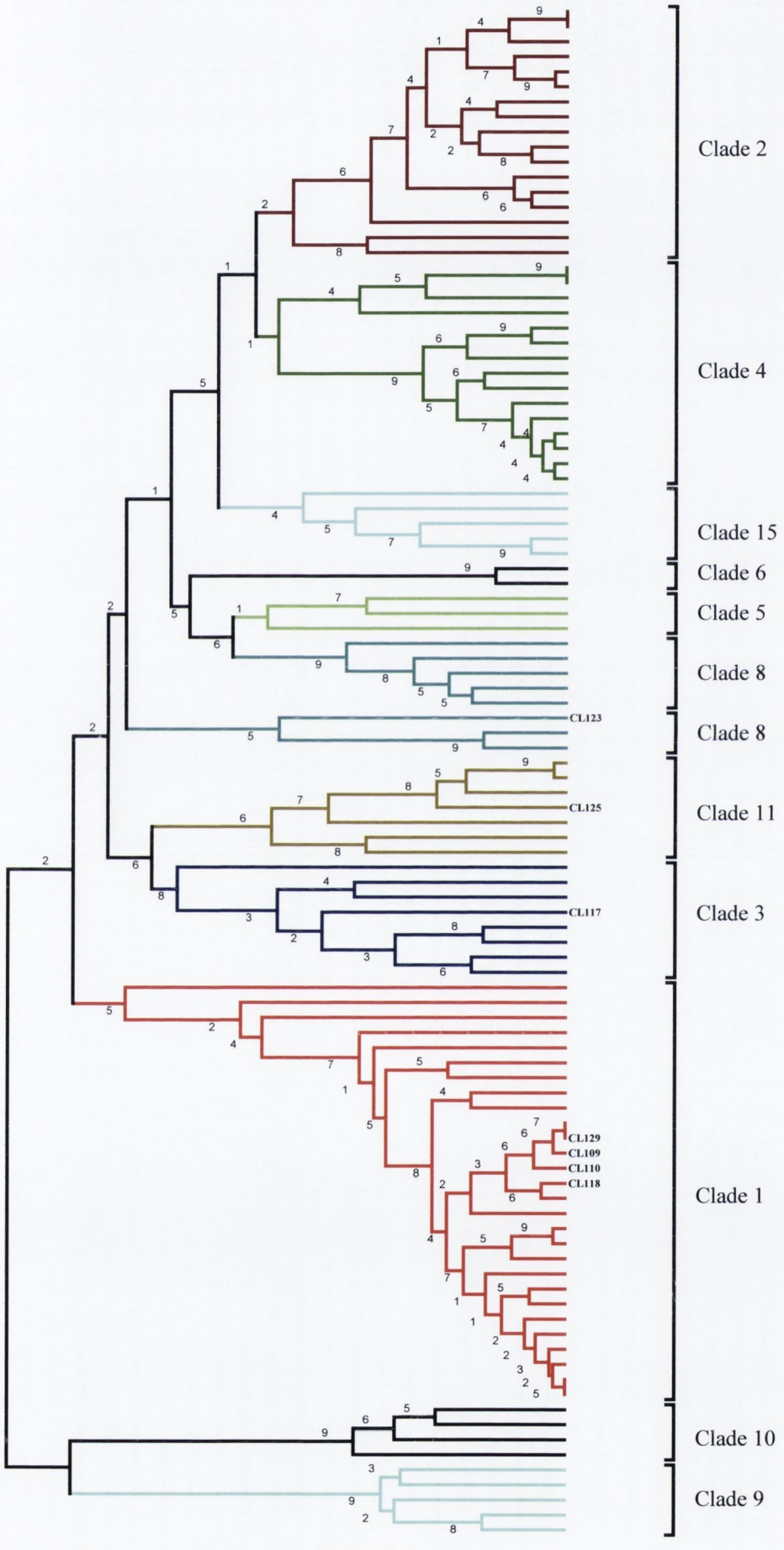

CL123
CL125
CL117
CL129
CL109
CL110
CL118
Clade 2
Clade 4
Clade 15
Clade 6
Clade 5
Clade 8
Clade 8
Clade 11
Clade 3
Clade 1
Clade 10
Clade 9

Figure 5.2 UPGMA dendrogram based on the the concatenated sequence of SNPs of the seven loci of the consensus *C. albicans* MLST scheme for the seventy-two *C. albicans* investigated. The number at the nodal points indicates the bootstrap value (as percentage) for 1000 replications. The scale bar at the bottom of the figure represents the *P* distance which is the proportion of nucleotide sites at which two sequences being compared are different. The MLST clades to which the isolates belong are shown on the right of the figure and clades were also delineated with different of the sub-tree branches. This diagram shows the agreement of clades distribution with isolates selected from Odds *et al.* population structure of *C.albicans* recovered from both oral leukoplakia and controls.

and six isolates from NCL), however, this was not statistically significant ($P>0.05$) (Table 5.7). The remaining isolates were from the healthy individuals. Clade 2 was the second largest clade with 13 isolates (13/70; 18.57%); 11 from HV and two from NCL. Clade 4 was the third largest with 10 isolates (13.9%), of which 7 were from HV, two from NCL and one isolate from CHC.

The same concatenated SNP sequences were used to construct a neighbour joining tree using Mega software. This included all oral leukoplakia and control *C. albicans* isolates (Fig. 5.3). Similarly to the previous UPGMA dendrogram this has revealed no genetic enrichment of clades with *C. albicans* isolates recovered from either CHC or NCL lesions. Fisher's exact testing supported this data ($P>0.05$) and showed no significant genotypic enrichment amongst CHC or NCL isolates.

5.3.3. Reproducibility of MLST

Reference *C. albicans* strain SC5314 was used to assess the reproducibility of the MLST as the sequence data of the strain has been already deposited in the MLST database. The sequence data of the test loci of this strain determined in the present study was 100% identical to that present in the database (www.mlst.net).

5.3.4. 25s rDNA Gene ABC Typing

ABC typing of the 72 *C. albicans* isolates investigated by MLST was also undertaken to augment the MLST data. The majority of the isolates tested belonged to genotype A (50 isolates, 69.4%). Eight isolates (11.1%) belonged to genotype B, and 14 isolates (19.4%) belonged to genotype C (Table 5.8). Genotype A isolates comprised the vast majority of the isolates from healthy individuals (29/35, 83%), but only 19/35 (54%) of the oral leukoplakia isolates (9 of CHC and 10 NCL) belonged to genotype A. Only three isolates (8.5%) from genotypes B and C were from HV. Genotype B was observed in 5 (14%) isolates from OL (3 CHC and 2 NCL). Interestingly, the oral leukoplakia group of isolates were highly enriched with genotype C (11/35, 31.4%), of which 8/20 (40%) were from CHC, which was highly significant ($P<0.02$ with Fisher's exact test) and 3 isolates from NCL. Genotype A isolates belonged predominantly to MLST clade 1 with 23 (92%) and clade 2 with 13 (100%), the distribution remaining clades with their ABC typing are shown in Tables 5.3-5.5.

Another UPGMA dendrogram was constructed based on the polymorphic sites identified throughout the MLST loci of the genotype C *C. albicans* isolates recovered from oral leukoplakia (CHC and NCL) lesions only. The purpose of this was to evaluate the genetic relatedness of the isolates specifically belonging to ABC genotype C (Fig. 5.4). This tree showed that isolates belonging to genotype C exhibited significant genotypic divergence according to the MLST sequence data, despite all belonging to the same ABC genotype.

Table 5.7 Multilocus sequences typing clade distributions of *C. albicans* from chronic hyperplastic candidosis and non-*Candida* leukoplakia lesions and from oral carriage in healthy individuals

Clade	CHC	NCL	HV
1	8 (32)	6 (24)	11 (44)
2	0	2 (15.3)	11 (84.6)
3	3 (75)	0	1 (25)0
4	2 (20)	1 (10)	7 (70)
others	7 (35)	6 (30)	7 (35)

CHC, chronic hyperplastic candidosis; NCL, non-*Candida* leukoplakia; HV, healthy individuals.

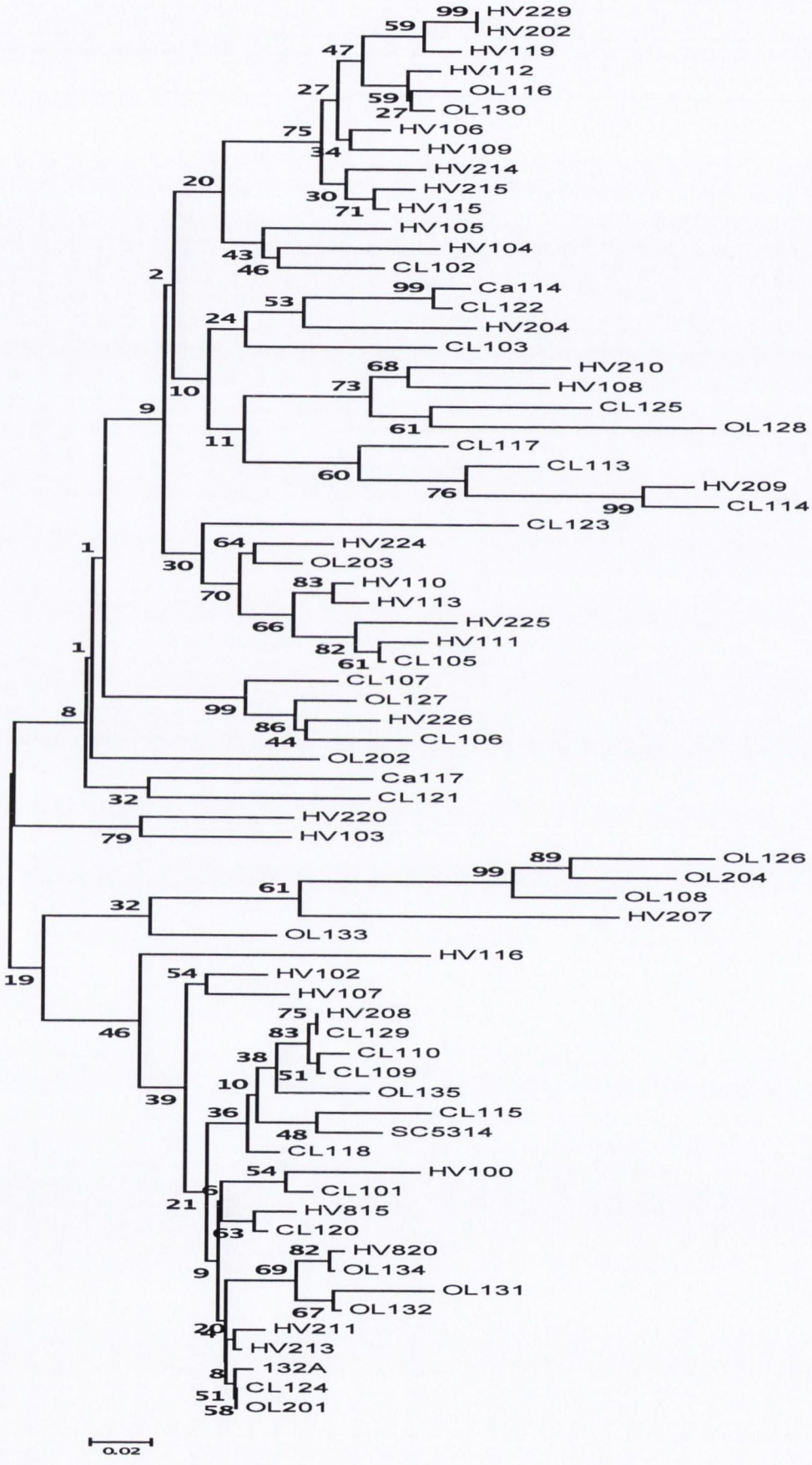

HV229
HV202
HV119
HV112
OL116
OL130
HV106
HV109
HV214
HV215
HV115
HV105
HV104
CL102
Ca114
CL122
HV204
CL103
HV210
HV108
CL125
OL128
CL117
CL113
HV209
CL114
CL123
HV224
OL203
HV110
HV113
HV225
HV111
CL105
CL107
OL127
HV226
CL106
OL202
Ca117
CL121
HV220
HV103
OL126
OL204
OL108
HV207
OL133
HV116
HV102
HV107
HV208
CL129
CL110
CL109
OL135
CL115
SC5314
CL118
HV100
CL101
HV815
CL120
HV820
OL134
OL131
OL132
HV211
HV213
132A
CL124
OL201
0.02

Figure 5.3 Neighbour joining tree for constructed from the concatenated sequence of the SNPs of MLST loci for the seventy two isolates of *C. albicans* isolates investigated in this study. This showed no significant genetic similarity among *C. albicans* isolates recovered from oral leukoplakia and control isolates. The number at the nodal points indicates the bootstrap value (as percentage) for 1000 replications.

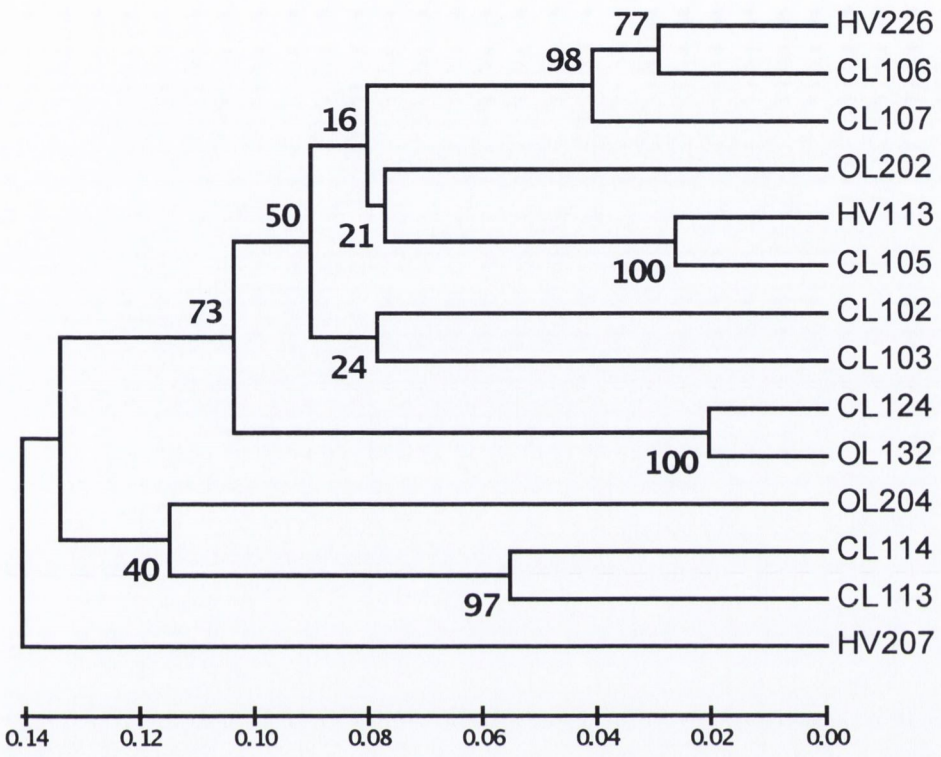


Figure 5.4. UPGMA dendrogram based on concatenated MLST SNPs of the eleven *C. albicans* genotype C recovered from CHC and NCL lesions and the three control isolates. This showed that the genotype C *C. albicans* do not belong to a genetically distinctive group of *C. albicans.* However, isolates CL124 and OL132 were closely related to each other as indicated by the bootstrap percentage and the *p* distance value 0.02, similarly, HV113 and CL105 isolates has the same trend.

Table 5.8 Distribution of ABC genotypes among *C. albicans* isolates recovered from healthy volunteers and oral leukoplakic lesions

Isolates	Genotype *n (%)*
HV	A=29 (83)
(*n=35*)	B =3 (8.5)
	C =3 (8.5)
NCL	A =10 (67)
(*n=15*)	B =2 (13)
	C =3 (20)
CHC	A =9 (45)
(*n=20*)	B =3 (15)
	C =8 (40)*

*$P<0.02$

Isolates assigned to ABC genotype A were more prevalent among the HV group, while genotype C were significantly associated with isolates recovered from CHC lesions. Genotype B isolates were observed relatively more frequently in oral leukoplakic lesions isolates than HV isolates but this finding was not statistically significant.

Abbreviations: HV, isolates from healthy volunteers; NCL, non-*Candida* leukoplakia; CHC, chronic hyerplastic candidosis.

5.4. Discussion

MLST provides an effective tool for investigating the population structure of *C. albicans* (Bougnoux *et al.*, 2002). In the present study, 72 isolates from CHC (n=20), and NCL lesions (n=15), and oral carriage isolates from HV (n=35) as well as the *C. albicans* reference strains SC5314 and 132A were investigated by *C. albicans* MLST. The results revealed that over half of the tested isolates had a previously unidentified MLST pattern (56/72, 77.8%). These included 25/35 (71.4%) of the oral leukoplakia isolates, of which 14/20 (70%) were from CHC and 11/15 (73.3%) from NCL lesions. new DSTs were identified in 31/37 (83.8%) of the oral carriage isolates tested and reference strain 132A (Tables 5.4, 5.5 and 5.6). Furthermore, four new alleles or genotypes were identified in this study. This high rate of unpublished novel DSTs among the investigated isolates relative to the large number of *C. albicans* isolates in the MLST database was surprising, but because the prevalence of new DSTs was approximately evenly distributed among leukoplakia and carriage isolates, this finding may reflect the fact that the isolates were recovered from Irish individuals. Ethnic associations between particular types of *Candida* isolates have been reported previously; for example *C. albicans* from South Africa were clustered in a distinct C3 fingerprint group (SA group) which was widely divergent from group I, II and III (Bilgnaut *et al.*, 2002). Furthermore, isolates in the SA clade were significantly more resistant to amphotericin B (Bilgnaut *et al.*, 2005). Similarly, *C. dubliniensis* isolates belonging to CD25 fingerprint group III that exhibit resistance to 5-fluorocytosine have been recovered exclusively from patients of Arab ethnic origin (Al Mosaid *et al.*, 2005; McManus *et al.*, 2008).

The percentage of heterozygosity identified amongst the total polymorphic sites identified throughout the MLST loci varied significantly amongst the 72 tested *C. albicans* isolates, from 1/86 (1.4%) – 28/86 (32.6%). This was also observed within *C. albicans* recovered CHC, NCL and oral carriage groups separately. The average of heterozygosity was higher in *C. albicans* recovered from CHC (15.9 sites) compared with that present in NCL and HV isolates (13.1 and 11.3 sites, respectively). This wide range exhibited by these isolates reflects the MLST sequence diversity of these groups of isolates that do not related from the process of microevolution (Odd *et al.*, 2006).

eBURST analysis showed that 39/72 of the isolates investigated were distributed along 11 clonal complexes from a total of 85 clonal complexes for the whole

C. albicans MLST database. Clonal cluster 1 was the most common observed among the isolates tested with a total of 17 isolates (8 CHC; 6 NCL and 9 HV). This was not unexpected as cluster 1 is the most common cluster identified previously in *C. albicans* MLST populations (Odds *et al.*, 2007). Unsurprisingly, DST69 (OL201) and its closely related DST37 (isolate HV213) and DST79 (isolate CL124) (both are SLVs to DST69) was identified among the isolates investigated in the present study. DST69 is the predicted ancestral founder of the eBURST cluster 1 which comprised of 24% of the isolates investigated in the present study (Fig. 5.1). This DST is the most commonly found DST worldwide. These findings indicate that cluster 1 isolates are derived from a common ancestral *C. albicans* type. However, the identification of a high proportion of isolates (45.8%) as singletons that did not belong to any of the 85 eBURST clusters present in the whole *C. albicans* MLST database contradicts the previous suggestion. However, the significance of these findings should be interpreted with caution as eBURST analysis of *C. albicans* is not definitive and only predicts the founders and patterns of evolutionary descent hypothetically based on allelic profile and DST data. The nature of sequence variations in diploid genomes makes eBURST analysis less relevant to that of haploid organism (Urwin and Maiden 2003).

The findings of this study support the previous finding of Odds *et al.* (2007) and Chen *et al.* (2006) that over two-thirds of *C. albicans* isolates belong to clades 1, 2 and 4. Clade 1 was also the largest with 25 isolates (34.7%, 14 of which were recovered from oral leukoplakia lesions), followed by clade 2 with 13 isolates (18%) and clade 4 with 10 isolates (13.9%). It is noteworthy here that the basis for clade differentiation of *C. albicans* were closely associated with geographical distribution, especially for clades 1-4. (Soll and Pujol 2003; Tavanti *et al.*, 2005a; Odds and Jacobsen 2008). These findings showed that the *C. albicans* isolates investigated in the present study were distributed similarly to that of *C. albicans* isolates recovered in Western Europe (Odds *et al.*, 2007), where most isolates (70%) belong to clades 1 to 4.

ABC typing was undertaken to enhance isolate discrimination. The majority (50/72, 69.4%) of the 72 *C. albicans* isolates investigated belonged to genotype A similar to previously reported findings (McCullough *et al.*, 1999; Tavanti *et al.*, 2003; Odds *et al.*, 2007; Wrobel *et al.*, 2008). The majority of genotype A isolates belonged to MLST clade 1 (23/50) and clade 2 (13/50). However, genotype C was observed more frequently than previously reported studies (19.4% of the total isolates tested in the present study and 40% of CHC the isolates compared to 11% of isolates in the study by

Table 5.9 Positions of polymorphic nucleotide sites associated and their allelic number at the seven MLST loci. The nucleotide base pair represented in a single code in case of homozygous sites while IUPAC nucleotide code where used for the heterozygous positions.

a) *AAT1*

Alleles	No. of isolates	Nucleotide positions*											Concatenated sequence[$]
		7	28	40	70	89	124	325	352	361	368	373	
AAT1a2	16	R	T	R	T	R	Y	C	C	C	C	C	GATTGATTGACTCCCCCCCCC
AAT1a3	5	G	T	A	T	A	C	C	C	C	C	C	GGTTAATTAACCCCCCCCCCC
AAT1a4	12	A	T	A	C	A	C	C	C	C	C	C	AATTAACCAACCCCCCCCCCC
AAT1a5	2	A	T	R	T	R	Y	C	C	C	C	C	AATTGATTGACTCCCCCCCCC
AAT1a8	9	A	T	G	T	G	T	C	C	C	C	C	AATTGGTTGGTTCCCCCCCCC
AAT1a11	2	A	T	R	Y	R	Y	C	C	C	C	C	AATTGACTGACTCCCCCCCCC
AAT1a13	5	A	T	A	T	A	C	C	C	C	C	C	AATTAATTAACCCCCCCCCCC
AAT1a14	3	A	C	G	T	A	T	T	C	C	C	C	AACCGGTTAATTTTCCCCCCC
AAT1a21	1	A	T	A	Y	A	C	C	C	C	C	C	AATTAACTAACCCCCCCCCCC
AAT1a28	3	A	Y	R	T	A	Y	C	C	C	C	C	AACTGATTAACTCCCCCCCCC
AAT1a32	1	G	C	R	T	A	Y	C	C	C	C	C	GGCCGATTAACTCCCCCTCCCC
AAT1a33	3	A	C	G	T	A	T	C	C	C	C	C	AACCGGTTAATTCCCCCCCCC
AAT1a35	3	A	T	A	C	A	C	C	C	C	Y	C	AATTAACCAACCCCCCCCCTCC
AAT1a36	3	A	T	A	C	A	C	C	C	C	T	C	AATTAACCAACCCCCCCCTTCC
AAT1a40	1	A	T	A	Y	A	C	C	C	C	Y	C	AATTAACTAACCCCCCCCCTCC
AAT1a62	2	R	Y	R	T	A	Y	C	Y	Y	C	Y	GACTGATTAACTCCCTCTCCCT
AAT1a67	1	A	T	A	T	A	Y	C	Y	Y	C	C	AATTAATTAACTCCCTCTCCCC
AAT1a73	1	R	T	R	T	R	C	C	C	C	C	C	GATTGATTGACCCCCCCCCCC

* Nucleotide position were numbered according to their position in the sequenced fragment of the correspondents alleles

The nucleotide base pair represented in a single code in case of homozygous sites while IUPAC nucleotide code where used for the heterozygous positions: R, G or A; Y, C or T; W, T or A; M, C or A; K,G or T and S, C or G. [$] In the concatenated sequences, each single base was replaced with its correspondent base pair.

b) *ACC1*

Alleles	No. of isolates	Nucleotide positions						Concatenated sequence
		8	29	211	281	317	392	
ACC12	16	G	T	C	C	T	C	GGTTCCCCTTCC
ACC13	13	G	T	A	C	C	C	GGTTAACCCCCC
ACC14	2	G	T	M	Y	Y	C	GGTTCACTCTCC
ACC15	13	G	T	M	C	Y	C	GGTTCACCCTCC
ACC16	1	G	T	A	Y	C	T	GGTTAACTCCTT
ACC17	6	G	T	A	T	C	C	GGTTAATTCCCC
ACC18	1	G	T	A	C	T	C	GGTTAACCTTCC
ACC110	2	R	T	A	Y	C	Y	GATTAACTCCCT
ACC112	1	G	W	A	C	C	C	GGTAAACCCCCC
ACC114	7	G	T	A	Y	C	C	GGTTAACTCCCC
ACC119	1	G	T	A	C	C	T	GGTTAACCCCTT
ACC123	2	G	T	M	C	C	C	GGTTCACCCCCC
ACC124	1	R	T	M	Y	Y	C	GATTCACTCTCC
ACC126	4	G	T	A	C	Y	C	GGTTAACCCTCC
ACC127	1	R	T	A	Y	C	C	GATTAACTCCCC
ACC131	1	R	T	A	C	C	C	GATTAACCCCCC
ACC132	1	R	T	A	C	C	Y	GATTAACCCCCT
ACC135	1	R	T	A	C	Y	Y	GATTAACCCTCT
ACC160	2	G	T	M	Y	C	C	GGTTCACTCCCC

c) *ADP1*

Alleles	No. of isolates	Nucleotide positions																		Concatenated sequence
		17	35	40	46	82	109	125	159	166	200	205	215	225	232	282	347	352	395	
ADP12	7	T	G	C	C	C	A	A	G	G	G	G	G	T	C	G	G	A	A	TTGGCCCCCCAAAAGGGGGGGGGGTTCCGGGGAAAA
ADP13	2	T	A	T	T	C	G	R	G	A	G	A	G	A	T	G	G	A	A	TTAATTTTCCGGGAGGAAGGAAGGAATTGGGGAAAA
ADP14	7	T	A	C	C	C	A	R	G	G	G	G	G	T	C	G	G	A	A	TTAACCCCCCAAGAGGGGGGGGGGTTCCGGGGAAAA
ADP15	16	T	R	C	C	C	A	A	G	G	G	G	G	T	C	G	G	A	A	TTGACCCCCCAAAAGGGGGGGGGGTTCCGGGGAAAA
ADP16	14	T	A	C	C	C	A	A	G	G	G	G	G	T	C	G	G	A	A	TTAACCCCCCAAAAGGGGGGGGGGTTCCGGGGAAAA
ADP18	3	T	A	C	C	C	A	A	G	G	G	G	R	T	C	G	G	A	A	TTAACCCCCCAAAAGGGGGGGGGATTCCGGGGAAAA
ADP110	3	T	A	T	T	C	G	G	G	A	G	A	G	A	T	G	G	A	A	TTAATTTTCCGGGGGGAAGGAAGGAATTGGGGAAAA
ADP114	4	T	A	C	C	C	A	G	G	G	G	G	G	T	C	G	G	A	A	TTAACCCCCCAAGGGGGGGGGGGGTTCCGGGGAAAA
ADP115	2	T	A	Y	Y	C	R	G	G	R	G	R	G	W	Y	G	G	A	A	TTAACTCTCCGAGGGGGAGGGAGGTACTGGGGAAAA
ADP116	1	T	A	Y	Y	C	R	A	G	R	G	R	G	W	Y	G	G	A	A	TTAACTCTCCGAAAGGGAGGGAGGTACTGGGGAAAA
ADP120	1	T	A	C	C	C	A	A	G	G	G	G	G	T	C	G	G	R	A	TTAACCCCCCAAAAGGGGGGGGGGTTCCGGGGGAAA
ADP121	2	T	A	Y	Y	C	R	R	G	R	G	R	G	W	Y	G	G	A	A	TTAACTCTCCGAGAGGGAGGGAGGTACTGGGGAAAA
ADP130	1	T	A	C	C	C	A	A	G	G	G	G	A	T	C	G	G	A	A	TTAACCCCCCAAAAGGGGGGGGAATTCCGGGGAAAA
ADP137	1	Y	A	Y	Y	C	R	R	G	R	G	R	G	W	Y	G	G	A	A	CTAACTCTCCGAGAGGGAGGGAGGTACTGGGGAAAA
ADP138	3	Y	A	C	C	C	A	A	G	G	G	G	G	T	C	G	G	A	A	CTAACCCCCCAAAAGGGGGGGGGGTTCCGGGGAAAA
ADP141	1	T	A	C	C	Y	A	A	G	G	G	G	G	T	C	G	G	A	A	TTAACCCCCTAAAAGGGGGGGGGGTTCCGGGGAAAA
ADP143	1	T	A	T	T	C	G	A	G	A	G	A	G	A	T	G	G	A	A	TTAATTTTCCGGAAGGAAGGAAGGAATTGGGGAAAA
ADP161	1	T	A	C	C	C	A	A	G	G	R	G	G	T	C	G	G	A	A	TTAACCCCCCAAAAGGGGGAGGGGTTCCGGGGAAAA
ADP165	1	T	A	C	C	C	A	R	R	G	G	G	G	T	C	G	R	A	A	TTAACCCCCCAAGAGAGGGGGGGGTTCCGGGAAAAA
ADP183	1	T	A	T	T	C	G	R	G	A	G	A	G	A	T	S	G	A	W	TTAATTTTCCGGGAGGAAGGAAGGAATTCGGGAAAT

d) *PMIb*

Alleles	No. of isolates	Nucleotide positions											Concatenated sequence
		21	27	34	36	66	72	88	234	237	276	289	
PMIb1	2	R	G	A	C	G	T	G	C	A	G	A	AGGGAACCGGTTGGCCAAGGAA
PMIb2	17	G	R	R	Y	K	W	R	C	A	G	A	GGGAGACTGTTAGACCAAGGAA
PMIb3	5	G	A	G	T	T	A	R	C	A	G	A	GGAAGGTTTTAAGACCAAGGAA
PMIb4	29	G	G	A	C	G	T	G	C	A	G	A	GGGGAACCGGTTGGCCAAGGAA
PMIb6	4	G	G	A	C	G	T	G	M	R	R	R	GGGGAACCGGTTGGCAGAGAGA
PMIb9	4	G	A	G	T	T	A	A	C	A	G	A	GGAAGGTTTTAAAACCAAGGAA
PMIb18	1	G	R	R	Y	K	W	R	M	R	R	R	GGAGGACTGTTAGACAGAGAGA
PMIb21	1	G	R	R	Y	G	T	G	C	A	G	A	GGAGGACTGGTTGGCCAAGGAA
PMIb24	1	G	G	A	C	G	T	G	M	R	R	G	GGGGAACCGGTTGGCAGAGAGG
PMIb27	1	G	A	R	Y	K	W	R	C	A	G	A	GGAAGACTGTTAGACCAAGGAA
PMIb31	4	G	R	R	C	K	W	R	C	A	G	A	GGAGGACCGTTAGACCAAGGAA
PMIb34	1	G	R	R	Y	K	W	G	M	R	R	R	GGAGGACTGTTAGGCAGAGAGA
PMIb102	2	G	R	R	Y	T	W	R	C	A	G	A	GGAGGACTTTTAGACCAAGGAA

e) *ALA1*

Alleles	No. of isolates	Nucleotide positions											Concatenated sequence
		1	25	61	71	100	142	160	185	307	351	352	
ALA12	25	T	A	G	G	C	AA	T	G	C	C	C	TTAAGGGGCCAATTGGCCCCCC
ALA13	1	Y	C	R	G	Y	R	Y	G	C	C	C	CTCCGAGGCTGACTGGCCCCCC
ALA14	6	T	M	A	G	Y	R	Y	K	C	T	C	TTACAAGGCTGACTGTCCTTCC
ALA16	2	Y	C	R	G	T	G	CC	G	C	T	C	CTCCGAGGTTGGCCGGCCTTCC
ALA17	4	T	M	R	G	Y	R	Y	G	C	Y	C	TTACGAGGCTGACTGGCCCTCC
ALA126	1	C	C	G	G	T	G	C	G	C	C	C	CCCCGGGGTTGGCCGGCCCCCC
ALA131	1	T	M	A	G	T	G	C	G	C	Y	C	TTACAAGGTTGGCCGGCCCTCC
ALA134	6	T	C	A	G	T	G	C	G	C	T	C	TTCCAAGGTTGGCCGGCCTTCC
ALA143	1	Y	M	R	G	T	G	C	G	M	Y	C	CTACGAGGTTGGCCGGCACTCC
ALA146	1	T	M	G	G	C	A	T	G	C	C	C	TTACGGGGCCAATTGGCCCCCC
ALA149	3	T	A	A	G	C	A	T	T	C	T	C	TTAAAAGGCCAATTTTCCTTCC
ALA150	1	T	M	R	G	Y	R	Y	G	C	C	C	TTACGAGGCTGACTGGCCCCCC
ALA156	4	T	C	R	G	Y	R	Y	G	C	Y	C	TTCCGAGGCTGACTGGCCCTCC
ALA157	1	Y	M	R	G	C	A	Y	K	C	C	C	CTACGAGGCCAACTGTCCCCCC
ALA158	1	T	C	A	G	T	G	C	G	C	Y	C	TTCCAAGGTTGGCCGGCCCTCC
ALA161	2	Y	C	R	G	T	G	C	G	C	Y	C	CTCCGAGGTTGGCCGGCCCTCC
ALA162	1	T	A	A	G	Y	R	Y	G	M	C	C	TTAAAAGGCTGACTGGCACCCC
ALA167	1	T	C	A	G	C	A	T	G	C	C	C	TTCCAAGGCCAATTGGCCCCCC
ALA172	1	T	M	A	R	C	G	C	G	M	C	C	TTACAAGACCGGCCGGCACCCC
ALA178	1	T	M	A	G	T	G	C	G	M	Y	Y	TTACAAGGTTGGCCGGCACTCT
ALA184	1	T	C	R	G	Y	R	Y	G	C	C	C	TTCCGAGGCTGACTGGCCCCCC
ALA197	1	C	C	A	G	C	A	C	T	C	C	C	CCCCAAGGCCAACCTTCCCCCC
ALA199	1	Y	M	A	G	T	G	C	G	M	C	C	CTACAAGGTTGGCCGGCACCCC
ALA1106	2	T	C	A	G	T	G	C	G	C	T	Y	TTCCAAGGTTGGCCGGCCTTCT
ALA1139	2	T	C	A	G	Y	A	Y	K	C	T	C	TTCCAAGGCTAACTGTCCTTCC
ALA1146	1	T	C	A	G	Y	A	C	G	C	C	C	TTCCAAGGCTAACCGGCCCCCC

f) *VPS13*

Alleles	No. of isolates	Nucleotide positions																Concatenated sequence
		33	49	134	170	212	217	241	281	282	320	322	326	328	334	370	375	
VPS133	5	G	C	A	C	G	T	A	A	G	G	T	G	G	G	T	C	GGCCAACCGGTTAAAAGGGGTTGGGGGGTTCC
VPS134	4	G	C	A	C	G	T	G	G	G	G	T	G	G	G	T	C	GGCCAACCGGTTGGGGGGGGTTGGGGGGTTCC
VPS136	8	G	C	A	C	G	Y	A	R	G	G	K	G	G	G	W	Y	GGCCAACCGGCTAAGAGGGGGTGGGGGGTACT
VPS138	2	G	C	A	C	G	Y	R	G	G	G	T	G	G	G	A	C	GGCCAACCGGCTGAGGGGGGTTGGGGGGAACC
VPS1310	1	G	C	W	C	G	T	A	A	G	G	T	G	G	G	T	C	GGCCTACCGGTTAAAAGGGGTTGGGGGGTTCC
VPS1313	1	G	C	T	C	G	T	A	A	G	G	T	G	G	G	T	C	GGCCTTCCGGTTAAAAGGGGTTGGGGGGTTCC
VPS1315	3	G	M	A	C	G	T	A	G	G	A	T	G	G	G	A	C	GGCAAACCGGTTAAGGGGAATTGGGGGGAACC
VPS1316	2	G	M	A	C	G	T	A	R	G	R	T	G	G	G	A	C	GGCAAACCGGTTAAGAGGGATTGGGGGGAACC
VPS1319	2	R	C	A	C	R	T	R	G	G	G	T	G	R	G	A	C	GACCAACCGATTGAGGGGGGTTGGGAGGAACC
VPS1320	2	G	C	A	C	G	C	A	G	G	G	T	G	G	G	A	C	GGCCAACCGGCCAAGGGGGGTTGGGGGGAACC
VPS1321	1	G	C	A	C	G	Y	A	R	G	G	K	G	G	G	T	Y	GGCCAACCGGCTAAGAGGGGGTGGGGGGTTCT
VPS1324	5	G	C	A	C	G	T	A	A	G	G	G	G	G	G	T	T	GGCCAACCGGTTAAAAGGGGGGGGGGGGTTTT
VPS1326	4	G	C	A	C	G	T	R	R	G	G	K	G	G	G	T	Y	GGCCAACCGGTTGAGAGGGGGTGGGGGGTTCT
VPS1330	1	G	C	A	C	G	Y	R	G	G	G	T	G	G	G	W	C	GGCCAACCGGCTGAGGGGGGTTGGGGGGTACC
VPS1332	4	G	A	A	C	G	T	A	G	G	A	T	G	G	G	A	C	GGAAAACCGGTTAAGGGGAATTGGGGGGAACC
VPS1337	1	G	M	A	C	G	T	R	G	R	R	T	G	G	G	W	C	GGCAAACCGGTTGAGGGAGATTGGGGGGTACC
VPS1341	4	G	C	A	C	G	T	R	R	G	G	K	G	G	G	T	C	GGCCAACCGGTTGAGAGGGGGTGGGGGGTTCC
VPS1344	1	G	C	W	Y	G	T	A	A	G	G	T	G	G	G	T	C	GGCCTACTGGTTAAAAGGGGTTGGGGGGTTCC
VPS1347	1	G	C	A	C	G	T	A	A	G	G	T	A	G	G	T	C	GGCCAACCGGTTAAAAGGGGTTAAGGGGTTCC
VPS1360	3	G	C	W	C	G	T	A	A	G	G	K	G	G	G	T	Y	GGCCTACCGGTTAAAAGGGGGTGGGGGGTTCT
VPS1363	2	G	C	A	C	G	T	A	A	G	G	G	G	G	G	W	T	GGCCAACCGGTTAAAAGGGGGGGGGGGGTATT
VPS1369	1	G	A	A	C	G	T	A	R	G	R	K	G	G	G	W	C	GGAAAACCGGTTAAGAGGGAGTGGGGGGTACC
VPS1376	2	G	C	A	C	G	T	R	R	G	G	T	R	G	G	T	C	GGCCAACCGGTTGAGAGGGGTTGAGGGGTTCC
VPS1378	1	G	C	A	C	G	Y	A	R	G	G	K	G	G	G	T	C	GGCCAACCGGCTAAGAGGGGGTGGGGGGTTCC
VPS1386	2	G	C	A	C	G	T	A	R	G	G	T	R	G	G	A	C	GGCCAACCGGTTAAGAGGGGTTGAGGGGAACC
VPS1394	1	G	C	A	C	G	Y	A	R	G	G	G	G	G	G	T	C	GGCCAACCGGCTAAGAGGGGGGGGGGGGTTCC
VPS13105	1	G	C	A	C	G	Y	R	G	R	G	T	G	G	G	W	C	GGCCAACCGGCTGAGGGAGGTTGGGGGGTACC
VPS13106	1	G	C	A	C	G	Y	A	R	G	G	G	G	G	G	T	Y	GGCCAACCGGCTAAGAGGGGGGGGGGGGTTCT
VPS13122	2	G	C	A	C	G	Y	A	R	G	G	T	R	R	G	W	C	GGCCAACCGGCTAAGAGGGGTTGAGAGGTACC
VPS13126	1	G	C	A	C	A	T	G	G	G	G	T	G	G	A	W	C	GGCCAACCAATTGGGGGGGGTTGGGGAAAACC

Continued overleaf

Cont.

Alleles	No. of isolates	Nucleotide positions																Concatenated sequence
		33	49	134	170	212	217	241	281	282	320	322	326	328	334	370	375	
VPS13127	1	G	C	A	C	G	T	A	A	G	G	G	G	G	G	W	Y	GGCCAACCGGTTAAAAGGGGGGGGGGGGTACT
VPS13140	1	G	C	A	C	G	T	R	G	R	R	T	G	G	G	W	C	GGCCAACCGGTTGAGGGAGATTGGGGGGTACC

g) *ZWF1b*

Alleles	No. of isolates	Nucleotide positions													Concatenated sequence
		23	31	43	49	55	62	97	175	262	274	337	379	482	
ZWF1b2	1	A	C	W	T	T	G	T	C	C	A	Y	Y	W	AACCTATTTTGGTTCCCCAACTCTTA
ZWF1b4	12	M	C	W	Y	Y	G	T	C	C	A	C	Y	W	ACCCTACTCTGGTTCCCCAACCCTTA
ZWF1b5	18	A	Y	W	T	T	G	T	C	C	A	Y	Y	W	AACTTATTTTGGTTCCCCAACTCTTA
ZWF1b6	1	A	Y	W	T	Y	G	T	C	C	R	T	T	A	AACTTATTCTGGTTCCCCGATTTTTA
ZWF1b8	4	M	C	W	C	C	G	T	C	C	A	C	T	A	ACCCTACCCCGGTTCCCCAACCTTTA
ZWF1b12	2	A	T	T	T	T	G	T	C	C	A	T	T	A	AATTTTTTTTGGTTCCCCAATTTTTA
ZWF1b14	1	A	C	A	Y	Y	G	T	C	C	R	C	Y	W	AACCAACTCTGGTTCCCCGACCCTTA
ZWF1b15	9	M	Y	T	Y	Y	G	T	C	C	A	Y	T	A	ACCTTTCTCTGGTTCCCCAACTTTAA
ZWF1b20	5	A	C	A	T	T	G	T	C	C	A	C	C	T	AACCAATTTTGGTTCCCCAACCCCTT
ZWF1b21	1	A	Y	W	T	T	G	T	C	C	A	Y	C	W	AACTTATTTTGGTTCCCCAACTCCTA
ZWF1b22	2	C	C	T	C	C	G	T	C	C	A	C	T	A	CCCCTTCCCCGGTTCCCCAACCTTAA
ZWF1b25	1	A	Y	W	T	T	G	T	C	C	A	Y	Y	T	AACTTATTTTGGTTCCCCAACTCTTT
ZWF1b27	1	A	T	T	T	T	G	T	C	C	A	C	C	T	AATTTTTTTTGGTTCCCCAACCCCTT
ZWF1b47	1	C	C	T	C	C	G	T	C	C	A	Y	T	A	CCCCTTCCCCGGTTCCCCAACTTTAA
ZWF1b55	1	A	C	A	T	T	G	T	T	C	G	T	T	A	AACCAATTTTGGTTTTCCGGTTTTAA
ZWF1b67	1	A	C	W	C	C	G	T	C	C	A	C	T	A	AACCTACCCCGGTTCCCCAACCTTAA
ZWF1b84	1	A	Y	W	T	T	G	T	Y	C	R	T	T	A	AACTTATTTTGGTTCTCCGATTTTAA
ZWF1b89	1	M	C	T	C	C	G	T	C	C	A	C	T	A	ACCCTTCCCCGGTTCCCCAACCTTAA
ZWF1b94	1	A	Y	W	Y	Y	G	T	Y	C	R	Y	T	A	AACTTACTCTGGTTCTCCGACTTTAA
ZWF1b95	2	A	Y	W	Y	Y	G	T	Y	M	R	Y	T	A	AACTTACTCTGGTTCTCAGACTTTAA
ZWF1b118	1	C	C	W	C	C	G	T	C	C	A	C	T	A	CCCCTACCCCGGTTCCCCAACCTTAA
ZWF1b119	1	M	C	W	C	C	R	T	C	C	A	C	T	A	ACCCTACCCCGATTCCCCAACCTTAA
ZWF1b200	1	M	C	T	C	C	R	T	C	C	A	C	T	A	ACCCTTCCCCGATTCCCCAACCTTAA
ZWF1b201	2	M	Y	T	Y	Y	G	Y	C	C	A	Y	T	A	ACCTTTCTCTGGCTCCCCAACTTTAA
ZWF1b202	1	A	Y	T	Y	Y	G	T	C	C	A	Y	T	A	AACTTTCTCTGGTTCCCCAACTTTAA

McCullough *et al.*, 1999, 6% of isolates in the study by Tavaniti *et al.*, 2003, 12.9% of isolates in the study by Odds *et al.*, 2007 and 4% of isolates in the study by Wrobel *et al.*, 2008), particularly, in *C. albicans* isolates recovered from CHC lesions which was highly significant ($P<0.02$). This association has not been previously reported. Furthermore, genotype C is considered the rarest genotype among the three ABC genotypes (McCullough *et al.*, 1999; Chen *et al.*, 2006; Odds *et al.*, 2007; Da Matta *et al.*, 2010). This finding suggests an enrichment of genotype C *C. albicans* isolates recovered from leukoplakia lesions, especially from CHC lesions. However, MLST data analysis of genotype C isolates showed that they were heterogenous group as they were distributed in MLST clade 1,3,4,8 and 15. The significance of these findings is currently unknown but warrants extensive further investigation with a larger population of isolates from more diverse patient groups in order to determine if this association between the high rate of genotype C in *C. albicans* recovered from CHC reflects a real enrichment of a specific subgroup of *C. albicans*.

The MLST data suggested that *C. albicans* isolates associated with oral leukoplakia lesions are not significant genetically different from those recovered from healthy individuals but are an even more heterogeneous group. The distribution of the oral leukoplakia isolates investigated in the present study overlapped with isolates recovered from healthy individuals on the basis of MLST analysis. Although oral leukoplakia isolates were more prevalent in MLST in clade 1 (14/35) than those recovered from carriers (9/35), this was not statistically significant ($p>0.5$). These findings are in agreement with a study by Bartie *et al.* (2001) who failed to find clonal restrictions among *C. albicans* strains recovered from chronic hyperplastic candidiasis, based on three Interrepeat (IR) PCR methods employing IR-PCR with two prokaryotic primers and one eukaryotic primer (primers 1245, 1246 and 1251, respectively) and M13 PCR fingerprinting. Furthermore this overlapping was also observed in the sequence data, the identification of DST 1659 in two isolates from two different sources; first one was recovered from CHC (CL129) and the other isolate was recovered from a healthy individual (HV208). Similarly, the MLST database contains a few examples of shared DSTs from different sources. Despite that, these isolates were indistinguishable by MLST, the genetic diversity may be present in other genes. (Wrobel *et al.*, 2008).

Based on MLST data, *C. albicans* isolates associated with CHC are not genetically distinctive from those isolates recovered from other sites. However, enrichment of genotype C in CHC isolates suggests the opposite. Further investigation with larger numbers of isolates recovered from different sources (both lesions and non-lesions) is required to assess the significance of this finding.

Chapter 6

General Discussion

6.1. General Discussion

In the clinical fields of oral medicine and oral surgery there is no oral condition surrounded by more controversy and uncertainty than oral leukoplakia. The condition is common, affecting perhaps 1% of the adult population, the natural history uncertain and unpredictable, but in a proportion of patients with this condition, the outcome is potentially life-threatening. Surgery and radiotherapy are the treatment of choice for oral cancer, which are both complex and expensive, and a significant burden on health services, since, in the Western world, highly technical microvascular reconstructive techniques are now the standard of care for advanced disease. The condition is associated with significant morbidity as well as mortality, since it involves an area of the body which is multifunctional, being involved not only with mastication and eating, but also with communication, (expression, appearance, speech) and taste. Significant psychological morbidity is seen in oral cancer patients following surgery, since the effects of extensive surgery often cannot easily be disguised, unlike other areas of the body. There have been major advances in functional reconstructive surgery for these patients in the last two decades, but the mortality rate has changed little. For about half the patients diagnosed with oral cancer, the diagnosis will be a death sentence within the next 5 years, despite optimal surgical intervention.

The key to improved prognosis is early diagnosis. Over 70% of patients with early disease will survive 5 years after surgery, but 70% of patients with advanced disease will die. Reports of malignant transformation of oral leukoplakic patches vary, but it is generally accepted that about 4% will become cancerous within a 10 year timeframe. The problem for clinicians is to identify those lesions which have high potential to transform, since this would justify more aggressive attempts at treatment, or ideally eradication, of the lesion. Unfortunately, oral leukoplakias are often multifocal, as the causative agents may affect the entire oral mucosal surface, or be very extensive, which adds to the clinical dilemma in treatment options, since aggressive treatment of a benign condition, would be associated with significant morbidity. Most clinicians adopt an observational approach to the management of extensive lesions. It would be helpful to identify markers for high risk lesions which would justify extensive treatment.

Syphilitic leukoplakia is recognised to have, perhaps, the highest malignant potential of all leukoplakias, but occurs in the tertiary stage of the disease and is very rarely seen in contemporary medical practice. Sublingual keratosis is considered by

many to be a high risk lesion, (Kramer *et al.*, 1978; Napier and Speight, 2008) Chronic hyperplastic candidosis is generally suggested to have a higher risk of malignant transformation than idiopathic leukoplakic lesions. The association between *Candida* and NCL and CHC is unclear. In CHC, two opposing schools of thought propose that *Candida* is either simply associated with a pre-existing keratotic lesion, or that it is causative of both the dysplasia and the keratosis seen in these lesions (Jepsen and Winther, 1965; Cawson and Lehner, 1968).

6.1.1. Clinical Studies and OL *Candida* Isolates

The findings of this study confirm that CHC is predominantly a disease of the over 50s (Arendorf *et al.*, 1983), which preferentially affects the buccal mucosa and commissure of the mouth along with the tongue, although other sites, such as the palate, can be affected. Wherever possible, attempts were made to control for this site predilection when recruiting the NCL cohort into the study. It may be that the local microenvironment can affect the mechanisms of carcinogenesis at these locations within the mouth. The sex prevalence finding is of interest because a ratio of 3:2, male:female was seen in the 31 CHC patients. This is in contrast to global data on leukoplakia sex prevalence where the lesion is seen predominantly in males, sometimes in a ratio of between 4:1 and 80:1 (Gupta *et al.*, 1980). Although this is a small CHC group, this may be of clinical significance, as there are no published data on the sex prevalence of biopsy-proven CHC. Support for the contention that CHC is a high risk lesion comes from the fact that two patients in the CHC developed carcinoma within their CHC lesions during a 2 year follow-up period, whereas none of the NCL patients developed malignancy. This suggests that pathogenic (as opposed to passenger) hyphal forms of *Candida,* invading the keratotic tissues, may be responsible for malignant transformation of these lesions, although this does not solve the question of dysplasia and keratosis induction by the microorganism. Animal studies have shown that *Candida* can induce hyperkeratosis, but the evidence for dysplasia induction is unclear (Franklin and Martin, 1986).

It could be argued in smokers with NCL, that the hyperkeratosis is a mucosal response to the chemical insult of the tobacco smoke and that it represents an attempted protective response, by increasing the mechanical barrier to carcinogens. If 4% of leukoplakias transform to malignancy, this infers that 96% do not, and malignant transformation could then represent failure of this protective response.

Wearing of an upper denture was found to predispose to increased candidal load, as would be expected as *Candida* adhere readily to dentures (Samaranayake *et al.*, 1980), *Candida* is an opportunistic pathogen and it is well known that the introduction of an upper denture into the mouths of healthy individuals promotes *Candida* growth, particularly in patients with poor denture hygiene and when denture stomatitis is present. However, there was also a significant association of upper denture wearing with CHC, compared to NCL. Perhaps in these patients, the increased candidal load over time encouraged the development of CHC.

Although smoking in this study was not significantly associated with *Candida* carriage (although statistically it did approach significance), there was an association between cigarette consumption and candidal load in those patients who did smoke. Tobacco as a predisposing factor for oral candidosis has been the subject of much research and debate, has been extensively reviewed by (Soysa and Ellepola, 2005) and the question is still unresolved. The data from this investigation add weight to both sides of this argument, and suggest that smoking does not predispose to candidal carriage in the mouth, but alterations in the oral environment induced by smoking, promote candidal growth, although the mechanisms by which this occurs are outside the scope of this thesis.

In the clinical environment, when there is a high index of suspicion that a lesion is CHC and therefore high risk, the discriminatory power of a lesional swab for *Candida* detection has been shown to be superior to an oral rinse (used by some clinicians to estimate the oral *Candida* burden), to distinguish CHC from NCL, even when there is *Candida* present on the surface of the NCL lesion. In this study surface colonisation by *Candida* was seen in 40% the NCL group and so simply detecting *Candida* on the lesional swab was not necessarily associated with a CHC diagnosis. In NCL, the surface *Candida* is planktonic, and it might be expected that this would be more easily sampled, in that pathogenic *Candida*, which might be more difficult to pick up on the swab, since they are more firmly attached to the mucosa. Furthermore, not every CHC lesional swab grew *Candida* in the study, even though histopathologically all the lesions were proven to be infected with hyphae. Nevertheless, lesional swabbing proved to be a sensitive test for the diagnosis of CHC, and probably this observed effect is due to the relatively low density of *Candida* on the surface of NCL lesions, because significance was demonstrated (as a measure of sensitivity of the test) at cfu densities below 100 cfu/swab and was still evident at 30 cfu/swab.

The most important finding of this study, however, was the significant association of candidal growth of significant density with higher (moderate and severe) histopathological dysplasia grade. All swabs taken from lesions exhibiting severe dysplasia in CHC, had cfu of over 150, which was not seen in NCL. Even though degree of dysplasia was matched in the CHC and NCL groups, (and, in fact, there were more severe dysplasia specimens in the NCL group), this association held firm. This evidence adds weight to the evidence to date that *Candida* is not only causative in CHC, but that dysplasia can be induced by invasive hyphal *Candida* in humans, but not when the *Candida* is associated with a NCL in planktonic form (i.e. loosely associated with the lesional surface only). This evidence is supported by analysis of the non-smoking subgroup, one of whom went on to develop cancer. Despite the absence of tobacco as a cause for their dyplasia, and only moderate or abstainant alcohol intake, these patients (in many of whom *Candida* was the only risk factor), had degrees of dysplasia at the very upper spectrum (severe dysplasia) of the diagnosis.

Although several non-*C. albicans* species were isolated from CHC (and NCL) lesions, they were always in low abundance, so that no one non-*C. albicans* species could be implicated as a co-factor.

Candida albicans was the predominant species among the lesional isolates recovered from CHC and was present in 100% of cases. This firmly implicates the *C. albicans* species in the pathogenesis of CHC. This prompted the question: could there be something different about the *C. albicans* isolates from CHC compared to NCL isolates and isolates from normal healthy individuals. This was alluded to in the studies by Krogh *et al.* in the 1980s who found unusual *C. albicans* biotypes associated with CHC and also increased nitrosamination potential in these unusual biotypes. At that time, strain identification by biotyping was a newly developed technique, but is somewhat subjective and has now been superceded by biotyping using commercially available systems like the API yeast identification system. Even with this system, discriminatory power is limited and only a relatively small number of biotypes can be discerned. The next part of the study addressed the question of whether CHC and NCL *C. albicans* isolates had different keratinocyte adhesion potential compared to isolates recovered from healthy controls, and whether, *C. albicans* isolates recovered from CHC and NCL differed genetically.

Adherence of a microorganism to host cells is a critical step in establishment of successful colonisation and subsequent infection (Calderone and Fonzi, 2001; Yang,

2003). Firstly, adherence of *C. albicans* recovered from oral leukoplakic lesions to BECs compared to well characterised reference *C. albicans* strains was investigated. Secondly, the effect of oral epithelial dysplastic changes on the adherence of *C. albicans* isolates was also investigated by comparing the adherence of *C. albicans* from CHC and NCL to both cultured normal keratinocytes and oral keratinocytes cell cultures derived from biopsies demonstrating dysplasia on histopathology, compared to reference strains.

6.1.2. Adherence of *C. albicans* Recovered from Oral Leukoplakia Lesions to Epithelial Cells

In order to assess the hypothesis that *C. albicans* species recovered from oral leukoplakic lesions were more virulent than other strains of *C. albicans*, the relative adherence capability of these isolates to human BECs and cultured normal and dysplastic oral epithelial cells *in vitro* was investigated. It was demonstrated that the binding capacity to BECs of half of the *C. albicans* recovered from CHC was significantly higher than that of oral *C. albicans* reference strain 132A but not to the *C. albicans* SC5314 strain recovered from disseminated human *Candida* infection. However, the adherence of the investigated isolates was variable, with a range from strongly adherent to weakly adherent binding capacity. Due to the multiplicity of the factors affecting the adherence process, one might speculate that host factors might contribute to this variation. Although, the use of BECs from pooled sources has been advocated in many literatures, use of a single source of BECs may be more suitable in such a comparison due to the homogenous nature of these cells. The difference in human BEC surface receptors, bacterial flora, life style, such as diet and more importantly the host immunity are other factors that might have an effect on the adherence assay. Use of human BECs, if feasible, from oral leukoplakic lesions to investigate the adherence of *C. albicans* recovered from similar lesions might be more appropriate to test the effect of the epithelial cellular change on binding to the yeast. This could show a different adherence capacity when human BECs exfoliated from leukoplakia lesions are used, adhesion of *C. albicans* recovered from CHC lesions may be greater than isolates recovered from non leukoplakia lesions. Human BECs from oral leukoplakic lesions may have different cell surface proteins which exhibit a different binding affinity to *C. albicans.*

In order to test the effect of epithelial dysplasia on the adhesion of *C. albicans* and to eliminate the effect of the heterogeneous nature of the BECs, an assay with more homogenous human epithelial cells was used. Human normal oral keratinocytes (NOK) and keratinocytes cultured from biopsies of histopathologically dysplastic (DOK cell line) lesions were grown in monolayers, to investigate the difference in the adhesion of *C. albicans* to both cell types. The result of this part of the present study has shown that *C. albicans* leukoplakia isolates differed in their adherence to NOK and DOK cells. although there was no significant difference in the adherence of individual *C. albicans* leukoplakia isolates and reference strains to the NOK cell line compared with the DOK cell line in each case, there was a general trend that the majority of leukoplakia isolates tended to be more adherent to the DOK cell line than NOK cell line. This finding could suggest that epithelial dysplasia has no or limited effect on the adherence of the *C. albicans*. However, there may be other factors that can promote the adherence of *Candida* to oral leukoplakia lesions, such as surface roughness of the lesion (Arendorf *et al.*, 1983). Other host and yeast factors such as state of immunity, phenotypic switching of the fungi and differential expression of *C. albicans* adhesins and invasins *in vivo* cannot be excluded. In contrast to this, the *C. albicans* might induce the epithelial cell atypia through two possible mechanisms by means of the carcinogens nitrosamine (Krogh *et al.*, 1987) and acetaldehyde (Tillonen *et al.*, 1999; Neiminen *et al.*, 2009)

6.1.3. Genotyping of *C. albicans* isolates by MLST and ABC Typing

The recent advances in DNA sequencing technology have improved the quality of genetic characterisation of microorganisms that facilitates the identification of outbreak-related strains based on genetic similarities of various strains of the causative microorganism. MLST has been shown to be an effective tool for epidemiological screening and population analysis of *Candida* species by analysing the nucleotide sequence variations within 7 housekeeping genes (Bougnoux *et al.*, 2002). Although, MLST was originally developed for haploid bacterial species (Maiden *et al.*, 1998), MLST has also been developed for diploid organisms such as *Candida* species (Bougnoux *et al.*, 2002 and 2003; Tavanti *et al.*, 2003 and 2005b; Dodgson *et al.*, 2003; Lan *et al.*, 2006; Jacobsen *et al.*, 2007; McManus *et al.*, 2008 and 2011). The genetic relatedness of *C. albicans* recovered from oral leukoplakic lesions and healthy individuals was investigated in order to determine if a genetically distinct group was

associated with oral leukoplakic lesions. MLST analysis of isolates recovered from CHC, NCL and oral carriage isolates from healthy volunteers showed that over half of the tested isolates had unidentified MLST patterns. However, these new DSTs were evenly distributed among all investigated oral leukoplakic and carriage isolates. This may reflect the geographical origin of the strains (i.e. recovered from Irish individuals). In this regard, previous studies using both MLST and the specific-specific complex DNA fingerprinting probe Ca3 have previously demonstrated geographic enrichment of certain clades of *C. albicans* isolates from certain geographical locations (Bilgnaut *et al.*, 2002; Soll and Pujol, 2003; Odds *et al.*, 2007).

In the present study, MLST analysis showed that the majority of isolates from all groups (healthy and leukoplakic lesions group), were distributed among the three major *C. albicans* MLST clades 1, 2 and 4 (nearly two thirds of the isolates investigated belonged to clades 1, 2, 4) and this is similar to previously reported *C. albicans* isolate populations from Western Europe (Tavanti *et al.*, 2005a; Odds *et al.*, 2007). This observation of the tendency of *C. albicans* isolates to geographical co-clustering is common to DNA fingerprinting with Ca3 probe analysis (Blingnaut *et al.*, 2002; Soll and Pujol, 2003) and has been confirmed (although imperfectly) by MLST analysis (Chen *et al.*, 2006; Odds *et al.*, 2007). However, it is worth noting that isolates investigated in previous studies were obtained from different body sites, in different clinical settings (i.e. blood, oral, wounds, vagina etc.) whereas the isolates of the present study were recovered only from the oral cavity. This is reflected by the high proportion of clade 1 isolates in the present study, which according to Odds and Jacobson (2008) are highly associated with *C. albicans* associated with mucosal surfaces either as pathogenic or commensal organisms. Other properties associated with MLST clades include resistance to antifungal drugs. A high proportion of *C. albicans* resistant to 5-fluorocytosine are found in Ca3/MLST clade 1 (Dodgson *et al.*, 2004; Tavanti *et al.*, 2005a).

Analysis of isolate differences based on allelic differences with eBURST, showed that a high proportion of the investigated isolates (from all groups) belonged to clonal cluster 1, which suggests that these isolates evolved from a common ancestor (putative founder of the clonal cluster). However, the identification of a significant proportion of singletons contradicts this suggestion. Many isolates designated as singletons by the eBURST test, were originally co-clustered in MLST clades. It is important to note that despite the efficiency of the eBURST analysis in studying

microevolution of the haploid organism, its use in diploid *C. albicans* should be limited to clonal clustering rather than evolutionary analysis (Odds *et al.*, 2006).

In addition to MLST analysis, ABC typing was undertaken on *C. albicans* isolates to augment the MLST data for each isolate using a different typing approach. ABC typing is based on the presence or absence of an intron in the internal transcribed spacer 1 (ITS1) region of DNA sequences encoding 25S ribosomal RNA. In accordance with previous reports (McCullough *et al.*, 1999; Tavanti *et al.*, 2003; Chen *et al.*, 2006; Odds *et al.*, 2007), the present study identified a high proportion (69.4%) of genotype A isolates within the investigated isolates of all groups (Table 5.8). ABC typing also showed near total predominance of genotype A in clades 1 (23/25) and 2 (13/13), which is an observation reported previously in other studies (McCullough *et al.*, 1999). Interestingly, genotype C was significantly enriched among *C. albicans* isolates recovered from CHC lesions investigated in the present study (19.4% of the total isolates tested in the present study and 40% of CHC the isolates compared to 11% of isolates in the study by McCullough *et al.*, 1999, 6% of isolates in the study by Tavaniti *et al.*, 2003, 12.9% of isolates in the study by Odds *et al.*, 2007 and 4% of isolates in the study by Wrobel *et al.*, 2008). However, MLST analysis of genotype C isolates did not show any distinct clustering of this group of isolates, as they were distributed in different MLST clades (Table 5.4 to 5.6). The nature of this association is still unknown. Whether this significant enrichment of genotype C isolates from CHC lesions reflects a real subgroup of *C. albicans* strongly associated with CHC lesions remains unclear. Assessment of the adherence capability of isolates from the genotype C group to both BEC and cultured epithelial cell lines did not reveal any significant difference to other isolates from other genotype groups. However, it is tantalising to speculate that genotype C isolates may have enhanced potential to induce pathological changes in mucosal tissue giving rise to CHC lesions. These findings and suggestions warrant further investigation with larger groups of patients from different geographic locations and ethnic backgrounds to determine the significance of the findings. MLST and ABC genotyping examine different parts of the *C. albicans* genome. If the strong association of genotype C isolates with CHC lesions is substantiated in larger studies, it may be worthwhile comparing the whole genome sequence of a genotype C isolates with genotype A and B isolates. Gene expression studies *in vitro* and *in vivo* would provide another potentially fruitful avenue of research to identify characteristics unique to genotype C isolates.

6.2. Conclusions

Chronic hyperplastic candidosis is a disease of the middle aged, and mostly over 50s, that may be more common in females than non-candidal leukoplakia (2:3 in this series), which is caused by active hyphal infection with the yeast species *C. albicans* in the cohort of patients examined in this thesis. Other non-*C. albicans* species were also isolated, but in low abundance and without sufficient consistency to be likely to be a part of the pathogenesis of the lesion. It may be predisposed by upper denture wear, but not smoking, *per se,* although heavy smoking was associated with increased oral candidal loads in the patients examined. A lesional swab has better discriminatory capacity than an oral rinse in identifying the lesion clinically and a lesional swab has discriminatory capacity between CHC and NCL, despite frequent recovery of *Candida* from NCL. Significant ($\geq$100 cfu) lesional candidal density, as determined by the lesional swab correlates with moderate and severe dysplasia grading in the CHC lesions sampled, but not NCL, and this finding supports the concept that *C. albicans* is the causative agent, rather than being a simple commensal passenger of CHC.

In the BEC, cultured normal keratinocyte and dysplastic cultured keratinocytes studies, *C. albicans* strains isolated from leukoplakias showed significantly increased adherence compared to the reference strains. Nearly half (8/18) of the CHC- associated isolates showed increased adherence (and one of the NCL-associated isolates) suggesting that strains causing CHC may have a survival advantage in the oral cavity. Adherence to cultured normal oral keratinocytes and dysplastic oral keratinocytes showed little difference between test and reference strains although a wide range of results for the oral leukoplakia isolates was noted. Adherence is a multifactorial process influenced by a number of yeast and host factors.

Relatedness of the leukoplakia-associated strains was investigated by MLST, eBURST analysis and genotyping. Unsurprisingly, clonal cluster 1 was most commonly observed. However, there were also a very high (45.8%) number of singletons in the leukoplakia-associated strains, but this should be interpreted with caution as eBURST identifies only founders and patterns of evolutionary descent and may be more appropriate to haploid rather than diploid organisms. Over two thirds of the isolates belonged to MLST clade 1, 2, or 4, consistent with the general clade distribution in Western Europe. ABC genotyping showed that over two thirds of the isolates belonged to genotype A and the majority of genotype A isolates belonged to MLST clade 1 and 2.

However, significant genotype C (the rarest genotype) enrichment (40%) was seen in the CHC-associated isolates, although MLST analysis of these isolates showed them to be heterogeneous and distributed in clades 1, 3, 4, 8, and 15. The MLST data suggest that leukoplakia-associated isolates are not significantly different from those recovered from healthy individuals, and are a heterogeneous group and is in agreement with Bartie *et al,* (2001), who analysed CHC-associated strains by PCR. However, the genotype C enrichment data suggests the opposite. Since MLST analysis is based on 7 household genes, but genotype analysis is based on 25S rDNA, *C. albicans* strains derived from CHC may, indeed, be clonally restricted, but this would need a further investigation on isolates from different geographical locations to assess.

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Appendix

SJH/AMNCH Research Ethics Committee Secretariat
Dan Lynch Ph: 4142860 email: Dan.Lynch@amnch.ie
Ursula Ryan Ph: 4142342 email: Ursula.Ryan@amnch.ie
Secretariat Fax 4142371

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TALLAGHT, DUBLIN 24, IRELAND
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Professor Stephen Flint
Department of Oral & Maxiofacial Surgery
Oral Medicine & Oral Pathology
Dublin Dental School & Hospital
Lincoln Place
Dublin 2

December 5th 2008

Re: The Biological Basis of *Candida* Leukoplakia

Please quote this reference in any follow up to this letter: 2008/47/01

Dear Professor Flint

Thank you for your letter and enclosures which you sent in response to the Committee's provisional approval of the above referenced study.
The Vice-Chair, on behalf of the Research Ethics Committee, has reviewed your response and has given ethical approval to this study.
Full ethical approval for this study is now in place.

Yours sincerely

Daniel R. Lynch,
Secretary,
SJH/AMNCH Research Ethics Committee

SJH/AMNCH RESEARCH ETHICS COMMITTEE

Information on requesting the opinion of the ethics committee including forms for completion

(Version 3 dated 2nd November, 2006)

SJH/AMNCH RESEARCH ETHICS COMMITTEE

APPLICATION FOR RECOGNISED ETHICS COMMITTEE OPINION

"The Biological Basis of *Candida* Leukoplakia"

Names of researchers:

Dr. Mohammed Abdulrahim
Prof. Stephen Flint
Prof. David Coleman

Dublin Dental School and Hospital.

A prospective study of patients with leukoplakia attending the Oral Medicine and Dysplasia Out-Patient clinics at the Dublin Dental School and Hospital, and a retrospective study of archival tissue from consented patients with leukoplakia, attending the same venue.

SJH / AMNCH RESEARCH ETHICS COMMITTEE.

Patient Information Sheet

Names of researchers:

Dr. Mohammed Abdulrahim
Prof. Stephen Flint
Prof. David Coleman

Working title of study:

"The Biological Basis of *Candida* Leukoplakia"

Introduction

Leukoplakia is a white lesion of the mouth with the potential to turn cancerous. The diagnosis of leukoplakia is made by taking a biopsy (sample) of the tissue and looking at it under a microscope. We cannot predict with any certainty which leukoplakias will become cancers. However, we do know that some leukoplakias which have the yeast, *Candida*, associated with them (*Candida* leukoplakias) are at higher risk of turning malignant. In *Candida* leukoplakia also know that, unfortunately, simple anti-fungal treatments do not reliably affect or cure the lesion. What is also not obvious is whether the yeast infects a pre-existing leukoplakia or the yeast causes the leukoplakia.

Description of study

- Aims and objectives of study

- 1) There are different types of *Candida* and the first part of the study is to look at the type or subtypes of *Candida* which are in the *Candida* Leukoplakia.

- 2) We then propose to characterise the interaction between *Candida* and the leukoplakia cells (keratinocytes) by culturing both together and looking at adhesion of the yeast and gene expression by both the yeast and keratinocytes.

- 3) We also propose to investigate the correlation of the distribution of abnormal cells with fungal invasion of the leukoplakia tissues in the biopsy specimen, the degree of abnormal changes with the above and the body's reaction to the infection.

Contribution required from participant

The study involves taking an oral rinse, a swab (to culture for *Candida*) and a biopsy of the leukoplakia. We perform all these tests anyway, as routine procedures to establish a definitive diagnosis. We are asking your permission to use a small piece of the biopsy specimen for our extra investigations 1) and 2). For part 3 we would like to investigate left-over biopsy material after your diagnosis has been fully established. We also ask your consent to further investigate (only in this field of research) the sample you have donated, depending on the results of our studies. Any further study will be subject to the granting of new ethical approval of the SJH/AMNCH Research Ethics Committee.

Possible benefits of the study.
The possible benefits of the study will be better understanding of the nature of the disease, and improvement of the treatment approaches to this disease, with an aim of cancer prevention.

Possible risks to participants and after effects
There are no more risks than if you were **not** in the trial, since we routinely take a biopsy of the lesion for accurate diagnosis. You are at no more risk of the normal side effects or complications of any biopsy procedure, namely: post-operative bleeding, swelling, pain and infection. We aim to prevent these through postoperative instructions, ensuring an atraumatic technique, pain killers and post-operative antiseptic mouthrinses.

Location of research.
Dublin Dental School and Hospital

What will happen to the results of the study
This project is part of PhD degree and the results of the study will be published in a scientific journal, with due consideration of confidentiality.

Confidentiality of information
Your identity will remain entirely confidential. Your name will not be published and will not be disclosed to anyone outside the study group. Your data and sample will be coded and anonymised.
The data will be stored securely at Dublin Dental School & Hospital in the data information system and protected by the use of security software. The DDSH has its own server, security and secure access systems. The anonymised samples will be kept for five years and then destroyed

Voluntary participation
You are **NOT obliged** to take part.

Permission
Ethical approval from Faculty Research Ethics Group – Faculty of Health Sciences, TCD.

Further information and how to take part
The procedures will be explained to you and an informed consent form must be signed by you if you agree to take part in this study. The research will be conducted in Dublin Dental School & Hospital and your *Candida* result will be routinely accessible through the researchers on follow-up appointments on Oral Medicine and Dysplasia clinics.

Contact details of researchers
Prof. Stephen Flint
Dr. Mohammed Abdulrahim
Department of Oral and Maxillofacial Surgery, Oral Medicine and Oral Pathology
Dublin Dental School & Hospital
Lincoln Place, Dublin 2
Tel: 01 6127200. Email: Mohammed.abdulrahim@dental.tcd.ie.

This study is covered by standard institutional indemnity insurance.
Nothing in this document restricts or curtails your rights.

SJH / AMNCH RESEARCH ETHICS COMMITTEE.

Patient Information Sheet

(Biopsy already taken)

Names of researchers:

Dr. Mohammed Abdulrahim
Prof. Stephen Flint
Prof. David Coleman

Working title of study:

"The Biological Basis of *Candida* Leukoplakia"

Introduction

Leukoplakia is a white lesion of the mouth with the potential to turn cancerous. The diagnosis of leukoplakia was made from your biopsy (sample) of the tissue in your mouth and looking at it under a microscope. We cannot predict with any certainty which leukoplakias will become cancers. However, we do know that some leukoplakias which have the yeast, *Candida*, associated with them (*Candida* leukoplakias) are at higher risk of turning malignant. We also know that, unfortunately, simple anti-fungal treatments do not reliably affect or cure the lesion. What is also not obvious is whether the yeast infects a pre-existing leukoplakia or the yeast causes the leukoplakia.

Description of study

- Aims and objectives of study

We propose to investigate the correlation of the distribution of abnormal cells with fungal invasion of the leukoplakia tissues in the biopsy specimen, the degree of abnormal cellular changes with the above and the body's reaction to the infection.

Contribution required from participant

We ask that we might investigate the stored, left-over material from your biopsy test, now that your diagnosis has been fully established. We also ask your consent to further investigate the sample (only in this field of research), depending on the results of our studies. Any further study will also be subject to the granting of new ethical approval of the SJH/AMNCH Research Ethics Committee.

Possible benefits of the study.

The possible benefits of the study will be a better understanding of the nature of this disease, and subsequently, improvement of the treatment approaches to this disease, with an aim of cancer prevention.

Possible risks to participants and after effects

There are no risks to you, since your biopsy test has already been done.

Location of research.
Dublin Dental School and Hospital

What will happen to the results of the study
This project is part of a PhD degree and the results of the study will be published in a scientific journal, with due consideration of confidentiality.

Confidentiality of information
Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the study group. Your data and sample will be anonymised.
The data will be stored securely at Dublin Dental School & Hospital in the data information system and protected by the use of security software. The DDSH has its own server, security and secure access systems.

Voluntary participation
You are **NOT** obliged to take part.

Permission
Ethical approval from Faculty Research Ethics Group – Faculty of Health Sciences, TCD.

Further information and how to take part
An informed consent form must be signed by you if you agree to take part in this study.

Contact details of researchers
Prof. Stephen Flint
Dr. Mohammed Abdulrahim
Department of Oral and Maxillofacial Surgery, Oral Medicine and Oral Pathology
Dublin Dental School & Hospital
Lincoln Place, Dublin 2
Tel: 01 6127200. Email: Mohammed.abdulrahim@dental.tcd.ie.

Nothing in this document restricts or curtails your rights.

SJH / AMNCH RESEARCH ETHICS COMMITTEE.

CONSENT FORM

Project Title:

"The Biological Basis of *Candida* Leukoplakia"

Principal Investigators:

Dr. Mohammed Abdulrahim
Prof. Stephen Flint
Prof. David Coleman

BACKGROUND: This project will investigate the relationship between the yeast, *Candida,* and the oral mucosal tissues in the disease *Candida* leukoplakia, a premalignant lesion. The procedure involves a biopsy and swab from the lesion and a mouth rinse (all of which are routinely taken as a part of the diagnostic procedure). A small piece of the biopsy tissue will be cultured with *Candida* and examined for adhesion and gene expression to establish the interaction of the two types of cells. We will then look at the biopsy itself to try to correlate the presence of *Candida* with abnormal cellular changes and the body's reaction to the infection. The benefit of the study will be better understanding of the nature of the disease and possible improvement of the treatment approaches to this disease, with an aim of cancer prevention.

Your data will be anonymised, confidential and securely stored.

DECLARATION:

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. And I agree that the tissue might be used in other future studies in this field of research (subject to approval of the SJH/AMNCH Research Ethics Committee) without need for additional contact. I understand I may withdraw from the study at any time. I have received a copy of this agreement.

Patient's Name .....
Contact Details:.....
.....

Participant's
Signature:.....**Date:**.....

I ..... the Researcher :

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Investigator's Signature:..... Date:.....

SJH / AMNCH RESEARCH ETHICS COMMITTEE.

CONSENT FORM

STORED BIOPSY INVESTIGATION

Project Title:

"The Biological Basis of *Candida* Leukoplakia"

Principal Investigators:

Dr. Mohammed Abdulrahim
Prof. Stephen Flint
Prof. David Coleman

BACKGROUND: This project will investigate the relationship between the yeast, *Candida,* and the oral mucosal tissues in the disease *Candida* leukoplakia, a premalignant lesion. We would like to look at your left-over biopsy tissue, now that your diagnosis has been established, to try to correlate the presence of *Candida* with abnormal cellular changes and the body's reaction to the infection. The benefit of the study will be a better understanding of the nature of this disease and possible improvement of the treatment approaches to this disease, with an aim of cancer prevention.

Your data will be anonymised, confidential and securely stored.

DECLARATION:

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. And I agree that the tissue might be used in other future studies in this field of research (subject to approval of the SJH/AMNCH Research Ethics Committee) without need for additional contact. I understand I may withdraw from the study at any time. I have received a copy of this agreement.

Patient's Name .....

Contact Details:.....
.....

Participant's
Signature:.....**Date:**.....

I ..... the Researcher :

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Investigator's Signature:..... Date:.....

SJH / AMNCH RESEARCH ETHICS COMMITTEE.

ADMINISTRATIVE APPLICATION

1. **Title of research project**:

   "The Biological Basis of *Candida* Leukoplakia"

2. **Name of local project supervisor(s)** – Professors Stephen Flint and David Coleman.

3. **Name and address of the person to whom the Committee's decision is to be communicated:**

Professor Stephen Flint,
Clinical Professor and Consultant in Oral Medicine
Head of Division
Division of Oral and Maxillofacial Surgery, Oral Medicine and Oral Pathology
Dublin Dental School and Hospital,
Lincoln place
Dublin 2

4. **Please give the name and telephone number of the person to be contacted if a query arises in connection with this study.**

   Professor Stephen Flint,
   Tel: 016127200, 016127314

5. **For each funded clinical trial a review fee of €1000.00 plus €150.00 per site is payable. Payment to the "Research Ethics Committee" should accompany the study documentation submitted to the SJH / AMNCH RESEARCH ETHICS COMMITTEE. Non-sponsored clinical trials are subject to a review fee of €150.00**
   If you believe the review fee should not be charged for the project now being proposed please give the reason(s) here:

   Please note that, for funded clinical trials, after the ethical opinion has been given, any amendment arising attracts a review fee of €200.00

   Payment should accompany the amendment documentation.

Signed _____ **Date**

_____

Project Supervisor.

SJH / AMNCH RESEARCH ETHICS COMMITTEE.

Confidential Research Protocol, 2006 Edition.

Please place an "X" or ✓ after the appropriate response in the boxed areas. NA is an abbreviation for Not Applicable.

1. Title of research project:

"The Biological Basis of *Candida* Leukoplakia"

2. Name of Chief Investigator – who should ordinarily be a hospital consultant:

Professor Stephen Flint,
Professor and Consultant in Oral Medicine.
Dublin Dental School and Hospital

DECLARATION BY SUPERVISOR

I confirm that the information provided in this protocol is correct. I also undertake to provide an annual report on the anniversary of Research Ethics Committee approval with details of the number of subjects who have been recruited, the number who have completed the study and details of any adverse effects.

Signed: Date:
(Chief Investigator)

Please PRINT name of signatory here
.....

Research Ethics Committee opinion:

Approved subject to :

Approved without conditions.

Signed:

(Chair)

Date:

3. What are the objectives of the research project?

The objectives of this study are

1) To speciate and subtype *Candida* isolates associated with *Candida* leukoplakia and commensal oral carriage.

2) To characterise *Candida* to host lesional keratinocyte adhesion and gene expression when co-cultured.

3) To investigate the correlation of the distribution of the inflammatory infiltrate with fungal intraepithelial invasion and to determine if there is any correlation with the degree of epithelial dysplasia.

4a. Does the design of the study allow a statistically significant conclusion to be reached?

YES

Statistical significance will be determined by multivariate analysis.

4b. Has statistical advice been sought?

YES

5. Will the conduct of the project conform to the principles of the Declaration of Helsinki? (Recommendations guiding Medical Doctors in Biomedical Research involving Human Subjects; the text of this Declaration is included on pages 3 to 8).

YES

If not, elucidate:

6. Please itemise here any ethical problems which you perceive to be associated with the research project:

There should be no ethical problems.

The biopsy procedure and mycological sampling are essential measures for definitive diagnosis and is the accepted standard of care for patients with leukoplakia. The procedures carry no more risk than if participants were not in the trial (i.e. the usual biopsy side effects of post-operative pain and swelling and complications of bleeding and infection).

Patient data will be kept confidential and anomymised, a secure access system will be used and no other individual outside of the research team will be allowed to have access to the collected data. DDSH have our own secure servers. The patient key code will not be kept on the server.

Patient explant cultures, DNA and RNA extracts will be anomymised and securely stored for five years. Thereafter, they will be autoclaved and disposed of through the DDSH clinical waste contractors. In the event that we would like to perform further investigations,

a reapplication for ethical approval will be made to the SJH/AMNCH Research Ethics Committee.

SECTION A

Details of project

7. Background:

A. What person or organisation devised this project?

Prof. Stephen Flint, Prof David Coleman and Dr Mohammed Abdulrahmen,
Dublin Dental School and Hospital.

B. Has a detailed research protocol been drawn up? (If so, such documentation must be submitted to the Committee.)

NO

C. Has the investigator who may be asked to present the project to the Committee studied all the documentation drawn up for the project, and will the documentation be studied by all the investigators before the project begins?

YES

D. Briefly describe the scientific rationale for the project:

Candida leukoplakia (CL) is a distinct variant of oral leukoplakia that is characterised by a white hyperkeratotic lesion, usually affecting non-keratinised mucosal sites although it may affect any site in the oral cavity, where superficial hyphal *Candida* infection is demonstrated histopathologically. CL is considered as a premalignant lesion by the WHO, (1978) with 9-40% of lesions undergoing malignant transformation compared to 2-6% of other idiopathic and tobacco-related oral leukoplakias in the same 10 year timeframe (Renstrup 1970, Samaranayake and MacFarlane 1990 and McCollough *et al* 2002).

There is a paucity of research in this area, regarding the nature of the condition, particularly with respect to aetiopathogenesis and therapeutic options.

The association of *Candida* with leukoplakia and increased malignant potential is well recognised. However, what is not known is whether the *Candida* infection generates the leukoplakia, whether a pre-existing idiopathic leukoplakia offers a favourable microenvironment for *Candida* super-infection, whether there is something different about the *Candida* species genotype or expression phenotypes in these lesions compared to *Candida* commensally populating other oral sites and the nature of the host response.

These are important questions because *Candida* leukoplakia represents an oral precancerous lesion where a therapeutic strategy could exist for prevention of malignant transformation, which may not be the case for other extensive idiopathic oral leukoplakias. Simple, blind antifungal therapy gives variable and very disappointing results. The lesions are often unresponsive, or, even if partial resolution occurs, generally recur. Antifungal therapy used repeatedly may generate resistant strains or species shift to azole resistant species, as has been seen in prophylaxis of candidosis in the immunocompromised. CL is often extensive and not amenable to surgery. If surgery is undertaken it is often extensive and may be disfiguring.

Unlike leukoplakias at other sites, such as the CIN classification in the uterine cervix, the degree of dysplasia **alone** does **not** reliably predict progression to malignant transformation. This creates a significant clinical dilemma, since often, prolonged, regular observation of the lesions (particularly when too large for surgical intervention) and a low clinical threshold for biopsy of suspicious areas is the management strategy of choice. This is the contemporary standard of care for most patients.

Patients with CL and other leukoplakias which cannot be excised, are usually followed-up closely with correction of predisposing factors, until such time as the lesion either resolves, reduces in size so that there is a surgical option or transforms (most other leukoplakias are in reality, tobacco-related keratoses, which may resolve or partially resolve on cessation of smoking).

A recent publication by Gaballah, *et al.* (2008) on the subject of tissue engineering in oral dysplasia and by Harrison *et al*, (2007) in tissue engineered skin, suggest that transglutaminase inhibitors may induce hyperproliferation and parakeratosis in oral mucous membrane and skin and are associated with oral dysplasia. When pathogenic, adhesion followed by invasion of epithelium by the flilamentous form of *Candida* occurs. Gene expression of virulence factors (secreted aspartyl proteases (SAPs), phospolipases, Hwp-1p etc.) in pathogenesis, as opposed to commensalism, is hypha-specific. Hwp-1p is a hypha-specific surface protein involved in *Candida* adhesion to epithelial cells. It is also a *Candida*-specific transglutaminase inhibitor (Staab *et al* 1999).

This project aims to investigate the interaction between *Candida*, the host and local keratinocytes with a view to elucidation of aetiopathogenesis of the disease, possible new therapeutic approaches to treatment for a premalignant lesion where currently the treatment is sub-optimal, and thereby, perhaps long-term, cancer prevention.

One hypothesis is particularly attractive: if Hwp-1p, by inhibition of host transglutaminase, is driving keratinocyte hyperproliferation and dysplasia in CL, a novel, non-surgical therapeutic avenue presents itself, if the protein could be blocked.

References

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8. Planning and organisational structure

Patients with leukoplakia who are referred to the DDSH are seen routinely in the Oral Medicine and multi-disciplinary Dysplasia Clinic attended by the Professor of Oral and Maxillofacial Surgery, Professsor Flint and another consultant Oral Physician. Patients with leukoplakia are routinely biopsied to assess the degree of dysplasia, the lesions are sampled for the presence of *Candida* and patients referred to our Smoking Cessation Counselling service if the lesions are felt to be tobacco-related.

Patients for the prospective part of the study (Parts 1 and 2) will be selected from patients with leukoplakia, attending consultant out-patient clinics. All these patients routinely require a biopsy and mycological sampling for definitive diagnosis. All are also regularly followed up in house, whilst the lesions are present, following surgery or until the lesions completely resolve.

Patients will also be recruited into part 3 of the trial, retrospectively, by identification of patients with biopsy-proven *Candida* leukoplakia and idiopathic leukoplakia at routine follow-up appointments.

An oral rinse and lesional swab will be collected from new patients at the time of biopsy to culture the yeast from the mouth generally and lesion specifically. The yeast isolates will be speciated, sub-typed and stored (all new patients with leukoplakias are routinely sampled for *Candida* presence). CL *Candida* isolates will be analysed for clane identity within species by Pulsed-field Gel Electrophoresis and Multilocus Sequence Typing and compared to isolates from other oral sites in the same patient and reference strains. These techniques are in regular use within the DDSH Candidology research facility.

The effect of the *Candida* (the strain isolated from the individual patient) on the patients' keratinocytes grown to confluence by primary cell culture of the biopsy obtained from the patient will be studied.

Adhesion studies will be carried out by incubation of the cultured keratinocytes from the leukoplakic lesion with the *Candida* strains isolated from the same patient. The plates are then washed in a standardized fashion and the number of *Candida* cells attached to the keratinocyes counted. The washing process is fairly robust, since true adhesion involves a covalent bonding of the yeast to the epithelial cell.

This will be compared with controls with typical laboratory strains of *Candida* incubated with the same keratinocytes and a commercial epithelial cell-line.

DNA and RNA will be extracted from the co-cultured cells. Gene expression profiling will be performed using qRT-PCR or micro-array analysis of biopsy tissue. *Candida* and host gene expression including virulence genes (particularly *Hwp-1*) will be analysed. These techniques are in regular use within the DDSH Candidology research facility.

The degree of epithelial dysplasia of the lesional tissue on conventional histopathology will be recorded, as routinely assessed and reported by our Consultant Oral Pathologist (Dr Mary Toner). Three parameters will be measured for correlation: *Candida* presence and standardised count, inflammatory infiltrate and degree of dysplaia. Archival tissue will be used for the patients with an established CL or non-*Candida* leukoplakia (NCL) diagnosis for *Candida*/dysplasia correlation studies.

9. What is the nature and extent of the medical examination that participants and controls are to undergo before participating in this project?
Dental and medical history and oral examination and follow-up as required in the Consultant clinics. Tissue samples will only be taken from patients with oral leukoplakia lesions.

10. How will the health of the participants and controls be monitored during and after the trial? (list clinical, laboratory and other examinations):
Patients will be undergoing a biopsy as part of the routine diagnostic procedures. Patients will be kept under regular periodic review, in house, which is the standard of care for patients with this condition

11. Will participants or controls undergo independent medical examination, before, during or after the trial?

NA

12. If a placebo group is to be used, will the group receive the best standard therapy?

NA

13. If the project involves the use of radioactive substances or of laser therapy has the approval of the Head of Medical Physics been obtained?

NA

If not, elucidate:

SECTION B

Investigators and Facilities

14. Name, qualification and position of each person associated with this project:

Name	Qualification	Position
a) Professor Stephen Flint.	MAjo(U Dubl), PhD(Brist), MBBS(Hons), BDS(Lond), FDSRCS(Eng), FFDRCSI, FICD, FTCD.	Professor and Consultant in Oral Medicine,
b) Professor David Coleman	PhD, FRCPath	Professor of Oral Microbiolgy
c) Mohammed Abdulrahim	BDS. MDentCh	PhD Student

15. Is each investigator a registered medical practitioner?

NO

Only a) is a registered medical practitioner.
a) and c) are registered dental practitioners.

16. Is each investigator a member of a major medical defence body?

NO

Only Prof Flint is a member of Medical Defence Body (Dental Protection). CIS protection applies to both Dr Abdulrahmin and Prof Flint.

17. What payments, monetary or otherwise, if any, are to be made to any of the investigators (include payments to any institution or research facility)?

Nil

18. What payments, whether monetary or otherwise, if any, are to be made to any person or institution providing facilities to be used for the purpose of the clinical trial?
Bench fees for PhD studentship form the Libyan Government.

19. In what hospitals or facilities will the trial / study take place?
The Dublin Dental School and Hospital

SECTION C

Participants

20. How many subjects and controls from this centre are expected to participate in this project?

Number

Subjects: 35 CL and 35 NCL

21. If this is a multicentre trial please indicate:

a) the expected overall number of subjects:

Number

NA

b) the number and geographical distribution of the centres involved in the study:

22. What criteria are to be used for the selection of participants?

Inclusion criteria:

a) Prospectively:
Patients with leukoplakia, attending the Dysplasia and Oral Medicine clinics in the Dublin Dental School and Hospital, who are to be routinely biopsied for diagnosis.

b) Retrospectively:
Patients with a confirmed histopathological diagnosis of CL attending the clinics for long-term follow-up.

23. Are women of childbearing potential included?

YES

If so, does the protocol/patient information sheet address the 8 points in the committee's checklist for studies involving women of childbearing potential (1–scientific justification, 2–negative teratogenic studies, 3–warning to subject that fetus may be damaged, 4–initial negative pregnancy test, 5–forms of contraception defined, 6–duration of use to exceed drug metabolism, 7–exclude those unlikely to follow contraceptive advice, 8–notify investigator if pregnancy suspected)?

NA

24. State the exclusion criteria (age, other illness, other medications etc.):

- Patients who cannot give informed consent.

25. What are the proposed methods by which participants and controls are to be recruited?

"Direct request to suitable patients attending investigator's clinic"

The participants will be recruited from patients with leukoplakia attending the Oral Medicine and Dysplasia Clinics in the Dublin Dental School & Hospital.
Prospective patients will be informed about their condition, consented for biopsy as usual and consented for participation in the trial.
Patients who have already been biopsied, will be asked for permission to investigate their archival biopsy material and consented, when they attend the clinic for routine follow-up.

26. What inducements or rewards, whether monetary or otherwise, are to be offered to participants and controls?

"None"

NA

If no, elucidate:

It is not appropriate.

27. What arrangements exist to provide compensation to each participant who may suffer injury or loss as a result of this research project?

"Participation in this study is covered by an approved policy of insurance in the name of (sponsor). In addition the medical practitioners involved in this study have current medical malpractice insurance cover. The sponsor (name) will comply with the ABPI guidelines and Irish Law (statutory and otherwise) in the unlikely event of your becoming ill or injured as a result of participation in this clinical study."

Is the Ethics Committee's standard compensation statement (above) being adopted ?

NO

There is no sponsor

If "NO" please give alternative wording.

The practitioners involved in this study have current medical/dental malpractice insurance cover.

28. Have you submitted to the committee, with this form, a patient information leaflet and consent form prepared by a sponsor or other external group, or a patient information leaflet and consent form based on the committee's guidelines (attached to this form) to be given to each participant and control?

YES

If no, elucidate:

29. What criteria are to be used to ensure that the identity of each participant and control remains confidential?

Only the investigator's group within the institution will know the identity of the subjects; codes will be used to conceal identities in all external communications. Samples and data will be anonymised, coded and securely stored and the key code will be kept totally separate from the stored data.

YES

If no, indicate the criteria used.

30. Give details of any risks to subjects or to controls from investigative or therapeutic procedures or from withholding of therapy?

No more than if participants were **not** in the trial (i.e. the usual biopsy side effects of post-operative pain, discomfort, and swelling and complications of bleeding and infection).
This is will be managed through preoperative instruction sheets, ensuring an atraumatic procedure, appropriate analgesia and post-operative antiseptic mouthrinses.

31. Indicate how adverse events are to be notified and evaluated:

If adverse events arise they will be carefully documented and patient will be managed suitably.
All patients are regularly followed up in the clinics.

SECTION D

Drugs and other Therapeutic Substances

32. Is the object of this project to assess the effect of a drug or therapeutic substance?

NO

If NO, skip to **END OF FORM**

33. Name of the substance or preparation which is the subject of the proposed project:

34. Name of the company or organisation which produces this substance or preparation:

35. Code number used by the company or organisation for this trial:

36. Does the organisation and performance of this trial conform to the International Conference on Harmonisation guidelines on Good Clinical Practice?

NA

37. Give details of the pharmacology, dosage, toxicity, and side effects of the substance or preparation:

NOTE: for the protection of both the investigator and the subject the list of side effects must be comprehensive and must also appear in full in the patient information leaflet.